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Loss of Cyclin E1 attenuates hepatitis and hepatocarcinogenesis in a mouse model of chronic liver injury

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Abstract

Chronic liver injury triggers liver fibrosis and hepatocellular carcinoma (HCC) the third leading cause of cancer-related mortality. Cyclin E1 (CcnE1, formerly designated Cyclin E) is a regulatory subunit of the Cyclin-dependent kinase 2 (CDK2). It is overexpressed in approximately 70 % of human HCCs correlating with poor prognosis, while the relevance of its orthologue Cyclin E2 (CcnE2) is unclear. Hepatocyte-specific deletion of NF-kappa-B essential modulator ($NEMO^{\Delta hepa}$) leads to chronic hepatitis, liver fibrosis and HCC as well as CcnE up-regulation. To this end, we generated $NEMO^{\Delta hepa}/CcnE1^{-/-}$ and $NEMO^{\Delta hepa}/CcnE2^{-/-}$ double knockout mice and investigated age-dependent liver disease progression in these animals. Deletion of CcnE1 in $NEMO^{\Delta hepa}$ mice decreased basal liver damage and reduced spontaneous liver inflammation in young mice. In contrast, loss of CcnE2 did not affect liver injury in $NEMO^{\Delta hepa}$ livers pointing to a unique, non-redundant function of CcnE1 in chronic hepatitis. Accordingly, basal compensatory hepatocyte proliferation in $NEMO^{\Delta hepa}$ mice was reduced by concomitant ablation of CcnE1, but not after loss of CcnE2. In aged $NEMO^{\Delta hepa}$ mice, loss of CcnE1 resulted in significant reduction of liver tumor-igenesis, while deletion of CcnE2 had no effect on HCC formation.

Conclusion: CcnE1, but not its orthologue CcnE2 substantially contributes to hepatic inflammatory response, liver disease progression and hepatocarcinogenesis in $NEMO^{\Delta hepa}$ mice.

Keywords

NEMO; HCC; CcnE1; CDK

Introduction

Hepatocellular carcinoma (HCC) represents the third leading cause of cancer-related mortality with increasing incidence and poor prognosis.[1] In general, HCC develops as a

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consequence of chronic hepatitis and continuous cycles of inflammatory cell death and compensatory proliferation, eventually resulting in genomic aberrations and instability of hepatocytes. Pre-malignant hepatocytes typically accumulate oncogenic mutations in cell cycle-related signaling molecules such as c-myc, p53, Rb and several cyclins.[2] For instance, several independent studies showed, that Cyclin E1 (CcnE1, formerly designated Cyclin E) is overexpressed in approximately 65–70% of human HCCs[3, 4] which was associated with poor prognosis.

CcnE1 and its orthologue Cyclin E2 (CcnE2) are the canonical regulatory subunits of Cyclin-dependent kinase 2 (CDK2). Activity of the CcnE/CDK2 kinase complex peaks at G1-/S-phase transition,[5] pointing to an important role of both E-type cyclins and CDK2 in this process. Surprisingly, single genetic inactivation of either CcnE1, CcnE2 or CDK2 in mice does not affect overall development, viability or organ homeostasis besides sterility in CDK2 and CcnE2 knockout mice,[6–8] indicating a high functional redundancy of interphase cyclins and CDKs for general cell cycle regulation.

Mice simultaneously lacking CcnE1 and CcnE2 are unable to undergo normal embryonic development and die during embryonic development at day 11.5. However, this is due to a failure of endoreplication in trophoblast giant cells affecting placenta development, but not related to impaired cell cycle progression. Accordingly, CcnE1/E2 double-deficient murine embryonic fibroblasts are able to proliferate, but are incapable to re-enter the cell cycle from starvation-induced quiescence and are also resistant to oncogenic transformation. This suggests that overall CcnE-activity is essential for the transition from quiescence into the active cell cycle, whereas CcnE1 and CcnE2 share at least some overlapping functions.[6] Besides its canonical function as a regulator of CDK2 activity, recent studies demonstrated CDK2-independent functions of E-cyclins. For instance, E-cyclins facilitate the licensing of replication origins in a CDK2-independent manner.[9, 10]

In the liver, we recently identified unique functions of CcnE1 and CcnE2 in liver regeneration and liver fibrosis. Following partial hepatectomy in mice, lack of CcnE1 had only a minor effect on hepatocyte proliferation and liver regeneration, while CcnE2$^{-/-}$ livers showed enhanced CcnE1 expression, accelerated and sustained DNA synthesis and hepatomegaly, defining CcnE2 as a negative regulator of CcnE1.[11] Moreover, we demonstrated that CcnE1 is essential for activation, proliferation and survival of hepatic stellate cells during liver fibrosis initiation. Accordingly, CcnE1$^{-/-}$ mice are protected from CCl_4-mediated liver fibrogenesis, whereas CcnE2$^{-/-}$ mice displayed even accelerated fibrogenesis.[12]

The role of Cyclin E during hepatocarcinogenesis was investigated in a study of Pok et al. (2013),[13] were they used dysplastic hepatocytes and HCCs obtained from DEN treated mice. Their finding shows that Cyclin E is expressed in dysplastic liver and HCCs at the time when wild-type p53 expression and function in livers was disrupted. By performing knockdown experiments in primary HCC cells, they were able to show that Cyclin E was responsible for regulating wild-type p53. Cyclin E - knockdown increased p53 and p21, diminished anti-apoptotic Bcl-XL and reduced cell viability. Whereas, blocking p53 enhanced Cyclin E, Bcl-XL expression and increased proliferation. p53 can induce the

expression of microRNA-34 (miR-34) and it is known that ectopic miR-34 expression recapitulates the biological effects of p53 on growth arrest and apoptosis by inhibition of Cyclin E. The study of Pok et. al. (2013) suggests that Cyclin E and p53 inversely regulate each other's expression very likely via the intermediary miR-34.

We recently established a novel mouse model of inflammatory hepatocarcinogenesis by deleting the NF-kappa-B essential modulator (NEMO) specifically in hepatocytes ($NEMO^{\Delta hepa}$).[14, 15] $NEMO^{\Delta hepa}$ mice display a complex phenotype with spontaneous liver apoptosis, compensatory hepatocyte proliferation, steatohepatitis, fibrosis and HCC as end stage disease, thereby perfectly reflecting chronic liver disease progression in humans.[16]

The aim of the present study was to explore the role of CcnE1 and CcnE2 *in vivo* for chronic liver disease progression and hepatocarcinogenesis. Hence, we deleted CcnE1 or CcnE2 in $NEMO^{\Delta hepa}$ mice. We provide evidence of a unique function of CcnE1 for inflammatory disease progression and hepatocarcinogenesis, and demonstrate that CcnE2 is dispensable for inflammatory HCC development in $NEMO^{\Delta hepa}$ mice.

