JMJD4-demethylated RIG-I prevents hepatic steatosis and carcinogenesis

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Article

Keywords: hepatocarcinogenesis, steatosis, HCC progenitor cell, RIG-I, JMJD4, methylation

Posted Date: April 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-410612/v1>

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Abstract

Hepatocarcinogenesis is driven by necroinflammation or metabolic disorders, and the underlying mechanisms remain largely elusive. Here, we interestingly found that DEN-induced hepatocarcinogenesis was enhanced while NASH-induced hepatocarcinogenesis was abolished by hepatocyte-specific RIG-I deficiency. Further, IL-6 decreased RIG-I expression in HCC progenitor cells (HcPCs), which then viciously promoted IL-6 effector signaling and drove HcPCs to fully established HCC. RIG-I expression was increased by HFD, which then enhanced cholesterol synthesis and steatosis, and the in-turn NASH and NASH-induced hepatocarcinogenesis. Mechanistically, RIG-I was constitutively monomethylated at K18 and K146, and demethylase JMJD4-mediated RIG-I demethylation suppressed IL6STAT3 signaling. The constitutive methylated RIG-I associated with AMPK α to inhibit HMGCR phosphorylation, thus promoting HMGCR enzymatic activity and cholesterol synthesis. Together with clinical data that RIG-I was decreased in hepatic precancerous dysplastic nodules while increased in NAFLD, we conclude that decreased RIG-I in HcPCs promotes necroinflammation-induced hepatocarcinogenesis, while increased constitutive methylated RIG-I enhances steatosis and NASH-induced hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and the leading causes of cancer-related death worldwide¹. Hepatic inflammation, caused by viral infection, alcohol abuse, or lipid accumulation, plays critical roles in hepatocarcinogenesis^{2,3}. Hepatocyte injury and death, activation of innate immune cells in the liver, and the subsequent compensatory proliferation of hepatocytes contribute to the hepatic malignant transformation, which goes through premalignant lesions containing HCC progenitor cells (HcPCs) to the development of fully established HCC⁴. However, the underlying mechanisms of this vicious process are still largely elusive. The regulation of inflammation in hepatocarcinogenesis is therefore of great scientific and clinical interest, especially in the stage from HcPCs to established HCC.

Mechanistically, hepatocyte injury and death release damage-associated molecular patterns (DAMPs), which activate hepatic resident and recruited macrophages through Toll-like receptor (TLR) signaling. Subsequently, the production of proinflammatory cytokines such as interleukin-6 (IL-6) initiates hepatic inflammation and the in-turn compensatory proliferation of remaining hepatocytes for repairing liver function. However, repeated hepatic injury causes chronic inflammation, which leads to inflammation-induced hepatocarcinogenesis, and proinflammatory cytokine IL-6 and its downstream oncogenic IL-6-signal transducer and activator of transcription 3 (STAT3) pathway play the critical role during this process^{5,6}. Especially in the stage from HcPCs to established HCC, autocrined IL-6 of HcPCs drives their progression to fully established HCC^{4,7}. Not only IL-6 production but also IL-6 effect or response are tightly regulated by multiple mechanisms, whether and how IL-6 effect on HcPCs is dysregulated as compared to that on normal hepatocytes are still unknown up to now.

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive hepatic lipid accumulation, including both triglyceride (TG) and cholesterol⁸. NAFLD patients progress through hepatic steatosis to nonalcoholic steatohepatitis (NASH), which is characterized by chronic liver injury, inflammation, and fibrosis. NAFLD and NASH may ultimately progress to cirrhosis and eventually HCC⁹. During these processes, hepatic lipid accumulation and steatosis are the first step, which are mediated by a set of mechanisms, including the activation of sterol-regulatory element binding proteins (SREBPs), and the expression and activation of downstream metabolic enzymes¹⁰. However, the regulation of hepatic lipid synthesis and their roles in NASH and NASH-induced hepatocarcinogenesis still need further investigation.

RIG-I is well-established to be one of the critical intracellular sensors for host recognition of RNA virus infection and subsequent induction of type I interferon (IFN) production in innate immune cells. It contains a C-terminal helicase domain to recognize cytoplasmic viral RNA and two N-terminal tandem caspase-recruiting domains (CARDs) to activate downstream type I IFN production^{11,12}. Other than RIG-I expression in immune cells, we previously found that RIG-I expression was mainly located in parenchymal hepatocytes in the liver, but not in mesenchymal cells¹³, suggesting the potential roles of RIG-I in liver functions and diseases. However, the roles of RIG-I in hepatic metabolism, steatosis, and carcinogenesis are still unknown, especially in the different stages of hepatocarcinogenesis, such as steatosis, liver injury, inflammation, compensatory proliferation, HcPCs, and eventually established HCC.

The function and stability of proteins are strictly controlled by multiple posttranslational modifications (PTM), such as phosphorylation, ubiquitination, acetylation, and methylation^{14,15}. For the PTM of RIG-I, phosphorylation at S8, S854, S855, T170, and T770, K63-linked ubiquitination at K99, K169, K172 and K788, K48-linked ubiquitination at K813, and acetylation at K909 have been presented to tightly regulate the function of RIG-I in recognition, activation, interaction, and degradation¹⁶. Moreover, as protein methylation is an important PTM manner, it has been linked to transcriptional activation or repression in histones, and to protein-protein interaction between non-histones^{17,18}. However, it is still unknown up to now whether and how RIG-I is methylated or demethylated, and the roles of methylation in the regulation of RIG-I function.

In this study, we focused on the roles of RIG-I in hepatocarcinogenesis, including the expression, modification, and function of RIG-I in different hepatocarcinogenesis mouse models as well as in human tissues, to provide mechanistic insight and potential target for preventing HCC.

Results

Decreased RIG-I expression in HcPCs is due to IL-6 stimulation in the late phase.

Chemical carcinogen DEN-induced hepatocarcinogenesis goes through HcPCs to fully established HCC, and we first examined RIG-I expression during hepatocarcinogenesis, including normal hepatocytes, nonaggregated hepatocytes and aggregates containing HcPCs from mice five months post DEN

injection^{4,19}, and established HCC cells. Both protein and mRNA levels of RIG-I was determined to be decreased in HcPCs and established HCC cells, while its expression between normal hepatocytes and nonaggregated hepatocytes were similar (Fig. 1a,b). Thus, RIG-I expression is decreased from HcPCs to established HCC during hepatocarcinogenesis, suggesting the potential role of RIG-I in the development of HcPCs.

The mechanism responsible for the decreased RIG-I in HcPCs was then investigated. As the initiated hepatocytes acquire the ability to autocrine IL-6 thus becoming HcPCs during hepatocarcinogenesis⁴, we analyzed whether IL6 mediated the decrease of hepatic RIG-I. By continuous injection of IL-6 twice a week *in vivo*, we found that hepatic RIG-I expression was increased in the early phase (within one week) while decreased in the late phase (at two months) (Fig. 1c). In hepatocyte-specific IL6 receptor knockout *IL-6ra^{hep-/-}* mice, continuous IL-6 injection failed to decrease hepatic RIG-I expression (Fig. 1d). Moreover, in *IL-6^{-/-}* or *IL6ra^{hep-/-}* mice, RIG-I expression in isolated HcPCs five months post DEN injection was not decreased as compared to that in nonaggregated hepatocytes (Fig. 1e). Together, although IL-6 increases hepatic RIG-I expression in the early phase, its expression is decreased in HcPCs, which is mediated by continuous IL-6 stimulation in the late phase.

As *Rig-I* is a typical Interferon-stimulated gene (ISG) and its promoter contains Interferon-sensitive response element (ISRE) sequence (AGTTTCNNTTTCNC/T)²⁰, we examined whether IL6induced STAT3 activation and nuclear translocation could bind the ISRE region of *Rig-I* gene. Using ChIP-qPCR analysis, IL6 was found to induce the binding of STAT3 and ISRE sequence of *Rig-I* gene (Extended Data Fig. 1a), which may initiate downstream *Rig-I* transcription. Moreover, we previously found that reduced trimethylation of histone H3 at K4 (H3K4me3) and increased H3K9me3 and H3K27me3 were linked to decreased RIGI in HCC¹³. These dysregulated histone modifications were also determined in HcPCs as well as in hepatocytes upon continuous IL-6 injection for two months (Extended Data Fig. 1b,c). Thus, IL6increased RIG-I expression in the early phase may be due to the activated STAT3initiated transcription, and IL-6-decreased RIG-I expression in the late phase may be due to the dysregulated histone methylations.

Decreased hepatic RIG-I expression promotes DEN-induced hepatocarcinogenesis.

