An inflammatory-CCRK circuitry drives mTORC1-dependent metabolic and immunosuppressive reprogramming in obesity-associated hepatocellular carcinoma

Hanyong Sun1,2, Weiqin Yang3, Yuan Tian1,4, Xuezhen Zeng3, Jingying Zhou3, Myth T.S. Mok3, Wenshu Tang3, Yu Feng3, Liangliang Xu3, Anthony W.H. Chan5, Joanna H. Tong5, Yue-Sun Cheung5, Paul B.S. Lai6, Hector K.S. Wang7, Shun-Wa Tsang7, King-Lau Chow7, Mengying Hu8, Rihe Liu9, Leaf Huang8, Bing Yang4, Pengyuan Yang4, Ka-Fai To5,10, Joseph J.Y. Sung1,11, Grace L.H. Wong1,11, Vincent W.S. Wong1,11 & Alfred S.L. Cheng3,11

Obesity increases the risk of hepatocellular carcinoma (HCC) especially in men, but the molecular mechanism remains obscure. Here, we show that an androgen receptor (AR)-driven oncogene, cell cycle-related kinase (CCRK), collaborates with obesity-induced pro-inflammatory signaling to promote non-alcoholic steatohepatitis (NASH)-related hepatocarcinogenesis. Lentivirus-mediated Ccrk ablation in liver of male mice fed with high-fat high-carbohydrate diet abrogates not only obesity-associated lipid accumulation, glucose intolerance and insulin resistance, but also HCC development. Mechanistically, CCRK fuels a feedforward loop by inducing STAT3-AR promoter co-occupancy and transcriptional up-regulation, which in turn activates mTORC1/4E-BP1/S6K/SREBP1 cascades via GSK3β phosphorylation. Moreover, hepatic CCRK induction in transgenic mice stimulates mTORC1-dependent G-CSF expression to enhance polymorphonuclear myeloid-derived suppressor cell recruitment and tumorigenicity. Finally, the STAT3-AR-CCRK-mTORC1 pathway components are concordantly over-expressed in human NASH-associated HCCs. These findings unveil the dual roles of an inflammatory-CCRK circuitry in driving metabolic and immunosuppressive reprogramming through mTORC1 activation, thereby establishing a pro-tumorigenic microenvironment for HCC development.
Hepatocellular carcinoma (HCC) is among the most lethal cancers that significantly correlate with obesity. The pathophysiology begins with obesity-induced hepatotoxicosis and non-alcoholic steatohepatitis (NASH), collectively known as non-alcoholic fatty liver disease (NAFLD), which can further develop into cirrhosis and HCC. Notably, HCC is characterized by strong sexual dimorphism in almost all geographic areas where male to female ratios average between 2.1 and 7.1. A prospective study of >900,000 US adults, men with a body mass index (BMI) of 35 kg/m² exhibited a dramatic 4.52-fold increase in relative risk of death from liver cancer, while a modest 1.68-fold increase was observed in women. A recent population-based cohort study of 5.24 million adults in the United Kingdom confirmed the significant modulation of HCC incidence by gender, in which higher BMI in men but not in women was associated with substantially increased risk of HCC. In addition, another population-based cohort study of 1.2 million Swedish men further showed that a high BMI (≥30 kg/m²) in late adolescence was associated with an increased risk of future severe liver diseases including HCC. These findings consistently underscore the sex disparity in obesity-associated HCC, but the molecular mechanisms underlying HCC development in obese men remain obscure.

Using obese mouse models exposed to the hepatic procarcinogen diethylnitrosamine (DEN), Park et al. demonstrated that obesity is a bona fide liver tumor promoter. The obesity-driven HCC development largely depends on a chronic pro-inflammatory state that results in elevated circulating levels of cytokines, such as tumor necrotic factor-α (TNF-α) and interleukin-6 (IL-6), and the latter of which has recently been shown to correlate with HCC progression in obese people. Chronic IL-6-mediated activation of signal transducer and activator of transcription 3 (STAT3) can cause hepatic insulin resistance critical for the development of glucose intolerance and steatotic HCC. Unlike early hepatocarcinogenesis which relies on paracrine nuclear factor kappa B (NF-κB)-regulated IL-6 production by inflammatory cells, HCC progenitor cells in premalignant lesions acquire autocrine IL-6-STAT3 signaling to stimulate cellular proliferation and transformation. Nevertheless, it is unclear how the hepatic IL-6-STAT3 cascade is activated and sustained during malignant transformation.

One of the major IL-6-driven signaling pathways in obesity and HCC development is mechanistic target of rapamycin (mTOR) which is a key signal transducer in the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Protein Kinase B (AKT) pathway. mTOR can assemble with Raptor and Rictor to form two functionally distinct complexes, mTORC1 and mTORC2, respectively. Activation of cap-dependent translation by phosphorylation of 4E-BP1 contributes to mTORC1-dependent carcinogenesis. Consistent with the increased de novo lipid synthesis in proliferating cancer cells, mTORC1 has been shown to activate the central lipogenic transcription factor, sterol regulatory element-binding protein 1 (SREBP1), through S6K1 to stimulate lipogenesis and cell proliferation. Animal model and human studies have confirmed the functional significance of mTORC1 activation in NAFLD pathogenesis. Stimulation of AKT-mTORC1 signaling, either alone or in combination with β-catenin, induces hepatic lipogenesis and tumorigenesis. Nonetheless, how mTORC1 remains constitutively active in the context of insulin resistance is unresolved. Additionally, mTORC1 was shown to be negatively regulated by glycogen synthase kinase 3β (GSK3β) via phosphorylation of tuberous sclerosis complex 2 (TSC2), which transmits diverse upstream signals including insulin to mTORC1. Moreover, inactivation of GSK3β was shown to inhibit hepatocellular apoptosis in dietary obesity-promoted HCC. While these findings implicate a causal effect of GSK3β dysregulation in obesity-related hepatocarcinogenesis, the upstream kinase that controls GSK3β/mTORC1 signaling in the obesity-induced inflammatory microenvironment has not been elucidated.

Genetic and biochemical studies have demonstrated the fundamental roles of androgen receptor (AR) in male predominance of HCC. Using genome-wide location and functional analysis, cell cycle-related kinase (CCRK), the latest cyclin-dependent kinase member (CDK20), was previously underpinned as a direct AR-regulated oncosene in hepatocarcinogenesis through concordant activation of GSK3β/β-catenin and AKT/EZH2 signaling. It was further shown that CCRK mediates virus-host signaling to promote hepatitis B virus (HBV)-associated hepatocarcinogenesis, and fosters an immunosuppressive microenvironment for HCC development by induction of myeloid-derived suppressor cells (MDSCs). Notably, overexpression and hyper-activation of CCRK distinguishes a subset of HCC patients with poor overall and disease-free survival. Based on the biological and clinical significance of CCRK in the male-predominant HBV-associated HCC, we investigated its potential role in obesity-associated HCC. Our findings demonstrated that knockdown of Ccrk dramatically suppressed hepatic lipid accumulation, inflammation and tumorigenicity in multiple murine NASH and HCC models. Mechanistically, obesity-induced pro-inflammatory STAT3 and AR-induced signaling cooperatively up-regulated CCRK expression, which in turn activated the mTORC1 pathway crucial for lipid/glucose homeostasis, immunosuppression, and tumorigenesis. These findings suggest that CCRK functions as a major signaling hub in obesity-associated hepatocarcinogenesis, providing insights into therapeutic strategies to reduce tumor burden from the worldwide obesity epidemic.

