Cyclin E1 and cyclin-dependent kinase 2 are critical for initiation, but not for progression of hepatocellular carcinoma

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E-type cyclins E1 (CcnE1) and E2 (CcnE2) are regulatory subunits of cyclin-dependent kinase 2 (Cdk2) and thought to control the transition of quiescent cells into the cell cycle. Initial findings indicated that CcnE1 and CcnE2 have largely overlapping functions for cancer development in several tumor entities including hepatocellular carcinoma (HCC). In the present study, we dissected the differential contributions of CcnE1, CcnE2, and Cdk2 for initiation and progression of HCC in mice and patients. To this end, we tested the HCC susceptibility in mice with constitutive deficiency for CcnE1 or CcnE2 as well as in mice lacking Cdk2 in hepatocytes. Genetic inactivation of CcnE1 largely prevented development of liver cancer in mice in two established HCC models, while ablation of CcnE2 had no effect on hepatocarcinogenesis. Importantly, CcnE1-driven HCC initiation was dependent on Cdk2. However, isolated primary hepatoma cells typically acquired independence on CcnE1 and Cdk2 with increasing progression in vitro, which was associated with a gene signature involving secondary induction of CcnE2 and up-regulation of cell cycle and DNA repair pathways. Importantly, a similar expression profile was also found in HCC patients with elevated CcnE2 expression and poor survival. In general, overall survival in HCC patients was synergistically affected by expression of CcnE1 and CcnE2, but not through Cdk2. Our study suggests that HCC initiation specifically depends on CcnE1 and Cdk2, while HCC progression requires expression of any E-cyclin, but no Cdk2.

HCC | liver | cell cycle | diethylnitrosamine | DNA repair

The mammalian cell cycle is controlled by cyclin-dependent kinases (Cdk) and cyclins acting as Cdk-regulatory subunits. Specific cyclin/Cdk complexes regulate transition through the distinct phases of the cell cycle by phosphorylation of phase-specific substrates (1). Extracellular mitogenic signals induce expression of D-type cyclins, which drive progression through G_1 phase via binding and activation of Cdk4 and Cdk6. These kinases phosphorylate and inactivate the retinoblastoma protein (Rb). Rb phosphorylation is completed by Cdk2 kinase in complex with E-type cyclins (CcnE1, CcnE2) shortly before S-phase entry, leading to the activation of E2F transcription factors and cell cycle-related genes. Despite their proposed important function for S-phase entry, genetic knockout studies in mice revealed that CcnE1, CcnE2, or Cdk2 are not essential for proliferation of normal tissue cells during development, homeostasis, and regeneration. Even compound inactivation of CcnE1 and CcnE2 in continuous growing murine embryonic fibroblasts allowed proper S-phase transition and proliferation but prevented at least cell cycle reentry after serum starvation and malignant transformation in vitro (2). We recently demonstrated that deletion of a single E-cyclin had only a modest effect on liver regeneration following partial hepatectomy (PH), whereas compound deletion of CcnE1 and CcnE2 substantially inhibited hepatocyte proliferation and liver mass restoration (3, 4).

Hepatocellular carcinoma (HCC) is one of the most frequent tumor diseases worldwide with growing incidence and poor prognosis. In most cases, HCC develops on the basis of chronic hepatitis in a multistep process involving frequent up-regulation or amplification of E-type cyclins and many other cell cycle-related proteins as reviewed elsewhere (5). However, the detailed contribution of the individual E-cyclins for HCC development has barely been investigated. We recently observed that inhibition of CcnE1 may attenuate HCC development in a genetic model of

Significance

The two E-type cyclins E1 and E2 are known to interact with Cdk2 and are thought to trigger cell cycle activity in carcinogenesis. However, the individual contributions of cyclin E1, cyclin E2, and Cdk2 for initiation and progression of hepatocellular carcinoma (HCC) are unknown. In the present study, we discovered that only cyclin E1—but not cyclin E2—is essential for initiation of liver cancer and requires Cdk2. Unexpectedly, advanced liver cancer progression can be mediated in presence of any E-cyclin, but in a Cdk2-independent manner. We identified the specific expression profiles of cyclin E1-dependent and cyclin E1-independent hepatoma cells. These signatures are useful for predicting patient prognosis and for developing novel cyclin E-based HCC therapies.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE111079).

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chronic hepatitis (6). Here, we aimed to genetically dissect the precise contribution of CcnE1, CcnE2, or Cdk2 for HCC initiation and/or progression. We show that CcnE1 has a Cdk2-dependent, unique, and critical function for initiation of liver cancer, while HCC progression is controlled by alternative mechanisms independent of Cdk2 activity.

Results

CcnE1—but Not CcnE2—Is Critical for DEN-Driven Hepatic Tumor Development. To assess the role of E-cyclins for hepatocarcinogenesis, 2-wk-old WT, CcnE1$^{-/-}$, and CcnE2$^{-/-}$ mice were treated with a single dose of the hepatocarcinogen diethylnitrosamine (DEN) and euthanized 22 wk or 38 wk later (*SI Appendix*, Fig. S1*A*). Similar expression levels of Cytochrome P450 2E1 (Cyp2E1) in all groups before and after treatment excluded an effect of E-cyclin ablation on DEN metabolism and its bioactivation (*SI Appendix*, Fig. S1*B*). Twenty-two weeks after DEN treatment, alanine transaminase (ALT) activities—an indicator of overall liver injury—and relative liver weights in all mice were at normal levels (*SI Appendix*, Fig. S1 *C* and *D*). However, small tumor nodules were macroscopically visible in 40% of WT and in 47% of CcnE2$^{-/-}$ mice, but not at all in CcnE1-deficient animals (*SI Appendix*, Fig. S1 *E* and *F*). Histologically, dysplastic lesions and precancerous tissue were identified in WT and CcnE2$^{-/-}$ livers but barely detectable in DEN-treated CcnE1$^{-/-}$ mice. Consistently, the proliferation marker PCNA was markedly expressed in WT and CcnE2$^{-/-}$ livers, while CcnE1$^{-/-}$ livers did not reveal excessive proliferation (*SI Appendix*, Fig. S1*G*).

