Capicua suppresses hepatocellular carcinoma progression by controlling ETV4-MMP1 axis

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List of abbreviations

HCC, Hepatocellular carcinoma; CIC, Capicua; TCGA, The cancer genome atlas; DEN, Diethylnitrosamine; PEA3, Polyoma enhancer activator 3; ETV4, ETS translocation variant 4; MMP, Matrix metalloproteinase; ERK, Extracellular signal–regulated kinase; RTK, Receptor tyrosine kinase; shCIC, shRNA against CIC; Cic LKO, liver-specific Cic null mice

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Abstract

Hepatocellular carcinoma (HCC) is developed by multiple steps accompanying progressive alterations of gene expression, which leads to increased cell proliferation and malignancy. Although environmental factors and intracellular signaling pathways that are critical for HCC progression have been identified, gene expression changes and the related genetic factors contributing to HCC pathogenesis are still insufficiently understood. In this study, we identify a transcriptional repressor Capicua/CIC as a suppressor of HCC progression and a potential therapeutic target. Expression of CIC is posttranscriptionally reduced in HCC cells. CIC levels are correlated with survival rates in patients with HCC. CIC overexpression suppresses HCC cell proliferation and invasion, whereas loss of CIC exerts opposite effects in vivo as well as in vitro. The levels of PEA3 group genes, the best-known CIC target genes, are correlated with lethality in patients with HCC. Among the PEA3 group genes, ETV4 is the most significantly upregulated gene in CIC-deficient HCC cells, consequently promoting HCC progression. Furthermore, ETV4 induces expression of MMP1, the MMP gene highly relevant to HCC progression, in HCC cells, and knockdown of MMP1 completely blocks the CIC deficiency-induced HCC cell proliferation and invasion. Conclusion: Our study demonstrates that the CIC-ETV4-MMP1 axis is a novel regulatory module controlling HCC progression.
Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide and the fifth most common malignancy, especially in East Asia and South Africa (1, 2). In most cases, human HCC is driven by chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, alcoholic abuse, non-alcoholic fatty liver disease, autoimmune hepatitis, diabetes mellitus, and several metabolic diseases (3).

Among multiple therapeutic strategies to overcome HCC, liver resection is still the best therapeutic strategy to treat HCC with a 5-year survival rate in approximately 70% (3). Another option of HCC treatment is the orthotopic liver transplantation, which has the lowest risk of tumor recurrence but is applied to very few patients. Radiofrequency ablation (RFA) and transarterial chemoembolization (TACE) are other therapeutic strategies but have marginal effects. Because of these limitations of HCC treatment, many studies are focused on finding molecular therapies for HCC. Sorafenib, a related multikinase inhibitor, is currently the only drug approved for advanced HCC management. Despite sorafenib treatment, overall survival is increased by only 37%, with several major side effects including acne-like rash, diarrhea, fatigue, and hypertension (4).

In search for a better understanding and efficacious treatment in HCC, many cancer drivers and molecular therapies have been reported (5). Some studies have explored HCC genomic alterations and identified frequently mutated genes, including TERT promoter, TP53, and CTNNB1 (β-catenin) (6, 7). In addition, chromosomal amplifications (1q, 6p, 8q, 11q, 17q, and 20q) and deletions (4q, 8p, 13q, 16q, and 17p) that affect important oncogenes and tumor suppressors have been identified in samples from patients with HCC (8). Moreover, several
signaling pathways are investigated to be targeted by novel therapies for HCC including RAS, TGF-β, FGF-19/FGFR-4, and MET signaling pathways (9). Importantly, RAS signaling is related to cell survival and proliferation and is activated in more than 50% of HCCs (10, 11).

Although HCC progression is considered as a multistep and a long-term progressive process, the precise molecular mechanism of HCC pathogenesis remains largely unknown (12).

Capicua/CIC is a transcriptional repressor that is highly conserved from C. elegans to humans (13). There are two main isoforms of CIC, the short (CIC-S) and long (CIC-L) form, which differ in their amino-terminal portions. CIC has two highly conserved domains: a DNA-binding high mobility group (HMG) box domain and a carboxy-terminal motif (C1) (13). CIC preferentially recognizes T(G/C)AATG(G/A)A sequences through the HMG-box and C1 domains to repress expression of its target genes in Drosophila and mammals (14-16). CIC activity can be regulated by receptor tyrosine kinase (RTK) signaling pathways in Drosophila and mammals (17-19). Activation of RTK-MAPK pathways phosphorylates CIC, resulting in degradation and/or cytoplasmic localization of CIC (20, 21).

Cic was firstly identified in a screen for mutations affecting tissue patterning in Drosophila embryo (17). In Drosophila, several studies revealed that Cic regulates not only anteroposterior and dorsoventral body patterning, but also intestinal stem cell proliferation (22), wing development (23), and other processes in development (24).