To analyze the role of RIG-I decrease in hepatocarcinogenesis, we constructed hepatocyte-specific RIG-I knockout *Rig-I^{hep-/-}* mouse (Fig. 1f and Extended Data Fig. 1d), and found that DEN-induced hepatocarcinogenesis was markedly promoted by hepatic RIG-I deficiency, including increased tumor incidence, number, and diameter (Fig. 1g,h). The DEN plus CCl₄-induced hepatocarcinogenesis model were applied, and it is also promoted by hepatic RIG-I deficiency (Extended Data Fig. 1e,f). Moreover, to exclude the potential effect of RIG-I as a viral RNA sensor in innate immune cells, we generated *Rig-I^{Mac-/-}* mice and determined that RIG-I in macrophages could not significantly influence DEN-induced hepatocarcinogenesis (Extended Data Fig. 1g). Together, hepatic RIGI deficiency promotes DEN-induced

hepatocarcinogenesis, suggesting that decreased RIG-I expression in HcPCs may participate in hepatocarcinogenesis.

Increased response to IL-6 in HcPCs is mediated by decreased RIG-I expression.

We next examined the underlying mechanism responsible for *Rig-I^{hep-/-}*-promoted hepatocarcinogenesis. The DNA damage, hepatocyte apoptosis, and serum ALT and AST representing liver damage following acute DEN injection were analyzed in *Rig-I^{hep-/-}* mice, and DEN-induced liver damage was not influenced by hepatic RIG-I deficiency (Extended Data Fig. 2a-e). The DEN-induced hepatic production of proinflammatory cytokines including IL-6 and TNF- α , infiltration of leukocytes, and compensatory hepatocyte proliferation were also not significantly influenced by *Rig-I^{hep-/-}* (Extended Data Fig. 2f-h). We then performed protein phosphorylation microArray to elucidate the altered intracellular signaling in *Rig-I^{hep-/-}* liver following acute DEN injection, and found that STAT3 phosphorylation was the most increased by hepatic RIG-I deficiency (Extended Data Fig. 2i). The increased DEN-induced STAT3 phosphorylation was then confirmed in *Rig-I^{hep-/-}* liver (Fig. 2a), suggesting that RIG-I deficiency may enhance STAT3 activation to promote hepatocarcinogenesis.

As IL-6 effects mainly through JAK2-STAT3 signaling and is the key proinflammatory cytokine driving hepatocarcinogenesis²¹, we examined whether hepatic RIG-I deficiency-promoted hepatocarcinogenesis was dependent on IL6STAT3 signaling. *Rig-I^{hep-/-}IL-6^{-/-}* double knockout (DKO) mice and *Rig-I^{hep-/-}IL6ra^{hep-/-}* DKO mice were generated respectively. Loss of IL-6 diminished DEN-induced HCC, and importantly, *Rig-I^{hep-/-}IL-6^{-/-}* mice and *IL-6^{-/-}* mice displayed a similar reduced induction of HCC by DEN (Fig. 2b), suggesting that hepatic RIG-I deficiency failed to promote hepatocarcinogenesis with IL-6 deficiency. Similar results were obtained in *Rig-I^{hep-/-}IL-6ra^{hep-/-}* DKO mice (Fig. 2c). Therefore, hepatocytespecific RIG-I deficiency-promoted hepatocarcinogenesis is dependent on the proinflammatory cytokine IL-6.

DEN-induced production of hepatic IL-6 was not influenced by RIG-I deficiency (Extended Data Fig. 2f), whether hepatic RIG-I deficiency promotes hepatocarcinogenesis by enhancing IL-6-STAT3 effector signaling was then examined. We found that IL6induced STAT3 phosphorylation was significantly promoted by hepatic RIG-I deficiency, both upon IL-6 injection *in vivo* and IL-6 stimulation in primary hepatocytes *in vitro* (Fig. 2d). The expression of IL6induced downstream gene *Saa1* was also enhanced by hepatic RIG-I deficiency (Fig. 2e). Thus, hepatic RIG-I deficiencypromoted hepatocarcinogenesis is mediated by the enhanced oncogenic IL6STAT3 effector signaling.

Since RIG-I expression is decreased in HcPCs and RIG-I deficiency promotes IL6-driven hepatocarcinogenesis, we presume that not only IL-6 is autocrined by HcPCs, but also their response to IL-6 is enhanced. IL-6-induced STAT3 activation is significantly enhanced in HcPCs as compared to that in nonaggregated hepatocytes (Fig. 2f). We also rescued RIG-I expression in HcPCs using AAV8-mediated gene delivery, and confirmed that IL-6-STAT3 signaling was compromised, to the level similar with that in

nonaggregated hepatocytes of control mice (Fig. 2g). Hence, the response to IL6 is enhanced in HcPCs, which is mediated by the decreased RIG-I expression.

To confirm that decreased RIG-I in HcPCs promotes hepatocarcinogenesis, especially in the stage from HcPCs to established HCC, we isolated CD44⁺ HcPCs from the livers of *Rig-1^{f/f}* and *Rig-1^{hep-/-}* mice five months post DEN injection, and determined that IL6-induced STAT3 activation was enhanced by RIG-I deficiency in HcPCs (Fig. 2h). These HcPCs were transplanted into wildtype mice, which were then injected weekly using CCl₄ to induce liver injury and inflammation for five months⁴.

Hepatocarcinogenesis of RIG-I deficient HcPCs were significantly increased as compared to that of HcPCs from *RIG-1^{f/f}* mice (Fig. 2i). Altogether, we conclude that IL6-induced RIG-I decrease and decreased RIG-I-enhanced IL-6 response in HcPCs may cause vicious feedforward progression from precancerous HcPCs to fully established HCC.

IL-6 induces RIG-I-STAT3 association to feedback impede JAK2-STAT3 interaction in the early phase.

The mechanism responsible for RIG-I-mediated inhibition of IL-6-STAT3 signaling was then examined. We found that IL-6 could induce the association between RIG-I and STAT3, both upon IL-6 injection *in vivo* and IL-6 stimulation in primary hepatocytes *in vitro* (Fig. 3a and Extended Data Fig. 3a). To determine how RIG-I binding to STAT3 suppresses STAT3 activation, we screened the kinases and regulators involved in IL6-STAT3 signaling, and found that STAT3 could bind to JAK1, JAK2, SHP1, SHP2, and SOCS3, but not SOCS1, PIAS1, or PIAS3, upon IL-6 stimulation in the liver (Extended Data Fig. 3b). Among them, IL6-induced JAK2-STAT3 interaction was significantly enhanced by hepatic RIG-I deficiency, both by IL-6 injection *in vivo* and IL-6 stimulation in primary hepatocytes *in vitro* (Fig. 3b and Extended Data Fig. 3c), while IL-6-induced JAK2 phosphorylation was not influenced (Extended Data Fig. 3d). As JAK2-phosphorylated STAT3 is the key pathway in IL-6 effects, hepatic RIG-I deficiency-promoted STAT3 activation is mediated by the enhanced JAK2-STAT3 interaction. Moreover, the tagged truncates of RIG-I, JAK2, and STAT3 were constructed, and both CARD domain of RIG-I and JH2 domain of JAK2 could bind the SH2-TA domain of STAT3, which contains the phosphorylation site tyrosine 705 (Extended Data Fig. 3e,f). Together with the IL6-induced early phase hepatic RIG-I expression, all these data determine that IL-6 induces RIG-I-STAT3 association and RIG-I expression to feedback inhibit JAK2-STAT3 interaction and IL6 effector signaling in the early phase.

IL-6 induces the demethylation of RIG-I to enhance RIG-I-STAT3 association.

We next examined the mechanism responsible for IL-6-induced RIG-I-STAT3 association. As protein PTM plays critical roles in their interaction, the PTM of RIG-I following IL-6 stimulation was analyzed by mass spectrometry, especially in the CARD domain which is responsible for RIG-I-STAT3 association. Methylation, acetylation, and phosphorylation of RIG-I were screened. Mono-methylation at K18, K146, and acetylation at K95, K172 were identified in untreated, while acetylation at K59, K154 were identified in IL-6-stimulated hepatocytes, and mono-methylation at K48 and phosphorylation at S8, T55 were identified in both. The mutants K48A, K59A, K95A, K154A, K172A, S8A, and T55A had no effect on the

RIG-I-STAT3 association (Extended Data Fig. 3g). However, the mutants K18M and K146M mimicking monomethylation of evolutionarily conserved K18 and K146 both significantly decreased RIG-I-STAT3 association, and K18M + K146M mutant abolished the association (Fig. 3c and Extended Data Fig. 3h,i). The K18A, K146A, K18R, K146R mutants of RIG-I mimicking demethylation were also constructed, and their association with STAT3 were significantly enhanced (Fig. 3d), suggesting that demethylation of RIG-I at both K18 and K146 may be important for RIG-I-STAT3 association. Furthermore, rabbit polyclonal antibodies specifically recognizing the K18 or K146 mono-methylated RIG-I were generated respectively (Extended Data Fig. 3j,k), and IL6-induced RIG-I demethylation at these sites was confirmed both in liver tissues *in vivo* and in primary hepatocytes *in vitro* (Fig. 3e and Extended Data Fig. 3l). Thus, IL6-induced RIG-I-STAT3 association was dependent on the induced demethylation at K18 and K146 of hepatic RIG-I.