Thirty-eight weeks after DEN injection, ALT values and relative liver weights were normal in CcnE1$^{-/-}$ mice, but significantly elevated in DEN-treated WT and CcnE2$^{-/-}$ mice (Fig. 1 *A* and *B*). Of note, ALT activity correlated with tumor number in WT and CcnE2$^{-/-}$—but not in CcnE1$^{-/-}$—mice (*SI Appendix*, Fig. S2*A*). Ninety-five percent of WT mice and 100% of CcnE2$^{-/-}$ mice developed liver tumors after DEN treatment, while 50% of CcnE1$^{-/-}$ animals remained tumor-free (Fig. 1 *C* and *D*). Importantly, CcnE1$^{-/-}$ mice displayed substantially reduced average tumor numbers and tumor size in comparison with WT or CcnE2$^{-/-}$ mice (Fig. 1*E*). Histologically, liver sections from DEN-treated WT and CcnE2$^{-/-}$ animals revealed strong dysplasia, abnormal tissue architecture, and excessive cell proliferation, whereas CcnE1$^{-/-}$ liver sections appeared mostly normal with infrequent small precancerous lesions and infrequent occurrence of proliferating hepatocytes (*SI Appendix*, Fig. S2*B*). In DEN-treated WT mice, CcnE1 was almost exclusively induced in hepatocytes within dysplastic areas, but barely detectable (<0.1%) in nonparenchymal cells such as CD45^{+} leukocytes or α-SMA^{+} myofibroblasts (*SI Appendix*, Fig. S3*A*). HCC formation in WT and CcnE2$^{-/-}$ mice was associated with up-regulation of CcnE1, but WT HCC did not show elevated CcnE2 expression (*SI Appendix*, Fig. S3*B*). On the cellular level, the few HCC nodules found in CcnE1$^{-/-}$ livers were characterized by small clusters of CcnE2-expressing cells at the edge of dysplastic lesions (*SI Appendix*, Fig. S3*C*). CcnE2 protein was predominantly localized in the cytoplasm and only occasionally detected in the nucleus. Importantly, we did not detect CcnE2 protein in livers of DEN-treated WT mice (*SI Appendix*, Fig. S3*D*). Altogether, these data indicate that CcnE1 is the dominant driver of DEN-induced HCC initiation, whereas CcnE2 expression during HCC formation seems to be a rare event.

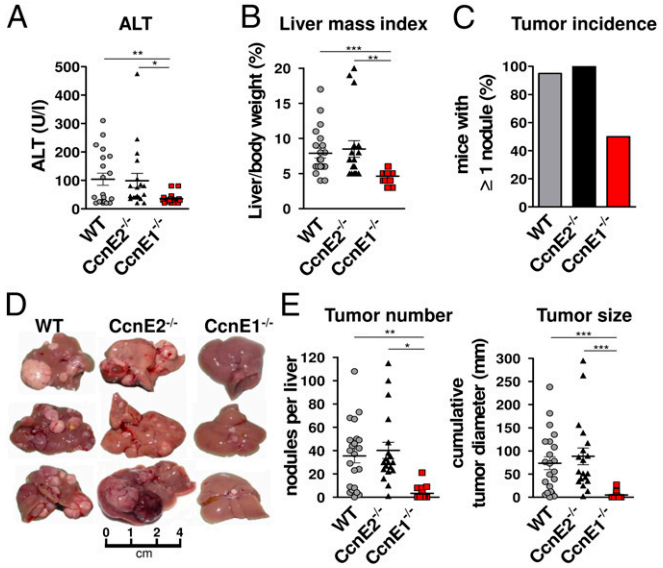

Fig. 1. CcnE1, but not CcnE2, is critical for DEN-driven hepatic tumor development. Analysis of 40-wk-old WT (n = 22), CcnE2$^{-/-}$ (n = 19), and CcnE1$^{-/-}$ (n = 16) mice after DEN treatment. (*A*) Serum ALT levels. (*B*) Liver: body weight ratio (%). (*C*) Tumor incidence. The proportion of mice with ≥1 liver nodule is shown. (*D*) Macroscopic appearance of livers and HCC. Three representative livers per group are shown. (*E*) Number of macroscopic HCC nodules and the cumulative liver tumor diameter (tumor size) for each mouse. *P < 0.05; **P < 0.005; ***P < 0.001.

CcnE1 Is a Key Factor for c-myc–Driven Hepatocarcinogenesis. To test whether CcnE1 is of general relevance for hepatocarcinogenesis, we verified our findings in a second independent HCC mouse model comprising overexpression of the proto-oncogene c-myc (myc^{tg}) in hepatocytes and crossed myc^{tg} transgenic animals with CcnE1$^{-/-}$ mice. In myc^{tg} mice, slow-growing HCCs usually develop with long latency (7, 8). Approximately 70% of myc^{tg} mice at the age of 70 wk (11/16) developed liver tumors that were mostly mononodular or two-nodular (Fig. 2 *A*–*C*). In contrast, most myc^{tg}CcnE1$^{-/-}$ livers were normal regarding morphology and relative liver mass, and only 2 of 11 mice (18%) developed liver tumors of small size (Fig. 2). Altogether, these data suggest a general key function of CcnE1 for hepatocarcinogenesis independent of the used tumor model.