In mammals, CIC has been implicated in pathogenesis of spinocerebellar ataxia type-1 neurodegenerative disease (25), as well as regulation of essential processes such as lung alveolarization (26), liver homeostasis (27), learning and memory (28), and follicular helper T cell differentiation (29). CIC has also been studied in several cancer contexts, and its mutations were found in soft tissue, brain, lung, gastric, prostate, and breast cancers (14, 21, 30-32).
Furthermore, CIC directly suppresses expression of \textit{PEA3} group genes (\textit{ETV1}, \textit{ETV4}, and \textit{ETV5}), which are known to be frequently overexpressed in many cancers and have tumor-promoting functions (14, 30, 33, 34).

Although increasing evidences have indicated that CIC functions as a tumor suppressor in various cancers (19, 21, 35, 36), no studies have examined its clinicopathologic significance and molecular functions in HCC. In this study, we present the first evidence that decreased level of CIC is associated with HCC progression and indicates poor prognosis. Both \textit{in vitro} and \textit{in vivo} assays demonstrate that CIC has a tumor suppressive function in the progression of HCC. Molecular studies reveal that CIC regulates \textit{ETV4} expression in HCC cells and that MMP1 acts as a key downstream target of the CIC-ETV4 axis in HCC context. Therefore, our findings suggest that CIC-ETV4-MMP1 regulatory axis might have a critical role in HCC progression.
Experimental procedures

Tissue microarray and immunohistochemistry

Two liver cancer tissue microarrays with liver tumors and adjacent normal liver tissues (LV1221 and LV6161) were purchased from Biomax (MD, USA). Formalin-fixed paraffin-embedded specimens were de-paraffinized and stained with rabbit polyclonal anti-CIC antibody. Each sample stained with anti-CIC antibody was scored as negative (-), weak (+), moderate (++) or strong (+++) according to the staining intensity. These scores were determined independently by two pathologists. The scoring by the pathologists was done in a blinded manner.

Induction of HCC in mice

To induce HCC, diethylnitrosamine (DEN, Sigma-Aldrich, MO, USA) was injected intraperitoneally (i.p.) into 2-week-old male mice (5 µg/g). For tumor formation analysis, mice were sacrificed to prepare liver tissues at 8 months after DEN treatment. Externally visible tumors (>1 mm) on liver were counted and measured. Livers were micro-dissected into tumor and non-tumor and stored at −80°C until analyzed by qRT-PCR.

Lung metastasis and survival were analyzed at 15 months after DEN treatment. The survival of the mice was recorded weekly. After 15 months, the mice were sacrificed, and their lungs were dissected, paraffin-embedded, and used for H&E staining. Serial sections of entire lung tissues were conducted. Four sections per each lung tissue were chosen for H&E staining. Total number of metastasized tumor lesions was counted from the H&E-stained sections and used for calculation of the average number of tumor foci in a lung tissue per each genotype.
Other assays used in this study are described in the Supporting Information.
Results

Inverse correlation between CIC protein levels and HCC severity

Since the role of CIC in HCC development and progression has not yet been determined, we analyzed The Cancer Genome Atlas (TCGA) data sets for patients with HCC (Table S1) in order to gain insight on the relevance between CIC and HCC pathogenesis. CIC mRNA levels were not downregulated, but rather increased in HCC tissue samples compared with normal liver tissues (Fig. 1A). However, the survival rate was significantly decreased in HCC patients with low levels of CIC (the lower 20%, n=74) compared with those with high levels of CIC (the upper 20%, n=74) (Fig. 1B and Table S2). Previous studies have demonstrated that activation of EGFR and its downstream signaling molecules, which promotes tumorigenesis and cancer metastasis, inactivates CIC via either degradation or cytoplasmic translocation (20, 21). Moreover, we have shown that CIC protein levels were dramatically decreased in prostatic adenocarcinoma (35). Thus, we examined CIC protein levels in normal liver and HCC tissues on tissue microarrays by immunohistochemistry using anti-CIC antibody. Reduced expression of CIC was more frequently observed in HCC samples than in normal liver tissues (Fig. 1C). To directly address whether CIC expression decreases in HCC tissues at protein level, but not at mRNA level, we examined expression profiles of both CIC protein and CIC mRNA in the same tissue samples of normal liver and HCC of different pathological stages (Table S3). Most HCC tissues showed reduction in CIC protein levels compared with normal liver tissues, whereas CIC mRNA level was not correlated with CIC protein level in each tissue sample (Fig. 1D), suggesting the posttranscriptional regulation of CIC expression in HCC cells. Taken together, these data indicate the association of CIC levels with HCC progression.
CIC suppresses HCC progression