To confirm the role of demethylated RIG-I in associating and suppressing IL6-induced STAT3 activation, we constructed the K18M + K146M mutant mouse mimicking mono-methylated RIG-I, and K18A + K146A mutant mouse mimicking demethylated RIG-I (Extended Data Fig. 3m). IL-6-induced hepatic STAT3 phosphorylation was significantly increased in K18M + K146M mice, while decreased in K18A + K146A mice, both by IL-6 injection *in vivo* and IL-6 stimulation in primary hepatocytes *in vitro* (Fig. 3f and Extended Data Fig. 3n). Similarly, hepatic RIG-I-STAT3 association was markedly decreased in K18M + K146M mice, while increased in K18A + K146A mice; IL6-induced JAK2-STAT3 association was enhanced in K18M + K146M mice, while decreased in K18A + K146A mice (Fig. 3g). Furthermore, DEN-induced hepatocarcinogenesis was increased in K18M + K146M mice, while decreased in K18A + K146A mice (Fig. 3h). Together, we conclude that IL6 induces demethylation of RIG-I at K18 and K146, which then associates with STAT3 and feedback inhibits IL-6-STAT3 effector signaling in the early phase.

Demethylase JMJD4 is responsible for IL-6-induced demethylation of RIG-I.

The mechanism responsible for IL-6-induced RIG-I demethylation was then investigated. We immunoprecipitated Flag-tagged RIG-I from lysates of IL6-stimulated L-02 hepatocyte cell line, then used mass spectrometry to identify RIG-I-associated proteins, and selected JMJD4 as the candidate because of its demethylase activity (Extended Data Fig. 4a). The IL-6-induced JMJD4-RIG-I association was confirmed by immunoprecipitation (Fig. 4a). The tagged truncates were constructed, and JMJC demethylase catalytic domain of JMJD4 also associated with the CARD domain of RIG-I, where K18 and K146 locate (Extended Data Fig. 4b). Thus, demethylase JMJD4 associates with RIG-I following IL-6 stimulation, which may participate in the IL6-induced RIG-I demethylation.

We next constructed hepatocyte-specific JMJD4 knockout mouse (Extended Data Fig. 4c,d), and determined that IL-6-induced hepatic RIG-I demethylation at K18 and K146 was abolished by JMJD4 deficiency, both by IL-6 injection *in vivo* and IL-6 stimulation in primary hepatocytes *in vitro* (Fig. 4b). IL6-induced RIG-I-STAT3 association was markedly decreased while STAT3 phosphorylation was enhanced by hepatic JMJD4 deficiency (Fig. 4c,d). Together, these data determine that IL6 induces JMJD4-erased methylation of RIG-I to enhance RIG-I-STAT3 association and feedback inhibit IL-6-STAT3 signaling in the early phase.

JMJD4 and RIG-I co-operatively suppress DEN-induced hepatocarcinogenesis.

As JMJD4-erased methylation of RIG-I feedback suppresses IL-6-STAT3 effector signaling, we further examined the role of JMJD4 in DEN-induced hepatocarcinogenesis. Hepatocyte-specific JMJD4 knockout significantly promoted both DEN and DEN plus CCl₄-induced hepatocarcinogenesis (Fig. 4e and Extended Data Fig. 4e), which is similar to those in *Rig-I^{hep-/-}* mice. JMJD4 expression was also analyzed in mouse HcPCs and HCC cells, and its expression moderately decreased as compared to that in normal hepatocytes and nonaggregates (Fig. 4f and Extended Data Fig. 4f). Together with the data that the markedly decreased RIG-I in HcPCs promotes hepatocarcinogenesis, this potentially decreased JMJD4 may further enhance IL-6 response and the corresponding hepatocarcinogenesis.

The hepatocyte-specific JMJD4 and RIG-I DKO mouse was then generated, and we found that the DEN-induced hepatocarcinogenesis in *Jmjd4^{hep-/-}Rig-I^{hep-/-}* mice was similar to that in *Rig-I^{hep-/-}* mice (Fig. 4g), suggesting that JMJD4 deficiency promoted hepatocarcinogenesis is dependent on RIG-I. Moreover, IL6-induced hepatic STAT3 phosphorylation was enhanced in *Jmjd4^{hep-/-}Rig-I^{hep-/-}* mice, which is similar to that in *Rig-I^{hep-/-}* mice (Fig. 4h). Altogether, we conclude that IL6-induced hepatic JMJD4-RIG-I association cooperatively feedback suppresses IL6-STAT3 effector signaling and DEN-induced hepatocarcinogenesis.

Opposite to DEN model, *Rig-I^{hep-/-}* abolished NASH-induced HCC.

Although the DEN-induced hepatocarcinogenesis model has been widely used for its ease and consistency in generating HCC as well as HcPCs, a recent study determined that tumors of Stelic Animal Model (STAM), by using streptozotocin (STZ) and highfat diet (HFD) to mimic NASH-induced HCC, were the most molecularly similar to human HCC among the available hepatocarcinogenesis mouse models²². We thus generated STAM tumors in *Rig-I^{f/f}* and *Rig-I^{hep-/-}* mice. Interestingly, hepatocyte-specific RIG-I deficiency nearly abolished NASH-induced hepatocarcinogenesis, suggesting the tumor-promotive role of RIG-I in NASH-induced HCC, which is opposite to the tumor-suppressive role of RIG-I in the DEN model (Fig. 5a,b and Extended Data Fig. 5a,b). The generated STAM tumors of *Rig-I^{f/f}* and *Rig-I^{hep-/-}* mice were then analyzed. Although the pathological features of tumors are similar, we interestingly found that the NASH features in nontumor liver tissues, including ballooning and inflammation, were induced in *Rig-I^{f/f}* mice, while abolished by hepatic RIG-I deficiency (Fig. 5c). Moreover, hepatic oil red O staining also determined that lipid accumulation was abolished by RIG-I deficiency in STAM model (Fig. 5d). Thus, these data indicate that hepatic RIG-I deficiency may suppress NASH progression, and then NASH-induced hepatocarcinogenesis.

The methionine & choline deficient (MCD) and choline deficient (CD) diets were then used to induce NASH in *Rig-I^{f/f}* and *Rig-I^{hep-/-}* mice to confirm the inhibited NASH progression by hepatic RIG-I deficiency. We determined that MCD and CD-induced NASH progression were both abolished in the liver of *Rig-I^{hep-/-}* mice as compared to those in controls (Fig. 5e and Extended Data Fig. 5c). Furthermore, hepatic oil red O staining also confirmed that lipid accumulation was abolished by RIG-I deficiency in these NASH models

(Fig. 5f and Extended Data Fig. 5d). Hence, we conclude that hepatic RIG-I deficiency suppresses NASH and NASH-induced HCC, and the tumor-promotive role of RIG-I in NASH-induced HCC is opposite to the tumor-suppressive role of RIG-I in DEN-induced hepatocarcinogenesis.

Hepatic RIG-I deficiency abolished the occurrence of steatosis, especially by the diminished hepatic cholesterol accumulation.

As NASH-induced HCC goes through steatosis, NASH, fibrosis, cirrhosis, and HCC, we next examined whether hepatic RIG-I deficiency could inhibit the development of the first step steatosis. Using HFD to induce simple steatosis but minor NASH, we found that hepatic RIG-I deficiency abolished HFD-induced steatosis as compared to that in *Rig^{f/f}* mice (Fig. 5g and Extended Data Fig. 5e). Hepatic oil red O staining also determined that lipid accumulation was abolished by RIG-I deficiency in the HFD model (Fig. 5h). Thus, hepatic RIG-I deficiency abolishes the occurrence of steatosis, which then abolishes the in-turn NASH and NASH-induced HCC. Moreover, we examined RIG-I expression in the liver of HFD-induced steatosis, and found the increased RIG-I in the steatosis liver (Extended Data Fig. 5f). Together, RIG-I expression is increased by hepatic steatosis, and RIG-I deficiency in hepatocytes abolishes steatosis development.