Results

E-Type Cyclins are over-expressed in $NEMO^{\Delta hepa}$ livers

We analyzed CcnE1 and CcnE2 expression in livers of 8 week old mice by qRT-PCR. CcnE1 was significantly over-expressed in $NEMO^{\Delta hepa}$ compared with $NEMO^{f/f}$ livers (Figure 1A). $NEMO^{\Delta hepa}$ /$CcnE2^{-/-}$ livers also showed higher CcnE1 mRNA levels but these were significantly lower compared to $NEMO^{\Delta hepa}$ livers (Figure 1A). However, CcnE2 mRNA was significantly up-regulated in $NEMO^{\Delta hepa}$/$CcnE1^{-/-}$ livers in comparison to $NEMO^{\Delta hepa}$ livers (Figure 1B). Interestingly, comparing the CcnE1 and CcnE2 RT-PCR results revealed that $NEMO^{\Delta hepa}$ livers predominantly over-express CcnE1 (Figure 1A, B).

To assess the impact of E-type cyclins for cell proliferation, we performed Ki67 staining. In all strains, hepatocyte-specific NEMO deletion triggered increased spontaneous cell proliferation in the liver (Figure 1C). At 8 weeks of age $NEMO^{\Delta hepa}$/$CcnE2^{-/-}$ mice revealed a higher proliferation rate compared to $NEMO^{\Delta hepa}$/$CcnE1^{-/-}$ mice, while $NEMO^{\Delta hepa}$ and $NEMO^{\Delta hepa}$ /$CcnE1^{-/-}$ mice showed the same basal proliferation (Figure 1C). Of note, the liver/body weight ratio was identical in all groups including controls at this age (Figure 1D).

Loss of CcnE1 attenuates overall liver injury and inflammation in $NEMO^{\Delta hepa}$ animals

Liver injury was assessed by measuring Alanine Aminotransferase (ALT) levels in the serum of the animals. In agreement with our previous reports, ALT activity was substantially elevated in $NEMO^{\Delta hepa}$ mice (Figure 2A), and ablation of CcnE2 in these animals had no effect on overall liver injury. In contrast, constitutive deletion of CcnE1 in $NEMO^{\Delta hepa}$ mice resulted in approximately 50% reduction of ALT activity hinting at a substantially improved liver injury due to lack of CcnE1.

Hepatic inflammation was further analyzed by immunostaining and qRT-PCR quantification of the pro-inflammatory factors CD45, TNFα, CCR2 and F4/80. These measurements

confirmed that ablation of CcnE1 acts anti-inflammatory in NEMO$^{\Delta hepa}$ livers showing reduced infiltration of CD45-positive and F4/80-positive leukocytes (Figure 2B,C, Supplementary Figure 1) in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ mice compared to NEMO$^{\Delta hepa}$ animals. Reduced inflammation in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ livers was further confirmed by RT-PCR and immunohistochemical staining for TNFα (Figure 2D,E) and by RT-PCR for CCR2 (chemokine receptor 2) (Figure 2F). In contrast, deletion of CcnE2 did not mediate an obvious protective effect in NEMO$^{\Delta hepa}$ mice, but even resulted in further elevated TNFα mRNA expression and accumulation of TNFα positive non-parenchymal cells (NPCs) compared to all controls (Figure 2D).

CcnE1 deletion attenuates liver cell proliferation in NEMO$^{\Delta hepa}$ mice during intermediate disease progression

Earlier studies indicated that livers of NEMO$^{\Delta hepa}$ mice at the age of 26 weeks represent a stage of intermediate disease progression comprising liver fibrosis and premalignant liver modifications. We thus investigated and compared hepatic proliferation in 26 week old NEMO$^{\Delta hepa}$ mice lacking either CcnE1 or CcnE2. Phosphorylation of histone H3 (pH3) is an established marker of mitosis. Immunofluorescence staining of pH3 in liver tissue revealed significantly more mitotic cells in NEMO$^{\Delta hepa}$ and NEMO$^{\Delta hepa}$ /CcnE2$^{-/-}$ livers compared to NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ livers (Figure 3), indicating that CcnE1 plays a unique role for spontaneous cell cycle activation and proliferation-related disease progression in NEMO$^{\Delta hepa}$ livers.

Hepatocarcinogenesis is ameliorated in the absence of CcnE1 expression

The previous experiments suggested a unique function of CcnE1 for early inflammatory response, overall liver injury and cell proliferation in the NEMO$^{\Delta hepa}$ model. We next assessed the role of CcnE1 for liver tumor formation and progression in 52 week old NEMO$^{\Delta hepa}$ mice. Deletion of CcnE1 resulted in a significant reduction of tumor numbers (a measure of tumor initiation, Figure 4A, B) and tumor size (indicating tumor progression, Fig. 4C) in NEMO$^{\Delta hepa}$ mice. Of note, NEMO$^{\Delta hepa}$ /CcnE1$^{-/-}$ livers still revealed multiple regenerative nodules (Fig. 4A), which are typical for the NEMO$^{\Delta hepa}$ model. In sharp contrast, we did not observe significant changes in tumor formation due to ablation of CcnE2 (Figure 4A-C). These findings were confirmed by determining total liver weights (Figure 4D) and liver weight indices (Figure 4E) of all experimental animals. Accordingly, absolute and relative weights of NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ livers were significantly reduced compared to both reference groups supporting the lower tumor load due to lack of CcnE1.

A recent study showed that heat shock protein 70 (hsp70) is a molecular marker for early HCC stages.[17] In order to characterize the tumorous liver tissue areas, we thus performed immunostaining for hsp70. In agreement with the previous data, liver tissue derived from NEMO$^{\Delta hepa}$ and NEMO$^{\Delta hepa}$/CcnE2$^{-/-}$ mice contained substantially larger areas of hsp70 positive cells compared to NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ mice (Figure 5).

