The Matricellular Protein CCN1 Suppresses Hepatocarcinogenesis by Inhibiting Compensatory Proliferation

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide, and is on the rise in the United States. Previous studies showed that the matricellular protein CCN1 (CYR61) is induced during hepatic injuries and functions to restrict and resolve liver fibrosis. Here we show that CCN1 suppresses hepatocarcinogenesis by inhibiting carcinogen-induced compensatory hepatocyte proliferation, thus limiting the expansion of damaged and potentially oncogenic hepatocytes. Consistent with tumor suppression, *CCN1* expression is down-regulated in human HCC. *Ccn1* ^{Hep} mice with hepatocyte-specific deletion of *Ccn1* suffer increased HCC tumor multiplicity induced by the hepatocarcinogen diethylnitrosoamine (DEN). Knockin mice (*Ccn1*$^{dm/dm}$) that express an integrin $\alpha_6\beta_1$-binding defective CCN1 phenocopied *Ccn1* ^{Hep} mice, indicating that CCN1 acts through its $\alpha_6\beta_1$ binding sites in this context. CCN1 effectively inhibits EGFR-dependent hepatocyte proliferation through integrin α_6-mediated accumulation of reaction oxygen species (ROS), thereby triggering p53 activation and cell cycle block. Consequently, *Ccn1*$^{dm/dm}$ mice exhibit diminished p53 activation and elevated compensatory hepatocyte proliferation, resulting in increased HCC. Furthermore, we show that a single dose of the EGFR inhibitor erlotinib delivered prior to DEN-induced injury was sufficient to block compensatory proliferation and annihilate development of HCC nodules observed 8 months later, suggesting potential chemoprevention by targeting CCN1-inhibitable EGFR-dependent hepatocyte proliferation. Together, these results show that CCN1 is an injury response protein that functions not only to restrict fibrosis in the liver, but also to suppress hepatocarcinogenesis by inhibiting EGFR-dependent hepatocyte compensatory proliferation.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide but the third leading cause of cancer-related deaths due to the lack of effective therapies ([1]). The incidence rate of HCC in the US is on the rise, with a CDC-estimated 3.5% increase annually ([2]). Being the largest organ that filters and detoxifies environmental toxins, the liver is constantly exposed to harmful chemicals and their metabolites that can cause DNA

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CONFLICT OF INTEREST

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damage and mutagenesis, leading to oncogenic initiation. Whole-exome sequencing of human HCC tumors revealed up to 121 mutational events per genome, suggesting that carcinogenesis from exposure to genotoxic agents contributes to human HCC induction ([3]). The progression of pre-neoplastic cells to HCC is facilitated by chronic liver inflammation, most commonly due to hepatitis viral infection, alcohol abuse, and metabolic disorders including obesity and type 2 diabetes ([2]). The rates of increase in obesity and type II diabetes have been particularly significant, potentially driving further increases in the development of HCC ([4]).

CCN1 (CYR61), a member of the CCN family of secreted matricellular proteins, regulates diverse cellular functions principally through engagement of distinct integrins in a cell type- and context-dependent manner ([5]). CCN1 is critical for placental angiogenesis and cardiac valvuloseptal morphogenesis during embryonic development ([6;7]). In adulthood, its expression is linked to inflammation and wound healing, and emerging data suggest that CCN1 serves a protective role in wound healing and tissue repair ([5]). For example, CCN1 functions to dampen and restrict tissue fibrosis in cutaneous wound healing by triggering cellular senescence in activated myofibroblasts, whereupon senescent myofibroblast express an anti-fibrotic phenotype ([8]). CCN1 also accelerates mucosal healing in colitis through the induction of IL-6 ([9]), and functions to limit and resolve liver fibrosis induced by cholestasis or exposure to hepatotoxin ([10]). Furthermore, recent studies have shown that CCN1 induces cholangiocyte proliferation and ductular reaction to promote biliary regeneration through integrin $\alpha_v\beta_5$-mediated activation of NFκB ([11]).

Aberrant *CCN1* expression has been associated with various types of cancer, and may either promote or inhibit the proliferation of specific cancer cells. For example, CCN1 promotes the proliferation and survival of established cell lines of breast cancer, ovarian cancer, pancreatic cancer, osteosarcoma, and glioma, and enhances their growth as tumors in xenografts ([12–16]), whereas overexpression of *CCN1* inhibits the proliferation of endometrial and lung cancer cell lines both in culture and in xenografts ([17;18]). Consistent with these observations, CCN1 is multifunctional and possesses activities that may either promote or inhibit tumor growth in a contextual manner, including the induction of angiogenesis, apoptosis, and cellular senescence ([8;19–21]). However, information on the role of CCN1 in HCC cell lines has been conflicting, with reports indicating that CCN1 either inhibits or promotes the proliferation and migration of these cells ([22;23]). To date, studies on proteins of the CCN family in cancer have focused on established cancer cell lines and xenografts, and no systematic study on CCN proteins in a carcinogenesis model has been reported.

Here we present the first evidence that CCN1 suppresses hepatocarcinogenesis induced by diethylnitrosoamine (DEN), a widely used model for HCC ([24]). When compared to several murine models of HCC, DEN-induced tumors have gene expression signatures that most closely reflect human HCC with poor prognosis ([25]), suggesting that DEN-induced tumorigenesis is an excellent model for HCC. Although DEN-induced tumors do not emerge in the context of cirrhosis, some 20% of all human HCC and up to 40% of HCC in HBV-infected individuals evolve in non-cirrhotic livers ([26;27]). The hepatocarcinogen DEN induces hepatocyte DNA damage and apoptosis, leading to compensatory proliferation of

hepatocytes, some of which may have suffered mutations and are at risk of neoplastic transformation ([28]). We show that CCN1 suppresses HCC tumorigenesis by inhibiting DEN-induced compensatory proliferation through integrin-mediated accumulation of reactive oxygen species (ROS), leading to activation of p53 and inhibition of EGFR-mediated hepatocyte proliferation. We further show that a single dose of the EGFR inhibitor erlotinib delivered prior to DEN exposure blocked compensatory proliferation and obliterated HCC formation. Our results support the notion that CCN1 is a response protein for hepatic injury that suppresses hepatocarcinogenesis through inhibition of EGFR-mediated hepatocyte compensatory proliferation.