To test whether CIC has a suppressive function in HCC progression, we examined the effect of CIC overexpression on HCC cell growth and invasion. We chose SK-HEP-1 and MHCC-97H cells, which are highly metastatic and aggressive HCC cell lines (37, 38), for the experiments, because they express relatively low levels of CIC compared with other HCC cell lines (Fig. S1). Forced expression of either CIC-S or CIC-L suppressed cell proliferation, invasion, and migration in both cell lines (Figs. 2A-D). Then, we tested whether suppression of CIC expression has the opposite effects. HCC cells that stably express shRNA against CIC (shCIC) had increased proliferation rate and invasive and migratory activity compared with control HCC cells (Figs. 2E-H). CRISPR-Cas9-mediated knockout of CIC also promoted HCC cell proliferation and invasion (Fig. S2). We confirmed these results in vivo using xenograft mouse models. Control and shCIC-expressing SK-HEP-1 or MHCC-97H cells were subcutaneously injected into either posterior flank of the same nude mice, respectively, and tumor volume was measured every week. The CIC-deficient HCC cells grew more rapidly and formed larger tumor mass than the control cells (Fig. 3A). To compare metastatic activity between control and CIC-deficient HCC cells, the cells were intravenously injected into nude mice followed by quantification of GFP signal, which is expressed from shRNA-expressing lentiviral vectors (pGIPZ), in lung tissues. The CIC-deficient HCC cells had higher degree and frequency of metastasis to lung than control cells (Figs. 3B, C). Taken together, these findings indicate that CIC functions as a negative regulator in HCC progression.

Increased lung metastasis and lethality in Cic-deficient mice treated with DEN
To better understand *in vivo* effect of CIC deficiency on HCC progression, we generated mice with a specific deletion of *Cic* alleles in hepatocytes (*Cic<sup>f/f</sup>;Alb-Cre, *Cic* LKO) (Fig. 4A) and induced liver cancer in these mice by treatment with diethylnitrosamine (DEN). WT (*Cic<sup>f/f</sup>*) and *Cic* LKO mice were intraperitoneally injected with DEN at 2 weeks of age and subjected to analyses of tumorigenesis, lung metastasis, and viability (Fig. 4B). Tumor formation on liver tissues was comparable between WT and *Cic* LKO mice at 8 months of age (Fig. 4C). However, lung metastasis was substantially increased in *Cic* LKO mice at 15 months of age (Fig. 4D). Moreover, about 30% of *Cic* LKO mice died after 1 year of age, whereas none of WT mice did (Fig. 4E). These results demonstrate that reduction in CIC expression can critically contribute to promotion of HCC progression, which is consistent with the finding that the survival rate was decreased in the HCC patients with low levels of *CIC* (Fig. 1B).

**ETV4 is a critical CIC target that promotes HCC progression**

Many studies have shown that *PEA3* group genes, which include *ETV1*, *ETV4*, and *ETV5*, are direct target genes of CIC (14, 19, 25, 26) and that overexpression of these genes promotes proliferation and invasion of various types of cancer cell (33). However, the role of PEA3 group transcription factors in HCC progression has not yet been comprehensively understood. Therefore, we analyzed expression profiles of *PEA3* group genes and association of these gene expression levels with lethality in patients with HCC using the TCGA database (Tables S1 and S4). Among three genes, only *ETV4* levels were increased in all stages of HCC cells with statistical significance compared with normal liver cells (Fig. 5A). On the other hand, overall survival rates of HCC patients were inversely correlated with the levels of all *PEA3* group genes.
(Fig. 5B), suggesting that PEA3 group transcription factors might also function as a tumor promoter in the context of HCC progression.

Next, we examined whether CIC regulates expression of PEA3 group genes in HCC cells. The levels of PEA3 group genes in control and shCIC-expressing HCC cells were determined by qRT-PCR analysis (Fig. 5C). Among three genes, ETV4 levels were most significantly upregulated in three different HCC cell lines with CIC RNAi (Fig. 5C). Knockdown of CIC did not increase levels of PEA3 group genes in HepG2 cells (Fig. 5C), suggesting that CIC differentially regulates its target gene expression in a cell-type dependent manner, which is consistent with previous findings (27, 35, 39). Furthermore, the upregulation of ETV4 expression in CIC knockdown HCC cells was confirmed at protein level (Fig. 5D). Increases in Evt4 levels were also most apparent in both normal liver and DEN-induced tumor tissues from Cic LKO mice compared with those in Ev1 and Ev5 (Figs. 5E-G). Overall, these data suggest that, among PEA3 group genes, ETV4 is a major target gene of CIC in hepatic cells.

