

Inhibition of hepatic cell proliferation through the systematic dissection of Cdk2/cyclin E complexes in mice

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“All difficult things have their origin in that which is easy, and great things in that which is small (大道易简).“

Lao Tzu

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Abbreviations

adv	adenovirus
AFP	α -fetoprotein
alb	albumin
ALT	Alanine-aminotransferase
APS	Ammonium-persulfate
AST	Aspartate-aminotransferase
ATP	Adenosine-5'-triphosphate
BSA	Bovine Serum Albumin
BrdU	5-bromo-2'-deoxyuridine
Ccn	Cyclin
Cdc2	Cell division cycle 2
Cdk	Cyclin-dependent kinase
cDNA	complementary DNA
CK19	Cytokeratin 19
Cre	Causes recombination
d	day(s)
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxy-nucleoside-triphosphate
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylene-diamine-tetraacetic acid
EGTA	Ethylene-glycol-tetraacetic acid
EGF	Epidermal Growth Factor
EtBr	Ethidium-bromide
GAPDH	Glycerinaldehyde-3-phosphate-dehydrogenase
h	hour(s)
HE	Hematoxylin Eosin
HGF	Hepatocyte Growth Factor
HRP	Horseradish Peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid
<i>i.p.</i>	intraperitoneal
kb	kilo-base
kDa	kilo-Dalton
loxP	locus of crossing over
m	minute(s)
MEF	Murine Embryonic Fibroblast
mRNA	messenger RNA
PCR	Polymerase Chain Reaction
PH	Partial Hepatectomy
qPCR	quantitative Realtime PCR
RNA	Ribonucleic acid
rpm	rotation per minute

RT	Room Temperature
s	second(s)
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline
TEMED	Tetramethyl-ethylene-diamine
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyethylene-20-sorbitan-monolaurate
V	Volt
WT	Wild Type

1. Introduction

1.1 The Liver

The liver is the largest internal organ in vertebrates. The earliest knowledge of liver anatomy dates back to approximately 2000 B.C. attributed to the Babylonians in the Assyro-Babylonian era (Townsend and Sabiston 2001). Thereafter, the ancient Greeks described for the first time the liver's ability to regenerate in the myth of Prometheus. Medical terms related to the liver often start in *hepato-* or *hepatic* from the Greek word for liver, *hēpar* (ἥπαρ).

1.1.1 Physiology and function of the liver

The liver exerts many functions including metabolism, production, storage and redistribution of nutrients, carbohydrates, lipids or fatty substances, and vitamins. In the first line, the liver serves as a central regulator of metabolic homeostasis for the body. It converts glucose into glycogen and stores it until required by the organism. The liver synthesizes plasma proteins such as clotting factors regulating blood clotting and albumin transferring water-insoluble substances and maintaining osmotic pressure. The metabolism and storage of many vitamins including vitamins A, D, K and B12 is another major function of the liver, which provides critical cofactors in enzymatic processes. Additionally, the liver produces bile acids which are essential for the processing of dietary fat. Other essential roles of the liver include detoxification of drugs, alcohol and xenobiotics, iron metabolism, clearance of by-products of metabolism such as bilirubin and elimination of numerous foreign antigens and microbes from the gut (Arias et al. 2009; Dufour and Clavien 2010).

1.1.2 Hepatocytes

To accomplish all these functions, distinct types of cells contribute to structure formation of the liver as a functional tissue. Among them, the hepatocyte, also called liver parenchymal cell, is the most abundant cell type by mass and number in the liver constituting approximately 60% of all liver cells and over 80% of the liver-cell mass under non-pathological conditions (Mitaka 1998; Gupta 2000).

Most functions within the liver are executed by hepatocytes characterized by high metabolic capacity. Therefore, hepatocytes are typically polyploid and polynuclear cells. Up to 40-50% hepatocytes contain two or more nuclei. Multinucleated hepatocytes develop mainly through endomitosis, in which mitosis is aborted prior to cytokinesis. Polyploidy in hepatocytes can further be induced by endoreplication – a process involving replication of DNA without nuclear division and subsequent mitosis. In addition, several studies demonstrated that polyploid hepatocytes can also be generated through fusion of a bone marrow-derived cell and a host hepatocyte *in vivo* (Wang et al. 2003; Willenbring et al. 2004; Duncan et al. 2010). Recently, it was also shown that cell fusion between hepatocytes is a physiological process in the murine liver (Faggioli et al. 2008; Faggioli et al. 2011).