The accumulation of triglyceride (TG) and total cholesterol (TC) in hepatocytes are the main features of hepatic steatosis, and they were then examined. In HFD-treated mice, both hepatic TG and TC were markedly suppressed in *Rig^{hep-/-}* mice (Fig. 5i). Remarkably, hepatic TC is equivalent between ND and HFD groups of *Rig^{hep-/-}* mice, suggesting the most markedly suppressed hepatic TC by RIG-I deficiency, and HFD could not increase hepatic cholesterol in *Rig^{hep-/-}* mice (Fig. 5i). Furthermore, the increased serum cholesterol in HFD-treated mice was abolished by hepatic RIG-I deficiency, while serum TG was not significantly influenced (Fig. 5j). Hence, RIG-I deficiency in hepatocytes abolished the HFD-induced increase of both hepatic and serum cholesterol, which may be responsible for the abolished steatosis in *Rig^{hep-/-}* mice. As atherosclerosis (AS) is the most threatening extrahepatic disease mediated by the increased serum cholesterol during hepatic steatosis, we also examined whether RIG-I deficiency could inhibit AS progression. Using the AS model of HFD-treated *ApoE* knockout mouse, we found that RIG-I deficiency markedly suppressed AS progression (Extended Data Fig. 5g,h). Thus, we conclude that RIG-I deficiency abolishes hepatic cholesterol accumulation to suppress both hepatic steatosis and high serum cholesterol-mediated extrahepatic disease.

Methylated RIG-I associates AMPK α to inhibit HMGCR phosphorylation, thus increasing HMGCR enzymatic activity and enhancing cholesterol synthesis.

To elucidate the mechanism responsible for RIG-I deficiency-mediated inhibition of cholesterol accumulation and steatosis, we performed the transcriptome analysis between the livers of *Rig^{f/f}* and *Rig^{hep-/-}* mice. The mRNAs of genes involved in lipid uptake, synthesis, transport, and excretion were not significantly influenced by hepatic RIG-I deficiency (Extended Data Fig. 6a). We then presumed that the protein levels of the cholesterol metabolic genes and/or their phosphorylation might be modulated by

hepatic RIG-I deficiency, and screened them using Western blot. Among them, the phosphorylation of HMGCR, the rate-limiting enzyme for cholesterol synthesis and its phosphorylation leads to inactivation, was the most markedly increased in *RigI^{hep-/-}* livers (Fig. 6a). Thus, the phosphorylation of HMGCR, leading to the inactivation of cholesterol synthesis, is increased by hepatic RIG-I deficiency, which may be responsible for the suppressed cholesterol accumulation and hepatic steatosis.

As HMGCR phosphorylation and inactivation are mediated by upstream activated kinase AMPK α ²³, we next examined the association between AMPK α and HMGCR, and downstream HMGCR phosphorylation. In *RigI^{hep-/-}* livers, both the basal level of HMGCR phosphorylation and its induction by fasting or Metformin treatment were significantly enhanced, while the phosphorylation of AMPK α was not influenced (Fig. 6b and Extended Data Fig. 6b). RIG-I was found to constitutively associate with AMPK α , and both the basal level and induced association between AMPK α and HMGCR were significantly enhanced by hepatic RIG-I deficiency (Fig. 6c and Extended Data Fig. 6c). Moreover, the N-terminal CARD domain of RIG-I associated with the kinase domain of AMPK α , which was also responsible for the association between AMPK α and C-terminal catalytic domain of HMGCR (Extended Data Fig. 6d,e). Together, we conclude that hepatic RIG-I constitutively associates with AMPK α to impede HMGCR, thus inhibiting HMGCR phosphorylation and enhancing its enzymatic activity to synthesize cholesterol.

Since PTM of proteins is important for their association, and methylation at K18, K146, and acetylation at K48, K95, K172 were found to be constitutively existed in RIG-I, we further examined whether these PTMs would influence its interaction with AMPK α . The respective mutants at these sites were used, and interestingly, only K18A and K146A mutants mimicking demethylation markedly inhibited RIG-I-AMPK α association, while K18M and K146M mutants mimicking mono-methylation enhanced their association, suggesting the constitutive K18 and K146 methylation of RIG-I is responsible for its interaction with AMPK α (Fig. 6d). Together with the result that IL6 induced the demethylation of RIG-I at these sites, we confirmed that the constitutive RIG-I-AMPK α association was inhibited by IL-6 administration (Extended Data Fig. 6f). In conclusion, K18 and K146 methylated RIG-I constitutively associates with AMPK α to inhibit HMGCR phosphorylation and enhance cholesterol synthesis, while JMJD4-demethylated RIG-I at K18 and K146 associates with STAT3 to feedback inhibit IL-6-induced STAT3 phosphorylation and hepatocarcinogenesis.

To confirm the enhanced cholesterol synthesis mediated by constitutive methylated RIG-I, we examined the HFD-induced hepatic steatosis in K18A + K146A mutant mice mimicking demethylated RIG-I, and in K18M + K146M mutant mice mimicking mono-methylated RIG-I. In contrast to those in wildtype mice, RIG-I K18A + K146A mice showed abolished hepatic steatosis and cholesterol accumulation following HFD treatment, while K18M + K146M promoted hepatic steatosis (Fig. 6e,f and Extended Data Fig. 6g). The STAM tumor models were also performed, and RIG-I K18A + K146A mice nearly abolished while K18M + K146M promoted STAM hepatocarcinogenesis (Extended Data Fig. 6h). Moreover, as JMJD4 is responsible for the demethylation of RIG-I at K18 and K146, we confirmed that HFD-induced steatosis and STAM hepatocarcinogenesis were both promoted in *Jmjd4^{hep-/-}* mice, which is similar to those in RIG-I K18M + K146M mutant mice (Fig. 6g,h and Extended Data Fig. 6i,j). Altogether, we conclude that both

steatosis and inflammation-induced hepatocarcinogenesis are promoted by the constitutive methylated RIG-I at K18 and K146, while suppressed by JMJD4-mediated RIG-I demethylation.

RIG-I expression and methylation may be correlated to human hepatocarcinogenesis, prognosis, and NAFLD progression.

In order to analyze the correlation of RIG-I expression to human hepatocarcinogenesis, we examined that in hepatic dysplastic nodules, which represent precancerous lesions of HCC. RIG-I expression was decreased while phosphorylated STAT3 was increased in human dysplastic nodules (Fig. 7a,b), which is consistent with those determined in mouse HcPCs. RIG-I expression in dysplastic nodules was significantly reverse correlated to STAT3 phosphorylation, implicating that decreased RIG-I may contribute to STAT3 activation in human dysplastic nodules (Fig. 7c). Additionally, hepatic JMJD4 expression differed between individuals, and was not statistically changed between normal livers and dysplastic nodules (Fig. 7d). Thus, decreased RIG-I in hepatic dysplastic nodules may be correlated to human hepatocarcinogenesis.

We next analyzed the correlation of RIG-I and JMJD4 expression in HCC tissues to the prognosis of patients. Using Cohort 1 (n = 152) and Cohort 2 (n = 140) HCC patients as we described previously¹³, we determined that high RIG-I in HCC predicts better disease-free survival and overall survival as compared to those in low RIG-I patients (Extended Data Fig. 7a,b). Moreover, patients with high RIG-I and high JMJD4 group had the significant best outcome (Fig. 7e and Extended Data Fig. 7c). The patients with high RIG-I and low JMJD4 in HCC had significant worse outcome than that of the high RIG-I and high JMJD4 group (Fig. 7e and Extended Data Fig. 7c), suggesting that JMJD4-mediated RIG-I demethylation may be important in the identification of HCC prognosis.

To examine the correlation of RIG-I expression and methylation to human NAFLD, we analyzed RIG-I in the liver tissues of NAFLD patients. RIG-I expression was significantly increased in the livers from at least 8 of 16 (50%) NAFLD patients, and the level of methylated RIG-I at K18 and K146 were also significantly increased in these 8 NAFLD livers (Fig. 7f-h and Extended Data Fig. 7d,e). Although HMGCR phosphorylation differed between individuals and was not significantly changed in NAFLD livers (Extended Data Fig. 7d), HMGCR phosphorylation was significantly lower while serum cholesterol was significantly higher in the 8 NAFLD livers with increased RIG-I than in those without significantly increased RIG-I (Fig. 7i,j). Furthermore, RIG-I expression in NAFLD livers was significantly reverse correlated to HMGCR phosphorylation (Fig. 7k), which is consistent with the RIG-I-mediated inhibition of HMGCR phosphorylation determined in mouse livers. Additionally, hepatic JMJD4 expression also differed between individuals and was not statistically changed in human NAFLD livers (Fig. 7l). Thus, we conclude that increased constitutive methylated RIG-I at K18 and K146 in NAFLD liver may be correlated to human NAFLD progression.