RESULTS

CCN1 expression is deregulated in HCC

The expression of *CCN1* in human HCC was evaluated by immunohistochemical staining of tumor tissue microarrays (Fig. 1A). Analysis of tissue cores from 194 HCC patients and 17 normal donors revealed that all normal liver tissues displayed low to moderate CCN1 staining (intensity score 2–3), whereas nearly half (49%) of HCC cases showed undetectable to barely detectable CCN1 (intensity score <2). However, ~44% of HCC cases had low to moderate levels of CCN1 similar to normal livers, and 7% of cases exhibited intense CCN1 staining (intensity score >3.5). Overall, CCN1 staining was significantly lower in HCC than in normal tissues (Mann-Whitney U test, p<0.001), although there was no significant difference among HCC of various TMN grades (Fig. 1A). Meta-analysis of Oncomine databases showed that *CCN1* mRNA was down-regulated in HCC by 70–80% compared to normal or adjacent cirrhotic liver tissues in 4 independent studies (Fig. 1B)([29]–[32]). Furthermore, *CCN1* is ranked within the top 4% of the least expressed genes in HCC (median rank 318, p=5.6E-8)(Fig. 1C). Thus, *CCN1* expression is significantly down-regulated in a large percentage of human HCC as measured on both protein and mRNA levels, suggesting that CCN1 may inhibit HCC development in these cases. We noted that ~7% HCC over-expressed *CCN1* (Fig. 1A); possibly a subset of tumors may have acquired mutations that mitigate the inhibitory effect of CCN1, allowing them to benefit from potentially tumor promoting function of CCN1, such as induction of angiogenesis ([19]).

Ccn1 mutant mice suffer enhanced susceptibility to chemical hepatocarcinogenesis

To study the effects of CCN1 on the genesis of HCC, we employed a well-characterized chemical carcinogenesis model in which postnatal day-15 mice injected with a single dose of the hepatocarcinogen DEN develop HCC at a high frequency in 8 months ([28]). Two genetically altered strains of mice were analyzed: one has the *Ccn1* gene deleted in hepatocytes (*Ccn1*^{Hep}) by intercross of *Ccn1*$^{flox/flox}$ and *Alb-Cre* mice ([10]), and the other has *Ccn1* replaced by the DM allele (*Ccn1*$^{dm/dm}$), which encodes a CCN1 mutant (DM) disrupted in its integrin $\alpha_6\beta_1$ binding sites ([21]). Consistent with the hypothesis that CCN1 suppresses HCC, both *Ccn1*^{Hep} and *Ccn1*$^{dm/dm}$ mice had on average 3 times more tumors than the wild type (*Ccn1*$^{wt/wt}$) and *Ccn1*$^{flox/flox}$ control mice (t-Test, p<0.001) (Fig. 2A–C), although individual variation in tumor incidence in both *Ccn1*$^{dm/dm}$ (4–21 tumors per mouse) and *Ccn1*^{Hep} (8–23 tumors per mouse) animals were greater than in *Ccn1*$^{wt/wt}$ and *Ccn1*$^{flox/flox}$ control animals (3–6 tumors per mouse) (Fig. 2C). The average tumor

diameters were comparable among all genotypes with similar individual variations (Fig. 2D). Interestingly, CCN1 is either undetectable or found at a much lower level in tumors than the surrounding parenchyma (Fig. 2E), consistent with lower CCN1 levels in human HCC (Fig. 1A). Immunohistochemical staining for PCNA and CD31 indicated no difference in tumor cell proliferation or angiogenesis among the genotypes (Fig. 2E). These results show that CCN1 suppresses carcinogenesis leading to HCC, and suggest that CCN1 inhibits the frequency of tumor initiation and/or early promotion but not the rate of tumor growth after neoplastic transformation. Since $Ccn1^{dm/dm}$ mice phenocopied $Ccn1^{Hep}$ mice, we surmised that the role of CCN1 in HCC suppression is mediated through its $\alpha_6\beta_1$ binding sites.

Enhanced hepatocellular compensatory proliferation in *Ccn1* mutant mice

DEN is metabolized by cytochrome P450 2E1 in hepatocytes to produce alkylating metabolites that form DNA adducts, leading to genetic mutations, genomic instability, and apoptotic cell death ([33;34]). Apoptotic hepatocytes stimulate Kupffer cells to produce hepatomitogens, which in turn induce compensatory proliferation of the remaining hepatocytes, some of which may have suffered mutations and are at risk of oncogenic transformation ([28;35]). Thus, compensatory proliferation after hepatic damage is thought to promote tumorigenesis. *Ccn1* expression is highly induced in the liver in response to injuries due to hepatotoxins and cholestasis ([10]), and is also elevated upon exposure to DEN (Supplementary Fig. S1). DEN-induced hepatic apoptosis reached >6 fold in $Ccn1^{wt/wt}$ mice within 24 hrs of DEN injection and declined thereafter, but DEN-induced apoptosis was significantly reduced in both $Ccn1^{Hep}$ and $Ccn1^{dm/dm}$ mice (Fig. 3A and Supplementary Fig. S2A). This observation is expected, since CCN1 synergizes with TNF family cytokines to induce hepatic apoptosis, and apoptosis induced by TNFα or FasL is substantially reduced in $Ccn1^{dm/dm}$ mice ([21;36]). DEN injury also induced compensatory hepatocyte proliferation, occurring with a peak at 24 hrs after DEN injection and declining thereafter (Fig. 3B and Supplementary Fig. S2B). Despite decreased apoptosis, both $Ccn1^{Hep}$ and $Ccn1^{dm/dm}$ mice showed 2–3 fold increase in hepatocellular proliferation after DEN injury ($p<0.05$)(Fig. 3B). Thus, compensatory hepatic proliferation is elevated in $Ccn1^{Hep}$ and $Ccn1^{dm/dm}$ mice, consistent with the notion that compensatory proliferation, rather than apoptosis *per se*, as the driving force for increased HCC in these *Ccn1* mutant mice. These results also suggest that CCN1 activities are mediated through its integrin $\alpha_6\beta_1$ binding sites.

CCN1 inhibits hepatocyte proliferation through α_6-ROS-p38 mediated activation of p53

To assess whether CCN1 may directly regulate hepatocyte proliferation, we isolated primary hepatocytes from $Ccn1^{wt/wt}$, $Ccn1^{Hep}$, and $Ccn1^{dm/dm}$ mice. The presence of EGF, a known hepatocyte mitogen, stimulated the incorporation of BrdU 4-fold (Fig. 4A). However, addition of purified CCN1 protein abrogated EGF-induced BrdU-labeling. The DM mutant protein has completely lost this inhibitory function, whereas D125A ([37]), a CCN1 mutant protein that is unable to bind α_v integrins maintained this activity, indicating that CCN1 acts through integrin α_6 and not α_v to inhibit hepatocyte proliferation (Fig. 4A). Hepatocytes from $Ccn1^{Hep}$ and $Ccn1^{dm/dm}$ mice responded similarly to those from $Ccn1^{wt/wt}$ mice, indicating that the EGF signaling pathway and cell cycle machinery in these hepatocytes are intact. Furthermore, knockdown of integrin α_6 by siRNA in hepatocytes abolished CCN1

inhibition of EGF-induced BrdU-labeling, whereas knockdown of α_v had no effect (Fig. 4B), further establishing that CCN1 acts through α_6 but not α_v integrins to inhibit hepatocyte proliferation.