DEN-Driven HCC Development Is Dependent on Cdk2 in Hepatocytes. To test the relevance of Cdk2 for HCC development, we generated conditional knockout mice with efficient hepatocyte-specific deletion of Cdk2 (Cdk2$^{\Delta hepa}$, Fig. 3*A*). We excluded an effect of the Cdk2 depletion on DEN bioactivation (*SI Appendix*, Fig. S4*A*). Two-week-old Cdk2$^{f/f}$ mice (representing WT controls) and Cdk2$^{\Delta hepa}$ mice were then treated with DEN and analyzed for HCC development after 38 wk. Cdk2$^{\Delta hepa}$ livers showed substantially reduced average tumor numbers in comparison with controls (Fig. 3 *B* and *C*). In addition, loss of Cdk2 resulted in significantly reduced cumulative tumor size and liver mass index (Fig. 3*C*). Consistently, Cdk2$^{\Delta hepa}$ mice showed improved liver histology with only a few dysplastic lesions and little hepatocyte proliferation (*SI Appendix*, Fig. S4*B*). CcnE1 was markedly overexpressed in DEN-treated WT and Cdk2$^{\Delta hepa}$ livers in comparison with untreated control mice, while CcnE2 expression was at best moderately induced by DEN. We also detected enhanced CcnA2 expression in DEN-treated Cdk2$^{f/f}$ livers, which was reduced to baseline levels when Cdk2 was deleted (Fig. 3*D*). Of note, Cdk2$^{\Delta hepa}$CcnE2$^{-/-}$ double knockout mice revealed almost identical inhibition of HCC development after DEN treatment as Cdk2$^{\Delta hepa}$ mice despite strong hepatic gene expression of CcnE1 (*SI Appendix*, Fig. S4 *C* and *D*). The data suggest that strong hepatic overexpression of CcnE1 alone is not sufficient for potent HCC initiation if Cdk2 is not available.

Different Requirements for CcnE1 in Precancerous Hepatoma Cells and Advanced HCC Cells. To further characterize the critical and unique function of CcnE1 for HCC formation, we generated

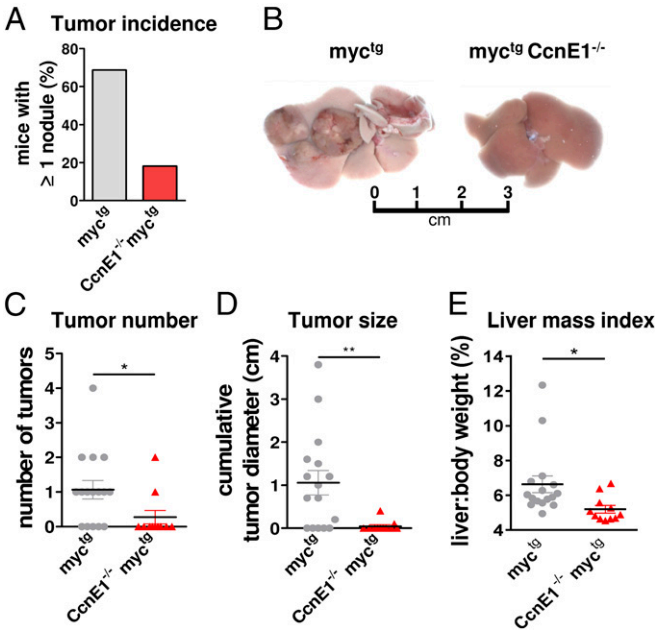

Fig. 2. CcnE1 is a key factor for c-myc–driven hepatocarcinogenesis. Mice with hepatocyte-specific overexpression of c-myc (myc^{tg}, n = 16) and myc^{tg}CcnE1$^{-/-}$ double-transgenic mice (n = 11) were euthanized at the age of 70 wk and analyzed for the number and size of macroscopic HCC nodules. (*A*) Tumor incidence. (*B*) Representative macroscopic appearance of livers from c-myc^{tg} and myc^{tg}CcnE1$^{-/-}$ mice. (*C*) Number of macroscopic HCC nodules for each mouse in the study. (*D*) Cumulative tumor diameter (mm) for each animal. (*E*) Liver:body weight ratio (%). *P < 0.05; **P < 0.005.

primary hepatoma cell lines with a floxed CcnE1 gene derived from CcnE1$^{f/f}$ donor mice 38 wk after DEN treatment (*SI Appendix*, Fig. S5*A*). We isolated both hepatocyte-derived cells from precancerous but tumor-free liver tissue (referred to as CcnE1$^{f/f}$preCL) and from large HCC nodules (referred to as CcnE1$^{f/f}$HCC). These cells were immortalized in vitro and analyzed at early passages. CcnE1$^{f/f}$HCC cells revealed typical characteristics of advanced hepatoma cells including rapid growth (Fig. 4*A*), loss of albumin expression, and sustained expression of the HCC marker α-fetoprotein (AFP, *SI Appendix*, Fig. S5*B*). In addition, these cells expressed excessive levels of CcnE1, CcnE2, and CcnA2 (*SI Appendix*, Fig. S5*B*). In comparison, CcnE1$^{f/f}$preCL cells grew rather slowly and revealed only moderate expression of AFP and E-type cyclins (Fig. 4*A* and *SI Appendix*, Fig. S5*B*). This data indicates that both cell types are of hepatocyte origin although with different grade of dedifferentiation toward liver cancer cells.

