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RAPID COMMUNICATION

Hepatocyte-specific deletion of *Mettl3* promotes hepatocellular carcinoma in mice

Hepatocellular carcinoma (HCC) is a key cause of morbidity and mortality, which is a global health problem. Elucidating the molecular and cellular mechanisms of HCC progression is helpful to find either early markers for diagnosis or novel drug targets for drug development. Some evidence showed that methyltransferase like 3 (METTL3), a central methyltransferase of the epi-transcriptomic complex that catalyzes m^{6}A mRNA modifications, is associated with HCC.[1] Based on the data collected from HCC cell lines and nude mouse models in which METTL3 was modulated,[2] researchers concluded that METTL3 promoted the pathogenesis of HCC,[2] and indicated that inhibition of METTL3 might be an effective approach for HCC treatment. However, the HCC cell lines and nude mouse models are not the ideal tools because they do not really reflect the function of METTL3 in the pathogenesis of HCC *in vivo*. The *in vivo* function of METTL3 in the pathogenesis of HCC by using a hepatocyte-specific *Mettl3* knockout (*Mettl3*-HKO) mouse model is largely unknown.

To fully elucidate whether METTL3 affects the pathogenesis of HCC *in vivo*, we administered male *Mettl3*-HKO and *Mettl3*$^{flox/flox}$ control mice with a single dose of diethylnitrosamine (DEN) (50 mg/kg) intraperitoneally at two weeks old, and then measured liver tumors at 35 weeks old. As shown in Figure 1A–D, *Mettl3*-HKO mice showed higher liver weight, more and larger HCCs than those in *Mettl3*$^{flox/flox}$ control mice. The cell proliferation was dramatically increased in *Mettl3*-HKO mice, as revealed by significantly increased Ki67 positive cells (Fig. S1A, B). These data demonstrate that hepatic deletion of *Mettl3* promotes HCC pathogenesis.

Overnutrition and hepatic steatosis has been shown to promote HCC development. Our recent study has shown that METTL3 negatively regulates the progression of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis.[3] It is important to examine whether hepatic METTL3 contributes to the promotion of HCC development during overnutrition. After a short time feeding (14 weeks) of a high-fat diet (HFD) following DEN treatment, *Mettl3*-HKO mice showed significantly more and larger HCC tumors (Fig. 1E–G). Consistently, the cell proliferation was dramatically increased in *Mettl3*-HKO mice, as revealed by significantly increased Ki67 positive cells (Fig. S1C, D). We next determined whether a much shorter time (8 weeks) of HFD feeding after DEN treatment was sufficient to cause HCC in *Mettl3*-HKO mice. Interestingly, we observed HCC tumors in *Mettl3*-HKO mice but not in *Mettl3*$^{flox/flox}$ control mice (Fig. 1H–J). No HCC tumors were detected in NC (8 weeks)-fed DEN-treated *Mettl3*$^{flox/flox}$ mice, whereas two HCC tumors were detected in NC (8 weeks)-fed DEN-treated *Mettl3*-HKO mice (Fig. 1H–J). The number of Ki67-positive cells was significantly increased in both NC- and HFD-fed DEN-treated *Mettl3*-HKO mice (Fig. 1K). These data demonstrate that hepatic deletion of *Mettl3* exacerbates DEN-induced HCC development under overnutrition conditions.

We next investigated whether hepatic deletion of *Mettl3* exacerbated DEN-induced HCC progression by promoting liver steatosis and inflammation. After DEN treatment, *Mettl3*-HKO mice under both NC diet (35 weeks)- and HFD (14 weeks)-feeding conditions displayed more severe steatosis and higher liver triglyceride levels than those in *Mettl3*$^{flox/flox}$ mice (Fig. 1L, M), which is likely due to the increased expression of *Cd36* (Fig. 1N–O; Fig. S1E). Tumor-promoting inflammatory microenvironment is also important to promote HCC development. We observed that the expression of key cytochemokines such as *Tnf*α, *Il6*, and *Ccl2* was significantly increased in *Mettl3*-HKO mice (Fig. 1N–O; Fig. S1E). However, the canonical TNF signaling cascade (phosphorylation of IκBα and p65) was not increased in DEN-treated *Mettl3*-HKO mice (Fig. 1O; Fig. S1E). Interestingly, the phosphorylation of signal transducer and activator of transcription-3 (STAT3) and extracellular signal-regulated kinase (ERK) was significantly elevated in DEN-treated *Mettl3*-HKO mice (Fig. 1O; Fig. S1E), which contributes to the increased cell proliferation. Elevated *Il6* expression (Fig. 1N) may contribute to