Given that ETV4 had the highest relevance to HCC progression (Figs. 5A, B) and that ETV4 expression was most significantly regulated by CIC in HCC cells (Fig. 5C), we focused on the role of ETV4 in HCC progression. We first tested whether ETV4 has HCC-promoting activity. Overexpression of ETV4 indeed increased cell proliferation, invasion, and migration in HCC cells (Figs. 6A-D and S3). We next examined whether the increased cell proliferation, invasion, and migration in CIC-deficient HCC cells were due to derepression of ETV4. Knockdown of ETV4 completely blocked the CIC deficiency-mediated promotion of HCC progression (Fig. 6E-H), demonstrating that ETV4 is a key target gene of CIC in regulation of HCC progression.

Regulation of MMP1 expression by the CIC-ETV4 axis in HCC cells
Matrix metalloproteinases (MMPs) promote cancer progression through various ways including destruction of extracellular matrix, activation of growth factors, suppression of apoptosis, and induction of angiogenesis (40). Therefore, MMPs are the principle mediators of cancer progression and frequently used as biomarkers for various types of cancer. There are 23 members of MMP in humans. Previous studies revealed that PEA3 group transcription factors activate expression of various MMP genes and that most MMP genes harbor ETS binding elements in their promoters (26, 41). To gain insight on which MMPs are critically involved in HCC progression, we analyzed the relevance of each MMP to HCC progression using the TCGA database (Tables S1 and S5), as we did for CIC and PEA3 group genes. Among 23 MMP genes, levels of MMP1, MMP9, MMP10, MMP11, MMP12, and MMP14 were significantly higher in HCC tissues than in normal liver tissues (Figs. 7A and S4). Analysis of survival rates revealed that levels of MMP1, MMP7, MMP10, MMP12, MMP16, and MMP26 were inversely correlated with survival rates of HCC patients with statistical significance (Figs. 7B and S5). Thus, these analyses identified MMP1, MMP10, and MMP12 as MMP genes strongly associated with promotion of HCC progression.

Next, we investigated which of the selected MMP genes are regulated by the CIC-ETV4 axis in HCC cells. Among MMP1, MMP10, and MMP12, only MMP1 expression was significantly induced by ETV4 overexpression in both SK-HEP-1 and MHCC-97H HCC cell lines (Fig. 7C). We confirmed that ETV4 enhances MMP1 promoter activity by luciferase assay using MMP1 promoter-containing reporter construct (Fig. 7D). We further examined regulation of MMP1 expression by CIC in HCC cells. Knockdown of CIC significantly upregulated levels of MMP1, but neither MMP10 nor MMP12, in SK-HEP-1 and MHCC-97H HCC cell lines (Fig. 7E). Consistent with this result, overexpression of CIC downregulated MMP1 expression (Fig. S6).
On the other hand, expression of \textit{MMP13}, which belongs to the interstitial collagenase family as 
\textit{MMP1} does and shares high amino acid identity (86\%) with \textit{MMP1} (42), was not significantly 
affected by either ETV4 overexpression or \textit{CIC} RNAi in HCC cells (Fig. S7), suggesting that the 
CIC-ETV4 axis might selectively regulate expression of \textit{MMP1} rather than all members of the 
MMP subfamily with similar biochemical and pathological properties. The levels of \textit{Mmp1a}, a 
mouse homolog of human \textit{MMP1}, were also upregulated in liver tumors from 15-month-old Cic 
LKO mice treated with DEN compared with those from WT controls, while the levels of other 
\textit{Mmp} genes including \textit{Mmp2}, \textit{Mmp9}, and \textit{Mmp12}, which have been implicated in the promotion 
of HCC progression (43, 44), were comparable (Fig. S8). Overall, these results suggest that 
\textit{MMP1} might be a key effector MMP protein that functions at the downstream of the CIC-ETV4 
axis in the context of HCC progression.

To directly address whether the increased expression of \textit{MMP1} in CIC-deficient HCC cells was 
due to derepression of \textit{ETV4}, we examined levels of \textit{MMP1} in control and shCIC-expressing 
HCC cells treated with either control siRNA or siRNA against \textit{ETV4}. Knockdown of \textit{ETV4} 
certainly restored \textit{MMP1} expression to the normal level in CIC-deficient HCC cells (Fig. 7F). 
Taken together, these data suggest that \textit{MMP1} expression can be regulated by the CIC-ETV4 
axis in the process of HCC progression.