1.1.3 Liver regeneration and two-thirds partial hepatectomy

In the healthy liver, mature hepatocytes are long lived quiescent cells with little turnover. However, in response to injury the liver displays remarkable regenerative capabilities for recovery of mass and functions. This fascinating process is best illustrated by liver growth after surgical resection of two-thirds of its mass, the standard partial hepatectomy (PH) procedure pioneered by Higgins and Anderson in 1931 and now commonly used to study liver regeneration (Higgins and Anderson 1931; Michalopoulos 2010). In this model, three liver lobes (left lateral, left middle and right middle lobe) constituting approximately 70% of liver mass are surgically removed. As a consequence, the remaining liver enlarges until the original liver mass is restored. This process is based on a compensatory hypertrophy and hyperplasia of the remaining tissue rather than on a true regenerative process, where the excised structure can reconstitute such as the regeneration of limbs in amphibian models. In a mouse model of 2/3 PH, original liver mass can be restored approximately within 7-10 days following surgery with a peak of DNA synthesis after approximately 42 h with some variation depending on the genetic mouse background (Fausto and Riehle 2005).

Liver regeneration following PH proceeds in a highly ordered fashion (Figure 1.1). The several steps of liver regeneration are regulated by integrated multiple pathways involving cytokines, growth factors and metabolic networks (Fausto et al. 2006). As a simplified model, liver regeneration can be divided into three distinctive phases: the initiation or priming phase, where quiescent hepatocytes are rendered in a state of

replicative competence; the proliferation phase, where up to 95% of previously quiescent hepatocytes re-enter cell cycle; and the termination phase, where cell proliferation in liver is suppressed in order to terminate regeneration at a defined time point (Zimmermann 2004; Fausto and Riehle 2005).

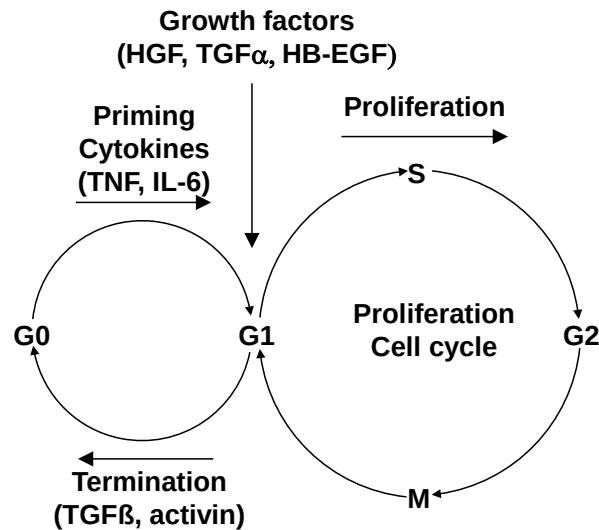


Figure 1.1: Multistep model of liver regeneration after PH (modified from Fausto et al. 2006). Liver regeneration can be divided into three phases: the priming phase, the proliferation phase and the termination phase. Cytokines and growth factors play a crucial role for regulating this process.

Cytokine pathways are largely responsible for the priming phase. The well-known key-players in the cytokine network are the pro-inflammatory molecules Tumor Necrosis Factor (TNF) and Interleukin-6 (IL-6), which are mainly produced by Kupffer cells, the resident macrophages of the liver. Expression of TNF and IL-6 is induced very rapidly after PH and primes hepatocytes to enter the cell cycle and to respond to the mitogenic effect of growth factors. Subsequent to priming, so called “immediate early genes” are induced within 2 hours after PH. These genes are predominantly transcription factors (e.g. c-fos, c-jun and c-myc), which activate the gene expression of downstream mediators involved in liver regeneration. Studies using microarray analysis revealed that approximately 185 genes are regulated during the priming phase of liver regeneration (Su et al. 2002).

In the proliferation phase at least three growth factors are of major importance: Hepatocyte Growth Factor (HGF), Transforming Growth Factor-alpha (TGF α) and Heparin-binding epidermal Growth Factor-like growth factor (HB-EGF). HGF is produced by non-parenchymal cells in the liver and also in other tissues and therefore stimulates hepatocytes in a paracrine or endocrine manner (Pediaditakis et al. 2001).