The tumor suppressor p53 is known to inhibit hepatocyte proliferation through exerting G1/S and G2/M cell cycle control ([38];[39]). Knockdown of p53 by siRNA eliminated the inhibitory effect of CCN1 on EGF-induced DNA synthesis, even though EGF alone still promoted DNA synthesis (Fig. 4C). Furthermore, siRNA knockdown of EGF receptor (EGFR) completely eliminated DNA synthesis, demonstrating that CCN1 inhibits EGFR-dependent hepatocyte proliferation (Fig. 4C). Incubation of CCN1 with primary hepatocytes increased p53 nuclear localization from being detectable in 4% to 20% of cells, showing that CCN1 itself can activate p53 in hepatocytes (Fig. 5A). This activity was abrogated in the DM mutant but not in the D125A mutant. Moreover, we isolated hepatocyte nuclear lysates and found that exposure to either the CCN1 or D125A protein increased nuclear p53 by >3-fold, whereas DM had no effect (Fig. 5B), indicating that CCN1 induces p53 nuclear localization through its $\alpha_6\beta_1$ binding sites.

One of the key signaling events induced by CCN1 is the accumulation of intracellular ROS ([21]), which can activate the stress-activated kinase p38 MAPK that, in turn, can directly phosphorylate p53 to increase its protein stability, nuclear localization, and transactivation ([40];[41]). To test whether CCN1 may activate p53 in hepatocytes through a ROS-p38 MAPK-dependent pathway, we assessed the effect of the ROS scavenger BHA. Pre-incubation of cells with BHA blocked CCN1-induced p53 nuclear localization as judged by fluorescence staining and nuclear fractionation (Fig. 5B,C), indicating a requirement for ROS. By contrast, the DNA-topoisomerase II inhibitor etoposide also activated p53 and induced p53 nuclear localization, but BHA had no effect (Supplementary Fig. S3), showing that CCN1-induced p53 activation is mediated through a ROS-specific pathway distinct from DNA damage induced by etoposide. Furthermore, treatment of hepatocytes with CCN1 increased the dually-phosphorylated, activated form of p38 in isolated nuclear lysates, and this activation was blocked by pre-incubation of cells with BHA (Fig. 5B). The DM-, but not D125A-mutation abolished the ability of CCN1 to activate p38 (Fig. 5B). Finally, siRNA knockdown of p38α, a major isoform of p38, abrogated CCN1-induced nuclear p53 localization (Fig. 5C), whereas non-targeting siRNA had no effect. These results show that CCN1 induces ROS accumulation in hepatocytes, thus activating p38 MAPK, which, in turn, mediates p53 nuclear localization.

Consistent with ROS-dependent activation of p38, CCN1 induced ROS accumulation in primary hepatocytes in ~10% of cells within 30 min. that was sustained for at least 5 hrs (Fig. 5D). This activity was abrogated in the DM but not the D125A mutant, and blocked by pre-incubation with a function-blocking mAb against integrin α_6 (GoH3)(Fig. 5E,F). Since CCN1 ligation to integrin $\alpha_6\beta_1$ can activate the small G-protein Rac1 to trigger NOX1 and NOX2 enzyme complexes to generate ROS ([8]), we further tested the involvement of Rac and NOX. Pre-incubation with NSC23766, a Rac GEF inhibitor, or apocynin, which prevents the assembly of Nox1 and Nox2 enzyme complexes, effectively blocked CCN1-induced ROS (Fig. 5F). Together, these results showed that CCN1 induces ROS accumulation in

hepatocytes through an integrin α_6-Rac-Nox-dependent pathway, which activates p38 MAPK and p53.

p53 activation in the liver upon DEN challenge is CCN1-dependent *in vivo*

Since CCN1 inhibits DNA synthesis in hepatocytes through p53 (Fig. 4C) and CCN1 enhances p53 activation in hepatocytes upon exposure to DEN in culture (Supplementary Fig. S4), we tested whether p53 is activated *in vivo* in a CCN1-dependent manner upon DEN challenge. $Ccn1^{wt/wt}$ and $Ccn1^{dm/dm}$ mice were injected with DEN, and liver protein lysates were analyzed 1–2 days thereafter. In $Ccn1^{wt/wt}$ mice DEN caused a transient ~4-fold increase in total p53 and its phosphorylation at Ser-15, a phosphorylation target of p38 MAPK that stimulates p53 transactivation ([40;41]), with concomitant increase in the p53 transcriptional target p21 within 1 day (Fig. 6A). By contrast, in $Ccn1^{dm/dm}$ mice total p53 level actually decreased by 70% and p21 level declined correspondingly (Fig. 6A). Furthermore, DEN injury in $Ccn1^{wt/wt}$ mice resulted in p53 nuclear localization in 20% of hepatocytes in the liver, compared to <3% without DEN treatment. This nuclear p53 localization was greatly reduced in $Ccn1^{dm/dm}$ mice (<8% of cells, $p<0.001$), indicating that p53 activation upon exposure to DEN is largely CCN1-dependent *in vivo*. Consistent with CCN1 action through ROS, DEN challenge led to a large increase in ROS positive cells (from <20 to ~300 cells/HPF) in C$cn1^{wt/wt}$ mice, but far fewer (<100 cells/HPF) in $Ccn1^{dm/dm}$ mice (Fig. 6C). These results show that CCN1 plays a key role in ROS accumulation and p53 activation in hepatocytes upon exposure to DEN *in vivo*.