\section*{CIC deficiency promotes HCC progression via \textit{MMP1} overexpression}

Since we identified \textit{MMP1} as a critical downstream target gene of the CIC-ETV4 axis in HCC 
cells, we finally determined whether the induction of \textit{MMP1} expression contributed to the 
enhanced cancer progression in CIC-deficient HCC cells. We transfected control and shCIC-
expressing HCC cells with either control siRNA or two different siRNAs against \textit{MMP1} and
examined cell proliferation, invasion, and migration. Knockdown of \textit{MMP1} completely suppressed the increased cell proliferation and invasive and migratory activity in \textit{shCIC}-expressing HCC cells (Figs. 8A-D), demonstrating that the increased expression of \textit{MMP1} indeed critically contributed to the CIC deficiency-mediated promotion of HCC progression.
Discussion

In this study, we demonstrated for the first time that CIC could function as a negative regulator of HCC progression via control of ETV4-MMP1 axis (Fig. 8E). Overexpression of CIC suppressed HCC cell proliferation and invasion, whereas CIC deficiency promoted HCC progression \textit{in vivo} as well as \textit{in vitro}. We found a discrepancy between CIC-deficient HCC cells and DEN-induced HCC in liver-specific \textit{Cic} null mice; CIC-deficient HCC cells had increased proliferation and invasive activity, while DEN-treated \textit{Cic}-deficient mice exhibited the enhanced lung metastasis, but not tumor formation in livers. This finding implies that loss of CIC might not be enough to facilitate the onset of HCC, but nevertheless could contribute to the promotion of HCC progression once HCC has occurred.

Analyses of the TCGA database and tissue samples from patients with HCC revealed that CIC expression is reduced in HCC cells at protein level, but not at mRNA level. These results suggest that HCC-promoting factors and/or signaling pathways might downregulate CIC expression in HCC cells at posttranscriptional level. It is well known that activation of RTK signaling suppresses CIC activity via degradation or cytoplasmic translocation of CIC in \textit{Drosophila} and mammals (13, 20, 21, 25). In this process, ERK, a downstream effector kinase of RTK signaling pathways, plays a pivotal role. ERK can interact with CIC (45) and the inhibition of ERK rescues CIC activity in the context of EGFR activation (21). These findings suggest that activation of ERK and its upstream signaling cascades might be involved in downregulation of CIC protein levels. Many studies have demonstrated that RAS/RAF/MEK/ERK signaling pathway is associated with HCC pathogenesis (46, 47). Levels of total or phosphorylated ERK are often higher in HCC cells than in normal liver cells (48, 49). Moreover, it was reported that ERK is
mainly found in the nucleus of HCC cells (46). Therefore, it would be conceivable that the
decrease in CIC levels in HCC cells was due to the enhanced ERK activity (Fig. 8E).

Our study demonstrated the significant relevance of MMP1 to HCC progression. In the context
of HCC, functional significance of other MMPs, such as MMP2, MMP9, and MMP12, have
been more appreciated than that of MMP1 (43, 44). However, our comprehensive analyses for
MMP genes in HCC patients using the TCGA datasets indicated that MMP1 is more significantly
associated with HCC pathogenesis than other MMPs previously recognized to promote HCC
progression. The in vitro experiments using HCC cell lines showed that MMP1 deficiency is
sufficient to suppress HCC cell growth and invasion, underlying the critical role of MMP1 in
HCC progression. We also provided several evidences that MMP1 is a critical downstream target
of the CIC-ETV4 axis that contributes to the CIC deficiency-mediated promotion of HCC
progression. MMP1 expression is under the control of ETV4 and knockdown of MMP1
completely blocks the increased cell proliferation and invasion in CIC-deficient HCC cells.
Nevertheless, it cannot be ruled out that other MMPs could also contribute to the enhanced cell
proliferation and invasion in CIC-deficient HCC cells, because most MMP genes have ETS
binding elements in their promoters (26). To better understand the molecular mechanism
underlying the CIC deficiency-mediated promotion of HCC progression, genome-wide
identification of target genes of CIC as well as ETV4 and studies on their roles in HCC
pathogenesis need to be followed.

This study suggests CIC-ETV4-MMP1 axis as a novel genetic module that controls HCC
progression. Patients with HCC have a poor survival rate mainly due to late diagnosis (50).
Therefore, it is very important to identify genetic alterations that can predict HCC development
and progression as early as possible. In this regard, our findings provide novel candidate
molecules that might be potentially developed as diagnostic markers as well as therapeutic targets for HCC.
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Author names in bold designate shared co-first authorship.
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Figure legends

Figure 1. Decreased CIC protein levels in HCC cells and its correlation with poor survival rates in HCC patients. A. Analysis of TCGA database for expression levels of CIC mRNA in normal liver (NL) and HCC samples of four different clinicopathologic stages (I, II, III, and IV). The numbers in parentheses mean the number of subjects in each group. ***P < 0.001. B. Kaplan-Meier analysis of overall survival for HCC patients with high (the upper 20%) or low (the lower 20%) CIC expression (74 patients per each subgroup). *P < 0.05. C. Analysis of CIC protein levels in normal liver (n=33) and HCC (n=554) tissues using tissue microarrays. Immunohistochemical staining of CIC was conducted. Representative immunohistochemistry images showing four different expression levels of CIC in normal liver and HCC tissues were presented in the left panel. Scale bars indicate 100 µm. The right panel shows the proportion of tissue samples with different CIC expression scores. D. Western blot and qRT-PCR analyses of CIC expression profiles in normal liver (NL) and HCC samples.