Both TGF α and HB-EGF are members of the Epidermal Growth Factor (EGF) family. TGF α is synthesized by hepatocytes and acts in an autocrine manner. HB-EGF is strongly induced after PH and further precedes expression of HGF and TGF α . There is evidence that HB-EGF links the priming and proliferation phase of liver regeneration (Mitchell et al. 2005). Following mitogenic stimulation by cytokines and growth factors, hepatocytes seem to autonomously undergo proliferation without extracellular stimuli. Details about the subsequent cell cycle progression will be described in chapter 1.2.

Once the restoration of liver functions and liver mass is completed, the induced regenerative response must finally be terminated. Major factors involved in the termination phase comprise TGF β and activins, which can inhibit DNA synthesis in hepatocytes. However, the precise molecular mechanisms responsible for the termination of liver regeneration are still poorly understood (Michalopoulos 2007; Böhm et al. 2010).

1.2 Cell cycle regulation in vertebrates

The cell cycle in eukaryotic cells consists of four distinct phases: G1 (Gap1)-phase, S-phase (DNA-synthesis), G2 (Gap2)-phase and M-phase (mitosis), respectively. The first three phases are collectively known as interphase. In addition, quiescent cells such as mature hepatocytes reside in a reversible non-replicative phase termed G0-phase.

From yeast to humans, the cell cycle is driven by a group of heterodimeric serine/threonine kinases known as cyclin-dependent kinases (Cdks). In yeast, a single Cdk (Cdc28 in *Saccharomyces cerevisiae* and Cdc2 in *Schizosaccharomyces pombe*) drives all phases of cell cycle, while in mammalian cells the sequential activation of at least four different Cdks (Cdk1, equivalent to Cdc2, Cdk2, Cdk4 and Cdk6) is responsible for the whole process. Accordingly, four cyclin families specifically activate corresponding Cdks *via* complex formation in different phases of cell cycle: CcnD¹ (CcnD1, CcnD2 and CcnD3) activate Cdk4 and Cdk6; CcnE (CcnE1 and CcnE2) activate Cdk2; CcnA (CcnA1 and CcnA2) activate Cdk2 and Cdk1; CcnB (B1, B2 and B3) activate Cdk1 (Satyanarayana and Kaldis 2009).

¹ Nomenclature for cyclin: Ccn stands for cyclin. CcnD is referred for all there D-type cyclins, it is valid as same as for CcnE, CcnA and CcnB. Single cyclin is referred as e.g. CcnD1 or CcnE1.

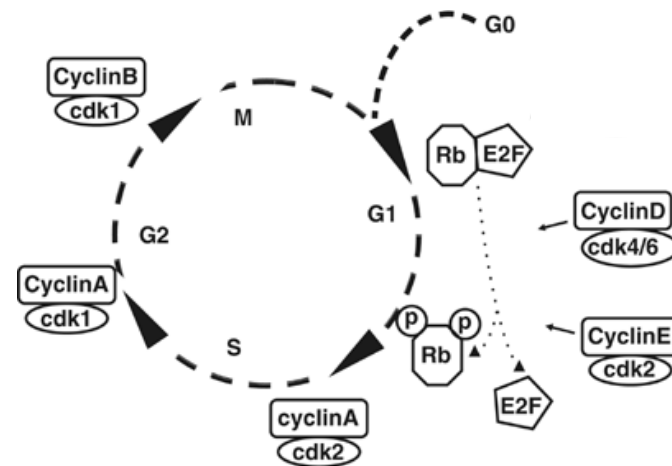


Figure 1.2: Cell cycle machinery and Rb-E2F pathway (modified from Giacinti and Giordano 2006). Specific Cdk/cyclin complexes regulate cell cycle progression. In G0 and early G1 phase, unphosphorylated Rb physically associates with the E2F transcription factors. In late G1 phase, p-Rb phosphorylated by Cdk4/6-CcnD and Cdk2/CcnE releases E2F, allowing the expression of cell cycle genes necessary for S-phase progression.

Extracellular stimuli such as cytokines and growth factors drive quiescent cells (G0-phase) into G1-phase as described previously. Subsequent progression of the cell cycle through each phase is precisely regulated by fluctuating activation of Cdk/cyclin complexes (Figure 1.2). The composition and regulation of Cdk/cyclin complexes vary depending on cell type and environment. Briefly, under normal conditions the mammalian cell cycle starts with expression of D-type cyclins, which are induced by extracellular mitogens. The key event in G1 phase is phosphorylation and thus inactivation of the retinoblastoma protein Rb and its homologues p107 and p130. Cdk4/CcnD and Cdk6/CcnD complexes phosphorylate specific residues of Rb, which leads to release of E2F and induction of early E2F responsive genes such as CcnE. CcnE activates Cdk2 in the later G1 phase resulting in completion of Rb phosphorylation and further induction of E2F responsive genes. Cells are then prepared to passage through the restriction point at boundary of G1/S phase. Until cells have reached the restriction point, the process of cell cycle initiation is fully revertible by depleting growth factors (Morgan 1997).