Blockade of EGFR inhibits HCC development

Based on the observation that CCN1 efficiently blocked EGF-induced hepatocyte proliferation through p53 in culture (Figs. 4), we hypothesized that CCN1 suppresses HCC by inhibition of EGFR-dependent hepatocyte compensatory proliferation. To test this hypothesis, $Ccn1^{dm/dm}$ mice were first injected with the specific EGFR tyrosine kinase inhibitor erlotinib or vehicle control, followed by DEN injection 3 hrs later. Immunostaining for PCNA in livers collected 24 hrs thereafter showed that erlotinib completely blocked DEN-induced compensatory proliferation, reducing PCNA-positive cells from 12% to background level (<1.6%, $p<0.001$)(Fig. 7A). Thus, DEN-induced compensatory hepatocyte proliferation is dependent on EGFR-signaling. Finally, we tested whether EGFR-mediated compensatory proliferation is essential for DEN-induced hepatocarcinogenesis. Mice were given a single injection of erlotinib 3 hrs before DEN as above, and sacrificed 8 months later to assess HCC development. Remarkably, the single dose of Erlotinib reduced the median number of HCC per mouse by 30-fold, from 15 to 0.5 ($p<0.001$)(Fig. 7B). Thus, blockade of compensatory proliferation, which peaks within the first 24 hr of DEN injury, is sufficient to obliterate HCC development. Together, these results show that EGFR-dependent hepatocyte mitogenesis, which is inhibited by CCN1 through p53 activation, is required for compensatory proliferation upon DEN-induced liver damage and for the development of HCC.

DISCUSSION

Although great strides have been made in understanding the pathobiology of HCC, this disease remains recalcitrant to available therapies and one of the leading causes of cancer-related deaths. Due to the lack of efficacious treatment options, chemoprevention in high-risk groups has been proposed as an alternative strategy for reducing HCC-related mortalities ([42]). Extensive studies by many laboratories have sought to unravel the molecular basis of the tumor initiating events and the role of inflammation in HCC tumor promotion ([43]). Exposure to carcinogens such as DEN causes hepatic damage and cell death, leading to compensatory proliferation of hepatocytes, some of which may have sustained damage and may be on their way to being transformed. Thus, DEN-induced HCC serves as an excellent model in which to dissect the regulation of compensatory proliferation, a critical early event in hepatocarcinogenesis ([28;35]). In this study, we present the first evidence that CCN1 suppresses hepatocarcinogenesis by inhibiting EGFR-dependent compensatory hepatocyte proliferation through ROS-mediated activation of p53. Remarkably, a single dose of EGFR inhibitor delivered prior to DEN exposure is sufficient to prevent development of HCC (Fig. 7). These findings highlight CCN1 inhibition of EGFR-dependent compensatory proliferation as an endogenous mechanism for suppressing hepatocarcinogenesis, and a potential target for chemoprevention.

We have delineated a molecular pathway through which CCN1 inhibits hepatocyte proliferation. CCN1 engagement of integrin α_6, most likely $\alpha_6\beta_1$, induces ROS accumulation in hepatocytes through NOX, leading to activation of p38 MAPK and p53, resulting in inhibition of hepatocyte proliferation (Figs. 4,5). It is interesting to note that p53 can be activated through both ROS-dependent and independent mechanisms ([44;45]), and CCN1 can by itself trigger p53 activation through the α6-ROS-p38 axis independent of DNA damage-inducing agents (Fig. 5). The role of p38α and p53 in exerting a cell cycle block through p21 in hepatocytes has been demonstrated in the context of liver regeneration ([39]). CCN1 induces p21 concomitant with p53, whereas the CCN1-DM mutant is unable to do so (Supplementary Fig. S5). These results suggest that p38 MAPK may have a protective role against HCC, consistent with the finding that hepatocyte-specific deletion of p38α enhanced thioacetamide-induced hepatocarcinogenesis in mice ([46]). However, due to the diverse mechanisms by which p53 can suppress tumorigenesis, it is possible that CCN1-activated p53 may contribute to HCC suppression through multiple pathways in addition to inhibiting compensatory proliferation ([47]). Our results also underscore the fact that ROS is a double-edge sword that may both drive tumorigenesis and inhibit tumorigenesis ([48]). Furthermore, *Ccn1* is regulated by the Wnt pathway and can in turn modulate Wnt signaling, which is implicated in the development of HCC ([5]). However, whether the interaction between CCN1 and Wnt signaling plays a role in hepatocarcinogenesis is currently unknown.

Apoptosis of damaged cells, often mediated through p53, can suppress tumorigenesis by preventing the proliferation of cells at risk of transformation. However, cell death can also stimulate oncogenesis through activation of inflammatory responses ([35]). In hepatocarcinogenesis, hepatocyte cell death due to DEN-induced damage results in activation of Kupffer cells to express and release hepatomitogens that promote compensatory

proliferation and HCC ([28]). We have previously shown that CCN1 can promote hepatic apoptosis in experimental hepatitis induced by concanavalin A, CD95-activating antibody, or alcohol gavage by acting in synergy with TNFα and FasL pathways ([21;49]). Thus, it is not surprising that $Ccn1^{Hep}$ and $Ccn1^{dm/dm}$ mice showed reduced apoptosis upon DEN-induced injury (Fig. 3). However, despite reduced apoptosis, these mutant mice exhibited enhanced compensatory proliferation, which is the driver of tumorigenesis. These findings suggest that CCN1 is a powerful inhibitor of hepatocyte proliferation and removing CCN1-mediated inhibition results in enhanced proliferation, overriding any shortfall in mitogenesis that may result from reduced apoptosis. Furthermore, reduced apoptosis in $Ccn1^{dm/dm}$ mice may also contribute to enhanced oncogenesis by allowing the proliferation of potentially damaged cells. Thus, CCN1 may suppress HCC through both apoptotic and growth inhibitory mechanisms to prevent tumor promotion in the very early stages of oncogenesis. It is interesting to note that heterozygosity of p53 in rats is able to reduce both DEN-induced hepatic apoptosis and compensatory proliferation, resulting in reduced hepatocarinogenesis ([50]). These results are consistent with a strong correlation between compensatory proliferation and hepatocarcinogenesis, and suggest that p53 heterozygosity is sufficient to reduce p53-dependent apoptosis but is not sufficient to obviate CCN1-mediated inhibition of hepatocyte proliferation.

The EGFR signaling pathway is often deregulated in epithelial cancers, and EGFR antagonists have been approved for the treatment of non-small cell lung cancer, pancreatic cancer, colorectal cancer, and squamous cell carcinoma ([51]), although clinical response in HCC patients is limited ([52]). In mouse models, EGFR inhibitors reduced HCC nodules by 4–5 fold when delivered daily for a total of 6 weeks beginning 12–13 weeks after initial DEN injection ([53;54]). Here we show that a single dose of erlotinib given 3 hrs before DEN injection strongly inhibited compensatory proliferation and reduced HCC nodules by 30-fold (Fig. 7). To our knowledge, this is the first demonstration that one dose of EGFR inhibitor is chemopreventive for DEN-induced HCC. Since erlotinib is metabolize quickly with a terminal half-life of 3.1 hrs in mice ([55]), these results allow us to conclude that EGFR-dependent compensatory proliferation occurring within a short period following DEN exposure, which is strongly inhibited by CCN1, plays an essential role in HCC development. These findings suggest that CCN1-regulated EGFR-dependent compensatory proliferation may be a potential target for chemoprevention.