Hepatocyte-specific deletion of CDK2 does not affect hepatocarcinogenesis in NEMO$^{\Delta hepa}$ mice

CcnE1 and CcnE2 are the prototypical regulatory subunits of CDK2. Hence, we investigated if the oncogenic effects of CcnE1 in NEMO-deficient hepatocytes were CDK2-dependent. Therefore, we generated NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ animals and analyzed the liver phenotype in 52 week old mice. Macroscopic analysis revealed a similar tumor formation of NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ livers compared to NEMO$^{\Delta hepa}$ mice (Figure 6A, compare Figure 4A). Further quantitative analysis of tumor initiation (Figure 6B) and tumor progression (Figure 6C) confirmed that deletion of CDK2 in hepatocytes does not affect overall liver tumor formation in NEMO$^{\Delta hepa}$ mice. In agreement, liver/body weight ratios were not significantly different between both groups (Figure 6D). Additionally, no significant differences in serum transaminases were found between NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ and NEMO$^{\Delta hepa}$ mice (Figure 6E, F).

We therefore tested CDK2 kinase activity during disease progression in NEMO$^{\Delta hepa}$ mice. Interestingly, we only detected elevated CDK2 kinase activity in 8-week old NEMO$^{\Delta hepa}$ mice representing the early inflammatory phase of disease progression, while CDK2 kinase was at best slightly above background in 52-week old animals. However, at this later stage comprising advanced liver tumors, we detected enhanced CDK1 kinase activity (Figure 6G).

Altogether the data suggest that the basal inflammation in NEMO$^{\Delta hepa}$ mice is associated with CDK2 kinase while in older mice in this model there is less CDK2 but increased CDK1 activation.

Discussion

The understanding of the molecular mechanisms involved in chronic liver diseases and HCC development is crucial, in order to develop novel therapeutic treatment options. Cyclin E1 (CcnE1, formerly designated Cyclin E) is overexpressed in approximately 65–70% of human HCCs[3, 4] and thus, understanding its functional role might have therapeutic implications.

An earlier study with HCC cell lines overexpressing the oncogene CcnE demonstrated that a siRNA approach reduced CcnE over-expression by 90 %.[4] This resulted in enhanced apoptosis of HCC cells and their proliferation was blocked (4). Additionally, when these cells were injected into nude mice, siRNA treatment also resulted in reduced growth of these cells *in vivo*.[4] These results demonstrate an essential role of Cyclin E for HCC growth. However, this initial siRNA approach targeted CcnE1 at a site, which has high homology with CcnE2 (data not shown) as CcnE2 was only discovered at this time.[18] Therefore, in their work the authors could not distinguish between the specific function of either CcnE1 or CcnE2 for the growth of HCC.

Therefore, the aim of the present study was to better define the role of CcnE1 or CcnE2 for the growth of HCC. As a model we used NEMO$^{\Delta hepa}$ mice as inactivation of NEMO is frequently found in human HCCs.[1] Hence the NEMO$^{\Delta hepa}$ model represents an interesting approach to study human-related mechanisms of chronic liver disease progression *in vivo*.

Our former studies showed that in 8 week old NEMO$^{\Delta hepa}$ livers a continuous apoptotic response triggers hepatocyte proliferation, which leads to chronic inflammation and consequently disease progression. These mechanisms are associated with an up-regulation of different cell cycle regulators.[14–16] Thus, we first aimed to differentiate between the relevance of both E-type cyclins for disease progression in this model and generated NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ and NEMO$^{\Delta hepa}$/CcnE2$^{-/-}$ animals.

Interestingly these *in vivo* experiments showed that CcnE1 but not CcnE2 is essential to determine disease progression in this model. The animals had less and smaller HCCs. These results suggest that CcnE1 is involved in tumor initiation and tumor progression in hepatocytes. We conclude that CcnE1 comprises a unique, non-redundant function for malignant hepatocyte transformation and growth, as our earlier results using the model of partial hepatectomy demonstrated that CcnE1 is dispensable for proliferation of non-malignant hepatocytes after tissue loss.[11]

One important question that was not addressed in the present study is the tissue-specific relevance of Cyclin E1 for tumor growth. Obviously besides hepatocytes other cells are relevant for HCC progression. However, from the initial experiments targeting Cyclin E via a siRNA approach specifically in HCC cells *in vitro* and *in vivo,* it is obvious that, Cyclin E in hepatoma cells contributes to the growth of these cells.

CcnE1 and CcnE2 both bind and activate CDK2, which is thought to be an essential step for cell cycle progression. Therefore, we next asked the question, if CDK2 alone might be essential to determine the effect on tumor growth as found in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ mice. Unexpectedly, NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ mice showed no significant difference in HCC initiation and progression compared to NEMO$^{\Delta hepa}$ mice. This experiment suggests that the protective effect observed in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ mice is independent of CDK2. To clarify this finding, we performed in young and old NEMO$^{\Delta hepa}$ mice CDK1 and CDK2 kinase assay and indeed the results suggest, that in the NEMO$^{\Delta hepa}$ model hepatocarcinogenesis is not alone dependent on CDK2 but also on CDK1.

Therefore, we conclude that CcnE1 has a unique, CDK2-independent oncogenic function in hepatocytes, which cannot be replaced by CcnE2. As CcnE1 is also dispensable for proliferation in healthy, non-transformed hepatocytes, it represents an attractive therapeutic target. Interestingly, a unique function for CcnE1 was recently also discovered in hepatic stellate cells (HSCs), were genetic inactivation of Cyclin E1 prevents activation, proliferation and survival of HSC and protects from liver fibrogenesis.[12] Therefore, these results suggest, that CcnE1 can potentially contribute to tumor growth in different non-parenchymal cells (NPCs) e.g. HSCs and immune cells. At present it is unclear, if CcnE1 works in NPCs alone or after binding to CDK2.

Besides the hepatocyte-specific function of CcnE1 our study also indicated that its expression in NPCs may contribute to tumor progression. Our study revealed that the absence of CcnE1 expression in 8 week old NEMO$^{\Delta hepa}$ animals had a minor impact on hepatocyte proliferation, but instead had a beneficial impact on liver injury. Furthermore, assessing inflammation in these mice by determining infiltration of CD45- and F4/80-

positive leukocytes as well as inflammation markers such as TNFα and CCR2 showed, that reduced liver injury in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ mice was associated with an amelioration of the inflammatory response.