Discussion

In this study, we interestingly find that hepatocarcinogenesis phenotypes modulated by hepatocyte-specific RIG-I deficiency are inverse in two commonly used mouse models, which are RIG-I promotes STAM HCC representing NASH-induced carcinogenesis whereas suppresses DEN-induced inflammation and carcinogenesis. Mechanistically, constitutive methylated RIG-I at K18 and K146 promotes hepatic cholesterol synthesis and lipid accumulation, whereas JMJD4-demethylated RIG-I suppresses IL-6 response and inflammation-induced hepatocarcinogenesis. More interestingly, JMJD4-demethylated RIG-I is beneficial in preventing both steatosis and inflammation-induced carcinogenesis, while the constitutive methylated RIG-I tends to promote them both, which are determined in RIG-I K18A + K146A mice and K18M + K146M mice *in vivo*. Thus, mutations at these two lysine sites of *RIG-I* gene may be correlated to human hepatic steatosis and carcinogenesis. Based on the public large sequencing data, we find that an SNP site exists at K18 (rs763359633, C > G, Lys > Asn), while no SNP is suggested at K146. However, it remains unknown whether this SNP site is correlated to human liver diseases, which is worth further analyzing intensively.

As an important PTM manner, protein methylation has been linked to transcriptional regulation in histones, and to protein-protein interaction between nonhistones^{17, 24}. Although a set of PTM of RIG-I have been identified, including phosphorylation, ubiquitination, and acetylation¹⁶, whether RIG-I is methylated and the potential role of methylation in regulating RIG-I function are still unknown currently. Here, the constitutive K18 and K146 methylation of RIG-I and IL-6-induced demethylation by demethylase JMJD4 are determined, which is responsible for the IL-6-induced RIG-I-STAT3 association. However, it is still unknown that the underlying mechanism and corresponding methylase for the constitutive K18 and K146 methylation of RIG-I, which may maintain the cellular response to IL-6 in the quiescent condition and the activity for cholesterol synthesis. Moreover, whether K18 and K146 of RIG-I return to be methylated when IL-6 is withdrawn, and which methyltransferase mediates this process remain to be investigated. The corresponding mechanism and biological significance of RIG-I methylation at K18 and K146 in the quiescent state may further reveal the entire roles of RIG-I in hepatocytes, and may also bear considerable potential for the prevention of steatosis and carcinogenesis.

During inflammation-induced hepatocarcinogenesis, we show that IL-6 induces RIG-I expression and RIG-I-STAT3 association to feedback suppress IL-6-STAT3 signaling in the early phase, while decreases RIG-I expression in HcPCs to enhance IL-6 response and hepatocarcinogenesis in the late phase. In this process, initiated hepatocytes acquire the ability to autocrine IL-6 and drive their malignant progression, thus becoming HcPCs, whose phenotype is CD44 positive also induced by IL-6-STAT3 signaling^{4, 25}. Here, not only IL-6 production but also IL-6 response are determined to be enhanced in HcPCs, and increased IL-6 response is mediated by the decreased RIG-I, which is also induced by IL-6 in the late phase, thus suggesting the feedforward vicious autocrined IL-6 and increased IL-6 response in the driving of hepatocarcinogenesis, especially in the stage from HcPCs to established HCC. Moreover, as IL-6 plays critical roles in the carcinogenesis of series types of cancer, such as hematologic tumors, gastrointestinal cancers, and urogenital neoplasms, whether decreased RIG-I-mediated vicious augment of IL-6 response is a common mechanism for IL-6-promoted malignancies may raise interesting future work in this field.

RIG-I is a well-established intracellular sensor for host recognition of invading RNA virus in the innate immune response, and is also important for the differentiation and activation of T cells in adaptive immune system^{11,26}. However, the roles of RIG-I methylation or demethylation at K18 and K146 in innate immune sensing and activation of immune response are still unknown, which may also be interesting and attractive. Moreover, RIG-I is determined to be expressed in the parenchymal cells of organs, such as in hepatocytes in this study, which may suggest its specific roles associating with the functions of the organs. Other immune sensors or immune molecules may be also expressed in the organs or systems, and their functions may be not restricted in the immune activation or regulation. Currently, as molecules or factors of other systems such as metabolism have been intensively investigated in the regulation of immune response²⁷, the immune molecules are probably to be also important in the regulation of other systems and progression of other diseases. For example, a current research reported that stress-induced IL-6 from brown adipocytes could enhance hepatic gluconeogenesis and hyperglycemia²⁸, which may be in line with our data that IL-6-induced demethylated RIG-I disassociates with AMPK α and may enhance HMGCR phosphorylation to suppress cholesterol synthesis. The potential interaction or cross talk between immune system and other systems especially metabolism at the level of intracellular molecules are promising topics in the future studies.

RIG-I, also known as DDX58, belongs to the DEAD/DEAH box helicase (DDX) protein family. DDX family is the largest family of RNA helicases and contains vital players of RNA metabolism. Members of this family share conserved helicase domain for RNA binding and unwinding properties, possessing roles in RNA transcription, splicing, modification, exporting, translation, and degradation²⁹. Other than helicase domain, DDX family members contain their specific domains for the interaction between proteins. For RIG-I, the N-terminal CARD domain mediates its interaction with downstream MAVS upon viral infection, and with STATs upon cytokine stimulation determined here and in our previous study¹³. Thus, the functions of DDX family members are not restricted in RNA binding, other domains contained in individual members may expand their roles in diverse properties and participate in multiple physiological and pathological processes. Additionally, as the helicase domain of RIG-I or other DDX members can constitutively associate with intracellular RNAs³⁰, the helicase domain binding host RNAs may also modulate their protein-protein interaction, which needs further investigation.

In the simplest model compatible with our findings, we propose that RIG-I is constitutively mono-methylated at K18 and K146, and increased methylated RIG-I during steatosis promotes cholesterol synthesis by inhibiting HMGCR phosphorylation. Upon hepatic inflammation, inflammatory circumstances such as IL-6 promote JMJD4-mediated RIG-I demethylation at K18 and K146, thus restricting STAT3 activation and oncogenic transformation, and decreased RIG-I expression in HcPCs enhances IL-6 effects and drives them to established HCC (Fig. 7m). Together, we find that JMJD4-demethylated RIG-I at K18 and K146 is an essential mechanism to inhibit hepatic steatosis and carcinogenesis.

Methods

Clinical Specimens. HCC patients involved in this study are Cohort 1, 152 patients from Sun Yat-sen University (Guangzhou, China), and Cohort 2, 140 patients from Guangxi Medical University (Nanning, China), who underwent radical resection of HCC as we described previously¹³. Human normal liver tissues were obtained from distal normal liver tissue of liver hemangioma patients. Liver dysplastic nodule tissues were from the corresponding patients during surgery, and these tissues were pathologically diagnosed. Human NAFLD liver tissues were collected from patients with NAFLD undergoing resection for liver hemangioma or cyst, which were pathologically confirmed, and the patients with liver steatosis due to excessive alcohol consumption, drug/toxin use, or viral infection were excluded from the study. Human normal liver tissues, dysplastic nodules, and NAFLD tissues were all obtained in Second Military Medical University (Shanghai, China). All the tissue samples in this study were collected with written informed consent from the patients and the experiments were approved by Institute Research Ethics Committee of each research center.

Animals. C57BL/6 mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). *Rig*^{f/f} mouse was constructed by ViewSolid Biotech (Beijing, China) using CRISPR/Cas9 techniques as previously described³¹, and then mated with Alb-Cre transgenic mouse (003574), obtained from The Jackson Laboratory (Bar Harbor, ME), to generate hepatocyte-specific deficiency. *Jmjd4*^{f/f} mouse, *Rig*-I K18A + K146A mutant mouse, and K18M + K146M mutant mouse were constructed by Cyagen (Suzhou, China) also using CRISPR/Cas9 strategy. *IL-6*^{-/-} (002650), *IL-6ra*^{f/f} (012944), and LyzM-Cre (004781) mice were obtained from The Jackson Laboratory. *ApoE*^{-/-} mouse (T001458) was from GemPharmatech (Nanjing, China). Mouse genotyping was done by PCR analysis on genomic DNA extracted from tails as previously described³². All animals were housed in a specific pathogen free facility and maintained in a standard temperature- and light-controlled animal facility. All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai, China.