Results

Dietary obesity induces hepatic CCRK to promote NASH. To explore the role of CCRK in obesity-induced NASH, male C57Bl/6 mice were fed with diet of saturated fat and fructose for 22 weeks (Fig. 1a). The high-fat high-carbohydrate (HFHC)-fed mice exhibited significant increases in body weight (p < 0.001; Fig. 1b) and circulating levels of triglyceride (p < 0.05) and non-esterified fatty acid (NEFA; p < 0.01; Fig. 1c) when compared to mice fed with chow diet (CD). They also showed impaired glucose tolerance and insulin resistance as measured by IPITT and IPITT, respectively (p < 0.001; Fig. 1d, e). Notably, CCRK was significantly up-regulated in the liver (p < 0.01; Fig. 1f), which was associated with hepatic steatosis, ballooning degeneration and spotty necrosis (p < 0.01; Fig. 1g). In contrast, the HFHC-fed female C57Bl/6 mice did not exhibit CCRK induction (Supplementary Fig. 1a). To explore the role of CCRK in obesity-promoted NASH, we next investigated whether CCRK promotes NASH development in male mice via administration of lentivirus expressing short-hairpin RNA (shRNA) in the dietary obesity model (Fig. 1a). Lentiviral shRNA-mediated down-regulation of Ccrk (shCcrk) in the livers of the obese mice nearly restored the plasma triglyceride and NEFA concentrations to the basal levels (p < 0.05; Fig. 1c), and reversed the glucose intolerance and insulin resistance when compared to the control mice treated with lentivirus expressing non-specific sequence control (shCtrl; p < 0.01; Fig. 1d, e). Moreover, Ccrk knockdown significantly reduced hepatic lipid accumulation, ballooning degeneration, and spotty necrosis (p < 0.05; Fig. 1g), thus supporting a key role of CCRK in promoting NASH.
Fig. 1 Dietary obesity-induced CCRK over-expression promotes lipid accumulation, glucose intolerance, and liver damages in male mice. a Schematic diagram of NASH mouse model with different diets (CD chow diet, HFHC high-fat high-carbohydrate) and lentivirus-mediated Ccrk knockdown (CD + shCtrl, n = 8; HFHC+shCtrl, n = 15; HFHC+shCcrk, n = 15). b Body weight, c blood triglyceride and non-esterified fatty acid (NEFA) levels in mice were increased by HFHC at 28 weeks, which could be reduced by Ccrk knockdown. d, e CCRK impaired insulin sensitivity in mice. d Intraperitoneal glucose tolerance test (IPGTT) and e intraperitoneal insulin tolerance test (IPITT) were performed on CD-fed and HFHC-fed mice, blood glucose was measured at indicated time points after glucose or insulin injection (left), and area under the curve (AUC) is shown in a bar chart (right). f CCRK protein expression was induced by HFHC in mouse livers, which could be reduced by shRNA-mediated knockdown. Quantification of CCRK protein levels (relative to β-actin) is shown in a bar chart (bottom). g Representative pictures of Oil Red O, ballooning degeneration, and spotty necrosis of liver tissues in different groups (image magnification = ×200 or ×400, scale bar = 20 μm). h, i Quantifications of Oil Red O, ballooning degeneration and j scoring of spotty necrosis showed increased lipid accumulation, ballooning and spotty necrosis in HFHC-fed mice, which were reduced by Ccrk knockdown. Data are presented as mean ± SD. *p < 0.05; **p < 0.01; and ***p < 0.001 as calculated by unpaired two-tailed Student’s t-test (b), one-way ANOVA followed by Bonferroni post-hoc test (c-f, h, j), two-way ANOVA followed by Bonferroni post-hoc tests (d, e), and Chi-square test (i).
CCRK mediates obesity-associated hepatocarcinogenesis. To further investigate the oncogenic function of CCRK, we employed an obesity-associated HCC model7,30 using HFHC-fed male mice with neonatal DEN treatment (Fig. 2a, b). In this model, hepatic CCRK was markedly up-regulated (p < 0.001; Fig. 2c), which was associated with much higher lipid accumulation and HCC tumorigenicity when compared to the DEN-treated CD-fed mice (>10-fold; p < 0.01; Fig. 2d–g). Strikingly, when compared to shCtrl controls, Ccrk knockdown significantly reduced >70% obesity-promoted tumor multiplicity and size in HFHC-fed mice (p < 0.01; Fig. 2e, f) but not in CD-fed mice (Supplementary Fig. 2a, b). Consistently, the livers of shCcrk-treated mice

Fig. 2 Knockdown of Ccrk improves glucose sensitivity, and reduces hepatic lipid accumulation and hepatocarcinogenesis. a Schematic diagram of NASH-HCC mouse model with carcinogen induction (DEN diethylnitrosamine), different diets, and lentivirus-mediated Ccrk knockdown (CD–shCtrl, n = 8; HFHC + shCtrl, n = 15; HFHC+shCcrk, n = 15). b Body weight of mice at 28 weeks. c Ccrk protein expression in mouse livers were increased by HFHC at 28 weeks, which could be reduced by shRNA-mediated knockdown. Quantification of CCRK protein levels (relative to β-actin) is shown in a bar chart (right). d Representative pictures of dissected livers (scale bar = 1 cm), and Oil Red O and Ki67 staining of liver tissues in different groups (image magnification = ×200 or ×400, scale bar = 20 µm). e Tumor multiplicity and f tumor sizes were higher in the livers of HFHC-fed mice, which could be reduced by Ccrk knockdown. g, h Dietary obesity-induced CCRK increased lipid accumulation and cell proliferation, which could be abrogated by Ccrk knockdown as shown by Oil Red O staining and Ki67 staining in the livers. i, j Blood triglyceride and NEFA levels were elevated by dietary obesity-induced CCRK at 28 weeks, which could be reduced by Ccrk knockdown. k CCRK over-expression impaired insulin sensitivity in mice. IPGTT was performed on CD-fed and HFHC-fed mice, blood glucose was measured at indicated time points after glucose injection (left), and AUC is shown in a bar chart (right). Data are presented as mean ± SD. *p < 0.05; **p < 0.01; and ***p < 0.001 as calculated by unpaired two-tailed Student’s t-test (b), one-way ANOVA followed by Bonferroni post-hoc test (c, e–k), and two-way ANOVA followed by Bonferroni post hoc test (k).
exhibited significant reduction in lipid accumulation (p < 0.05) and hepatocellular proliferation (p < 0.01; Fig. 2g, h). The reduced tumorigenicity was also associated with significant restoration of normal circulating triglyceride/NEFA levels (p < 0.05; Fig. 2i, j) and glucose tolerance (p < 0.05; Fig. 2k). Taken together, our results suggest that CCRK plays a key role in promoting obesity-associated hepatocarcinogenesis.

**IL-6-mediated STAT3 and AR cooperatively induce CCRK.** We next investigated the molecular mechanism underlying the up-regulation of CCRK in obesity-associated hepatocarcinogenesis. As IL-6 represents a crucial HCC-promoting pro-inflammatory cytokine in obese state, we treated both HepG2 and HuH7 HCC cell lines with IL-6, and found increased phosphorylation of STAT3 at Tyr705 (p-STAT3 Tyr705) and elevated mRNA and protein expressions of CCRK, which were abrogated by short-interfering RNA (siRNA)-mediated knockdown of STAT3 (Fig. 3a, b). Luciferase reporter assay further demonstrated that IL-6 transcriptionally up-regulated CCRK in a STAT3-dependent manner (Fig. 3c). Consistently, we found that the serum IL-6 levels were significantly elevated (p < 0.05) and positively correlated with CCRK protein expression in both dietary obesity-induced NASH and HCC models (p < 0.01; Supplementary Fig. 2c, d).

Since CCRK is a direct transcriptional target of AR, we determined the potential interaction between IL-6/STAT3 and AR signaling in CCRK up-regulation. Knockdown of AR by siRNA abrogated IL-6-induced CCRK transcriptional activity and expression in HepG2 and HuH7 cells (Fig. 3a–c). Notably, deletion of the AR response element (ARE) in the CCRK promoter completely abolished the CCRK transcriptional activation by IL-6 (Fig. 3c). Moreover, ectopic AR expression in STAT3 knockdown cells restored CCRK expression (Fig. 3d). Conversely, in CCRK-low-expressing LO2 immortalized hepatocytes and SK-Hep1 liver sinusoidal endothelial cells, ectopic expression of constitutive active STAT3 (STAT3C), but not dominant negative STAT3 (DN-STAT3) up-regulated AR and CCRK expression (Supplementary Fig. 3a), which could be abrogated by knockdown of AR (Supplementary Fig. 3b). Collectively, these data demonstrate a concerted action by IL-6/STAT3 and AR signaling to induce CCRK transcription and expression in liver and HCC cells, which was further supported by in vivo data from IL-6 neutralization experiment (Supplementary Fig. 3c, d).