During the last decades evidences have been obtained showing a link between inflammation and tumorigenesis.[19] The most confirmed tumor-promoting cytokines are “M1 cytokines” such as TNFα.[19] TNFα was shown to promote the growth of HCCs in mice lacking the P-glycoprotein Mdr2, which develop cholestatic inflammation followed by hepatocellular carcinoma (HCC).[19, 20] In the present study, the reduced inflammatory environment was also associated with reduced HCC growth in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$, but not in NEMO$^{\Delta hepa}$/CcnE2$^{-/-}$livers. Therefore, the reduced inflammatory response found in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ animals could of course also be a result of the impaired response in hepatocytes. However as indicated by the divergence between hepatocyte proliferation and inflammation in 8 week old animals these results strongly suggest that CcnE1 in NPCs, *e.g.* HSCs or monocytes also contribute to reduced HCC growth in CcnE1-deficient NEMO$^{\Delta hepa}$ livers. For example, it was recently demonstrated that a pre-clinical targeting of Cyclin E1 using stabilized siRNA has a high anti-fibrotic therapeutic potential[21] suggesting that this might also be relevant to prevent the growth of HCCs.

In summary our study demonstrates that CcnE1, but not CcnE2, is essential for initiation and progression of HCCs. This mechanism in hepatocytes is CcnE1-specific and CDK2-independent. Additionally, our results suggest that CcnE1 in NPCs contributes to the growth of HCC and hence makes CcnE1 to an attractive therapeutic target for chronic liver diseases.

Material and Methods

Animal experimentation

Mice were treated in accordance with the guidelines of the National Academy of Sciences (NIH publication 86–23 revised 1985) and maintained in the animal facility of the University Hospital RWTH Aachen. Animal studies were approved by the regional authorities for nature, environment and consumer protection of the state North Rhine-Westphalia (LANUV, Germany).

Hepatocyte-specific NEMO$^{\Delta hepa}$ and CDK2$^{\Delta hepa}$ knockout mice were generated as recently described.[15, 22] The generation of constitutive CcnE1$^{-/-}$ and CcnE2$^{-/-}$ knockout mice has been previously described.[6] Double knockout mice (NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$, NEMO$^{\Delta hepa}$/CcnE2$^{-/-}$ and NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ mice) were generated by crossing NEMO$^{\Delta hepa}$ mice with the corresponding single knockout mice. All strains were kept on a C57BL/6 background. Progression of chronic liver disease was examined in 8, 26 and 52 week old animals. Liver weight and body weight were recorded; serum and liver tissue were collected.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA from liver tissue was isolated using the peqGOLD RNAPureTM Kit (Peqlab, Erlangen, Germany). Reverse-transcription was performed as previously described.[23] Relative quantitative gene expression was measured *via* Real-Time PCR using a 7300 Real Time PCR System with SDS software 1.3.1 (Applied Biosystems, Foster City, CA) and a

SYBR Green PCR Kit (Invitrogen, Carlsbad, CA). GAPDH expression was used as internal standard. The primer sequences are given in supplementary Table 1.

Immunofluorescence staining

Immunofluorescence staining was performed on frozen liver sections. Hepatocyte proliferation was quantified by counting the number of nuclei positive for either Ki67 or pH3 relative to the total nuclei per power field (200x) stained with 4', 6-diamidino-2-phenylindole (DAPI).

Stained microscopic images were acquired with a Zeiss Axio Imager.Z1 microscope, AxioCam MRm camera using Axiovision 4.8 software (all from Carl Zeiss, Inc., Oberkochen, Germany).

In vitro kinase assay

Immunoprecipitations and kinase assays were performed as described previously[11] with minor modifications. Briefly, 500 μg of liver protein extract was incubated with antibodies against CDK2 (sc-6248, Santa Cruz) or CDK1 (sc-54; Santa Cruz Biotechnology) overnight at 4 °C in Nonidet P-40 lysis buffer containing protease inhibitors (cOmpleteTM Mini, Roche, Mannheim, Germany). Antibody/protein complexes were coupled to protein A/G agarose beads (Santa Cruz) for 1 h at 4 °C and subsequently washed three times in NP40-lysis buffer and in Kinase buffer (50 mM HEPES pH7.5; 10 mM $MgCl_2$; 10 mM β-Glycerophosphate, 1 mM DTT), respectively. Kinase assays were performed by incubating the immunoprecipitated protein complexes in kinase buffer containing 15 μM ATP, 160 Bq [γ−32P] ATP (Hartmann, Braunschweig, Germany) and 2 μg of recombinant histone H1 (Roche) in a total volume of 30 μl for 30 min at 4 °C. The reaction was inactivated with SDS/PAGE sample buffer and resolved by electrophoresis on polyacrylamide gels. Immobilized gels were exposed to autoradiography (Hyperfilm, GE Healtcare, Freiburg, Germany).