S-phase of the cell cycle is defined as the time interval necessary for DNA-synthesis. In S-phase, CcnA2 together with Cdk2 and Cdk1 orchestrates the process of DNA-replication. Once DNA replication is finished, cells enter G2 phase, where Cdk1/CcnA2 kinase complexes prepare the cells for mitosis. For progression through mitosis the association of Cdk1 with B-type cyclins is essential. Along with the cell cycle, diverse checkpoints (G1/S, intra S-phase, G2/M and intra mitosis)

determine further progression or arrest. For this control, Cdk inhibitors (CKIs) involving Ink4 family (p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c} and p19^{Ink4d}) and Cip/Kip (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}) family play crucial roles by preferentially inhibiting Cdk4/6 and Cdk2 complexes, respectively (Sherr and Roberts 1999).

1.2.1 Origin licensing and G1/S-transition

In S-phase, the chromosomal DNA has to be precisely replicated without errors exactly once per cell cycle. To accomplish this challenging task for the cell, a tightly coordinated process for initiation of replication involving more than 20 proteins has been evolved in eukaryotic cells (Bell and Dutta 2002). The mechanism of initiation comprises origin recognition, assembly of pre-replication complexes (pre-RC), helicase activation and replisome loading.

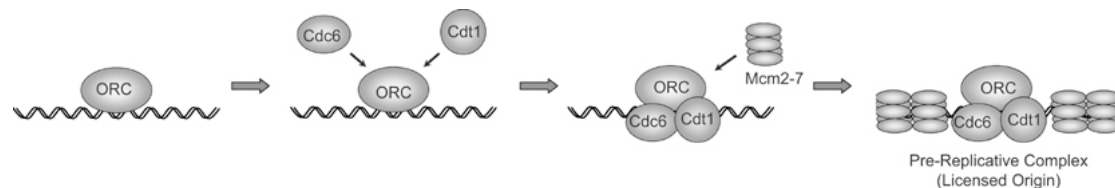


Figure 1.3: Assembly of the pre-RC (adapted from Takeda and Dutta 2005). Origin licensing begins with the loading of ORC onto replication origins. ORC recruits Cdc6 and Cdt1, which are essential for the subsequent loading of multiple Mcm2-7 complexes at each origin.

This process already starts normally during early G1 phase with recruitment of a group of six related proteins collectively referred to as the origin recognition complex (ORC) to replication origins prior to S phase (Figure 1.3). The proteins constituting the ORC complex are conserved from yeast to humans, whereas the recruitment mechanisms vary largely between simple and higher eukaryotes. However, the subsequent steps are highly conserved among all eukaryotes. ORC recruits the initiation factors Cdc6 and Cdt1 to origins, which promotes the loading of the heterohexameric MCM2-7 complex. It has been shown that the MCM2-7 complex serves as the replicative helicase during DNA-synthesis and is required for unwinding the origin (Walter and Newport 2000; Labib and Diffley 2001; Labib et al. 2001; Forsburg 2004). A recent study using CcnE knockout mice showed that E-type cyclins facilitate the loading of MCM2-7 complexes on chromatin through physical interaction with Cdt1 (Geng et al. 2007). Once MCM2-7 complexes are loaded onto chromatin, the assembly of pre-RC is completed. From then on, the other components of the pre-RC are dispensable for replication initiation.

The loading of the MCM complex represents the license for replication. The precise regulation of pre-RC formation is of high relevance to ensure that DNA replication occurs only once per cell cycle. In this scenario, Cdk/cyclin complexes belong to the primary regulators besides their well-established role in driving cell cycle progression. For pre-RC formation in G1-phase, overall low Cdk activity is required. In contrast, high Cdk activity is responsible for both removing the licence and preventing relicensing in S-phase. High Cdk activity persists until the next round of cell cycle to avoid re-replication (Coverley et al. 2002).

1.2.2 Functional redundancy of interphase Cdk and associated cyclins

The presence of phase-specific activation of Cdk by multiple cyclins in higher eukaryotic cells led to the prediction that each Cdk/cyclin complex carries unique phase-related functions. Experiments blocking kinase activity of different Cdk *in vitro* nicely demonstrated the correlation between cell cycle arrest in one specific phase and inhibition of the responsible kinase (Th'ng et al. 1990; Yasuda et al. 1991; van den Heuvel and Harlow 1993).