CCRK, STAT3, and AR form a positive feedback loop. Given the pivotal roles of STAT3 and AR feedback circuits in HCC initiation, we speculated that CCRK feedback regulates STAT3 and AR signaling to form a positive loop. Ectopic CCRK expression in AR knockdown cells rescued p-STAT3 Tyr705 (Fig. 3e), while knockdown of CCRK in AR-over-expressing cells abrogated p-STAT3 Tyr705 (Supplementary Fig. 3e). Thus, AR activates STAT3 signaling in a CCRK-dependent manner. To further determine whether CCRK activates STAT3 signaling, we first performed CRISPR/Cas9-mediated CCRK deletion in HuH7 cells, which suppressed p-STAT3 Tyr705 and AR expression (Fig. 3f). Next, ectopic expression of wild-type (WT) CCRK, but not its kinase-defective (KD) mutant, increased p-STAT3 Tyr705 and AR expression in both LO2 and CCRK knockout (KO) HuH7 cells (Fig. 3f), suggesting a CCRK autoregulatory loop.

We next investigated whether the auto-regulation occurs at the transcriptional level. Indeed, CCRK induced its own promoter activity, which was abolished by knockdown of either STAT3 or AR in CCRK-expressing LO2 and SK-Hep1 cells (Fig. 3g and Supplementary Fig. 3f). Deletion of ARE in the CCRK promoter also prevented the transcriptional activation, indicating that the auto-regulation was dependent on direct AR binding (Fig. 3g and Supplementary Fig. 3f). We next conducted co-immunoprecipitation to determine whether STAT3 physically interacts with AR for transcriptional regulation. WT but not KD CCRK induced a robust physical interaction between p-STAT3 Tyr705 and AR in LO2 and SK-Hep1 cells (Fig. 3h and Supplementary Fig. 3g). Furthermore, pairwise sequential ChIP assays showed that CCRK--induced co-localization of STAT3 and AR interaction in the ARE of its own promoter in both cell lines (Fig. 3i and Supplementary Fig. 3h). Taken together, these findings demonstrate that CCRK induces STAT3-AR physical interaction for transcriptional activation via promoter co-occupancy, thus forming an inflammatory-CCRK circuitry (Fig. 3j).
**Fig. 3** IL-6 activates STAT3 and AR signaling to stimulate CCRK expression. **a** Western blot and **b** qRT-PCR analysis of HepG2 and Huh7 cells treated with or without IL-6, combined with siRNA-mediated knockdown of STAT3 or AR. **c** Luciferase reporter assay was used to measure CCRK promoter activity in HepG2 and Huh7 cells expressing either WT or AR response element (ARE)-deleted mutant CCRK promoter, treated with or without IL-6, combined with siRNA-mediated knockdown of STAT3 or AR. **d** CCRK expression was blocked by knockdown of STAT3, but rescued by over-expression of AR. **e** STAT3 phosphorylation suppressed by AR knockdown could be rescued by over-expression of CCRK. **f** WT but not kinase-defective (KD) CCRK-induced STAT3 phosphorylation and AR expression. **g** CCRK induces its own promoter activity, which was abolished by deletion of ARE or knockdown of either STAT3 or AR in CCRK-expressing cells. **h** Co-immunoprecipitation of p-STAT3 Tyr705 and AR in L02 cells transfected with WT CCRK, but not in those transfected with empty vector or KD CCRK. IgG is a control for non-specific immunoprecipitation. **i** ChIP-re-ChIP assay demonstrated an increased co-occupancy of STAT3 and AR at the ARE of CCRK promoter in L02 cells transfected with WT CCRK relative to those transfected with vector only. IgG is a control for non-specific immunoprecipitation. The antibodies used in the 1st and 2nd ChIP are as indicated. **j** Schematic representation of the IL-6/STAT3/AR/CCRK circuitry in hepatocarcinogenesis. Data are presented as mean ± SD. *p < 0.05 and **p < 0.01 as calculated by one-way ANOVA followed by Bonferroni post hoc test (i).
expression of CCRK contributes to the pathophysiology of NAFLD via mTORC1 signaling. To test this, we first constructed a CCRK-inducible LO2 liver cell model (LO2-CCRK) in which doxycycline treatment readily induced CCRK, leading to increased p-GSK3βSer9, p-mTORSer2448, p-4E-BP1Thr37/46, p-S6KThr389, and mSREBP1 (Fig. 5a). Exposure of doxycycline-induced LO2-CCRK cells with fatty acid mixture of oleic and palmitic acids (2:1 ratio), which mimics the liver steatosis of NAFLD patients, significantly increased hepatocellular lipid accumulation compared to non-induced cells ($p < 0.001$; Fig. 5b, c). Notably, inhibition of the mTORC1 signaling by shRNA-mediated knockdown of the positive regulatory subunit Raptor (shRaptor) or, rapamycin treatment (Fig. 5a), abolished the lipid over-accumulation in CCRK-expressing liver cells ($p < 0.001$; Fig. 5b, c). In parallel, doxycycline-induced LO2-CCRK cells also exhibited significantly higher glucose uptake ($p < 0.001$; Fig. 5d), which was completely abolished by mTORC1 inhibition ($p < 0.001$; Fig. 5d).

To determine whether CCRK promotes hepatic insulin resistance, we utilized CCRK KO Huh7 cells, which exhibited reduced GSK3β/mTORC1 signaling compared to the WT cells (Fig. 5e). We treated both WT and CCRK KO Huh7 cells with high dose of insulin for 24 h, followed by assessment of insulin sensitivity by phosphorylation of Akt at Ser473 (p-Akt Ser473) stimulation 7,30,39. High insulin exposure reduced subsequent insulin-stimulated p-AktSer473 in Huh7 WT cells (Fig. 5f, lane 4 vs. lane 2), which could be restored in CCRK KO cells (Fig. 5f, lane 6 vs. lane 4). To investigate whether mTORC1 signaling mediates CCRK-promoted insulin resistance, we treated LO2-CCRK cells in hyperinsulinemia condition and found that induction of CCRK further impaired insulin sensitivity (Fig. 5g, lane 6 vs. lane 4). Notably, both genetic and pharmacological inhibition of
mTORC1 signaling rescued insulin sensitivity in CCRK-expressing cells (Fig. 5g, lane 7 and 8 vs. lane 6). Collectively, our findings suggest that hepatic CCRK fosters lipid accumulation, glucose uptake and insulin resistance through mTORC1 signaling.

**CCRK promotes mTORC1-dependent HCC tumorigenicity.** Next, we investigated whether mTORC1 signaling is required for CCRK-induced tumorigenicity by xenograft experiments. In contrast to non-induced LO2-CCRK cells, doxycycline-induced CCRK-expressing cells formed significantly larger and heavier...
tumors (p < 0.01; Fig. 5h, i), whereas inhibition of mTORC1-signaling by either Raptor knockdown or Rapamycin treatment abrogated the induced tumorigenicity in CCRK-expressing cells (p < 0.01; Fig. 5h, i). Consistently, tumor nodules from doxycycline-induced LO2-CCRK cells showed significantly more proliferating cells when compared with non-induced, Raptor-ablated and Rapamycin-treated groups as assessed by Ki-67 staining (p < 0.05; Fig. 5j). Western blot analysis on the xenograft tumor tissues further confirmed that the reduced tumorigenicity was associated with the inactivation of GSK3β/mTORC1 signaling and its downstream molecules, and the cellular proliferation marker PCNA (Fig. 5k). Taken together, we demonstrate that CCRK promotes hepatic metabolic dysregulation and tumorigenicity in an mTORC1-dependent manner.