Statistical Analysis

Data are presented as mean ± standard deviation of the mean. Statistical significance was determined by a two tailed-Students t test. For each experiment control mice were compared to each other and NEMO-deficient mice were compared among each other.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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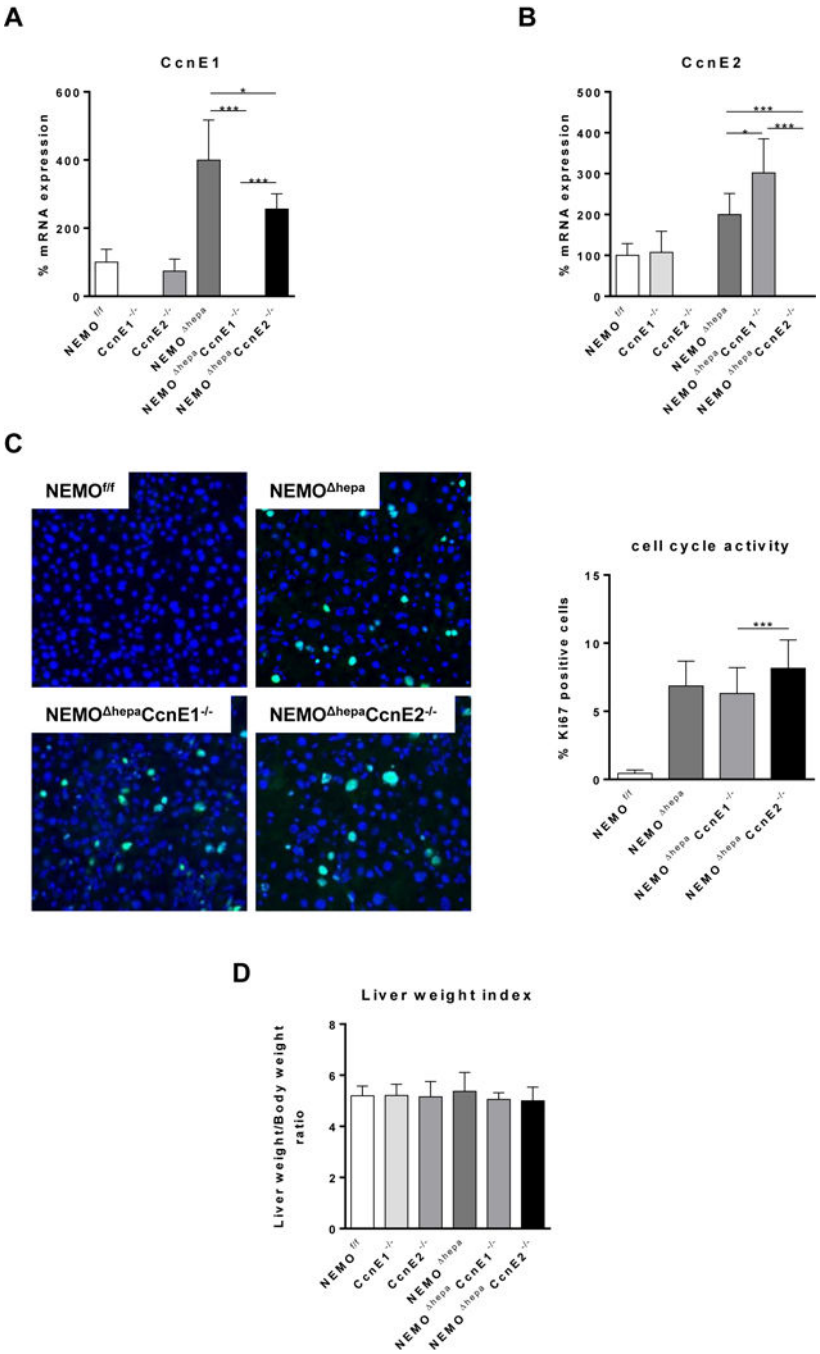

Figure 1: NEMO$^{\Delta hepa}$ livers of 8 week old mice display up-regulation of E-type Cyclins.

(A) CcnE1 and **(B)** CcnE2 qRT-PCR of liver mRNA was determined for NEMO$^{f/f}$ (n=5), CcnE1$^{-/-}$ (n=6), CcnE2$^{-/-}$ (n=6), NEMO$^{\Delta hepa}$ (n=5), NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=5) and NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=5). **(C)** Ki67 stainings are depicted (group/n=5) (green: Ki67-positive cells, blue: nuclei which are stained with DAPI, magnification: ×200). **(D)** Liver weight/Body weight ratio for NEMO$^{f/f}$ (n=14), CcnE1$^{-/-}$ (n=11), CcnE2$^{-/-}$ (n=7), NEMO$^{\Delta hepa}$ (n=10), NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=11) and NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=9). (Values are mean ± SD, *P<0.05, **P<0.01, ***P<0.001.)

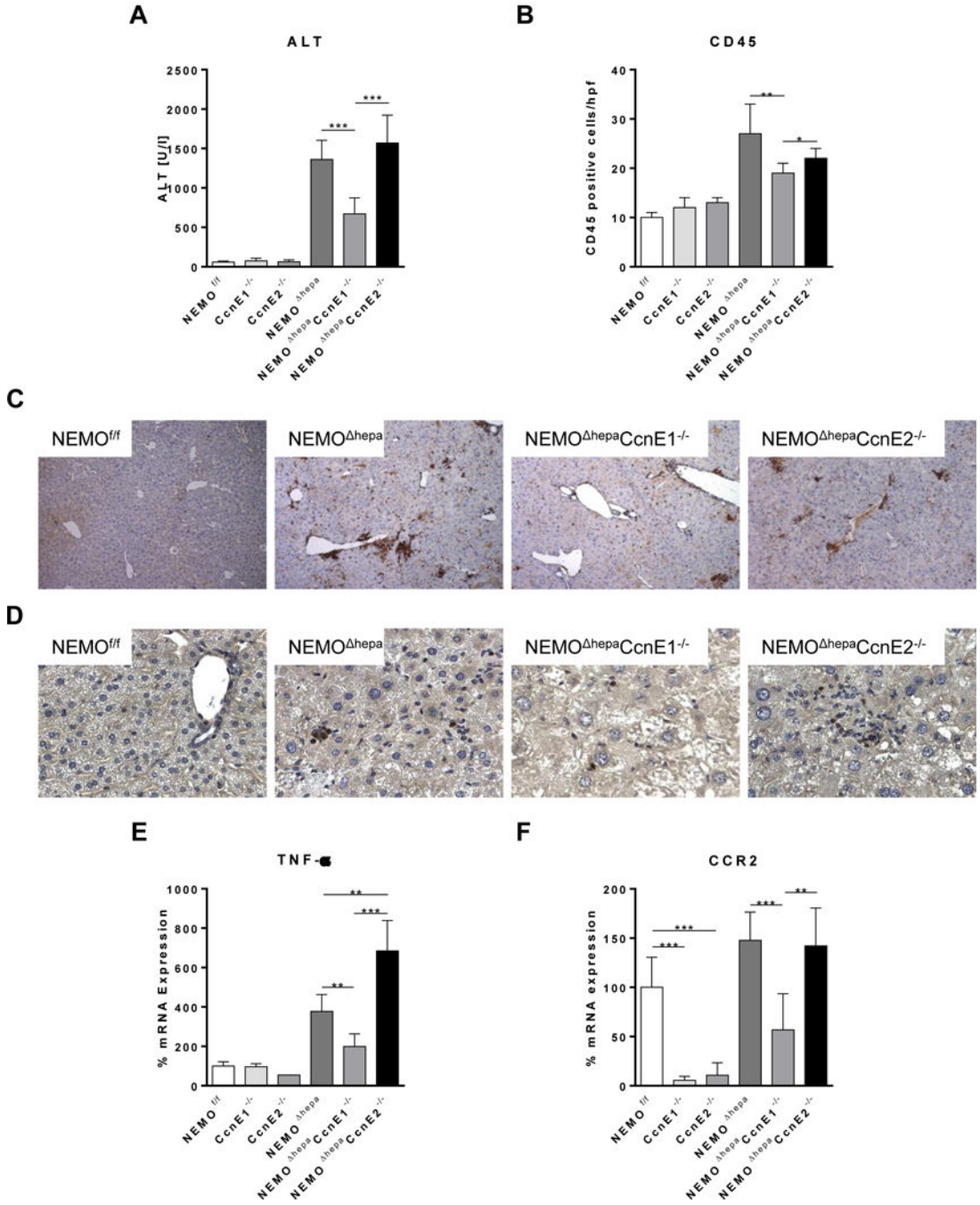

Figure 2: Amelioration of liver injury in 8 week old CcnE1-deficient NEMO$^{\Delta hepa}$ animals.