Intriguingly, *in vivo* studies using gene targeting in mice revealed widespread redundancy among mammalian Cdk and also of cyclins in most cell types. The first evidence came from studies using knockout mice with depletion of individual D-type cyclins, which displayed overall normal development besides some abnormalities in individual cell types (Fantl et al. 1995; Sicinski et al. 1995; Sicinski et al. 1996; Huard et al. 1999; Lam et al. 2000; Sicinska et al. 2003). It appears that deletion of a single D-type cyclin can be substituted by its siblings in the majority of cell types. In line with this idea, expression of the CcnD2 gene knocked into the original CcnD1 locus rescued the phenotype of CcnD1^{-/-} mice (Carthon et al. 2005). However, each D-type cyclin also performs essential functions in specific cell types, e.g. CcnD1 in retina, mammary glands and neurons, CcnD2 in B lymphocytes and pancreatic β -cells, and CcnD3 in T lymphocytes (Huard et al. 1999; Solvason et al. 2000; Kowalczyk et al. 2004). Further investigation in cells and mice with combined depletions of two or all three D-type cyclins revealed that loss of D-type cyclins may also be compensated by E- or A-type cyclins (Ciemerych et al. 2002; Kozar et al. 2004; Pagano and Jackson 2004). For instance, murine embryonic fibroblasts (MEFs) lacking all D-type cyclins can still go through cell cycle and divide suggesting that E- or A-type cyclins can take over functions of D-type cyclins at least in some cell types (Kozar et al. 2004). In line

with this hypothesis, expression of human CcnE1 knocked into the original CcnD1 locus also rescued the phenotype of CcnD1^{-/-} mice (Geng et al. 1999).

The catalytic partners of the D-type cyclins, Cdk4 and Cdk6, share 71% structure identity and can both interact with all three D-type cyclins to phosphorylate Rb. Mice lacking either Cdk4 or Cdk6 are viable and display similar abnormalities as observed in mice lacking D-type cyclins (Rane et al. 1999; Tsutsui et al. 1999; Malumbres et al. 2004). It appears that Cdk4 and Cdk6 can compensate for each other in most cells. However, specific roles of Cdk4 or Cdk6 were also observed in several cell types, which might be attributed to distinct substrate specificity and subcellular localization (Rane et al. 1999; Franklin et al. 2000; Martin et al. 2003; Malumbres et al. 2004). In the case of combined deletion of both Cdk4 and Cdk6, Cdk2 can probably take over their functions *via* complex formation with D-type cyclins (Malumbres et al. 2004).

Similar to the case of CcnD associated complexes, none of the components of the Cdk2/CcnE (i.e. CcnE1 or CcnE2) complexes are absolutely required for mouse development and cell cycle progression, which is surprising as the Cdk2/CcnE complexes were once believed to be essential for G1/S transition in all mammalian cells (Berthet et al. 2003; Geng et al. 2003; Ortega et al. 2003). However, combined deletion of both CcnE1 and CcnE2 results in embryonic lethality although this is rather due to placental defects involving impaired endoreplication of trophoblast giant cells. Surprisingly, CcnE-deficient MEFs proliferate actively under conditions of continuous cell cycling but are unable to re-enter the cell cycle from the quiescent G0 state (Geng et al. 2003).

Further investigations uncovered a novel function of CcnE in assembly of the pre-replication complex (pre-RC) during initiation of DNA-synthesis, which is essential for endoreplication of trophoblast giant cells and megakaryocytes as well as for re-entering the cell cycle from quiescence (Geng et al. 2007). The deletion of Cdk2, which presents the canonical binding partner of E-type cyclins, does not reproduce any of the phenotypes observed in CcnE1^{-/-}CcnE2^{-/-} double knockout mice indicating that at least some of the functions of E-type cyclins are Cdk2-independent (Berthet et al. 2003; Ortega et al. 2003; Geng et al. 2007). Several studies suggest that Cdk4 and Cdk1 might contribute to the compensatory mechanism in the absence of Cdk2 (Aleem et al. 2005; Jablonska et al. 2007; Santamaria et al. 2007; Satyanarayana et al. 2008b). In this line of evidence, Cdk2^{-/-}Cdk4^{-/-} double knockout mice die during embryonic development due to heart defects indicating overlapping function between Cdk2 and Cdk4 at least in cardiomyocytes (Berthet et al. 2006). Another study

showed that Cdk4 compensates for loss of Cdk2 in neural progenitor cells of the subventricular zone (Jablonska et al. 2007).