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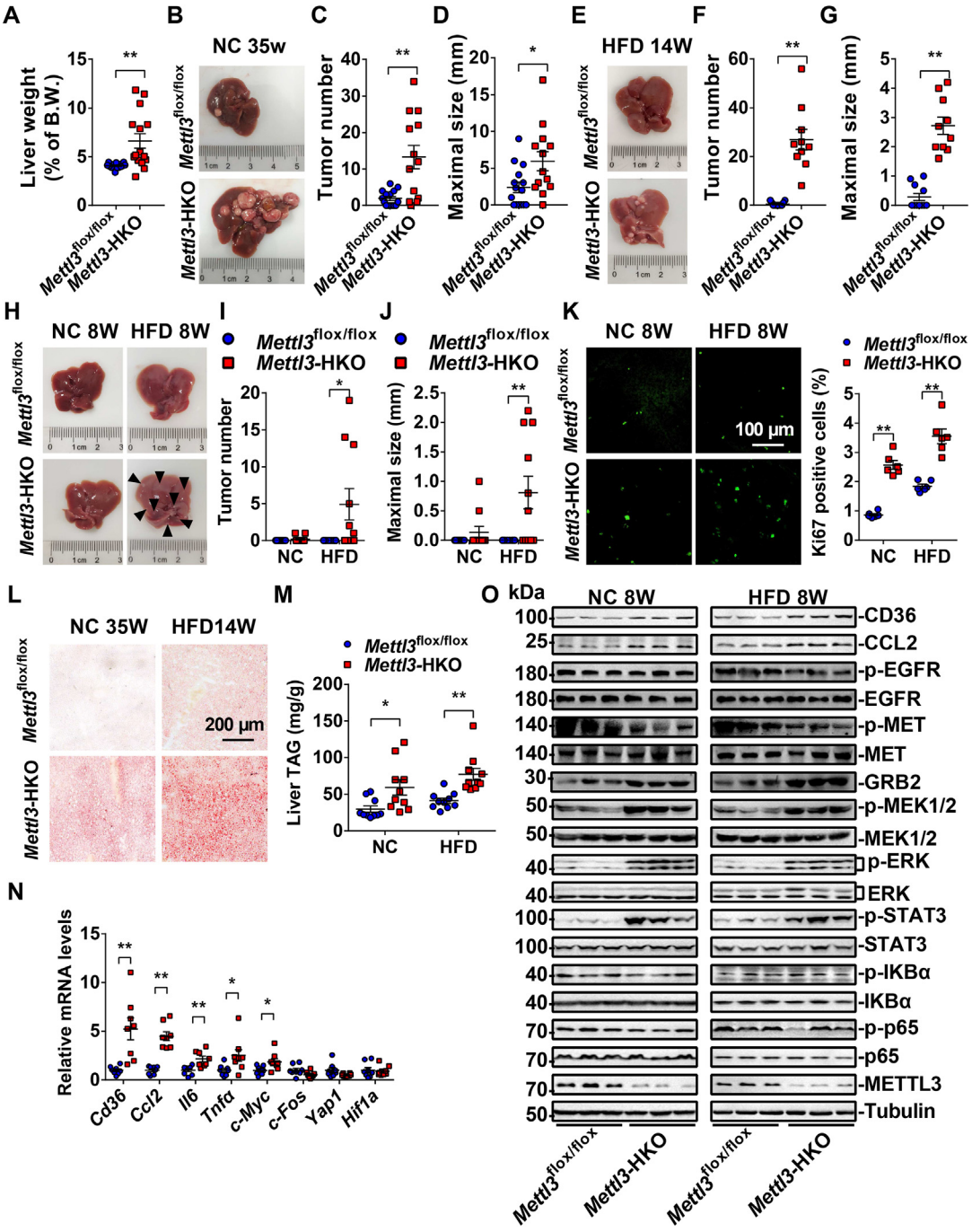