We infected both CcnE1$^{f/f}$ hepatoma cell lines with recombinant adenoviruses either expressing EGFP (adv-EGFP, control) or cre-recombinase and EGFP together (adv-EGFP/cre) to delete the CcnE1 gene and track infected cells. Importantly, CcnE1$^{f/f}$preCL cells did not proliferate after cre-mediated CcnE1 deletion. In contrast, CcnE1$^{f/f}$HCC cells were basically able to proliferate in the absence of CcnE1 although with a significant delay compared with CcnE1-proficient cells (Fig. 4*B*). Rb phosphorylation was strongly diminished in CcnE1$^{f/f}$preCL cells but only marginally reduced in CcnE1$^{f/f}$HCC cells upon CcnE1 deletion (Fig. 4*C*), pointing to the need of a CcnE1-dependent kinase activity specifically in preCL cells. Expression of the downstream S-phase cyclin, CcnA2, was reduced to similar levels in both cell types upon CcnE1 deletion. CcnE1$^{f/f}$preCL control cells were less frequent in S phase compared with CcnE1$^{f/f}$HCC cells. Surprisingly, deletion of CcnE1 had no major influence on S-phase progression in both cell lines (*SI Appendix*, Fig. S5*C*). Of note, CcnE1$^{f/f}$preCL and CcnE1$^{f/f}$HCC cells frequently accumulated DNA double-strand breaks (DSB), which was significantly more pronounced in CcnE1$^{f/f}$preCL cells (*SI Appendix*, Fig. S5*D*). CcnE1 deletion triggered a considerable amount of cell death specifically in CcnE1$^{f/f}$preCL cells, as evidenced by activation of caspase-3 (Fig. 4*C*) and occurrence of large (~20%) sub-G_1 populations, which was not found in HCC cells (Fig. 4*D*). Confocal live cell imaging revealed that adenoviral infection per se had no major influence on cell survival and cell division in CcnE1$^{f/f}$preCL cells (Fig. 4*E* and Movie S1). However, CcnE1 deletion resulted in substantial cell death with apoptosis-like morphology specifically in infected (green) cells within 48–72 h, while noninfected cells survived and underwent proper cell division (Fig. 4*E* and Movie S2).

Cdk2-Kinase Activity Is Critical for Proliferation of Precancerous Hepatocytes but Dispensable for HCC Progression. Based on our genetic data from Cdk2$^{\Delta hepa}$ mice (Fig. 3), we further analyzed the role of Cdk2 for HCC initiation versus progression. To this end, we isolated hepatocyte-derived cells from DEN-treated Cdk2$^{f/f}$ mice 38 wk after HCC initiation. Again, we isolated cells from tumor-free precancerous liver tissue (referred to as Cdk2$^{f/f}$preCL) and from solid tumor nodules (Cdk2$^{f/f}$HCC). Precancerous liver cells showed decreased CcnE1 gene expression compared with HCC cells, while CcnE2 and CcnA2 gene

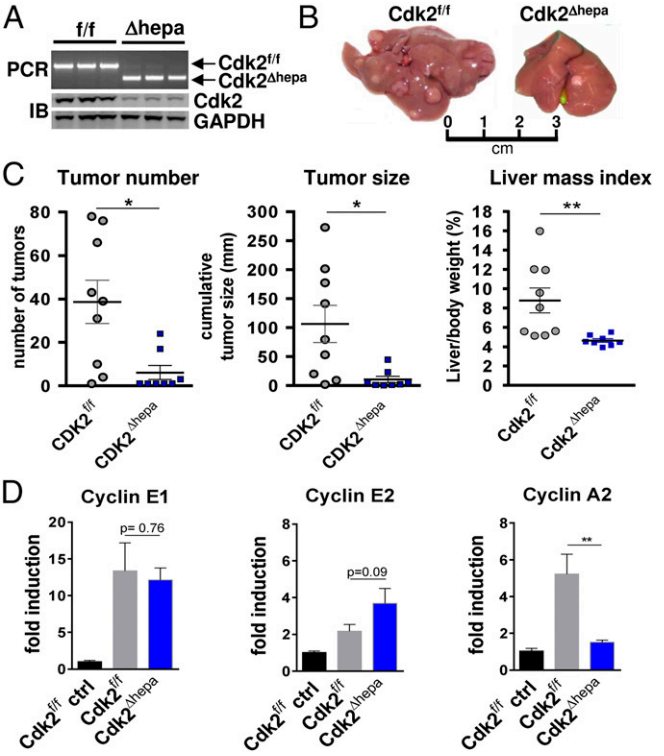

Fig. 3. HCC development is attenuated by hepatocyte-specific inactivation of Cdk2. Cdk2$^{f/f}$ (n = 9) and Cdk2$^{\Delta hepa}$ (n = 8) mice were treated with DEN at the age of 14 d and euthanized at the age of 40 wk. (*A*) Cdk2 deletion efficiency in the liver (Δhepa) was verified by PCR using hepatic cDNA and by immunoblot (IB). (*B*) Macroscopic appearance of representative livers from 40-wk-old Cdk2$^{f/f}$ and Cdk2$^{\Delta hepa}$ animals. (*C*) Scatter plots showing the number of macroscopic HCC nodules per mouse (*Left*), cumulative tumor diameter (mm, *Middle*) and liver:body weight ratio (%, *Right*) for each animal. (*D*) Gene expression analysis of cyclin E1 (*Left*), cyclin E2 (*Middle*), and cyclin A2 (*Right*) in livers of DEN-treated Cdk2$^{f/f}$ (n = 9) and Cdk2$^{\Delta hepa}$ (n = 8) animals. Expression levels were calculated as fold induction in comparison with untreated WT controls (Cdk2$^{f/f}$ ctrl, n = 3). *P < 0.05; **P < 0.005.