**Hepatic CCRK-mTORC1 signaling recruits MDSCs.** Since the liver environment plays an important role in tumor initiation and growth, we next employed an inducible and liver-specific CCRK transgenic (TG) mouse model to investigate the pro-tumorigenic effect of CCRK via the hepatic immune microenvironment. At day 10 post-tamoxifen stimulation, CCRK expression was induced in the liver of pTYL-LSL-CCRK/+; Rosa26CreER2/+; TG compared to Rosa26CreER2/+ control mice (Fig. 6a). Consistent with the in vitro findings, the hepatic p-GSK3βSer and p-mTORSer2448 levels as well as the downstream p-4E-BPThr37/46, p-S6KThr389, and mSREBP1 expressions were up-regulated (Fig. 6a), which were confirmed using TG mouse-derived primary hepatocytes (Fig. 6b). In accordance with the mTORC1/mSREBP cascade activation, CCRK TG mice developed extensive liver steatosis as compared to the control mice when fed with HFHC diet (p < 0.05; Supplementary Fig. 4a–b).

We then assessed the tumor growth in control and CCRK TG mice using an orthotopic model via intrahepatic injection of syngeneic Hepa1–6 HCC cells (Fig. 6c). To exclude the influence of hepatoma-intrinsic CCRK on tumor growth and immunoregulatory function, we generated Ccrk KO Hepa1–6 cells via CRISPR/Cas9-mediated depletion (Fig. 6c). Notably, the hepatic tumorigenicity of Ccrk KO Hepa1–6 cells was significantly enhanced in CCRK TG compared to control mice (p < 0.01; Fig. 6d). Consistent with the oncogenic role of CCRK signaling via MDSC-mediated immunosuppression (Supplementary Fig. 5a), we found that CCRK TG mice exhibited significantly higher liver-infiltrating level of CD11b+Ly6CintLy6G+ polymorphonuclear MDSCs (PMN-MDSCs; p < 0.05; Fig. 6e), but not CD11b+Ly6C+Ly6G– monocyteic MDSCs (M-MDSCs; Supplementary Fig. 5b). We next investigated whether the activated mTORC1 signaling promotes the enhanced tumorigenicity in CCRK TG mice. Lentiviral shRNA-mediated down-regulation of Raptor (shRaptor) in the livers of TG mice (Supplementary Fig. 5c) abolished tumor growth (p < 0.05; Fig. 6d), which was associated with significant reduction in PMN-MDSCs (p < 0.05; Fig. 6e). Moreover, the level of PMN-MDSCs, but not M-MDSCs, in the liver positively correlated with tumor weight in the orthotopic HCC model (p < 0.05; Fig. 6f and Supplementary Fig. 5b). To determine its functional significance, we depleted PMN-MDSCs by intraperitoneal injection of anti-Ly6G antibody after tumor cell implantation in CCRK TG mice (Supplementary Fig. 5d). Administration of anti-Ly6G antibody significantly suppressed CCRK-induced PMN-MDSC accumulation in liver (p < 0.01), leading to a borderline significant reduction in HCC tumorigenicity (p = 0.0555; Supplementary Fig. 5e, f).

We further validated these findings in the dietary obesity-HCC model (Fig. 2a). Consistently, the level of PMN-MDSCs, but not M-MDSCs, was also significantly increased in HFHC-fed mice (p < 0.05) but normalized to basal level by Ccrk knockdown (p < 0.05; Fig. 6g and Supplementary Fig. 5g). Overall, the level of PMN-MDSCs but not M-MDSCs significantly correlated with tumor multiplicity in the dietary obesity-HCC model (p < 0.0001; Fig. 6h and Supplementary Fig. 5g). To examine how hepatic CCRK expression induces PMN-MDSCs, we performed cytokine profiling and found a significant induction of granulocyte-colony stimulating factor (G-csf) in the peri-tumoral liver tissues of CCRK TG mice (p < 0.05; Supplementary Fig. 5h). Consistently, the hepatic G-csf expression was significantly increased in HFHC-fed compared to CD-fed mice (p < 0.05), which was abrogated by Ccrk knockdown (p < 0.01; Fig. 6i). Using both ectopic expression and KO cell models, we further demonstrated that CCRK positively regulated G-CSF expression, which could be abolished by Raptor knockdown (Fig. 6j, k). Taken together, these results suggest that hepatic CCRK signaling recruits pro-tumorigenic PMN-MDSCs to liver microenvironment through mTORC1-dependent G-CSF expression.

**Co-activation of CCRK and mTORC1 in human NASH-HCCs.** To investigate the clinical relevance of our findings, the protein levels of CCRK, p-GSK3βSer, GSK3β, p-mTORSer2448, mTOR, p-4E-BPThr37/46, 4E-BP1, p-S6KThr389, S6K, mMREB1, G-CSF, p-STAT3 Tyr705, STAT3, and AR were examined by Western blot (Fig. 7a) in 23 pairs of human NASH-associated HCCs with neither viral hepatitis nor alcoholic liver disease (Supplementary Table 1). Compared with the paired non-tumor liver tissues, significant up-regulation of CCRK, p-GSK3βSer/GSK3β, p-mTORSer2448/mTOR, p-4E-BPThr37/46/4E-BP1, p-S6KThr389/S6K, mMREB1, G-CSF, p-STAT3 Tyr705/STAT3, and AR were detected in HCC tissues (p < 0.05; Fig. 7b). Quantitative RT-PCR also demonstrated significant elevation of the mRNA levels of IL-6, CCRK, and human MDSC markers CD33 and OLR1 in the clinical specimens (p < 0.05; Supplementary Fig. 6a).

Association analysis further showed positive correlations between IL-6 and CCRK (r = 0.5403), CCRK and CD33 (r = 0.4473), CCRK and OLR1 (r = 0.4684) (p < 0.05, Supplementary Fig. 6b), p-STAT3 Tyr705/STAT3 and CCRK (r = 0.3691), and AR and CCRK (r = 0.6507), CCRK and p-GSK3βSer/GSK3β (r = 0.3066),
Fig. 6 Hepatic CCRK-mTORC1 signaling recruits MDSCs to form a tumor-prone liver microenvironment. a mTORC1 signaling was activated in the livers of CCRK TG mice. The downstream molecules of mTORC1 signaling were detected by Western blot. b mTORC1 signaling components were activated in the primary hepatocytes isolated from CCRK TG mice. c Schematic diagram of CCRK TG mouse model with intrahepatic injection of Ccrk KO Hepa1–6 cells by CRISPR/Cas9, and lentiviral-mediated Raptor knockdown. d CCRK TG mice (n = 8) developed significantly larger tumors compared to controls (n = 11), which was abolished by down-regulation of Raptor (shRaptor; n = 5) (scale bar = 1 cm). e PMN-MDSCs were induced in CCRK TG mice, but were reduced after Raptor knockdown, and f PMN-MDSC levels positively correlated with tumor weight. g PMN-MDSCs were induced by HFHC diet, but were reduced by Ccrk knockdown in NASH-HCC model (CD + shCtrl, n = 8; HFHC + shCtrl, n = 15; HFHC + shCcrk, n = 15), wherein h PMN-MDSC levels positively correlated with tumor multiplicity. i G-csf mRNA levels were induced by HFHC diet, but were reduced by Ccrk knockdown in NASH-HCC model. j G-CSF mRNA level was augmented by CCRK over-expression in LO2 cells, which could be attenuated by Raptor knockdown. k G-CSF mRNA expression was down-regulated in CCRK KO Huh7 cells. Data are presented as mean ± SD. *p < 0.05 and **p < 0.01 as calculated by one-way ANOVA followed by Bonferroni post hoc test (d, e, g, j), Pearson correlation (f, h), and unpaired two-tailed Student’s t-test (k).
Fig. 7 CCRK over-expression correlates with mTOR signaling activation in patients with NASH-associated HCCs. a Western blots showed the induced expressions of CCRK/mTOR signaling proteins in HCC tissues relative to matched non-tumor tissues (23 pairs), and representative results from three patients are shown. b The Western blot results of all patients from a were quantified and shown in dot plots, wherein the fold enrichments of target proteins relative to their corresponding controls are shown. c The positive correlations of STAT3/AR/CCRK/GSK3β/mTORC1 signaling components were confirmed in the patient specimens. d Schematic diagram showing the IL-6-triggered self-reinforcing circuitry of STAT3/AR/CCRK (highlighted in the yellow panel), which activates mTORC1 signaling (highlighted in the green panel) through GSK3β/TSC2 to promote NASH and HCC development. Data are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001 as calculated by unpaired two-tailed Student’s t-test (b), and Pearson correlation (c).
p-GSK3βSer9/GSK3β and p-mTORSer2448/mTOR (r = 0.3877), p-mTORSer2448/mTOR and p-4E-BP1Thr37/46/4E-BP1 (r = 0.3409), p-mTORSer2448/mTOR and p-S6KThr389/S6K (r = 0.3853), p-mTORSer2448/mTOR and mSREBP1 (r = 0.3218), and p-mTORSer2448/mTOR and G-CSF (r = 0.4335) at protein levels (p < 0.05; Fig. 7c). Taken together, our findings in clinical HCC specimens consolidate our model of IL-6-triggered self-reinforcing STAT3/AR/CCRK circuitry, thereby activating mTORC1 signaling cascades to promote NASH-associated HCC development (Fig. 7d).