Figure 1 Hepatocyte-specific depletion of *Mettl3* promotes hepatocellular carcinoma (HCC) in mice. Male *Mettl3*-HKO mice and *Mettl3*$^{flox/flox}$ control mice were administered with a single dose of diethylnitrosamine (50 mg/kg) intraperitoneally at two weeks old. **(A—D)** Mice were maintained on an NC diet (n = 13—15 for each group). Mice were sacrificed at 35 weeks old for HCC analysis. Liver weights were measured (A). Representative images of mouse livers from the indicated genotype were shown (B). Tumor number (C) and maximal size of tumors (D) were measured. **(E—G)** Mice were fed a high-fat diet (HFD) for 14 weeks (n = 10—11 for each group). Representative images of mouse livers from the indicated genotype (E) were shown. Tumor number (F) and maximal size of tumors (G) were determined. **(H—K)** Mice were fed an NC diet or HFD for 8 weeks (n = 11—12 for each group). Representative images of mouse livers from the indicated genotype were displayed (H). Tumor number (I), the maximal size of tumors (J), and Ki67-positive cells (K) were measured. **(L, M)** Oil red O staining and liver TAG levels were measured in the livers of *Mettl3*-HKO mice and *Mettl3*$^{flox/flox}$ mice fed with an NC diet for 35 weeks or an HFD for 14 weeks (n = 10 for each group). **(N)** Quantitative RT-PCR analysis of the indicated genes in livers (n = 8 for each group). **(O)** Immunoblotting analysis of CD36, CCL2, p-EGFR, EGFR, p-MET, MET, GRB2, p-MEK1/2, MEK1/2, p-STAT3, STAT3, p-p65, p65, p-IKBα, IKBα, METTL3, and tubulin in the livers of *Mettl3*-HKO and *Mettl3*$^{flox/flox}$ mice fed with NC or HFD for 8 weeks after diethylnitrosamine treatment (n = 6 for each group; n = 3 for representative images). $^{*}P < 0.05$, $^{**}P < 0.01$. Data represent the mean ± standard error of the mean.

the activation of STAT3, whereas increased GRB2 and p-MEK1/2 contributed to the activation of ERK (Fig. 1O; Fig. S1E). However, the activation of upstream receptors (EGFR and HGFR/MET) were unchanged or decreased (Fig. 1O; Fig. S1E), respectively, indicating that EGFR and HGFR/MET unlikely contribute to the activation of the GRB2/MEK/ERK signaling pathway. The liver oncogene c-*Myc* but not *Yap1*, *c-Fos*, or *Hif1α* was up-regulated in *Mettl3*-HKO mice (Fig. 1N), which may contribute to the pathogenesis of HCC. The up-regulation of c-*Myc* was likely due to the increased chromatin accessibility based on the data from our published GEO dataset GSE141325.[3] Our recent study also showed that hepatic deletion of *Mettl3* promoted the expression of *Cd36* and *Ccl2* due to the increased chromatin accessibility independent of its enzyme activity.[3] METTL3 still regulated a large amount of mRNA m^6A modification in the liver,[3] which may also contribute to the pathogenesis of HCC. These data demonstrate that hepatic deletion of *Mettl3* exacerbates DEN-induced HCC progression by activating multiple signaling pathways.

Hepatic deletion of *Mettl3* promoted liver steatosis, inflammation, and activation of STAT3 and ERK signaling pathways, which further exacerbated DEN-induced HCC progression. Deletion of *Mettl3* in other tissues or cell types such as brown adipose tissue, and islet β cells also causes tissue/cell injury and diseases. These results indicate that METTL3 is essential for maintaining tissue homeostasis.[4,5] In contrast, METTL3 is highly expressed in many tumors including HCC, and the knockdown of *Mettl3* in HCC cell lines decreases HCC cell proliferation, cell survival, migration, and colony formation, which leads to the suppression of HCC tumorigenesis in nude mice models,[2] indicating that METTL3 is also required for survival and proliferation of tumor cells. These data suggest that METTL3 is essential for both maintaining liver function and promoting HCC cell proliferation. These results also indicate that hepatic METTL3 may play distinct roles at different stages of HCC pathogenesis. For example, down-regulation or inactivation of METTL3 at the first stage may contribute to the initiation of HCC, whereas up-regulation or activation of METTL3 at a later stage may contribute to the acceleration of HCC. Current data support this hypothesis. Down-regulation of nuclear METTL3 has been shown to be associated with nonalcoholic steatohepatitis, and hepatic deletion of *Mettl3* promotes liver injury and nonalcoholic fatty liver disease,[3] whereas METTL3 is abnormally up-regulated in HCC,[2] promoting HCC progression.

Author contributions

C.L. and X.L. performed most of the experiments. Z.Y. provided research materials and analyzed data. X.L. analyzed data. Z.C. conceived and designed the project, researched data, and wrote the manuscript.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.02.048.

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