In addition, it was demonstrated that Cdk1 can take over the function of Cdk2 under several conditions (Aleem et al. 2005; Santamaria et al. 2007). Most strikingly, mouse embryos lacking Cdk2, Cdk4 and Cdk6 develop normally until mid-gestation. Except for some cell types such as hematopoietic cells and cardiomyocytes, other cells do not display obvious cell cycle defects in absence of all interphase Cdks. It seems that Cdk1 alone is sufficient to drive the cell cycle in the majority of mammalian cell types, which mimics the situation in yeast (Santamaria et al. 2007).

Owing to the essential role of Cdk1 for mitosis, deletion of Cdk1 leads to early embryonic lethality (Diril et al. 2012). No evidence so far indicated that other Cdks can compensate for the loss of Cdk1. Even when Cdk2 is directly expressed from the Cdk1 locus, it still leads to early embryonic lethality (Satyanarayana et al. 2008a). Cdk1 can interact with either A-type cyclins (CcnA1 and CcnA2) or B-type cyclins (CcnB1, CcnB2 and CcnB3). Expression of CcnA1 is restricted to testes and CcnA1^{-/-} mice are viable and develop normally (Liu et al. 1998). In contrast, CcnA2 is expressed ubiquitously and genetic ablation of CcnA2 leads to early embryonic lethality indicating an essential role for early embryogenesis (Murphy et al. 1997). A recent study reported that acute ablation of CcnA alone in MEFs led to prolonged expression of CcnE without affecting cell proliferation, while CcnA seems to be indispensable for cell proliferation of hematopoietic and embryonic stem cells. Concomitant deletion of both E- and A-type cyclins completely extinguished cell division (Kalaszczyńska et al. 2009).

Similar to CcnA1, expression of CcnB3 is also restricted to testes and fetal ovaries (Nguyen et al. 2002). The precise functions of CcnB3 in mouse embryogenesis and development are still unknown since CcnB3^{-/-} mice have not been described so far. However, CcnB1^{-/-} and CcnB2^{-/-} mice have been generated. CcnB1^{-/-} mice died before day 10.5 p.c., while CcnB2^{-/-} mice are viable suggesting that CcnB1 can fully compensate the loss of CcnB2 (Brandeis et al. 1998).

Taken together, among the Cdk/cyclin network in mammals Cdk1 is the only cyclin-dependent kinase that cannot be replaced by other interphase Cdks and its binding partners (CcnA2 and CcnB1) appear to be the most non-redundant cyclins. Functional redundancy of other interphase Cdks and cyclins complicates the analysis of cell cycle mechanisms *in vivo* and requires their conditional inactivation in mice to study their role during embryogenesis and in adult organs.

1.3 Cell cycle inhibition by the kinase inhibitor Roscovitine as a potential anti-cancer therapy

Cdk activity is often increased in proliferative diseases such as cancer, owing to deregulation of cell cycle pathways frequently leading to overexpression of Cdk activators (cyclins) and/or inactivation of Cdk inhibitors. Thus, the development of small-molecule Cdk inhibitors for cancer therapy has been an intense area of research in the last decades (Malumbres et al. 2008).

(R)-Roscovitine (also referred to as Seliciclib or CYC202) is a second-generation Cdk kinase inhibitor. It belongs to a group of substituted purines with a basic ring structure, which can mimic the ATP molecule therefore act by directly competing with ATP for binding to the catalytic site of the kinase (Meijer and Raymond 2003). Compared to other broad-range kinase inhibitors such as Flavopiridol and Olomoucine, Roscovitine displays a high specificity towards Cdk2, Cdk1, Cdk7 and Cdk5 (Meijer et al. 1997).

An abundance of biochemical studies on a wide variety of cultured cells demonstrated that Roscovitine basically mediates two major effects: cell cycle arrest and cell death. Roscovitine inhibits cell cycle progression in different cell cycle phases depending on the cell line, the dose and the duration of treatment (Wesierska-Gadek et al. 2009b). This effect is mainly attributed to the direct inhibition of Cdks (Alessi et al. 1998; Lacrima et al. 2005; Payton et al. 2006). However, indirect effects of Roscovitine cannot be excluded. For instance, Roscovitine inhibits the Cdk-activating kinase (CAK), Cdk7, which in turn impedes other Cdk activities (Wesierska-Gadek et al. 2009a). As a consequence, direct and indirect inhibition of Cdks by Roscovitine results in impairment of many essential events for cell cycle progression such as DNA replication initiation, centrosome duplication and nuclear envelope breakdown (Matsumoto et al. 1999; Sirri et al. 2002).