We have previously shown that CCN1 is highly induced in human cirrhotic livers and upon hepatic injury in mice, and CCN1 functions to limit and resolve liver fibrosis resulting from exposure to the hepatotoxin carbon tetrachloride or cholestasis due to bile duct ligation by triggering cellular senescence in activated myofibroblasts ([10]). Senescent myofibroblasts express an anti-fibrotic phenotype that includes expression of matrix metalloproteinases, and delivery of CCN1 accelerates fibrosis resolution even in mice with established fibrosis ([10]). Our current study revealed that CCN1 can also suppress hepatocarcinogenesis by inhibiting compensatory hepatocyte proliferation. These findings indicate that CCN1 functions as an injury response protein in the liver that not only limits and resolves inflammation-induced liver fibrosis, but also inhibits the genesis of liver cancer.

MATERIALS AND METHODS

Mice and HCC Induction

$Ccn1^{dm/dm}$ and $Ccn1^{flox/flox}$ mice were generated in svJ129-C57BL/6 mixed background and backcrossed >10 times into C57BL/6 background ([8,10,21]). $Ccn1^{Hep}$ mice were generated by intercross of $Ccn1^{flox/flox}$ and Alb-Cre mice (Jackson Laboratory)([10]). To induce HCC, male pups at 15 days old were injected i.p. with DEN (25 mg/Kg body weight) and sacrificed for analysis 8 months later. For $Ccn1^{wt/wt}$ mice, 11 animals were used (n=11); $Ccn1^{dm/dm}$, n=10; $Ccn^{flox/flox}$, n=7; $Ccn1^{Hep}$, n=11. Where indicated, mice were injected i.p. with Erlotinib [N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine hydrochloride, 50 mg/Kg body weight] 3 hrs before DEN injection. Healthy mice bred and maintained in our facility were randomly segregated for all experiments, and all were processed and included in final analysis since there was no observable sign of disease or abnormality during experiments that would have caused exclusion from analysis. No special animal randomization was needed. The investigator was not blinded to mice genotype allocation during the experiment. All animal procedures including estimation for numbers of animals used (Mead's resource equation, based on two components, control & DEN-treated, and 5% critical value of Student's t-Test) were approved by the University of Illinois Animal Care Committee.

Human HCC Tissue Microarray and Histology

HCC tissue microarray (US Biomax, #LV20812 and LV2161) were processed according to manufacturer's protocol. Mouse liver tissues were formalin-fixed, paraffin-embedded, and sectioned (6-μm) before immunostaining with CCN1 antibodies ([10]), mouse anti-PCNA monoclonal antibody (mAb; PC-10, AbCam #ab29), mouse anti-p53 mAb (PAb 240, AbCam #ab26), or rabbit polyclonal anti-CD31 (AbCam #ab28364). Secondary antibodies recognizing mouse and rabbit IgG (GE Healthcare #NA931 and NA934) were conjugated to horseradish peroxidase, using 3,3′-diaminobenzidine (DAB) as chromogen (brown). Samples were counterstained with hematoxylin.

Hepatocyte isolation and BrdU-labeling

Mouse hepatocytes were isolated and cultured as described ([56]). For BrdU incorporation assay, cells were incubated with 0.1 mM BrdU at 37°C for 3 hrs, fixed and stained with mouse anti-BrdU mAb (clone BU-1, Millipore #MAB3510). After further staining with fluorescent (Alexa-488) secondary antibody recognizing mouse IgG (Invitrogen #A11029) and counterstaining with DAPI, samples were examined by fluorescent microscopy.

ROS detection

Intracellular ROS was determined by fluorescent microscopy using dihydrocalcein AM (DHC-AM 10 μM in PBS, Invitrogen #D23805), which turns fluorescent green when oxidized, as described ([36]). ROS-positive cells were counted in 5-randomly selected HPFs and numbers were normalized by total cells counted. To assess ROS in liver tissues ([28]), flash frozen samples in OCT were cryo-sectioned to 20 μm and immediately incubated with

dihydroethidium (5 μM in PBS) at 37°C for 10 mins followed by examination by fluorescent microscopy.

RNA Interference

Hepatocytes were transfected with 10 nM targeting siRNA or a non-targeting control (Integrated DNA Technologies) with Lipofectamine RNAiMAX (Invitrogen #13778-075). Cells were used for experiments 2 days after knockdown. The sequences (5′ to 3′) used are: CCN1, TAACTCATTGTTTCTCGTTAACTCCAC; EGFR, GCTTTGTTCTGCCACGTATTATTTCTA; p38α, CACCAGGTACACGTCATTGAATTCCTC; p53, AGGCTGATATCCGACTGTGACTCCTCC; integrin α6, AAAGGGTAACATCACCTTCTATTGCAC; integrin αv, GTCATATTTAGATATGATTTCTGCCAC.

Proteins and reagents

Recombinant CCN1 protein (wild-type, DM and D125A mutants) were produced in insect cells using a baculovirus system and purified as described ([8]). Immuno-blotting employed rabbit polyclonal anti-phospho-p53 (Ser15) and mAb against phospho-p38 MAPK (Thr180/ Tyr182, D3F9), lamin A/C (4C11), and cytochrome c (D18C7) were from Cell Signaling Technology. Rabbit anti-EGFR (EP38Y) mAb was from AbCam. Rat anti-integrin α_6 mAb (GoH3) was from Beckman Coulter.