**Discussion**

The rising prevalence of metabolic risk factors, especially obesity and diabetes, among HCC patients underscores the urgent need for novel therapeutic strategies. Strikingly, the risk of obesity-associated HCC is even higher in males. Here we show that hepatic CCRK, cooperatively induced by the pro-inflammatory IL-6/STAT3 and AR signaling promotes HCC development by reprogramming lipid metabolism and immune microenvironment. Mechanistically, STAT3-AR co-binding stimulated by CCRK transcriptionally activates its own promoter, which in turn triggers the mTORC1/4E-BP1/S6K/mSREBP1 cascade via GSK3β phosphorylation to augment hepatic lipid accumulation, glucose intolerance, insulin resistance, and tumorogenicity. Moreover, over-expression of CCRK reshapes the liver microenvironment by up-regulating mTORC1-dependent G-CSF expression to recruit pro-tumorigenic PMN-MDSCs, resulting in immune evasion. As we also showed concordant STAT3-AR-CCRK-mTORC1 signaling activation in patients with NASH-associated HCC, our findings unveil a central role of CCRK as a molecular hub of metabolic and immunosuppressive cascades, and offer a novel therapeutic target for HCC intervention in the era of obesity epidemic.

Mounting evidence suggests that obesity is associated with chronic low-grade systemic inflammation leading to metabolic syndrome and insulin resistance. The pro-inflammatory IL-6 levels are higher in mice and human NASH patients, and a positive correlation between hepatic IL-6 expression and disease severity was evident in patients with NASH. Naugler et al. have previously shown that estrogen effectively suppressed IL-6 expression in female mice via MYD88/NF-kB signaling, and thereby reduced liver damages and HCC multiplicity. In this murine HCC model, the IL-6 response was transient that occurred only during the first 48h after DEN stimulation, whereas hepatocarcinogenesis was a long process that took almost 8 months. These results implied that a sustainable oncogenic signal is required but not clearly defined. In this study, we addressed this enigma by showing in the male NASH context (Figs. 1 and 2) that the IL-6 can induce a self-reinforcing STAT3/AR/CCRK oncogenic circuitry, in which CCRK facilitates the physical interaction of phosphorylated STAT3 and AR and their co-occupancy at CCRK promoter for sustained transcriptional activation (Fig. 3). As we also observed CCRK up-regulation in male HFFH-fed mice (Supplementary Fig. 1a) and NASH patients (Supplementary Fig. 6c), the IL-6/STAT3 and androgen/AR crossstalk in CCRK activation may explain why obese men are at greater risk of developing HCC.

The diverse functional effects of mTOR pathways in liver metabolism and hepatocarcinogenesis are well-established, however, the upstream drivers have not been fully defined. We found that in both NASH and HCC murine models, mTOR signaling was consistently activated and driven by CCRK (Fig. 4). Mechanistically, CCRK phospho-inactivates GSK3β, thereby subverts the mTOR inhibitor TSC2 to up-regulate p-4E-BP1, p-S6K, and mSREBP1 levels. As a negative regulator of a key rate-limiting initiation factor eIF4E, 4E-BP1 phosphorylation by mTORC1 leads to the dissociation of 4E-BP1 from eIF4E, allowing the formation of cap-dependent translation initiation complex at 5' end of mRNAs. Once phosphorylated and activated by mTORC1, p-S6K1/2 is another major effector that regulates RPS6, as well as other regulators of translation initiation. Notably, both of these critical pathways for protein synthesis have been shown to play indispensable roles for mTORC1-dependent hepatocarcinogenesis. Additionally, increased de novo lipogenesis via the mTORC1/S6K/mSREBP1 axis has pathogenic and prognostic significance in HCC. A recent study has further shown that either genetic or pharmacological inhibition of the SREBP pathway dramatically reduced HCC progression. Importantly, we demonstrated that in vivo ablation of Ccrk simultaneously circumvents these metabolic and lipogenic mTORC1-dependent cascades to diminish hepatic lipid accumulation, glucose intolerance, insulin resistance, and HCC tumorigenicity.

Both cellular and non-cellular components of the tumor microenvironment determine tumor development and progression, as well as anti-tumor immunity and response to cancer therapy. Interestingly, in addition to the tumor-intrinsic roles, CCRK also exerts tumor-extrinsic functions in the liver microenvironment. Using a CCRK TG model, we showed that CCRK effectively induced mTORC1-dependent G-csf expression to recruit PMN-MDSCs to the liver, leading to enhanced tumorigenicity via immune escape (Fig. 6). In the dietary obesity HCC model, we further found that the PMN-MDSC level (Fig. 6g) and hepatic p-p65Ser536 expression (Supplementary Fig. 7) were positively regulated by CCRK. As PMN-MDSCs were also increased in male CCRK TG mice under HFFH diet (Supplementary Fig. 4c), our data suggest that the obesity-triggered AR/CCRK signaling may establish an immunosuppressive barrier through induction of MDSC expansion and recruitment via NF-kB/IL-6 and mTORC1/G-CSF pathways, respectively. Ma et al. have recently depicted the role of lipid dysregulation on adaptive immune responses, especially the selective loss of intrahepatic CD4+ T lymphocytes in NAFLD-promoted HCC. Thus, whether and how CCRK-driven signaling network modulates and impairs T cell-mediated tumor immune surveillance warrant further investigation.

Our findings of both intrinsic effects on hepatocytes and hepatocyte-extrinsic functions in the liver microenvironment highlight the therapeutic potential of targeting CCRK in HCC. Pharmacological inhibition of the aberrantly activated mTOR in HCC has been proposed, but the in vivo effects have not been clearly elucidated. Rapamycin was even shown to produce unwanted adverse effects in HCC pre-clinical models. As an upstream driver of mTOR pathway, Ccrk inhibition is sufficient to reverse the metabolic and oncogenic phenotypes in multiple NASH and HCC models. Moreover, a recent study showed that co-blockade of CCRK improved immune-checkpoint therapy for large hepatoma eradication. There are currently a number of pan-CDK inhibitors that display binding to CCRK, but CCRK-specific inhibitor has yet been developed. Given the functional and clinical significance of CCRK in both viral and NASH-related HCCs, the development of CCRK-targeted agents and characterizations of their effects in combination immunotherapy will ultimately lead to more effective treatment for patients with HCC.
Methods

Patient specimens. Paired tumor/non-tumor tissues from 23 patients with NASH-associated HCCs who underwent hepatectomy (Supplementary Table 1), and liver biopsy tissues from 23 NAFD patients (Supplementary Table 2), were collected at the Prince of Wales Hospital (Hong Kong) for analyses. All HCC patients reported a history of metabolic syndrome involving diabetes, hypertension, dyslipidemia, and hepatic steatosis. HCC patients with chronic hepatitis B and C, or record of excess alcohol intake, were excluded from the study. The research protocol was obtained from all human subjects, and the study was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee. Liver tissues were granted by the NASH Clinical Research Network scoring protocol.