The cell death-inducing effect of Roscovitine was initially thought to result from cell cycle arrest. However, increasing evidence indicates that rather inhibition of Cdk7/9-dependent transcription by Roscovitine triggers cell death mechanisms. Both Cdk7 and Cdk9 contribute to activation of RNA-polymerase II *via* phosphorylation of its C-terminal domain. The lack of phosphorylation reduces mRNA synthesis, which eventually leads to cell death in several investigated cell lines (Ljungman and Paulsen 2001; MacCallum et al. 2005). Subsequent studies demonstrated that Roscovitine

inhibits transcription of essential cell survival factors such as Mcl-1, XIAP and Survivin (Kim et al. 2004; Alvi et al. 2005; Lacrima et al. 2005; Mohapatra et al. 2005).

For its ability to inhibit cell cycle progression and induce cell death, Roscovitine treatment becomes an ideal candidate for anti-tumor therapy. Moreover, the short half-life of Roscovitine and the lack of activity of its metabolites might sensitize tumor cells to cell death but prevent normal cells from an irreversible inhibition. Due to its anti-tumor effect, Roscovitine has reached phase 2 clinical trials for B-cell malignancies, lung and breast cancer (Aldoss et al. 2009).

1.4 Aim of this study

Previous studies using gene-targeting techniques demonstrated that mice lacking Cdk2 develop normally besides meiotic failure. Cdk2 appears dispensable for cell proliferation of most cell types. However, cell type specific functions of Cdk2 are not well characterized. Previous studies revealed conflicting results about the role of Cdk2 for liver regeneration. Therefore, the first aim of the current study was to examine the role of Cdk2 for hepatocyte proliferation and liver regeneration in the well-established model of partial hepatectomy (PH) in mice. For this purpose, conditional, hepatocyte-specific Cdk2 knockout mice should be generated for the first time as part of this study and subjected to PH.

Based on the initial result that loss of Cdk2 in hepatocytes does not significantly impair liver regeneration, the second aim of this study was to identify molecules and mechanisms that compensate the loss of Cdk2 in the murine liver. This work package was based on the hypothesis that CcnE1 mediates important, Cdk2-independent functions in the regenerating liver and should analyze the expression, localization and function of CcnE1 in the Cdk2-deficient liver.

The last aim was to precisely dissect the contributions of all components of the Cdk2/CcnE complexes (Cdk2, CcnE1 and CcnE2) for cell cycle progression of hepatocytes *in vitro* and *in vivo*. For this purpose, mouse mutants harbouring all possible combinations of Cdk2 and CcnE1/CcnE2 knockout alleles should be generated with the ultimate aim to substantially block hepatocyte proliferation *in vitro* (in *ex vivo* isolated primary hepatocytes) or *in vivo* (after PH). This knowledge gained from the liver regeneration model will also be of clinical relevance as it could help to block aberrant proliferation of hepatocytes in hepatocellular carcinomas or in other liver diseases with extensive cell proliferation such as chronic hepatitis or liver fibrosis.

2. Material and Methods

2.1 Material

2.1.1 Chemicals

Name of the product	Company
Ammoniumpersulfate (APS)	AppliChem
Acetone	J.T.Baker
Agarose	Invitrogen
Acetic acid (glacial) 100%	AppliChem
Ammoniumacetate	AppliChem
Adenosin-5'-triphosphate (ATP)	Sigma-Aldrich
[³² P]-γ-ATP (10mCi/ml)	Hartmann Analytic
Bromophenol Blue	Serva
Bovine Serum Albumin(BSA)	Sigma-Aldrich
Calciumchloride (CaCl ₂)	Sigma-Aldrich
Chloroform	Merck
Dithiotreitol (DTT)	Sigma-Aldrich
Dodecylsulfate (SDS)	Roth
Ethylene-diamine-tetraacetic acid (EDTA)	Calbiochem
Ethylene-glycol-tetraacetic acid (EGTA)	Fluka
Eosine	Sigma-Aldrich
Ethanol 100%	AppliChem
Ethidiumbromide (EtBr)	Sigma-Aldrich
Formaldehyde 37%	Roth
Glucose	Merck
Glutaraldehyde	AppliChem
Glycerol	Sigma-Aldrich
Glycine	AppliChem
Haematoxyline / Eosin (HE)	Merck
Hydrochloric acid (HCl)	AppliChem
4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES)	Merck
Magnesiumchloride (MgCl ₂)	Sigma-Aldrich
100% Methanol	VWR
Nonidet P-40	Sigma-Aldrich
Paraformaldehyde	Merck