Statistical analysis

The difference of CCN1 immunohistochemical staining intensities in human HCC tissue microarray was analyzed by non-parametric Mann-Whitney U-test with no adjustment for multiple comparisons. All other experiments, including the numbers and diameter of tumors in mice, were analyzed by unpaired two-tail t-Test, with data variation showing a normal distribution. All experiments were repeated three times with similar results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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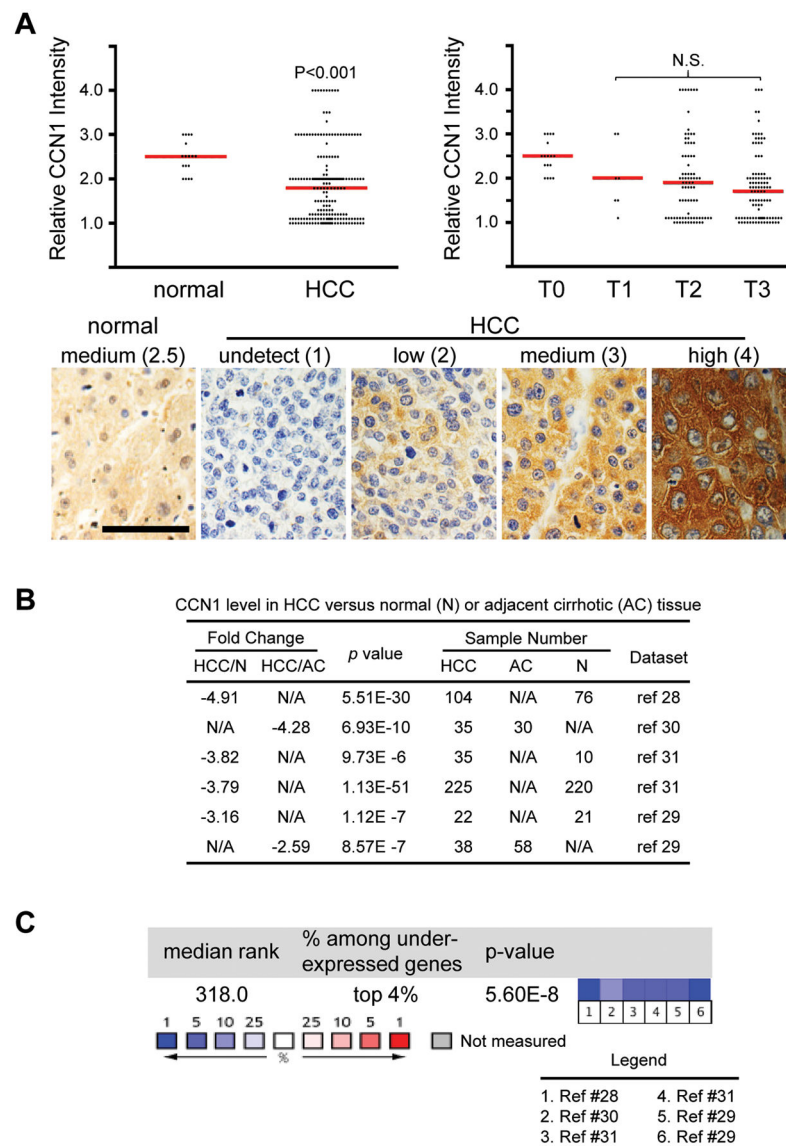
Figure 1. CCN1 is down-regulated in human HCC tissue

A, human HCC tissue microarray was stained with anti-CCN1 antibodies (brown) and counter stained with hematoxylin (blue). CCN1 staining intensity was graded as undetectable (1), low (2), moderate (3), and high (4) and expressed by scatter plots. Difference between normal vs. HCC, and different grades of HCC (T1 to T3) was analyzed by Mann-Whitney U-test. Median value of each group was shown as a red line. Bar: 50 μm. B, table summarizing *CCN1* mRNA expression analysis in human HCC tissues in Oncomine database. N: normal tissue; AC: tumor adjacent cirrhotic tissue. C, meta-analysis of the six data sets in Oncomine.
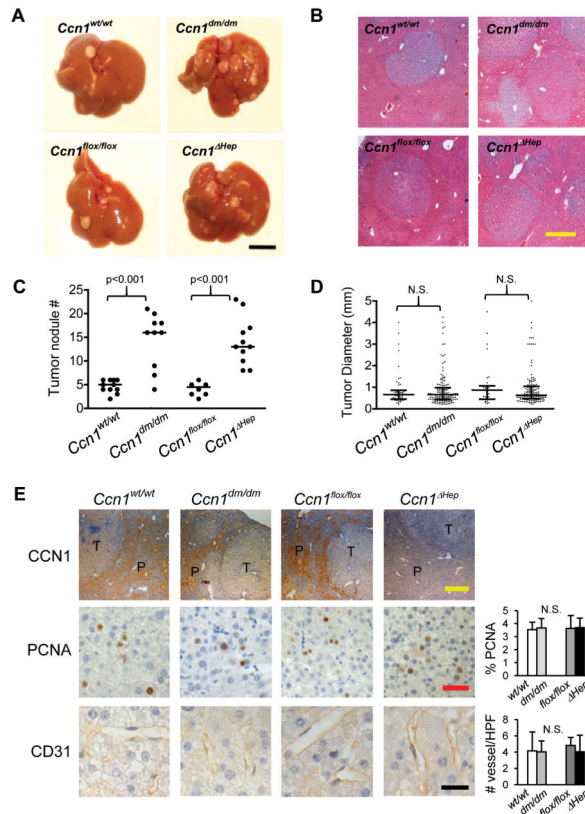
Figure 2. Increased DEN-induced HCC in *Ccn1*^{Hep} and *Ccn1*^{dm/dm} mice