(A) ALT serum levels in 8 week old animals for NEMO$^{f/f}$ (n=5), CcnE1$^{-/-}$ (n=14), CcnE2$^{-/-}$ (n=9), NEMO$^{\Delta hepa}$ (n=5), NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=8) and NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=8). **(B)** Quantification of the CD45-staining (group/n=5). **(C)** CD45-staining on paraffin liver sections (brown: CD45-positive cells, blue: nuclei, magnification: ×100). **(D)** TNFα-staining on paraffin liver sections (brown: TNFα-positive cells, blue: nuclei, magnification: ×200). **(E)** TNFα and **(F)** CCR2 qRT-PCR of liver mRNA was determined (group/n=5). (Values are mean ± SD, *P<0.05, **P<0.01, ***P<0.001.)

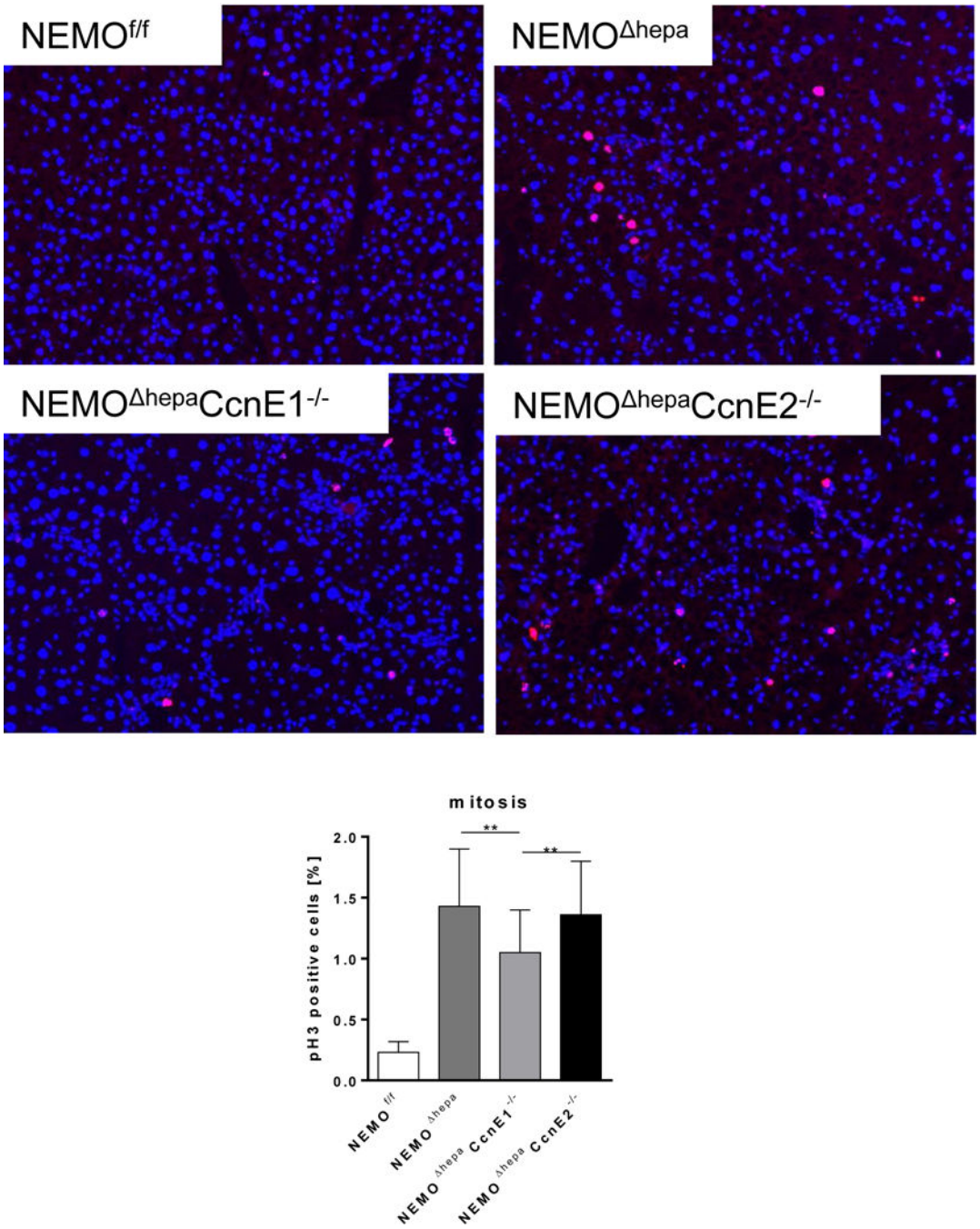

Figure 3: Absence of CcnE1 expression reduces proliferation in 26 week old NEMO$^{\Delta hepa}$ mice.
(A) Phospho-histone H3 (pH3) was stained on liver cryosections (group/n=5) (red: pH3-positive cells; nuclei are counter stained with DAPI (blue); magnification: ×200). (Values are mean ± SD, ** P <0.01.)