Reagents. Antibodies specific to RIG-I (3743), pSTAT3 Y705 (4113), STAT3 (9139), JAK1 (3344), pJAK2 (3771), JAK2 (3230), SHP1 (3759), SHP2 (3397), SOCS1 (3950), SOCS3 (52113), PIAS1 (3550), PIAS3 (4164), pACC (11818), ACC (3676), pAMPK α (50081), AMPK α (5832), cleaved-caspase 3 (9661), pH2AX (9718), and horseradish peroxidase-coupled secondary antibodies (7074 and 7076) were from Cell Signaling Technology (Danvers, MA). Antibodies specific to Flag-tag (F1804), β -actin (A5441), and HMGR (SAB4200529) were from Sigma-Aldrich (St. Louis, MO). Antibodies specific to RIGI (ab45428), Ki67 (ab16667), Ly6G (ab238132), and V5-tag (ab9116) were from Abcam (Cambridge, MA). Antibody specific to pHMGR (orb251472) was from Biorbyt (United Kingdom). Antibodies specific to H3K4me3 (07-473), H3K9me3 (05-1242), and H3K27me3 (07449) were from Millipore (Shanghai, China). Antibody specific to JMJD4 was constructed and purified by Proteintech Group (Wuhan, China). Antibodies specific to mono-methylated RIG-I at K18 or K146 were developed using the indicated synthesized antigenic peptides, and rabbit polyclonal antibodies to these peptides were constructed and purified by Abmart (Shanghai, China) as we described previously^{33,34}. The antibody specificity was validated by dot blot

analysis. Antibody specific to CD44-PE (130-102-606) and Anti-PE MicroBeads (130-048-801) were from Miltenyi Biotec (Germany). Protein G Agarose (P4691), Anti-Flag M2 Affinity Gel (A2220), and DEN (N0258) were from SigmaAldrich. Recombinant mouse IL-6 (575706) was from BioLegend (San Diego, CA). Fetal Bovine Serum (FBS, 10099141C), DMEM (11965092), and RPMI 1640 (11875093) were from Gibco (Shanghai, China).

Mouse Models. For DEN-induced hepatocarcinogenesis model, male mice at postnatal day 15 were injected intraperitoneally with DEN (25 mg/kg), and livers were examined eight months after the initial injection. For DEN plus CCl₄ model, male mice were injected weekly with CCl₄ (0.5 ml/kg body weight, dissolved in olive oil at a ratio of 1:3) starting from four weeks post the initial DEN injection and lasting for 15 weeks, and were sacrificed eight weeks after the last CCl₄ injection as described^{35,36}. For acute DEN, IL-6, or Metformin administration, eight-week-old male mice were injected intraperitoneally with DEN (100 mg/kg) or IL-6 (20 ug/kg), and liver tissues were examined in the indicated time post injection^{13,31}. For Metformin administration, eightweek-old male mice were fasted overnight, refed for two hours, and injected intraperitoneally with Metformin (250 mg/kg) for one hour, and liver tissues were then examined. For STAM hepatocarcinogenesis model, male mice at postnatal day two were injected subcutaneously with STZ (200 ug per mouse), and fed with HFD (D12492, Research Diets, New Brunswick, NJ) for five months starting from onemonth-old²². For NASH models, one-month-old male mice were fed with MCD (A06071301B, Research Diets) for one month or with CD (D05010403, Research Diets) for two months; and male mice were fed with HFD for five months to generate steatosis³⁷.

Cell lines and transfection. The human hepatocyte cell line L-02 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured using RPMI 1640 with 10% FBS as routine. Cells were seeded and then transfected with plasmids using jetPRIME transfection reagent (114 – 15, Polyplus-transfection, France) according to the manufacturer's protocol as we described previously³⁸.

Isolation of primary hepatocytes and HcPCs. Primary hepatocytes from eight-week-old male mice were isolated using two-step liver perfusion method including perfusion and digestion as described³⁹. Primary hepatocytes were separated by centrifugation at 50 g for 5 minutes and purified on 50% Percoll solution (P4937, Sigma-Aldrich), and then resuspended in DMEM supplemented with 10% FBS and seeded into six-well plates at 1×10^7 cells per well. For isolating HcPCs, livers of male mice five months post the initial DEN injection at postnatal day 15 were perfused, and nonaggregate and aggregate cells were isolated by filtration through 70 and 40 μ m sieves. After dispersing the aggregates into single cells, HcPCs were enriched for CD44⁺ cells using magnetic beads as reported⁴. 1×10^3 HcPCs were transplanted through intrasplenic injection to the male mice undergone Retrorsine (PHL84274, Sigma-Aldrich) injection before and CCl₄ injection after transplantation as reported⁴. Tumor multiplicity and size were evaluated five months post transplantation.

Molecular cloning of genes. The genes and their various truncates in this study were amplified by PCR from the cDNA of human liver tissue, and subsequently cloned into pcDNA vectors with Flag or V5 tag. Each construct was confirmed by sequencing. Genes with site mutations were constructed by Fast Mutagenesis System (FM111-02, TransGen Biotech, Beijing, China) and confirmed by sequencing. The rAAV (serotype 8) vector expressing RIG-I under promoter CAG was constructed as we previously described^{38,40}. For AAV8 administration, 1×10^{12} vg AAV8 was injected through tail vein two weeks before the sacrifice.

MS Analysis. RIG-I and its associated proteins were immunoprecipitated from L-02 hepatocyte cell line treated with IL-6 (100 ng/ml) for 30 minutes, and the precipitates were washed, boiled, and loaded to SDS-PAGE. After Coomassie Blue staining, the RIG-I specific bands and the selected bands were cut and then analyzed in reverse-phase nanospray liquid chromatography-tandem mass spectrometry. The MS and spectra analysis were performed by PTM BIO (Hangzhou, China) as we described previously^{33,34,41}.

RNA-seq. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and subjected to high throughput sequencing as we previously described⁴². Genes with FPKM values above 5.0 were used for subsequent analysis. The mRNA levels of genes associated with lipid uptake, synthesis, transport, and excretion were gathered and analyzed by Heml 1.0 software.

RNA extraction and Real-time PCR. Total RNA was extracted from frozen HCC tissues, mouse liver tissues, and primary cells using TRIzol reagent following the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) analysis was performed using LightCycler (Roche, Switzerland) and SYBR RT-PCR kit (RR430B, Takara, Dalian, China) as previously described³⁸. The qPCR primers for gene expression analysis were human *RIG-I* (forward: 5'-TGT GCT CCT ACA GGT TGT GGA-3', reverse: 5'-CAC TGG GAT CTG ATT CGC AAA A-3'); human *JMJD4* (forward: 5'-CTG CTA CGG ACC TAC GGA GAC-3', reverse: 5'-GCC CGC CTG TAT GTA CTC TTT C-3'); internal control human β -actin (forward: 5'-ACA ATG AGC TGC TGG TGG CT-3', reverse: 5'-GAT GGG CAC AGT GTG GGT GA-3'); mouse *Rig-I* (forward: 5'-AGA GTG TCA GAA TCT CAG TCA-3', reverse: 5'-CTG CTG CTC ATA GAC AGG AA-3'); mouse *Saa1* (forward: 5'-AGG GTT TTT TTC ATT TGT TC-3', reverse: 5'-TCT GAG TTT TTC CAG TTA GC-3'); mouse *IL-6* (forward: 5'-TGA TGC ACT TGC AGA AAA CA-3', reverse: 5'-ACC AGA GGA AAT TTT CAA TAG GC-3'); mouse *TNF- α* (forward: 5'-CAG GCG GTG CCT ATG TCT C-3', reverse: 5'-CGA TCA CCC CGA AGT TCA GTA G-3'); internal control mouse β -actin (forward: 5'-AGT GTG ACG TTG ACA TCC GT-3', reverse: 5'-GCA GCT CAG TAA CAG TCC GC-3'). The relative expression of the individual genes was normalized to that of internal control using $2^{-\Delta\Delta Ct}$ cycle threshold method in each sample⁴³.

ChIP assay and real-time PCR. The cells or tissues were cross-linked and processed as we described previously^{13,38}. Antibodies to H3K4me3, H3K9me3, H3K27me3, STAT3, or control IgG (sc2025, Santa Cruz, Dallas, TX) were used at 5 μ g per immunoprecipitation for the specific immunoprecipitation of respective histone residues or protein. 10 μ l sonicated but preimmunoprecipitated DNA from each sample was used as input control. ChIP results were analyzed by real-time PCR and normalized as we described

previously^{13,38}. The primers for ISRE region of *Rig-I* gene were forward: 5'-GGT TCA GAA ACG AAA CTG GAT3' and reverse: 5'-TGG CCA TAG GAA ATC GAA ACT-3'.