Figure 2. CIC suppresses cell proliferation, migration, and invasion in HCC cells. A. Western blot analysis for ectopic expression of CIC-S and CIC-L in HCC cells (SK-HEP-1 and MHCC-97H). B. Cell growth assay of control and CIC-overexpressing HCC cells. C. Matrigel invasion assay of control and CIC-overexpressing HCC cells. The bottom panel is a bar graph for quantification of cell invasiveness. D. Transwell migration assay of control and CIC-overexpressing HCC cells. The bottom panel is a bar graph for quantification of cell migration. E. Western blot analysis showing knockdown efficiency of shCIC in HCC cells. shNC is for a negative control shRNA. F. Cell growth assay of control and shCIC-expressing HCC cells.
Matrigel invasion assay of control and shCIC-expressing HCC cells. The right panel is a bar graph for quantification of cell invasiveness. H. Transwell migration assay of control and shCIC-expressing HCC cells. The right panel is a bar graph for quantification of cell migration. Three independent experiments were performed. All error bars show s.e.m. \( *P < 0.05, **P < 0.01, \text{ and } ***P < 0.001. \)

**Figure 3. CIC deficiency promotes tumor growth and metastasis in vivo.** A. *In vivo* subcutaneous tumor growth curves of SK-HEP-1 and MHCC-97H cells with shNC- or shCIC-lentiviral infection. n=12 per each group. The middle panel is a representative image of xenograft tumors dissected from the mice after the last measurement of tumor size. The right panel is a graph for average weights of the dissected tumors. B. *In vivo* imaging of lung tissues into which intravenously-injected control or shCIC-expressing SK-HEP-1 and MHCC-97H cells metastasized. GFP signals expressed in the injected cells are shown as dot plots. The right panel is a bar graph for quantification of GFP signals. n=10 per each group. All error bars show s.e.m. \( *P < 0.05 \text{ and } **P < 0.01. \) C. A bar graph for the incidence (black area) of lung metastasis in each group of the nude mice.

**Figure 4. Enhanced lung metastasis in liver-specific Cic null mice treated with DEN.** A. Western blot images of CIC and \( \beta \)-actin (loading control) in liver and lung tissues of wild-type (WT) and liver-specific Cic null (Cic LKO) mice. B. Experimental scheme for analysis of tumorigenesis and lung metastasis of HCC in mice treated with diethylnitrosamine (DEN). C. Representative images of tumor-bearing livers from 8-month-old WT and Cic LKO mice treated with DEN. Arrows indicate tumor foci. The right panel is a bar graph for the average numbers of
tumor foci on liver tissues of the DEN-treated WT and Cic LKO mice at 8 months of age. n=6 per each group. D. Representative images of H&E-stained lung tissues of DEN-treated WT and Cic LKO mice at 15 months of age. The right panel is a bar graph for the average numbers of tumor lesions in randomly selected lung tissue sections. Numbers of WT and Cic LKO mice used for this analysis are 10 and 8, respectively. E. Kaplan–Meier survival curve of the DEN-treated WT (n=11) and Cic LKO mice (n=11). All error bars show s.e.m. *P < 0.05.

Figure 5. \textit{ETV4} is a major CIC target with the most significant relevance to HCC among PEA3 group genes. A. Analysis of TCGA database for expression levels of \textit{ETV1}, \textit{ETV4}, and \textit{ETV5} in normal liver (NL) and HCC samples of four different clinicopathologic stages (I, II, III, and IV). The numbers in parentheses mean the number of subjects in each group. B. Kaplan-Meier analysis of overall survival for HCC patients with high or low expression of PEA3 group genes (74 patients per each subgroup). C. qRT-PCR analysis of PEA3 group gene expression levels in control and CIC knockdown HCC cells. Experiments were performed more than three times, independently. D. Western blot analysis for levels of ETV4 in control and CIC knockdown HCC cells. E. qRT-PCR analysis for levels of Cic, Etv1, Etv4, and Etv5 in livers of 7-week-old WT and Cic LKO mice. n=6 per each genotype. F and G. qRT-PCR analysis for levels of Cic, Etv1, Etv4, and Etv5 in liver tumors harvested from the DEN-treated WT and Cic LKO mice at 8 months of age (F) and at 15 months of age (G). n=5 per each genotype. All error bars show s.e.m. *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 6. CIC deficiency-mediated promotion of HCC progression is due to derepression of \textit{ETV4}. A. Western blot analysis showing ectopic expression of ETV4 in SK-HEP-1 and MHCC-
97H cells. **B-D.** Cell growth assay (**B**), matrigel invasion assay (**C**), and transwell migration assay (**D**) of control and ETV4-overexpressing HCC cells. **E.** qRT-PCR analysis for *ETV4* mRNA levels in control and *CIC* knockdown HCC cells transfected with either control or *ETV4* siRNA. **NN:** control HCC cells transfected with control siRNA, **N4:** control HCC cells transfected with *ETV4* siRNA (siETV4), **CN:** *CIC* knockdown HCC cells transfected with control siRNA, and **C4:** *CIC* knockdown HCC cells transfected with siETV4. **F-H.** Cell growth assay (**F**), matrigel invasion assay (**G**), and transwell migration assay (**H**) of control and *CIC* knockdown HCC cells treated with either control or *ETV4* siRNA. Three independent experiments were performed. All error bars show s.e.m. *P < 0.05, **P < 0.01, and ***P < 0.001.