A, mouse livers 8 months after DEN injection, showing surface HCC nodules. Bar: 1 cm. B, H & E stained liver tissue sections showing tumor nodules. C, number of tumor nodules in left lateral lobe of each mouse was counted (2 cm^2) and plotted. Horizontal lines show median tumor number per mouse in each group, p<0.001; unpaired t-test. D, diameter of each tumor nodule from every mouse was measured and plotted. Data shown include 51 tumors in *Ccn1*$^{wt/wt}$ mice, 145 tumors in *Ccn1*$^{dm/dm}$ mice, 33 tumors in *Ccn1*$^{flox/flox}$ mice, and 156 tumors in *Ccn1*^{Hep} mice. Horizontal lines represent median and sub-median tumor diameters. N.S., no statistical difference. E, liver tissue sections immunostained (brown) for CCN1, PCNA, and CD31, and counter stained with hematoxylin (blue). N=5 for all genotype groups with all tumor nodules examined in every mouse, and pictures shown are representative for each group. P, parenchyma; T, tumor. Yellow bar: 0.5 mm, red bar: 50 μm, black bar: 20 μm.
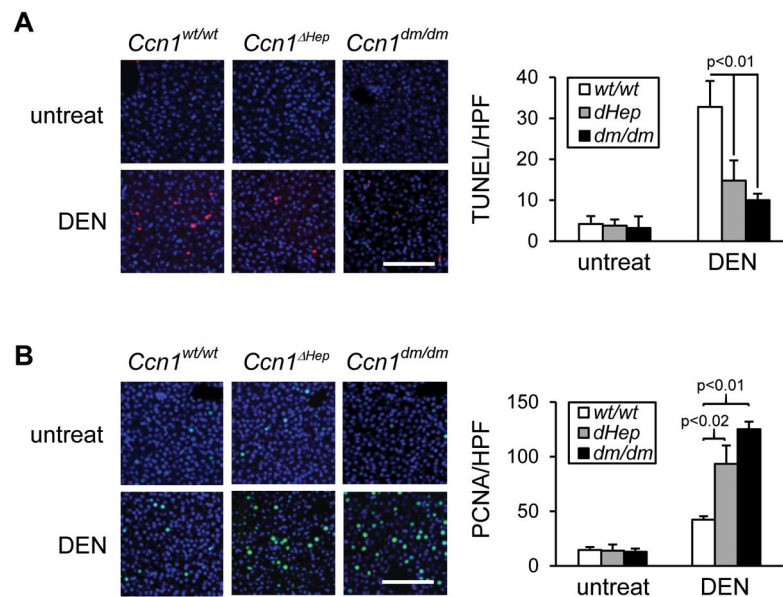
Figure 3. CCN1 regulates hepatocyte proliferation and apoptosis after DEN injection
Male mice (15 days old) were injected with DEN and sacrificed after 1 day and examined for liver apoptosis and cell proliferation. A, livers were sectioned and stained for TUNEL (red) and DAPI (blue). Apoptotic cells were counted in 5 random high power fields for each mouse, and average value expressed as mean ± s.d. (n=3 for each group). Experiment was repeated 3X with similar results. B, liver sections were immunostained for PCNA (green) and with DAPI. PCNA-positive cells were counted as above. Bar: 100 μm.
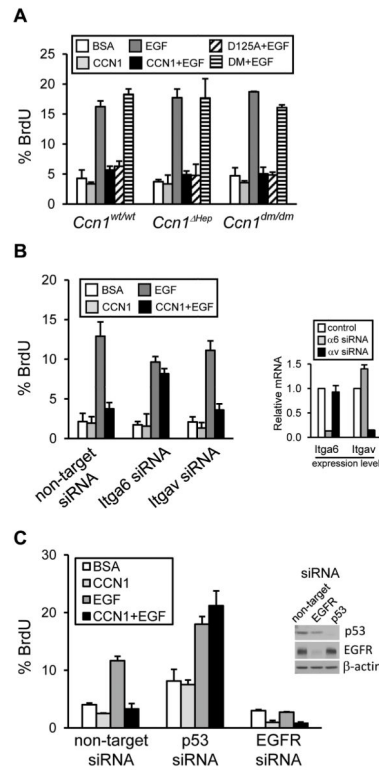
Figure 4. CCN1 inhibits hepatocyte DNA synthesis through integrin α_6 and p53

A, freshly isolated hepatocytes from adult $Ccn1^{wt/wt}$, $Ccn1^{\Delta Hep}$ and $Ccn1^{dm/dm}$ mice were incubated with purified wild type, DM, and D125A CCN1 proteins (10 μg/ml each) with or without EGF (0.1 μg/ml) at 37°C for 2 days, and BrdU incorporation determined. Percentage of BrdU-positive cells were counted in 5 random HPF and presented as mean ± s.d. of triplicate determinations. Experiments were repeated three times with similar results. B, hepatocytes from $Ccn1^{wt/wt}$ mice were incubated with siRNAs targeting integrin α_6 or α_v subunits, or a non-targeting control, and treated with CCN1, EGF, or both, and BrdU incorporation assessed as above. Knockdown was confirmed by qPCR of the target integrin mRNA (right). C, p53 and EGFR expression was knocked-down by siRNAs in hepatocytes (inset), and cells were further incubated with CCN1 with or without EGF, and BrdU incorporation assayed as above.
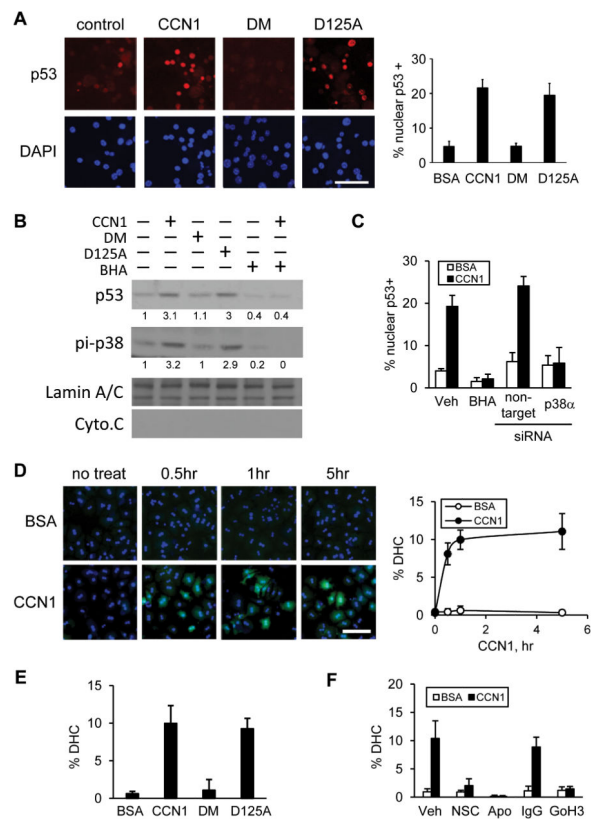
Figure 5. CCN1 induces p53 nuclear localization through ROS and p38 MAPK