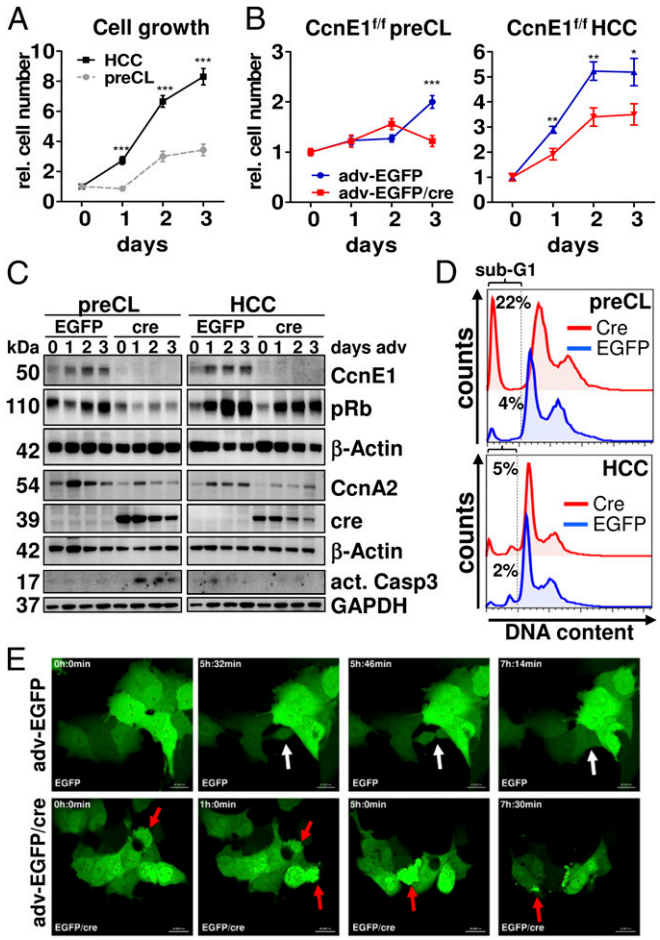

Fig. 4. Different requirements for CcnE1 in precancerous hepatoma cells and advanced HCC cells. (*A* and *B*) Growth curves. Relative (rel.) values are shown. (*A*) untreated CcnE1$^{f/f}$preCL and CcnE1$^{f/f}$HCC cells. (*B–E*) CcnE1$^{f/f}$preCL and CcnE1$^{f/f}$HCC cells were infected with adv-EGFP or adv-EGFP/cre and analyzed at indicated time points after infection. (*B*) Growth curves of CcnE1$^{f/f}$preCL and CcnE1$^{f/f}$HCC cells after infection. (*C*) Immunoblot analysis of CcnE1, phospho-Rb, CcnA2, cre-Rekombinase (cre) and activated Caspase-3 (act. Casp3). (*D*) Infected CcnE1$^{f/f}$preCL and CcnE1$^{f/f}$HCC cells were gated for EGFP expression and analyzed for DNA-content by FACS. Sub-G1, Apoptotic cells. (*E*) Infected CcnE1$^{f/f}$preCL cells (green) were subjected to confocal live cell fluorescence microscopy. A time window of 7 h is shown. White arrow: dividing cell; Red arrows: apoptosis-like cell death. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$.

expression were strongly up-regulated without significant differences between the groups (Fig. 5*A*).

Following adv-cre infection, proliferation was completely abolished in precancerous Cdk2$^{-/-}$ cells, while HCC cells were basically able to proliferate in the absence of Cdk2 although with a significant delay (Fig. 5*B*). We detected considerable expression of CcnE1 and phosphorylated Rb in both cell types independent of Cdk2 expression (Fig. 5*C*). In vitro kinase assays confirmed that cre-mediated deletion of Cdk2 completely abolished Cdk2 kinase activity in both Cdk2$^{f/f}$preCL and Cdk2$^{f/f}$HCC cells (Fig. 5*D*). Both cell types revealed a strong CcnE2-associated kinase activity, which was largely blocked after deletion of Cdk2 (Fig. 5*D*). In contrast, CcnE1 contributed differentially to kinase activity in both cell lines: In Cdk2$^{f/f}$preCL cells, we detected a strong CcnE1-associated kinase activity even after Cdk2 deletion pointing to interaction of CcnE1 with an alternative Cdk. However, these cells did not proliferate, suggesting that they specifically require Cdk2. In sharp contrast, CcnE1-associated kinase activity was hardly detectable in Cdk2$^{f/f}$HCC cells despite strong CcnE1 expression (Fig. 5 *C* and *D*). Thus, advanced hepatoma cells can proliferate in the absence of any Cdk2- or CcnE-associated kinase activity.

CcnE1-Independent Growth of Hepatoma Cells Is Characterized by a Gene Signature Comprising Elevated Expression of Cell Cycle and DNA Repair Genes. To investigate the mechanisms of CcnE1 addiction in hepatoma cells in a systematic approach, we collected data from four independent CcnE1$^{f/f}$preCL cell lines (requiring CcnE1) and three CcnE1-independent HCC-derived cell lines (CcnE1$^{f/f}$HCC). Gene expression analysis revealed significantly elevated CcnE1 expression in all CcnE1$^{f/f}$HCC cells and confirmed efficient knockout of CcnE1 after adv-cre infection in all groups. In addition, we found substantially increased CcnE2 and CcnA2 expression in CcnE1$^{f/f}$HCC and CcnE1$^{-/-}$HCC cells (*SI Appendix*, Fig. S5*E*). We subjected all CcnE1-expressing cells and their CcnE1-deleted counterparts to whole transcriptome shotgun sequencing (RNA-Seq). CcnE1-expressing CcnE1$^{f/f}$preCL and CcnE1$^{f/f}$HCC cells revealed similar clusters and numbers of deregulated genes compared with untreated WT hepatocytes