**Figure 7.** *MMP1* expression is regulated by the CIC-ETV4 axis. **A.** Analysis of TCGA database for expression levels of *MMP1* in normal liver (NL) and HCC samples of four different clinicopathologic stages (I, II, III, and IV). The numbers in parentheses mean the number of subjects in each group. **B.** Kaplan-Meier analysis of overall survival for HCC patients with high or low *MMP1* expression (74 patients per each subgroup). **C.** qRT-PCR analysis for levels of *MMP1, MMP10,* and *MMP12* in control and ETV4-overexpressing HCC cells (SK-HEP-1 and MHCC-97H). **D.** Dual luciferase assay for regulation of *MMP1* promoter activity by ETV4. The left panel is a schematic illustration for the luciferase reporter construct harboring *MMP1* promoter region (-663/+1), in which there are two putative ETV4 binding sites (-323/-319 and -155/-149). The right panel is a bar graph for relative luciferase activity in the presence or absence of ETV4 overexpression. **E.** qRT-PCR analysis for levels of *MMP1, MMP10,* and *MMP12* in control and *CIC* knockdown HCC cells. **F.** qRT-PCR analysis for *MMP1* levels in
control and CIC knockdown HCC cells treated with either control or ETV4 siRNA. NN: control HCC cells transfected with control siRNA, N4: control HCC cells transfected with siETV4, CN: CIC knockdown HCC cells transfected with control siRNA, and C4: CIC knockdown HCC cells transfected with siETV4. Three independent experiments were performed. All error bars show s.e.m. *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 8. Upregulation of MMP1 expression contributes to the increased cell proliferation and invasion in CIC knockdown HCC cells. A. qRT-PCR analysis for MMP1 levels in control and CIC knockdown HCC cells treated with either control or MMP1 siRNAs. NN: control HCC cells transfected with control siRNA, N1-1: control HCC cells transfected with MMP1 siRNA-1 (siMMP1-1), N1-2: control HCC cells transfected with MMP1 siRNA-2 (siMMP1-2), CN: CIC knockdown HCC cells transfected with control siRNA, C1-1: CIC knockdown HCC cells transfected with siMMP1-1, and C1-2: CIC knockdown HCC cells transfected with siMMP1-2. B-D. Cell proliferation assay (B), matrigel invasion assay (C), and transwell migration assay (D) of control and CIC knockdown HCC cells treated with either control or MMP1 siRNAs. Three independent experiments were performed. All error bars show s.e.m. *P < 0.05, **P < 0.01, and ***P < 0.001. E. Proposed model for the regulation of HCC progression by CIC-ETV4-MMP1 axis.
Figure 1. Decreased CIC protein levels in HCC cells and its correlation with poor survival rates in HCC patients. A. Analysis of TCGA database for expression levels of CIC mRNA in normal liver (NL) and HCC samples of four different clinicopathologic stages (I, II, III, and IV). The numbers in parentheses mean the number of subjects in each group. ***P < 0.001. B. Kaplan-Meier analysis of overall survival for HCC patients with high (the upper 20%) or low (the lower 20%) CIC expression (74 patients per each subgroup). *P < 0.05. C. Analysis of CIC protein levels in normal liver (n=33) and HCC (n=554) tissues using tissue microarrays. Immunohistochemical staining of CIC was conducted. Representative immunohistochemistry images showing four different expression levels of CIC in normal liver and HCC tissues were presented in the left panel. Scale bars indicate 100 µm. The right panel shows the proportion of tissue samples with different CIC expression scores. D. Western blot and qRT-PCR analyses of CIC expression profiles in normal liver (NL) and HCC samples.
Figure 2. CIC suppresses cell proliferation, migration, and invasion in HCC cells. A. Western blot analysis for ectopic expression of CIC-S and CIC-L in HCC cells (SK-HEP-1 and MHCC-97H). B. Cell growth assay of control and CIC-overexpressing HCC cells. C. Matrigel invasion assay of control and CIC-overexpressing HCC cells. The bottom panel is a bar graph for quantification of cell invasiveness. D. Transwell migration assay of control and CIC-overexpressing HCC cells. The bottom panel is a bar graph for quantification of cell migration. E. Western blot analysis showing knockdown efficiency of shCIC in HCC cells. shNC is for a negative control shRNA. F. Cell growth assay of control and shCIC-expressing HCC cells. G. Matrigel invasion assay of control and shCIC-expressing HCC cells. The right panel is a bar