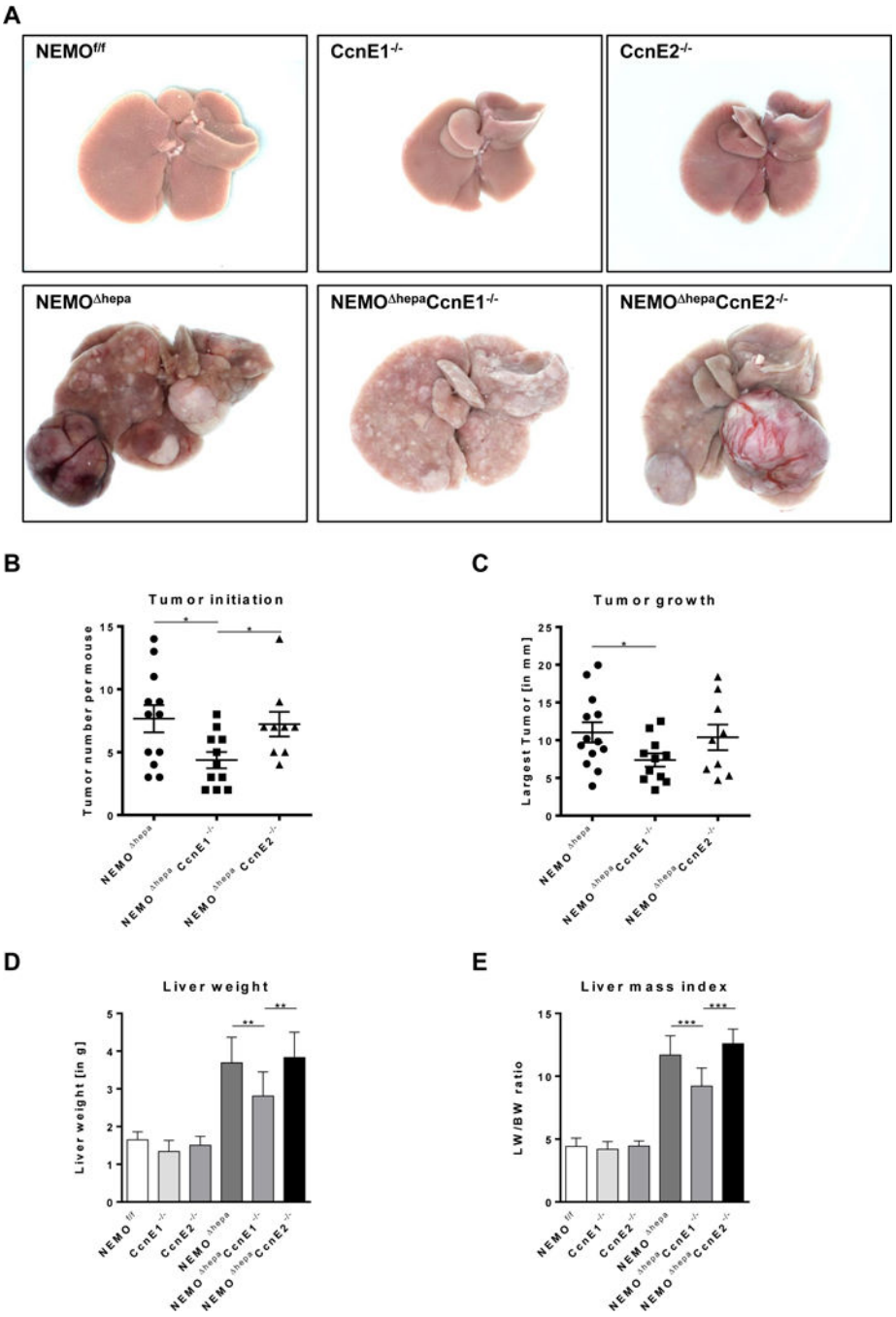

Figure 4: Attenuated hepatocarcinogenesis in NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ mice.

(A) Macroscopic images of livers derived from the different strains at the age of 52 weeks are displayed. **(B)** Tumor number per mice and respective strain is presented for NEMO$^{\Delta hepa}$ (n=12), NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=11) and NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=9). **(C)** The size of the largest tumor per liver and strain is indicated serving as a measure of tumor progression for NEMO$^{\Delta hepa}$ (n=13), NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=11) and NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=9). **(D)** Liver weight and **(E)** Liver weight/Body weight ratio was calculated for NEMO$^{f/f}$ (n=8),

CcnE1$^{-/-}$ (n=7), CcnE2$^{-/-}$ (n=9), NEMO$^{\Delta hepa}$ (n=10), NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=11) and NEMO$^{\Delta hepa}$/CcnE1$^{-/-}$ (n=9). (Values are mean ± SD, *P<0.05, **P<0.01, ***P<0.001.)

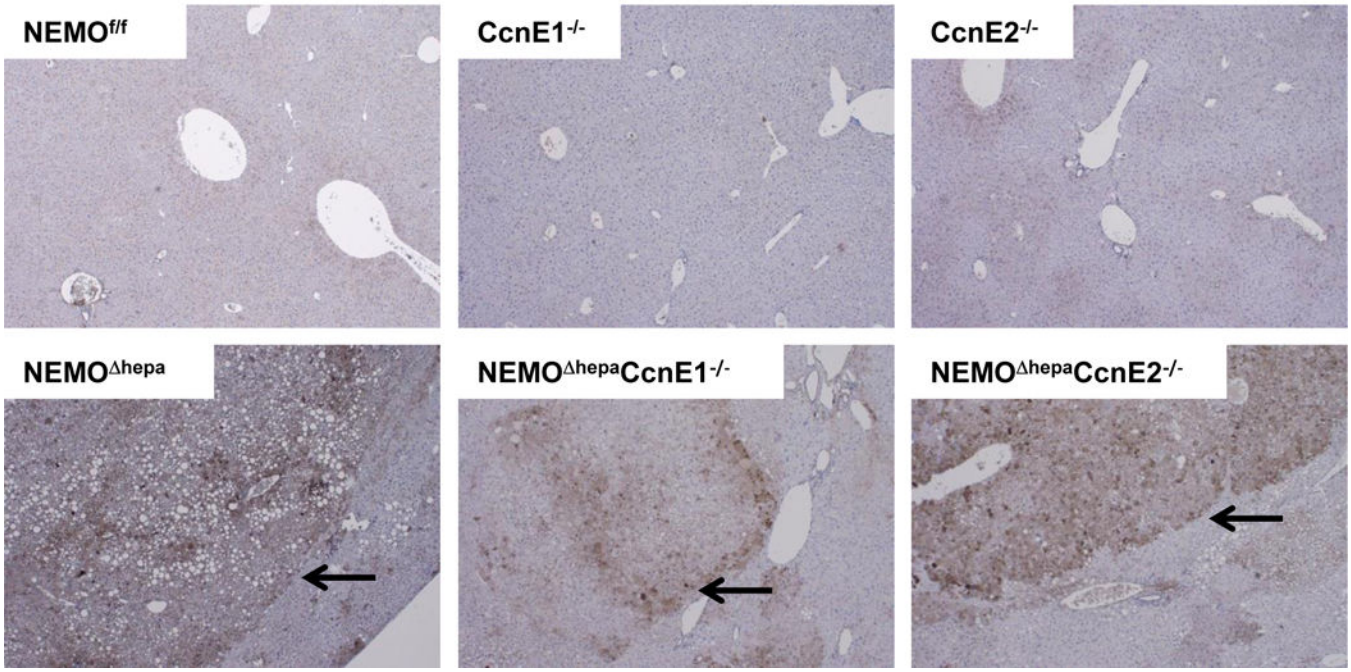

Figure 5: Livers of NEMO$^{\Delta hepa}$ and NEMO$^{\Delta hepa}$/CcnE2$^{-/-}$ mice display larger areas of hsp70 positive cells.