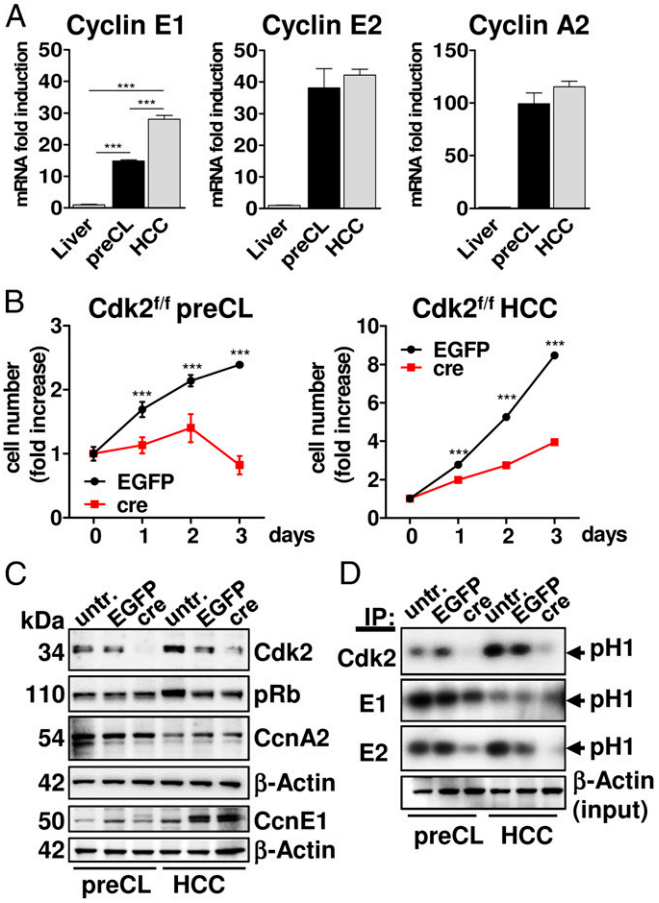

Fig. 5. Cdk2-kinase activity is critical for proliferation of precancerous murine hepatoma cells but dispensable for tumor progression. (*A*) Basal gene expression of CcnE1, CcnE2 and CcnA2 in Cdk2$^{f/f}$preCL and Cdk2$^{f/f}$HCC cells in comparison with total liver RNA (Liver) from untreated donor mice of the same genetic background (Cdk2$^{f/f}$). (*B–D*) Cdk2$^{f/f}$preCL and Cdk2$^{f/f}$HCC cells were treated with adv-EGFP (EGFP) or adv-EGFP/cre (cre) or left untreated (untr.) (*B*) Growth curves of Cdk2$^{f/f}$preCL and Cdk2$^{f/f}$HCC cells. (*C*) Immunoblot analysis of CcnE1, CcnA2, Cdk2 and Rb phosphorylation 3 d after Cdk2 deletion. (*D*) Cdk2 (IP:Cdk2), CcnE1 (IP:E1) and CcnE2 (IP:E2) kinase complexes were immunoprecipitated and subjected to in vitro kinase assay using recombinant histone H1 as substrate. Input: 10% of the supernatant (Sn) was analyzed for β-Actin expression. ***$P < 0.001$.

involved e.g., in cell cycle, PI3K-Akt, Ras, TGF-β, and Wnt signaling (*SI Appendix*, Fig. S6 *A–C*). However, HCC cells specifically showed up-regulation of pathways involved in base excision repair, mismatch repair, and ribosome biogenesis compared with preCL cells or WT hepatocytes (*SI Appendix*, Fig. S6*C*), and this was still evident after CcnE1 was deleted (*SI Appendix*, Fig. S6*D*). Differential regulation of representative genes involved in DNA repair (e.g., Rad21, Msh2, Npm1), cell cycle control (e.g., Ect2, E2F1, GADD45a) and TGF-beta signaling (Bmp2) was confirmed by quantitative real-time PCR (Fig. 6*A* and *SI Appendix*, Fig. S7). Importantly, deletion of CcnE1 per se had no major effect on these gene expression profiles. However, the gene signature identified in this experiment indicates that CcnE1-independent hepatoma cell growth is associated with elevated expression of DNA repair and cell cycle genes such as Rad21, Ect2, Msh2, CcnE2, E2F1, and Rbpp7.

HCC Patients with Poor Survival Are Characterized by Elevated Expression of E-Type Cyclins and a Gene Signature Similarly to Murine CcnE1-Independent Hepatoma Cells. We next analyzed the relationship between CcnE1/CcnE2 and Cdk2 expression and survival in a comprehensive dataset from 369 HCC patients based on data generated by the The Cancer Genome Atlas Research Network (https://cancergenome.nih.gov/) (9). High expression of CcnE1 (irrespective of CcnE2) or CcnE2 (irrespective of CcnE1) was significantly associated with poor patient prognosis ($P = 0.0236$, Fig. 6*B*). The additive overexpression of both E-type cyclins additionally affected overall survival ($P = 0.0064$, Fig. 6*B*), which was reinforced by a more detailed comparison (*SI Appendix*, Fig. S8*A*). This finding indicates that CcnE1 and CcnE2 can partially replace each other during HCC progression. In sharp contrast, the overall level of Cdk2 expression (i.e., not considering CcnE1/CcnE2 level) was not significantly associated with patient survival ($P = 0.0919$, Fig. 6*B*). Detailed analysis of selected patient subcohorts with defined expression levels of CcnE1 and CcnE2 further excluded any significant effect of Cdk2 on patient survival (*SI Appendix*, Fig. S8*B*), suggesting that advanced HCC progression in patients is strongly affected by CcnE1 and CcnE2 in a Cdk2-independent manner.