graph for quantification of cell invasiveness. H. Transwell migration assay of control and shCIC-expressing HCC cells. The right panel is a bar graph for quantification of cell migration. Three independent experiments were performed. All error bars show s.e.m. *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 3. CIC deficiency promotes tumor growth and metastasis in vivo. A. In vivo subcutaneous tumor growth curves of SK-HEP-1 and MHCC-97H cells with shNC- or shCIC-lentiviral infection. n=12 per each group. The middle panel is a representative image of xenograft tumors dissected from the mice after the last measurement of tumor size. The right panel is a graph for average weights of the dissected tumors. B. In vivo imaging of lung tissues into which intravenously-injected control or shCIC-expressing SK-HEP-1 and MHCC-97H cells metastasized. GFP signals expressed in the injected cells are shown as dot plots. The right panel is a bar graph for quantification of GFP signals. n=10 per each group. All error bars show s.e.m. *P < 0.05 and **P < 0.01. C. A bar graph for the incidence (black area) of lung metastasis in each group of the nude mice.
Figure 4. Enhanced lung metastasis in liver-specific Cic null mice treated with DEN. A. Western blot images of CIC and β-actin (loading control) in liver and lung tissues of wild-type (WT) and liver-specific Cic null (Cic LKO) mice. B. Experimental scheme for analysis of tumorigenesis and lung metastasis of HCC in mice treated with diethylnitrosamine (DEN). C. Representative images of tumor-bearing livers from 8-month-old WT and Cic LKO mice treated with DEN. Arrows indicate tumor foci. The right panel is a bar graph for the average numbers of tumor foci on liver tissues of the DEN-treated WT and Cic LKO mice at 8 months of age. n=6 per each group. D. Representative images of H&E-stained lung tissues of DEN-treated WT and Cic LKO mice at 15 months of age. The right panel is a bar graph for the average numbers of tumor lesions in randomly selected lung tissue sections. Numbers of WT and Cic LKO mice used for this analysis are 10 and 8, respectively. E. Kaplan–Meier survival curve of the DEN-treated WT (n=11) and Cic LKO mice (n=11). All error bars show s.e.m. *P < 0.05.
Figure 5. ETV4 is a major CIC target with the most significant relevance to HCC among PEA3 group genes. A. Analysis of TCGA database for expression levels of ETV1, ETV4, and ETV5 in normal liver (NL) and HCC samples of four different clinicopathologic stages (I, II, III, and IV). The numbers in parentheses mean the number of subjects in each group. B. Kaplan-Meier analysis of overall survival for HCC patients with high or low expression of PEA3 group genes (74 patients per each subgroup). C. qRT-PCR analysis of PEA3 group gene expression levels in control and CIC knockdown HCC cells. Experiments were performed more than three times, independently. D. Western blot analysis for levels of ETV4 in control and CIC knockdown HCC cells. E. qRT-PCR analysis for levels of Cic, Etv1, Etv4, and Etv5 in livers of 7-week-old WT and Cic LKO mice. n=6 per each genotype. F and G. qRT-PCR analysis for levels of Cic, Etv1, Etv4, and Etv5 in liver tumors harvested from the DEN-treated WT and Cic LKO mice at 8 months of age (F) and at 15 months of age (G). n=5 per each genotype. All error bars show s.e.m. *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 6. CIC deficiency-mediated promotion of HCC progression is due to derepression of ETV4. A. Western blot analysis showing ectopic expression of ETV4 in SK-HEP-1 and MHCC-97H cells. B-D. Cell growth assay (B), matrigel invasion assay (C), and transwell migration assay (D) of control and ETV4-overexpressing HCC cells. E. qRT-PCR analysis for ETV4 mRNA levels in control and CIC knockdown HCC cells transfected with either control or ETV4 siRNA. NN: control HCC cells transfected with control siRNA, N4: control HCC cells transfected with ETV4 siRNA (siETV4), CN: CIC knockdown HCC cells transfected with control siRNA, and C4: CIC knockdown HCC cells transfected with siETV4. F-H. Cell growth assay (F), matrigel invasion assay (G), and transwell migration assay (H) of control and CIC knockdown HCC cells treated with either control or ETV4 siRNA. Three independent experiments were performed. All error bars show s.e.m. *P < 0.05, **P < 0.01, and ***P < 0.001.

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