Cell culture and transfection. HepG2 (ATCC, HB-8065), Huh7 (ICRR, 0403), LO2 (Celsusaurus, CVCL_6926), and SK-HeP1 (ATCC, HTB-52) human cell lines and Hepal-6 (ATCC, CRL-1830) mouse cell line were cultured in DMEM supplemented with 10% FBS ( Gibco). Cell transfection was conducted using FuGENE® HD Transfection Reagent (Promega) or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Plasmid, RNA interference, and transfection. For plasmid transfection, concomitantly active and Y705F-DN-STAT3 (STAT3C and DN-STAT3) were a gift from Linzhao Cheng (Addgene plasmid #24983, #24984). CCRK-expressing plasmid, RNAi, and transfection were performed as previously described58. CCRK-expressing vector was provided by Dr. Marie Lin (Chinese University of Hong Kong). A (kinase-defective) KD mutant of CCRK in which the T-loop threonine-161 was substituted with alanine was constructed as previously described59. A dominant-negative phosphorylation-defective GSK-3β mutant in which the serine-9 was replaced with alanine (GSK-3β(S9A)) was a gift from Brendan Manning (Addgene plasmid #11428). Raptor siRNA (shRaptor) was a gift from David Sabatin (Addgene plasmid #18560). The non-silencing shRNA mimics control (shCtrl), RH545346 (Invitrogen). shRNA vectors targeting mouse Ccrk (shCcrk) was constructed as previously described58. Cell transfection was conducted using FuGENE® HD Transfection Reagent (Promega) according to the manufacturer’s instructions. Briefly, plasmids were mixed with transfection reagent at the 1:2 µg (plasmids): 3–6 µl (reagent) ratio for 15 min at room temperature and then evenly dropped onto the cells. The transfected cells were cultured in the incubator until needed.

Sense and anti-sense strands of siRNA oligonucleotides were synthesized and purified by Techdragon Company. The lipoxyphilized powder of siRNAs was dissolved in DNease/RNase-free water and aliquoted for storage at ~80 °C. siRNAs specifically targeting human CCRK, AR, STA3, and TSC2 were designed. A scrambled siRNA (scsiR) was synthesized as a negative control, which does not target any human gene mRNA. Cells were transfected with siRNAs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. In brief, at 24 h before transfection, cells were seeded (in antibiotic-free media) at 70–80% confluence. siRNAs and Lipofectamine 2000 was individually incubated with serum-free MEM medium ( Gibco) for 5 min at room temperature, then they were mixed together at room temperature for 20 min. The mixtures were then pipetted onto the cells evenly, and the cells were cultured in the incubator until indicated time point.

Construction of inducible and stable cell lines. Generation of the doxycycline-inducible stable cell line was performed as described81. Bref, CCRK cDNA was amplified by PCR and used to replace the PAF gene of the vector PB-T-PAF to form the new construct PB-T-CCRK. LO2 cells were seeded and grown in 175 flasks overnight to reach 60–70% confluence. On the following day, cells were co-transfected with pCycL43, PB-RN together with or without PB-T-CCRK using Lipofectamine 2000 following the manufacturer’s instructions. Two days after transfection, cells were subjected to dual drug selection, i.e. 1 µg/ml puromycin (Gibco) and 800 µg/ml G418 (Roche), until drug-resistant colonies formed and stabilized.

A stable expressing shRaptor and shControl (shCtrl) stable cell lines, doxycycline-inducible CCRK-expressing LO2 cells (LO2-CCR) were seeded and grown in six-well plates overnight to reach 50–70% cell confluence. Cells were transfected with shRaptor or shCtrl plasmid using FuGENE® HD Transfection Reagent and cultured for 48 h. After that, transfected cells were detached from the plates with 0.25% trypsin EDTA, and transferred to a 100-mm tissue culture plate and maintained in 400 µg/ml hygromycin (Life Technologies Corporation) for 4 weeks until resistant colonies formed. Then, the stable cells (shRaptor or shCtrl-LO2) were isolated and transferred into new dishes and cultured in antibiotics-containing medium for further experimental use.

CCRKO Huh7 cell line was constructed as previously described82. Briefly, human CCRK-targeted sgRNA was designed online (http://crispr.cos.uni-heidelberg.de) and cloned into the vector lentiCRISPR v2 (Addgene Plasmid #52961). This construct was confirmed by sequencing. Next, Huh7 cells were transfected with this construct or control vector. Two days after transfection, puromycin (1 µg/ml) was added to select transfected cells; 2 weeks later, individual cells were plated in 96-well plates and cultured for 4 weeks. CCRK KO Huh7 cells were confirmed by sequencing and Western blot.

Quantitative reverse transcription PCR (qRT-PCR). Total RNAs were extracted using TRIzol Reagent (Invitrogen). In each reaction 500 ng of total RNA was used. cDNA were synthesized using cDNA synthesis kit (Takara), and then cDNA were used to perform qRT-PCR. Primers were designed using Primer3 software. qRT-PCR was performed in triplicate and GAPDH was used as an internal control. The primers are listed in Supplementary Table 3.

Western blot. The primary antibodies for western blotting used in this study are CCRK (74185, Abcam, 1:1000), pSTAT3 (9131, Cell Signaling Technology, 1:1000), STAT3 (D322G, 1:1000, Cell Signaling Technology, 1:1000), p-AR (53C13, 2, sc-52458, Santa Cruz Biotechnology, 1:1000), AR (6C1, 1:2000, EMD millipore, 1:1000). The anti-β-actin (C49F9, 1:1000, Roche) was used as an internal control. The protein interaction was then detected by western blot using indicated antibodies.

Chromatin immunoprecipitation (ChIP) assay. Chromatin immunoprecipitation assay was performed as previously described83. At 48 h post-transfection of CCRK and control plasmids, cells grown at the confluency of 80% in 150-mm dishes were cross-linked with 1% formaldehyde for 10 min at room temperature on the shaker, followed by adding 1 mg glycine to a final concentration of 125 mM for 2 min to quench formaldehyde. Next, cells were washed twice using PBS at 4 °C and harvested. A total of 1 × 108 cells were collected by centrifugation at 700× g, 5 min, 4 °C, and then the cells were resuspended with 3.6 ml lysis buffer III (10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1% SDS, 0.5% Na-deoxycholate) and T-PER (10 mg/ml Protease Inhibitor cocktails) and rotated at 4 °C for 6 h at 4 °C. The complexes were then washed with Co-IP lysis buffer four times for 10 min each at 4 °C and collected by centrifuging at 2500 rpm, 5 min, 4 °C. Proteins were detached from beads by denaturing with 5 × protein loading buffer at 100 °C for 10 min. Protein interaction was then detected by western blot using antibodies.

Co-immunoprecipitation. Cells were collected and lysed in 1 ml co-IP lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, and protease inhibitor cocktail) on ice. The lysates were centrifuged at 12,000g. 15 min, 4 °C and immunoprecipitated with IgG, anti-pSTAT3 (52706) or anti-AR antibodies at 4 °C overnight. On the following day, 20 µl of protein A/G beads (Santa Cruz) were added into the protein–antibody complex and incubated for 1 h at 4 °C, then the complexes were washed with Co-IP lysis buffer four times for 10 min each at 4 °C and collected by centrifuging at 2500 rpm, 5 min, 4 °C. Proteins were detached from beads by denaturing with 5 × protein loading buffer at 100 °C for 10 min. Protein interaction was then detected by western blot using antibodies.