Finally, we investigated if the murine gene signature describing CcnE1-independent hepatoma cells (compare Fig. 6*A*) might also be relevant in HCC patients. To this end, we evaluated the coexpression of several candidate genes with CcnE2 in all 369 HCC patients. Importantly, we observed a high positive Spearman correlation of Ect2, Msh2, Rad21, and E2F1 gene expression with CcnE2 (adjusted P value <0.000005, Fig. 6*C*), indicating that a subpopulation of HCC patients comprises a similar gene signature as CcnE1-independent murine hepatoma cells. Altogether, the bioinformatic analyses point to an important role of CcnE1 and CcnE2 for HCC progression and increased patient mortality, independent of Cdk2.

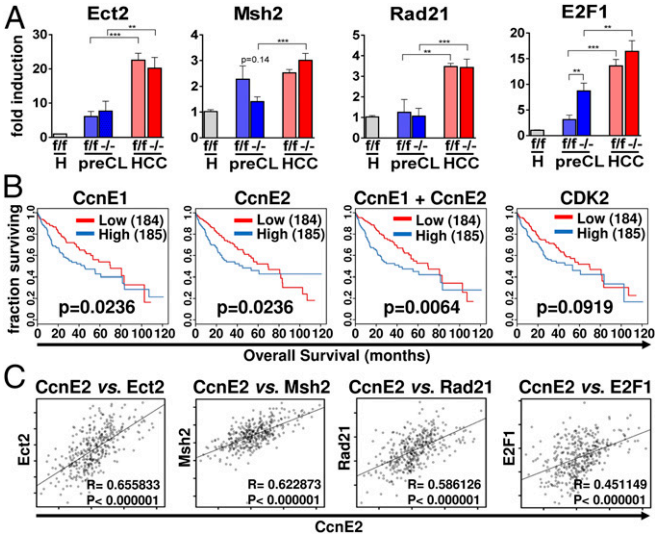

Fig. 6. Common properties and gene signatures of CcnE1-independent mouse hepatoma cells and HCC patients with high CcnE2 expression. (*A*) Four independent CcnE1$^{f/f}$preCL cell lines (preCL) and three independent HCC-derived cell lines (HCC) were infected with adv-cre to delete CcnE1 or treated with adv-EGFP control virus. qPCR gene expression analysis for CcnE1, CcnE2, and CcnA2 in seven cell lines with (f/f) or without ($^{-/-}$) CcnE1 expression is shown. H, primary hepatocytes from CcnE1$^{f/f}$ mice ($n = 3$) were used as reference. (*B* and *C*) Bioinformatic analysis of 369 human HCC samples based on data generated by the Cancer Genome Atlas (TCGA) Research Network. (*B*) Overall survival comparison analysis of patient cohorts with high (i.e., above median) or low (i.e., below median) expression of CcnE1, CcnE2, CcnE1, and CcnE2 or Cdk2. *P* values are indicated within diagrams. (*C*) Scatter plots showing high positive Spearman Correlation of Ect2, Msh2, Rad21, E2F1, and Rbbp7 with CcnE2 expression in HCC patients. p, adjusted *P* value; R, Spearman coefficient. $^{**}P < 0.005$; $^{***}P < 0.001$.

Discussion

Several earlier studies suggested that the E-cyclins CcnE1 and CcnE2 are functionally equivalent (10). However, more recent work also identified nonredundant functions of either CcnE1 or CcnE2 (11). HCC is associated with strong CcnE1 overexpression correlating with poor prognosis of patients, while the contribution of CcnE2 for liver cancer has barely been investigated so far (5). In a recent and elegant study, Sicinski and coworkers (12) showed that compound deletion of CcnE1 and CcnE2 prevented HCC progression in mice and halted proliferation of human HCC cells. However, this important work does not answer the question of whether CcnE1 or CcnE2 may mediate individual functions in distinct stages of hepatocarcinogenesis, which was now extensively addressed in our present study.

In our present study, ablation of CcnE1 largely prevented the development of HCC in two independent tumor models, and previous analysis indicated that inhibition of CcnE1 may also attenuate hepatocarcinogenesis in an inflammatory environment (6). In sharp contrast, CcnE2 was fully dispensable for HCC formation. Importantly, the tumor-initiating function of CcnE1 depends on its canonical binding partner Cdk2. We therefore conclude that CcnE1 and Cdk2 mediate a unique and essential function specifically for early HCC initiation.

A more detailed in situ analysis of residual liver dysplasia in DEN-treated CcnE1$^{-/-}$ mice revealed focal overexpression of CcnE2, which was not found in HCC-bearing WT mice. Similarly, we found low or moderate CcnE2 expression in preCL cells, whereas CcnE2 expression in HCC-derived cells was substantially higher. Based on this data, we conclude that induction of CcnE1 during hepatocyte transformation is an early key event, whereas CcnE2 overexpression occurs at a later stage of hepatocarcinogenesis. This best explains why CcnE2$^{-/-}$ mice (with strong CcnE1 expression) frequently develop HCC while carcinogenesis in CcnE1$^{-/-}$ livers is restricted to a few foci with spontaneous CcnE2 expression. However, this interpretation also implicates that expression of basically any E-type cyclin is sufficient to drive hepatocarcinogenesis. This is in perfect agreement with our bioinformatics analysis of 369 HCC patients showing an additive effect of CcnE1 and CcnE2 expression on patient survival.