A, primary hepatocytes were incubated with CCN1, DM, or D125A (10 μg/ml) for 1 day, then probed with anti-p53 antibody and counter stained with DAPI. Cells with nuclear p53 staining were counted in 5 random HPF, and shown as mean ± s.d. of triplicate determinations. B, cells were incubated with wild type or mutant CCN1 proteins with or without BHA (0.1 mM) for 1 day. Isolated nuclear lysates were electrophoresed and probed with antibodies against p53, dually phosphorylated (Thr180/Tyr182) p38 MAPK, lamin A/C, and cytochrome C. C, hepatocytes were incubated with BHA (0.1 mM) or siRNA against p38α, with or without CCN1 (10 μg/ml) for 1 day before being stained for p53 localization as above. D, hepatocytes were incubated with BSA or CCN1 (10 μg/ml) for times indicated before being loaded with DHC-AM and counterstained with Hoechst 33342 (blue). Cells positive for ROS (green) were counted in 5 random HPF and shown as mean ± s.d. from triplicate determinations. E, cells were incubated for 1 hr with BSA, WT, DM and D125A CCN1 (10 μg/ml each), loaded with DHC-AM, and examined for ROS as above. F, hepatocytes were pre-incubated with NSC23766 (NSC, 0.1 mM), apocynin (0.3 mM), or GoH3 (0.1 mg/ml) for 30 mins before addition of CCN1 for 1 hr and assay for ROS. Control cells were pre-incubated with vehicle (DMSO) or non-immune rat IgG.
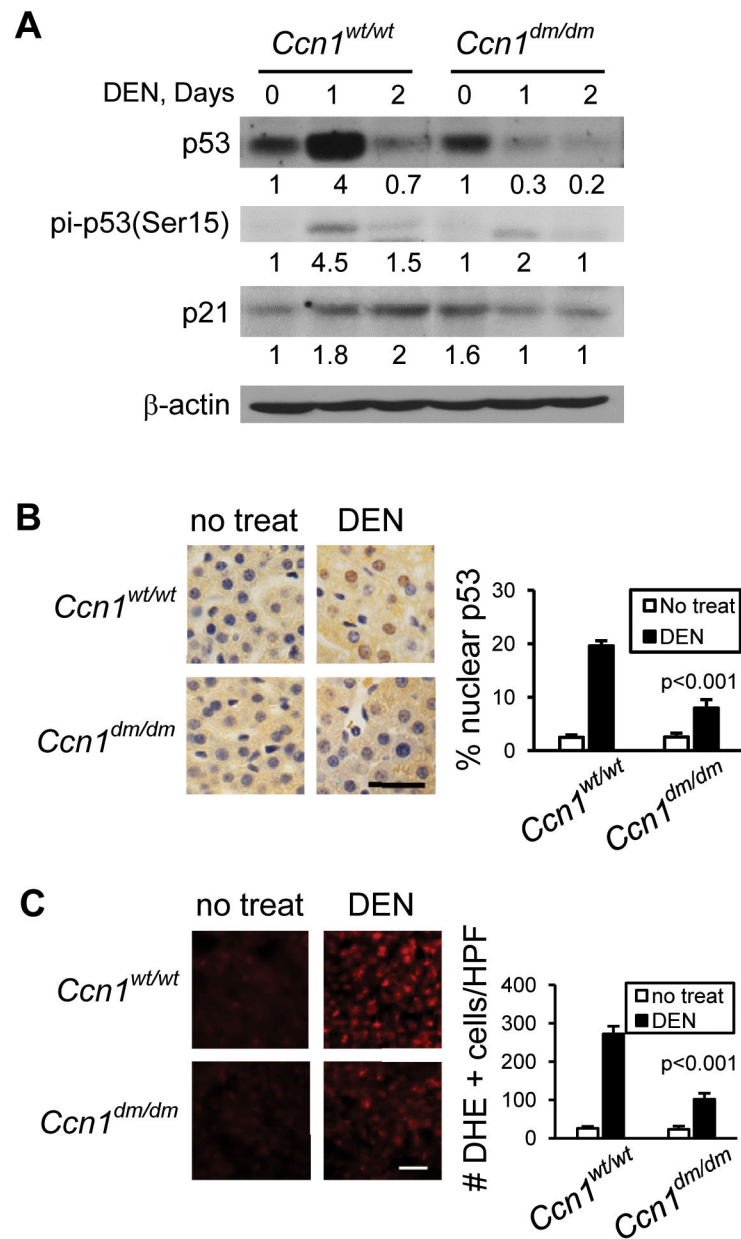
A

B

C

Figure 6. CCN1 regulates p53 activation and ROS production *in vivo*

A, total liver protein lysates from $Ccn1^{wt/wt}$ and $Ccn1^{dm/dm}$ mice (n=3 for each group) 1 or 2 days after DEN injection were probed with antibodies against total and Ser-15 phosphorylated p53, p21, and β-actin by Western blotting. Band intensity was normalized against β-actin. B. Mouse livers collected 1 day after DEN injection were sectioned and immunostained for p53 (brown) and counterstained with hematoxylin (blue). Bar: 50 μm. Cells with nuclear p53 localization were counted in 5 random HPF and expressed as mean ± s.d. (n=3; right). C. mouse livers collected 1 day after DEN injection were incubated with dihydroethidium at 37°C for 5 min. and examined by fluorescence microscopy. Cell nuclei with ethidium staining were counted from 5 random HPF, and expressed as mean ± s.d. (n=3; right). Bar: 50 μm.
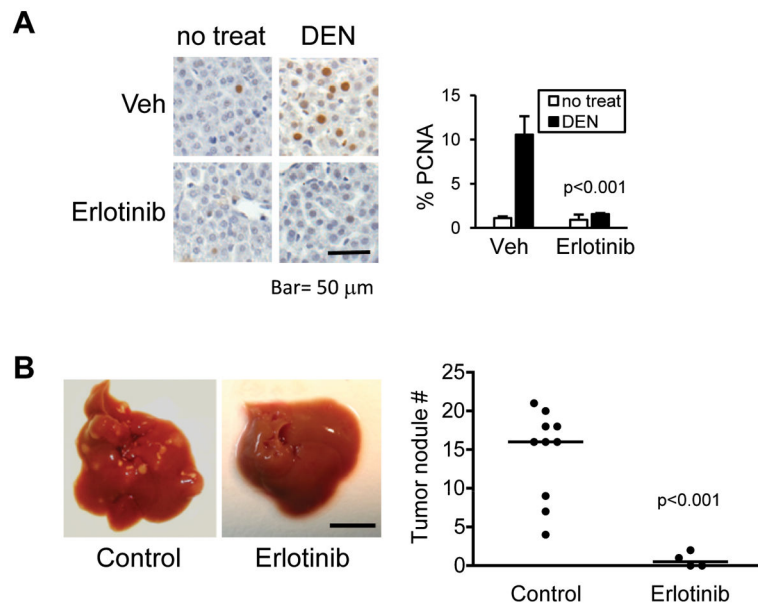
Figure 7. Erlotinib inhibits DEN-induced hepatocyte compensatory proliferation and HCC
A, *Ccn1*^dm/dm mice (n=3) were injected with erlotinib (50 mg/Kg) 3 hrs before DEN
injection, and livers were collected 1 day thereafter. PCNA positive cells were counted after
immunostaining in 5 random HPFs (right). Bar=50 µm. B. *Ccn1*^dm/dm mice were treated
with erlotinib and DEN as above, and livers were collected and examined for tumors 8
months later. Numbers of HCC were counted in tissue sections (2 cm^2) of each mouse
(right). Horizontal lines show median tumor number per mouse: control mice without
erlotinib, 15 (n=10); erlotinib treated, 0.5 (n=4). p<0.001; unpaired t-test. Bar=1 cm.