Chromatin immunoprecipitation (ChIP)-re-ChIP assay. ChIP-re-ChIP assay was performed as previously described84. At 48 h post-transfection of the CCRK and control plasmids, cells grown at the confluency of 80% in 150-mm dishes were cross-linked with 1% formaldehyde for 10 min at room temperature on the shaker, followed by adding 1 mg glycine to a final concentration of 125 mM for 2 min to quench formaldehyde. Next, cells were washed twice using PBS at 4 °C and harvested. A total of 1 × 108 cells were collected by centrifuging at 700× g, 5 min, 4 °C and lysed with 10 mM cold lysis buffer I (50 mM HEPES–KOH, pH 8.0, 1 mM EDTA, 140 mM NaCl, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, supplemented with protease inhibitors), and rotated at 4 °C for 10 min. Then, the cell debris was harvested through centrifugation at 1350×g, 5 min, 4 °C and then the supernatant were eluted and supplemented with protein–antibody complexes and incubated for 1 h at 4 °C. The complexes were then washed with Co-IP lysis buffer four times for 10 min each at 4 °C and collected by centrifuging at 2500 rpm, 5 min, 4 °C. Proteins were detached from beads by denaturing with 5 × protein loading buffer at 100 °C for 10 min. Protein interaction was then detected by western blot using antibodies.
(Millipore), STAT3 antibody (Cell signaling), or IgG antibodies (Santa Cruz) were used as controls. SYBR Green-based detection (Applied Biosystems).

Luciferase reporter assay. The WT and androgen-responsive element (ARE)-deleted CCRK promoter luciferase reporters were constructed as previously described38. For IL-6-induced CCRK transcription, cells were transiently transfected with WT or ARE-deleted CCRK promoter constructs and Renilla luciferase reporters. After 24 h, the cells were further treated with human recombinant IL-6 for 3 h. The CCRK self-reinforced transcriptional activity was co-transfected with CCRK construct, WT or ARE-deleted CCRK promoter constructs and Renilla luciferase reporters for 48 h. The treated cells were harvested using passive lysis buffer for 15 min and assayed by the Dual Luciferase Reporter Assay System (Promega) using GloMax microplate luminometer (Promega). The experiments were replicated three times in two independent experiments.

Lipid accumulation assay. A free fatty acid (FFA) mixture was prepared at 2:1 ratio of oleic acid (OA) to palmitic acid (PA) that mimics benign chronic steatosis with low toxicity described previously39. Briefly, 100 mM PA (Sigma) and 100 mM OA (Sigma) stocks were prepared in absolute ethanol at 70 °C and filter-sterilized. Five percent of the OA solution was mixed with an equal volume of FBS and filter-sterilized. A 5 mM stock solution for each fatty acid was prepared in 5% BSA solution in distilled water at 37 °C then the mixture was stored at 4 °C for further experiments. Nile Red (Sigma) is a dark purplish-red powder, the stock solution was prepared in DMSO at 1 mg/ml and then diluted 1:500 in PBS for further experiments. Nile red staining was performed with 1 ml (for each well) of 3.7% formaldehyde/PBS for 15 min at room temperature. After this, cells were washed with PBS twice and stained with Nile-red solution for 30 min (samples were covered with foil to avoid bleaching by room light). Nuclei were counterstained by Hoechst (H33342, Calbiochem). The positive cells were calculated at random at ×400 under the fluorescence microscope and more than 1000 nuclei were scored.

Insulin resistance assay. Cells were plated in six-well plates and grown overnight. On the following day, cells were treated by standard medium (DMEM supplemented with 10% FBS containing doxycycline or control solution with or without 10 mM rapamycin) for 36 h and starved for 12 h with FBS-free standard medium. The starved cells were co-cultured with 200 µM FFA for 24 h to induce steatosis, and the BSA solution was used as control. Cells grown on coverslips were washed with PBS twice and fixed with 1 ml (for each well) of 3.7% formaldehyde/PBS for 15 min at room temperature. After this, cells were washed with PBS twice and stained with Nile red solution for 30 min (samples were covered with foil to avoid bleaching by room light). Nuclei were counterstained by Hoechst (H33342, Calbiochem). The positive cells were calculated at random at ×400 under the fluorescence microscope and more than 1000 cells were assessed. The ratio of positive cells in every groups was used for statistical analysis. The experiments were replicated three times in three independent experiments.

Generation of conditional activation of CCRK TG mouse. To overexpress CCRK specifically in adult mouse liver, a liver-specific transferrin promoter (ptT) together with transcription stopping cassette flanked by loxP sites (LSL) were used to control the expression of human CCRK. The 0.6 kb mouse transferrin promoter from C57BL/6J genome DNA was amplified and subcloned in front of the 1.5 kb human CCRK cDNA in pCDNA3.1. The LSL stopping cassette was amplified from the genomic DNA of Rosa26Laiz mouse, and was inserted between the 0.63 kb transferrin promoter and the human CCRK cDNA. The linearized DNA carrying ptT-LSL-CCRK was released by PmlI, digested into 1 ng/µl by TE buffer (10 mM Tris pH 7.5, 0.1 mM EDTA), before being subjected to Pronuclear injection. The expression of CCRK in Tg(ptT-LSL-CCRK) was blocked by transcription stopping cassette stopping removal. Removal of the stop cassette by tamoxifen-induced Cre recombinase will turn on the expression of CCRK in a time-specific manner in mouse liver. To achieve this, Rosa26-CreERT2 flanked by loxP (Tg(ptT-LSL-CCRK)) was co-crossed with Tg(Rosa26-CreERT2/FRT/+) to establish the mouse line Rosa26-CreERT2/FRT/+. Primary hepatocytes were isolated from control and CCRK TG mouse as previously described. Adenovirus coding with Cre was used to infect primary hepatocytes for 4 h and then washout. Cells were collected 48, 96, and 144 h post infection for further analysis.

NASH and NASH-HCC mouse model. The obesity-promoted NASH and HCC models were constructed as described previously30.39. For the dietary obesity-NASH model, 6-week-old C57BL/6 male mice were randomly assigned to receive regular CD or high fat high carbohydrate diet (HFHC, Surwit diet) and drinking water enriched with high-fructose corn syrup for 22 weeks. For the dietary obesity-HCC model, 6-week-old male mice were injected with DEN (25 mg/kg; Sigma-Aldrich) at 14 days of age. After 4 weeks, mice were separated into two dietary groups and fed with CD or HFHC and drinking water enriched with high-fructose corn syrup for 22 weeks. Lentiviruses encoding shRNA against Ccrk (shCcrk) or control sequence (shCtrl) were packaged according to the manufacturer’s instructions (Dharmacon) for lentiviral infection in the dietary obesity model. At the age of 18 weeks, 5 × 10^6 transducing units of lentiviruses in 100 ml PBS were administered via tail vein injection as previously described. Intraperitoneal glucose or insulin tolerance test (IPGTT/IPITT) was performed at the age of 26 weeks. All mice were sacrificed at the age of 32 weeks. All animal experimental ethics approvals had been obtained from the Chinese University of Hong Kong (CUHK) Animal Experimentation Ethics Committee.

Intraperitoneal glucose and insulin tolerance tests. For intraperitoneal glucose tolerance test (IPGTT), 26-week-old mice were transferred to new clean cages and fasted overnight for ~16 h. On the following day, 1.5 g glucose per kg body weight was treated intraperitoneally. For intraperitoneal insulin tolerance test (IPITT), 26-week-old mice were transferred to new clean cages and fasted for 6 h. 75 U insulin per kg body weight was treated intraperitoneally. Tail blood was then collected under normal condition at indicated time-points. Glucose levels were measured using one touch blood glucose strips (Johnson and Johnson). Metabolic profiling. Blood was collected from heart or tail vein, and kept at room temperature for not more than 4 h. The blood was centrifuged at 3000 rpm, 15 min, room temperature, and the serum was transferred to new tubes and stored at −20 °C until use. The concentration of serum insulin, triglyceride, and non-essential fatty acid (NEFA) were measured using respective ELISA kits (LabAssay) according to the manufacturer’s instructions.

Xenograft mouse models. For the xenograft mouse model, 6-week-old female athymic nude mice were injected with 5 × 10^3 LO2-CCRK-shRaptor and LO2-CCRK-shCtrl lentiviruses (Promega) using GloMax microplate luminometer (Promega). The experiments were replicated three times in two independent experiments.