Our experiments on the role of Cdk2 for hepatocarcinogenesis revealed complex insights. Cdk2$^{\Delta hepa}$ mice revealed significantly reduced tumor burden following DEN treatment, while the requirements for Cdk2 in preCL or HCC hepatoma cells were different. Our experiments using Cdk2$^{f/f}$HCC cells suggest that advanced HCC progression can occur largely in a kinase-independent fashion, as HCC cells lacking Cdk2 proliferated normally and did not reveal considerable levels of CcnE1- or CcnE2-associated kinase activities. Of note, Geng et al. (12) received similar results in human HCC cell lines, demonstrating

that the dispensability of Cdk2 for advanced HCC progression is species-independent. In good agreement, we could exclude any significant effect of Cdk2 expression on survival of HCC patients. However, our data from murine preCL cells demonstrate an exclusive need for Cdk2 kinase activity for early tumorigenesis.

We finally aimed to understand the genetic differences between CcnE1-addicted and CcnE1-independent hepatoma cells besides CcnE2 expression levels. As expected, CcnE1-depleted preCL cells revealed significant down-regulation of cell cycle-related pathways and genes, and most of these genes such as Ect2 or E2F1 have been implicated in progression of HCC (13, 14). Unexpectedly, we also found significant deregulation of several DNA repair pathways. Recent work suggested that inactivation of DNA repair could be related to impaired tumor growth (15), and some of our candidate genes such as Msh2, Rad21, and Npm1 were shown to be involved in HCC progression (16–18). Of note, down-regulation of the DNA repair machinery in preCL cells was associated with strong accumulation of DSBs and predisposition to Caspase-3–mediated apoptosis. It can be speculated that CcnE1 expression enables cell cycle progression of precancerous cells despite excessive DSBs by overcoming checkpoints that would usually resulting in arrest or apoptosis. It is remarkable that the gene signature, which describes CcnE1-independent mouse hepatoma cells, is also of importance in HCC patients. Accordingly, we found a significant correlation of CcnE2 expression with Ect2, Msh2, Rad21, E2F1, and Rbbp7 in HCC patients. It is therefore tempting to speculate that HCC patients with low expression of these factors would particularly benefit from therapeutic CcnE1 inhibition similar to preCL cells.

Our current model on the role of CcnE1 and CcnE2 for initiation and progression of HCC is illustrated in *SI Appendix*, Fig. S8*C*: Precancerous hepatoma cells reflect the early stage of HCC initiation, are characterized by DSB accumulation, and require CcnE1/Cdk2 kinase activity for proliferation. At this early stage, these cells with low overall activation of the cell cycle and DNA repair machinery can be considered CcnE1-addicted, and patients with such a signature have a better prognosis. Inhibition of CcnE1 under these conditions is sufficient to prevent HCC in mice and might be also beneficial in patients. In contrast, CcnE1-independent hepatoma cells are characterized by a gene signature comprising high CcnE2 expression, elevated expression of DNA repair genes, and overall strong cell cycle activity even in absence of CcnE1 or Cdk2, and we identified patient cohorts with a similar expression pattern. We predict that inhibition of CcnE1 and/or Cdk2 in such patients will not be a promising treatment option at all. However, determination of gene signatures in HCC tissue from patients could, in general, help to predict the response toward an anti-CcnE1 therapy. We recently showed that therapeutic targeting of CcnE1 in vivo using RNA interference is technically possible and capable to halt the progression of liver fibrosis (19). Thus, our present data suggest that targeting CcnE1 in HCC patients with the appropriate gene signature (i.e., CcnE2 low, cell cycle moderate, DNA repair low) could be therapeutically beneficial. Altogether, our study identified individual contributions of CcnE1, CcnE2, and Cdk2 for either initiation or progression of HCC and provided markers for the classification into CcnE1-dependent or CcnE1-independent liver tumors in mice and man.

Materials and Methods

Animal Experimentation. For our study, we used mice of male gender with constitutive deletion of CcnE1 ($CcnE1^{-/-}$) and CcnE2 ($CcnE2^{-/-}$) as reported (2). The generation of hepatocyte-specific Cdk2 knockout mice ($Cdk2^{\Delta hepa}$) and the proof of efficient Cdk2 deletion in liver has been described in our previous study (3). Transgenic mice carrying a c-myc transgene under the control of the hepatocyte-specific albumin promoter (alb-myc^{tg}) were provided by S. Thorgeirsson, National Cancer Institute, Bethesda (8). For HCC induction, 14-d-old male mice were injected once intraperitoneally (i.p.) with 25 mg of DEN/kg of body weight and euthanized after 22 or 38 wk. Animal experiments were approved by the authority for environment conservation and consumer protection of the state North Rhine-Westfalia (State Agency for Nature, Environment and Consumer Protection, Recklinghausen, Germany).

Generation of Primary Murine Hepatoma Cells with Floxed Cyclin E1 and Cdk2 Genes. For the isolation of cyclin E1 or Cdk2 floxed primary hepatoma cells, we used mice with a floxed allele of cyclin E1 ($CcnE1^{f/f}$; ref. 20) or $Cdk2^{f/f}$ (21) and a recently described protocol (22) with some modifications as specified in *SI Appendix*.

Statistical Analysis. Data were presented as mean ± SEM unless otherwise stated. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by a Student's *t* test.

Availability of Whole Transcriptome Shotgun Sequencing Data. Data has been submitted to the Gene Expression Omnibus (GEO) repository, accession no. GSE111079.

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