Primer sequences for mice identification. *Rig-I^{f/f}* forward: 5'-AGG ATT GCT CTT GGT CAG-3', reverse: 5'-CTG TGA AGT ATA GTT GAG TAG G-3'; *Rig-I*K18A + K146A WT forward: 5'-TGA GGC AGT TTC GAT TTC CTA TGG-3', mutant forward: 5'-CTG CTG TCC ATT CCT TAT TCC ATA3', common reverse: 5'-GTG GAA ACA CCC AGT GCA ATT TAC-3'; *Rig-I*K18M + K146M WT forward: 5'-TGA GGC AGT TTC GAT TTC CTA TGG-3', WT reverse: 5'-GCT GTC TAC TCT AAT ATG GCC TGG-3', mutant forward: 5'-GGC AGT TTC GAT TTC CTA TGG C-3', mutant reverse: 5'-AGT GTC TCG GAT CTG TCT GAT TTC-3'; *Jmjd4^{f/f}* forward: 5'-GCA GAG GAA GCC ATA CCT GAG AA-3', reverse: 5'-ATC AAT GGG CCT CCA AAT GAT GG-3'; *IL-6^{-/-}* common forward: 5'-TTC CAT CCA GTT GCC TTC TTG-3', WT reverse: 5'-TTC TCA TTT CCA CGA TTT CCC AG-3', mutant reverse: 5'-CCG GAG AAC CTG CGT GCA ATC C-3'; *IL6ra^{f/f}* forward: 5'-GAA GGA GGA GCT TGA CCT TGG-3', reverse: 5'-AAC CAT GCC TAT CAT CCT TTG G-3'; Alb-Cre WT forward: 5'-TGC AAA CAT CAC ATG CAC AC-3', mutant forward: 5'-GAA GCA GAA GCT TAG GAA GAT GG-3', common reverse: 5'-TTG GCC CCT TAC CAT AAC TG-3'.

Liver function and lipid test. Liver function and serum lipid were evaluated by determining the activities of ALT and AST in the serum and serum lipid using an automatic biochemical analyzer FDC-7000i (Shanghai, China) according to the manufacturer's instructions. Hepatic TC content was examined using Cholesterol Assay Kit (ab65390, Abcam), and hepatic TG content was examined using Triglyceride Determination Kit (TR0100, Sigma-Aldrich) according to the respective standard protocols.

Histological analysis. Paraffin-embedded tissue sections were stained with HE to visualize liver pathology, and were immunostained for the examination of pH2AX, cleaved-caspase 3, Ly6G, Ki67, and TUNEL staining as we described previously^{13,31,35}. Oil red O staining of frozen liver sections was performed to assess lipid droplet accumulation. Images were acquired and scanned with a light microscope (Pannoramic DESK, 3D HISTECH, Hungary).

Immunoprecipitation and Western blot. Cells or tissues were harvested and lysed with cell lysis buffer (9803, Cell Signaling Technology) supplemented with protease inhibitor cocktail (539134, Millipore). Protein concentrations of the lysates were measured with bicinchoninic acid (BCA) protein assay kit (ab102536, Abcam) and equalized with the lysis buffer. Equal amount of the extracts was used for immunoprecipitation, or loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as we described previously⁴⁴. β -actin was used as a loading control. Protein levels were quantified using Image J software and normalized to the internal control β -actin.

Statistical Analysis. Data are presented as mean $\pm$ s.d. from one representative of at least three independent experiments. Statistical comparisons between experimental groups were analyzed by Student's *t*-test, chi-square test, or one-way ANOVA in SPSS 17.0 (Chicago, IL), and *P* < 0.05 was taken to indicate statistical significance. Correlation was analyzed using Pearson's correlation coefficient assay in

SPSS 17.0 with *r* and *P* values shown. For Kaplan-Meier survival analysis of HCC patients, logrank test in SPSS 17.0 was used with the *P* values shown.

Declarations

Acknowledgments

We thank Prof. Bao-Liang Song from Wuhan University for kindly providing HMGCR plasmid, and Ms. Tingting Fang from our laboratory for technical assistance.

This work was sponsored by grants from National 135 Major Project of China (2018ZX10302205 and 2017ZX10102032), National Natural Science Foundation of China (91842104, 81871229, 81671564, 81701566, 82073184), Program of Shanghai Academic Research Leader (19XD1424900), Shanghai Sailing Project (17YF1424600), Shanghai Shuguang Program (20SG39), and Shanghai Chenguang Program (18CG39).

Author contributions

Z.L., Y.Z., and K.J. performed the experiments and contributed equally for the whole study. Y.Y., L.Z., S.W., M.W., and Y.L. provided reagents and performed experiments. N.L. and Y.Y. provided reagents and analyzed the data. J.H. and X.C. analyzed the data and wrote the manuscript. J.H. designed and supervised the study.

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Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Figure 1

IL-6-induced RIG-I decrease in HcPCs promotes hepatocarcinogenesis. a,b, RIG-I expression in isolated normal hepatocytes, nonaggregated hepatocytes and HcPCs from male mice five months post DEN injection, and established HCC cells was examined by Western blot (a) and qRT-PCR (b, n = 4, one-way ANOVA and Tukey's multiple comparisons test). c, RIG-I expression was examined in male mouse livers in the indicated time periods post continuous intraperitoneal IL-6 injection twice a week. d, RIG-I expression

was examined in liver tissues of IL-6^{rahep}^{-/-} mice treated as in c. e, RIG-I expression was examined in nonaggregate hepatocytes and HcPCs isolated from male IL-6^{-/-} or IL 6^{rahep}^{-/-} mice five months post DEN injection. f, RIG-I expression in liver tissues and isolated hepatocytes from RIG-I^{hep}^{-/-} mice was confirmed by Western blot. g, Representative livers of DEN induced HCC in male RIG-I^{f/f} and RIG-I^{hep}^{-/-} mice. h, Tumor incidence (chi-square test), number and maximum diameter (unpaired t-test) in g were analyzed (n = 12). Data are shown as mean ± s.d. or photographs from one representative of three independent experiments. *P < 0.05, **P < 0.01. The unprocessed gels and source data are available at <https://data.mendeley.com/datasets/jvn3t64p8j/1>

Figure 2

Figure 2

Increased response to IL-6 in HcPCs is mediated by decreased RIG-I. a, STAT3 phosphorylation and activation were examined in liver tissues from male *Rig-I^{ff}* and *Rig-I^{hep-/-}* mice 48 hours post DEN administration. b, Tumor incidence (chi square test), number and maximum diameter (unpaired t-test) of DEN-induced HCC in *Rig-I^{ff}IL-6^{-/-}* and *Rig-I^{hep-/-}IL-6^{-/-}* mice were analyzed (n = 12). c, Tumor incidence (chi-square test), number and maximum diameter (unpaired t-test) of DEN induced HCC in male *IL-6ra^{hep}*

/- and Rig l^{hep} /-IL-6^{ra} hep /-mice were analyzed (n = 12). d, IL-6-induced STAT3 phosphorylation in the indicated time periods was examined in liver tissues and isolated hepatocytes from male Rig-lf/f and Rig l^{hep} /- mice. e, IL-6-induced Saa1 mRNA expression in the indicated time periods was examined in liver tissues and isolated hepatocytes from male Rig-lf/f and Rig l^{hep} /- mice (n = 4, unpaired t-test). f, Isolated nonaggregated hepatocytes and HcPCs were stimulated with IL-6 for the indicated time periods, and STAT3 phosphorylation was examined. g, Isolated nonaggregated hepatocytes and HcPCs from control AAV8 or AAV8 RIG I treated male mice were stimulated as in f, and STAT3 phosphorylation was examined. h, Isolated HcPCs from male Rig-lf/f and Rig l^{hep} /- mice were stimulated with IL-6 for 30 minutes, and STAT3 phosphorylation was examined. i, Tumor number and maximum diameter were analyzed in male mice transplanted with isolated HcPCs from Rig-lf/f and Rig l^{hep} /- mice by intrasplenic injection (n = 6, unpaired t-test). Data are shown as mean ± s.d. or photographs from one representative of three independent experiments. *P < 0.05, **P < 0.01, ▲P > 0.05. The unprocessed gels and source data are available at <https://data.mendeley.com/datasets/jvn3t64p8j/1>