CD and hepatic steatosis were evaluated histologically in paraffinembedded liver tissues were prepared with a microtome (Leica). Then, the sections were deparaffinized, rehydrated, and washed in distilled water. Hematoxylin and eosin (H&E) were used to stain the liver sections. The sections were then blocked using blocking buffer for 30 min at room temperature and stained with polyclonal antibody against Ki-67 (1:100, Labvision) at 4 °C for 16 h. The universal HRP Multimer Ultraview Kit on Benchmark XL (Ventana Medical System) was used for chromogen development. For the nuclei staining, the sections were stained with the alu haematoxylin for 2 min, differentiated with 0.3% acid alcohol for several seconds and blue-up with Scott’s tap water substitute for 1 min. The Ki-67 indexes were assessed by counting the percentages of nucleus-stained (brown) cells/loci per total number of cells. At least 10 fields (magnification ×400) were counted per each liver section and the average indexes were calculated.

Crispr/Cas9-mediated genome editing. Ccrk-depleted Hepa1-6 cells were generated by transfection with specific sgRNAs to Ccrk (sgRNA1, CGGCCTCCCGAGATGCG; sgRNA2, TGCCGTGGAGAAGATGCC; sgRNA3, AGCCAGAAGGCCGGACATG) and selected by G48. Single clone was sorted by FACSArial Fusion (BD Biosciences) as previously described. Ccrk depletion was confirmed by sequencing and Western blot analysis.

Orthotopic HCC mouse model and MDSC expansion analysis. Six-week-old CCRK transgenic mice were injected with tamoxifen (100 mg/kg body weight) daily for 3 consecutive days. At 30-day post tamoxifen injection, 5 × 10^5 Ccrk-depleted Hepa1-6 cells were intrahepatically injected into the liver of male Ccrk TG mice. Mice were sacrificed at one month post-tumor injection. Blood, tumor, and matched non-tumor liver tissues were harvested. Single cell was isolated from
At 2-week post tamoxifen injection, 5 × 10^7 transducing units of shRaptor lentivirus were injected with tamoxifen (100 mg/kg body weight) daily for 3 consecutive days. Raptor proliferating cells were determined and calculated by FlowJo software (Tree Star). FACS Fortessa/FACSAria Fusion (BD Biosciences). The percentages of proliferating cells were determined and calculated by FlowJo software (Tree Star).

**Raptor knockdown in CCRK TG mouse model.** Six-week-old male CCRK TG mice were injected with tamoxifen (100 mg/kg body weight) daily for 3 consecutive days. At 2-week post tamoxifen injection, 5 × 10^7 transducing units of shRaptor lentivirus were injected to CCRK TG mice by tail vein injection. Two weeks later, 5 × 10^6 Ccrk-depleted Hepa-6 cells were intraperitoneally injected into the liver of mice. Second dose of lentivirus was given to mice 2 weeks after surgery. Mice were sacrificed at one-month post-tumor injection. Blood, tumor, and matched non-tumor liver tissues were harvested. Single cell was isolated from tumor or matched non-tumor tissues by gentleMACSTM dissociator (Miltenyi Biotec). Cells were stained with anti-mouse myeloid markers CD11b, Gr-1, Ly6C, Ly6G (eBioscience and BD Biosciences) and analyzed by flow cytometry using FACSBD (BD Biosciences). The percentages of proliferating cells were determined and calculated by FlowJo software (Tree Star).

**MDSC depletion in CCRK TG mouse model.** Six-week-old male CCRK TG mice were injected with tamoxifen (100 mg/kg body weight) daily for 3 consecutive days. At 30-day post tamoxifen injection, 5 × 10^6 Ccrk KO Hepa-6 cells were intraperitoneally injected into the liver of mice. For MDSC depletion, three doses of anti-Ly6G antibody (Bio X Cell) were intraperitoneally injected to mice every 5 days. Mice were sacrificed at one-month post-tumor injection. Blood, tumor, and matched non-tumor liver tissues were harvested. Single cell was isolated from tumor or matched non-tumor tissues by gentleMACSTM dissociator (Miltenyi Biotec). Cells were stained with anti-mouse myeloid markers CD11b, Gr-1, Ly6C, Ly6G (eBioscience and BD Biosciences) and analyzed by flow cytometry using FACSBD (BD Biosciences) and FlowJo software (Tree Star).

**Nanoparticle-mediated hepatic IL-6 depletion.** A lipid/calcium/phosphate (LCP) nanoparticle optimized for delivering plasmid DNA to the nucleus of liver hepatocytes was delivered via asialoglycoprotein receptor (ASGPR). The coding sequence of IL-6 receptor-binding domain was used to generate plasmid DNA and subsequently encapsulated into LCP nanoparticle. The LCP loaded with plasmid DNA of control GFP (pGFP) or IL-6 (pIL-6) trap was prepared using a modified protocol as described previously65. The formulation of pGFP and pIL-6 trap, including a His-tag(-)-Tag at the C-terminal end, was injected (0.25 ml, balanced in osmolarity) to the orthotopic liver tumor-bearing C57Bl/6 mice at a dose of 30 μg/ea through tail vein at a 5-day interval. Mice were then sacrificed at day-28 post-tumor implantation. The IL-6 concentration in liver tissues was detected by IL-6 high sensitive ELISA kit (R&D). Liver and tumor tissues were collected for further analysis.

**Statistical analysis.** Unless otherwise indicated, data are presented as mean ± SD from three independent experiments. The GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for statistical analysis. The independent Student’s t-test or non-parametric test was used to compare gene expression and functional parameters between two selected groups. ANOVA followed by Bonferroni post hoc tests were performed when more than two groups were included in the experimental results. The transcript levels of IL-6, CCRK, CD33, OLR1 and the protein levels of CCRK, p-SGSK3β/S9/GSK3β, p-mTOR/pSer2448/mTOR, p-e3BPiThr37/46, p-S6KThr389/pS6K, mSRPBP1, p-STAT3 Tyr705/STAT3, and AR in the paired tumor and non-tumor tissues were compared using nonparametric Wilcoxon matched pairs test. The correlation between mRNA or protein expression was analyzed using Pearson’s Chi-square test. A two-tailed p-value of <0.05 was regarded as statistically significant.

**Data availability** All data supporting the findings of this study are available within the article and Supplementary Information, or from the corresponding author upon request.

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Author contributions
Study concept and design: G.L.H.W., V.W.S.W., and A.S.L.C.; acquisition of data: H.S., W.Y., Y.T., X.Z., J.Z., M.T.S.M., W.T., Y.F., L.X., A.W.H.C., and B.Y.; analysis and interpretation of data: H.S., W.Y., Y.T., X.Z., J.Z., M.T.S.M., W.T., Y.F., L.X., A.W.H.C., and A.S.L.C.; acquisition of patient specimens: A.W.H.C., J.H.T., Y.S.C., and P.B.S.L.; drafting of the manuscript and preparation of figures: H.S., W.Y., X.Z., M.T.S.M., and A.S.L.C.; critical revision of the manuscript: K.F.T., J.I.Y.S., G.L.H.W., V.W.S.W., and A.S.L.C.; obtained funding: K.L.C., K.F.T., J.I.Y.S., G.L.H.W., V.W.S.W., and A.S.L.C.; administrative, technical or other material support: H.K.S.W., S.W.T., K.L.C., M.H., R.L., M.T.S.M., W.T., Y.F., L.X., A.W.H.C., and A.S.L.C.; obtaining funding: K.L.C., K.F.T., J.I.Y.S., G.L.H.W., V.W.S.W., and A.S.L.C.; administrative, technical or other material support: H.K.S.W., S.W.T., K.L.C., M.H., R.L., and P.Y.; study supervision: G.L.H.W., V.W.S.W., and A.S.L.C.

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Competing interests: G.L.H.W. has served as an advisory committee member for Gilead Sciences, and as a speaker for Abbott, Abbvie, Bristol-Myers Squibb, Echosens, Furiui, Gilead Sciences, Janssen and Roche. V.W.S.W. has served as an advisory board member for AbbVie, Allergan, Center for Outcomes Research in Liver Diseases, Gilead Sciences, Janssen, Perspectum Diagnostics and Pfizer, and received lecture fees from Bristol-Myers Squibb, Echosens, Gilead Sciences, and Merck. The remaining authors declare no competing interests.

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