Hsp70-staining is demonstrated (brown: hsp70-positive cells, blue: nuclei, magnification: ×100).

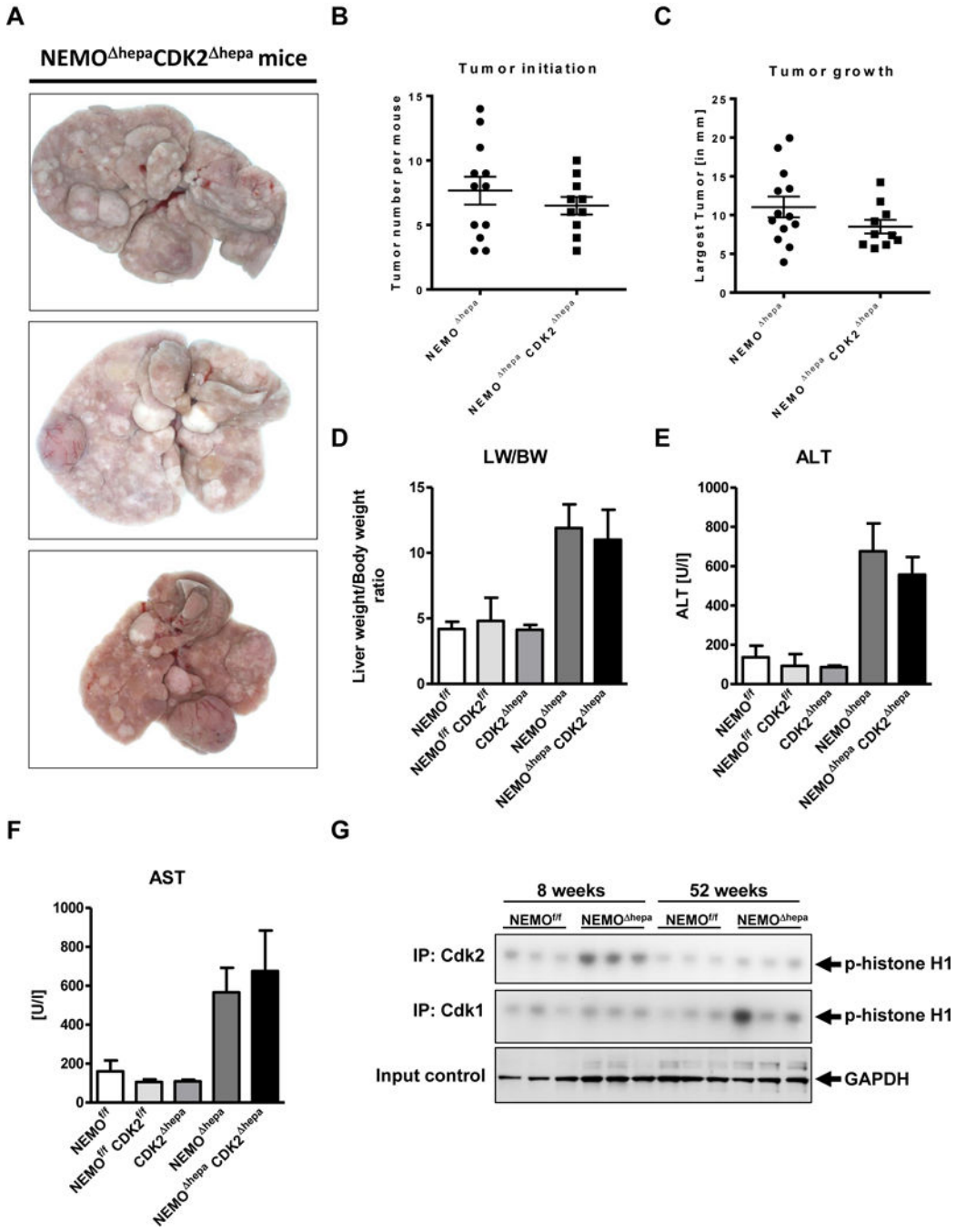

Figure 6: Deletion of CDK2 in hepatocytes does not affect tumor progression in NEMO$^{\Delta hepa}$ livers.

(A) Macroscopic images of livers obtained from 52 week old NEMO$^{\Delta hepa}$ and NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ mice are shown. **(B)** Tumor number per mouse is shown for NEMO$^{\Delta hepa}$ (n=12) and NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ (n=10). **(C)** The size of the largest tumor per mouse is presented for NEMO$^{\Delta hepa}$ (n=13) and NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ (n=10). **(D)** Liver weight/Body weight ratios are depicted for NEMO$^{f/f}$ (n=5), NEMO$^{f/f}$/CDK2$^{f/f}$ (n=5), CDK2$^{\Delta hepa}$ (n=5), NEMO$^{\Delta hepa}$ (n=10) and NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ (n=7). **(E)** ALT and **(F)** AST serum

levels were determined for NEMO$^{f/f}$ (n=5), NEMO$^{f/f}$/CDK2$^{f/f}$ (n=5), CDK2$^{\Delta hepa}$ (n=5), NEMO$^{\Delta hepa}$ (n=10) and NEMO$^{\Delta hepa}$/CDK2$^{\Delta hepa}$ (n=7). **(G)** CDK2 and CDK1 kinase activities were analyzed in livers of NEMO$^{\Delta hepa}$ mice at the age of 8 and 52 weeks (n=3). Cre-negative littermates (NEMO$^{f/f}$) of matching age were used as controls (n=3). IP: Immunoprecipitation of native CDK2 or CDK1 kinase complexes. Phosphorylated histone H1 (p-histone H1) is highlighted by arrows. Input control: 5% of the supernatant from immunoprecipitations was probed for GAPDH expression to prove equal starting amounts of protein. (Values are mean ± SD, *P <0.05, **P <0.01, ***P <0.001.)