Figure 3

Figure 3

IL-6 induces RIG-I demethylation to enhance RIG-I-STAT3 association and feedback impede JAK2-STAT3 interaction. a, RIG-I-STAT3 association induced by IL-6 was examined by immunoprecipitation in male mouse liver tissues. b, IL 6 induced JAK2-STAT3 interaction was evaluated by immunoprecipitation in liver tissues from male *Rig-I^{fl/f}* and *Rig-I^{hep-/-}* mice. c,d, V5 tagged STAT3 and Flag tagged RIG-I mutants as indicated were transfected into L 02 hepatocyte cell line, and their association was tested by

immunoprecipitation. e, Methylated RIG-I at K18 and K146 were examined by the specific antibodies in the precipitates by total RIG-I antibody from the liver tissues upon IL-6 stimulation. f, IL 6 induced STAT3 phosphorylation was evaluated in liver tissues from wildtype, RIG-I K18M+K146M or K18A+K146A mutant male mice upon IL-6 stimulation. g, IL-6-induced RIG I STAT3 association and JAK2-STAT3 interaction were examined using immunoprecipitation in the liver tissues from male wildtype, RIG-I K18M+K146M or K18A+K146A mutant mice upon IL-6 stimulation. h, Tumor incidence (chi-square test), number and maximum diameter (unpaired t-test) of DEN induced HCC in male wildtype, RIG-I K18M+K146M or K18A+K146A mutant mice were analyzed (n = 12). Data are shown as mean $\pm$ s.d. or photographs from one representative of three independent experiments. *P < 0.05, **P < 0.01. The unprocessed gels and source data are available at <https://data.mendeley.com/datasets/jvn3t64p8j/1>

Figure 4

Figure 4

JMJD4-demethylated RIG-I inhibits DEN-induced hepatocarcinogenesis. a, JMJD4-RIG-I association induced by IL-6 was examined by immunoprecipitation in male mouse liver tissues. b, Methylated RIG-I at K18 and K146 upon IL-6 stimulation was examined in male *Jmjd4f/f* and *Jmjd4hep-/-* liver tissues and isolated primary hepatocytes as indicated. c, IL-6-induced RIG-I-STAT3 association was evaluated by immunoprecipitation in liver tissues from male *Jmjd4f/f* and *Jmjd4hep-/-* mice. d, IL 6 induced STAT3 phosphorylation was evaluated in liver tissues from male *Jmjd4f/f* and *Jmjd4hep-/-* mice upon IL-6 stimulation. e, Tumor incidence (chi-square test), number and maximum diameter (unpaired t-test) of DEN-induced HCC in *Jmjd4f/f* and *Jmjd4hep-/-* mice were analyzed (n = 12). f, JMJD4 expression in isolated normal hepatocytes, nonaggregated hepatocytes and HcPCs from male mice five months post DEN injection, and established HCC cells was examined by Western blot. g, Tumor incidence (chi-square test), number and maximum diameter (unpaired t-test) of DEN induced HCC in male *Jmjd4f/fRig-I^{f/f}*, *Rig-I^{hep-/-}* and *Jmjd4hep-/-Rig-I^{hep-/-}* mice were analyzed (n = 12). h, IL-6-induced STAT3 phosphorylation was evaluated in liver tissues from male *Jmjd4f/fRig-I^{f/f}*, *Rig-I^{hep-/-}* and *Jmjd4hep-/-Rig-I^{hep-/-}* mice upon IL 6 stimulation. Data are shown as mean $\pm$ s.d. or photographs from one representative of three independent experiments. *P < 0.05, **P < 0.01, $\blacktriangle$ P > 0.05. The unprocessed gels and source data are available at <https://data.mendeley.com/datasets/jvn3t64p8j/1>

Figure 5

Figure 5

Hepatocyte-specific RIG-I deficiency abolishes steatosis, NASH, and NASH-induced hepatocarcinogenesis. a, Tumor incidence (chi-square test), number and maximum diameter (unpaired t-test) of STAM HCC in male *Rig-I^{fl/f}* and *Rig-I^{hep-/-}* mice were analyzed (n = 12). b, Representative livers of STAM HCC in male *Rig-I^{fl/f}* and *Rig-I^{hep-/-}* mice. c,d, HE (c) and oil red O (d) staining were analyzed in nontumor liver tissues of STAM model in male *Rig-I^{fl/f}* and *Rig-I^{hep-/-}* mice. Scale bars: 20 μ m. e,f, HE (e) and oil red O (f) staining were analyzed in liver tissues of MCD model in male *Rig-I^{fl/f}* and *Rig-I^{hep-/-}* mice. Scale bars: 20 μ m. g,h, HE (g) and oil red O (h) staining were analyzed in liver tissues of HFD model in male *Rig-I^{fl/f}* and *Rig-I^{hep-/-}* mice.

Rig I^{hep} ^{-/-} mice. Scale bars: 20 μ m. i,j, Hepatic TG and TC (i), and serum TG and TC (j) were examined in HFD treated male Rig I^{f/f} and Rig-I^{hep}^{-/-} mice (n = 4). Data are shown as mean $\pm$ s.d. or photographs from one representative of three independent experiments. *P < 0.05, **P < 0.01, $\blacktriangle$ P > 0.05. The unprocessed images and source data are available at <https://data.mendeley.com/datasets/jvn3t64p8j/1>

Figure 6

Methylated RIG-I associates AMPK α to inhibit HMGCR phosphorylation and enhance cholesterol synthesis. a, HMGCR, ACC, AMPK α , and their phosphorylation were examined in liver tissues of male Rig-I^{f/f} and Rig-I^{hep} ^{-/-} mice. b, HMGCR, AMPK α , and their phosphorylation were examined in liver tissues of male Rig-I^{f/f} and Rig-I^{hep} ^{-/-} mice fasting for 16 hours. c, The AMPK α -HMGCR interaction and AMPK α -RIG-I interaction upon fasting for 16 hours were evaluated using immunoprecipitation in liver tissues from male Rig-I^{f/f} and Rig-I^{hep} ^{-/-} mice. d, V5 tagged AMPK α and Flag-tagged RIG-I mutants as indicated were transfected into L-02 hepatocyte cell line, and their association was tested by immunoprecipitation. e,f, HE (e) and oil red O (f) staining were analyzed in the liver tissues of HFD model in male wildtype, RIG-I K18M+K146M or K18A+K146A mutant mice. Scale bars: 20 μ m. g,h, HE (g) and oil red O (h) staining were analyzed in the liver tissues of HFD model in male Jmjd4^{f/f} and Jmjd4^{hep} ^{-/-} mice. Scale bars: 20 μ m. Data are shown as photographs from one representative of three independent experiments. The unprocessed gels and images, and source data are available at <https://data.mendeley.com/datasets/jvn3t64p8j/1>

Figure 7

Figure 7

RIG-I expression and methylation may be correlated to human hepatocarcinogenesis, prognosis, and NAFLD progression. a, RIG-I, JMJD4, and STAT3 phosphorylation were examined in human normal liver tissues and dysplastic nodule tissues from the indicated patients. b, Quantified RIG-I expression and STAT3 phosphorylation in human normal liver (N) tissues and dysplastic nodule (DN) tissues were shown ($n = 12$, unpaired t-test). c, The correlation between RIG-I expression and STAT3 phosphorylation in

human dysplastic nodule tissues was analyzed by Pearson's correlation coefficient assay. d, Quantified JMJD4 protein level in human normal liver (N) tissues and dysplastic nodule (DN) tissues were shown (n = 12, unpaired t-test). e, Kaplan-Meier survival curves of overall survival based on dichotomized RIG-I and JMJD4 mRNA expression in HCC tissues of Cohort 1 and 2. The median levels of RIG-I and JMJD4 expression in each cohort were used as the cutoff, with log-rank test for significance. f, RIG-I, JMJD4, and HMGCR phosphorylation were examined in human normal liver tissues and NAFLD tissues from the indicated patients. g, Methylated RIG-I at K18 or K146 were examined in the precipitates using RIG I antibody from human normal liver tissues and NAFLD tissues as in f. h,i, Quantified RIG-I protein level (h) and HMGCR phosphorylation level (i) in human normal liver tissues (N, n = 12) and the indicated NAFLD tissues (n = 8, unpaired t test) were shown. j, Serum TC of the indicated NAFLD patients were shown (n = 8, unpaired t-test). k, The correlation between RIG-I expression and HMGCR phosphorylation in human NAFLD tissues was analyzed by Pearson's correlation coefficient assay. l, Quantified JMJD4 protein level in human normal liver (N, n = 12) tissues and NAFLD (n = 16, unpaired t-test) tissues were shown. m, Working model for the mechanism of constitutive methylated RIG I at K18 and K146 inhibits HMGCR phosphorylation to promote cholesterol synthesis and NAFLD progression, while IL-6-induced and JMJD4 mediated RIG-I demethylation inhibits STAT3 phosphorylation to suppress hepatocarcinogenesis, and decreased RIG I in HcPCs enhances IL-6 effects and drives them to established HCC. Data are shown as mean $\pm$ s.d., survival curves, or photographs as indicated. The unprocessed gels and source data are available at <https://data.mendeley.com/datasets/jvn3t64p8j/1>

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