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Ponceau Red	Sigma-Aldrich
Potassiumchloride (KCl)	Merck
2-Propanol	Merck
Sodiumfluoride (NaF)	Roth
Sodiumvanadate (Na ₃ VO ₄)	Sigma-Aldrich
Tetramethyl-ethylene-diamine	TEMED
Tris Buffer grade	AppliChem
Triton X-100	Sigma-Aldrich
TWEEN-20	Serva

2.1.2 Enzymes, reagents and kits

Name of the product	Company
Acrylamide Solution 30%	Biorad
Accutase	eBioscience
Bacteriophage T4 polynucleotide kinase	NEB
BioRad Protein Assay	Biorad
Collagen Type I from rat tail	Sigma-Aldrich
Collagenase Type II	Worthington
cOmplete Mini, Protease Inhibitor Cocktail Tablets	Roche
Covering solution for cryo sections (Tissue-Tek®)	Sakura
Diaminobenzidine (DAB)	Sigma-Aldrich
DNA marker 1kb plus Ladder®	Invitrogen
ECL™ Prime Western Blotting Detection Reagent	GE Healthcare
EGF	Sigma-Aldrich
Hot Star Taq Master Mix Kit	Qiagen
Insulin	Novo Nordisk
Omniscript RT PCR Kit	Qiagen
Proteinase K	AppliChem
QIAzol® lysis reagent	Qiagen
Red Taq	Sigma-Aldrich
RNeasy Lipid Tissue Mini Kit	Qiagen
SuperSignal® West Pico Chemiluminescent Substrate	Pierce
SYBR® GreenER qPCR SuperMix	Invitrogen
Vectashield Mounting Medium with DAPI	Vector

2.1.3 Antibodies**2.1.3.1 Primary antibodies**

Antibody	Company
Anti-BrdU	GE Healthcare
Anti-Cdc2 (p34, H-297; sc-747)	Santa Cruz Biotechnology
Anti-Cdk2 (M2; sc-163)	Santa Cruz Biotechnology
Anti-Cdk4 (C-22; sc-260)	Santa Cruz Biotechnology
Anti-Cdk6 (C-21; sc-177)	Santa Cruz Biotechnology
Anti-Cdt1 (H-300; sc-28262)	Santa Cruz Biotechnology
Anti-HNF4 α (C-19; sc-6556)	Santa Cruz Biotechnology
Anti-Cyclin A (C-19; sc-596)	Santa Cruz Biotechnology
Anti-Cyclin D1 (72-13G; sc-450)	Santa Cruz Biotechnology
Anti-Cyclin E (M20; sc 481)	Santa Cruz Biotechnology
Anti-Cyclin E1	MILLIPORE
Anti-GAPDH	AbD SeroTec
Anti-Histone H3	Cell signaling Technology
Anti-Ki-67 (NCL-Ki67p)	Leica
Anti-MCM2 (N-19; sc-9839)	Santa Cruz Biotechnology
Anti-p16 (M-156; sc-6397)	Santa Cruz Biotechnology
Anti-p21 ^{Kip1} (C-19; sc-6397)	Santa Cruz Biotechnology
Anti-p27 ^{Kip1}	BD Pharmingen
Anti-PCNA	Invitrogen
Anti-pRb (Ser807/811)	Cell signaling
Anti-Rb (M-153, sc-7905)	Santa Cruz Biotechnology

2.1.3.2 Secondary antibodies

Antibody	Company
chicken anti-mouse IgG-HRP	Santa Cruz Biotechnology
chicken anti-rat IgG-HRP	Santa Cruz Biotechnology
rabbit anti-goat IgG-HRP	Santa Cruz Biotechnology
goat anti-rabbit IgG-HRP	Cell Signaling Technology
Alexa 488 donkey anti-mouse	Invitrogen
Alexa 488 rabbit anti-rat	Invitrogen
Alexa 546 goat anti-rabbit	Invitrogen
Alexa 546 rabbit anti-goat	Invitrogen

2.1.4 Solutions, buffers and media

Name of product	Company
DMEM	Invitrogen
EBSS negative	Gibco
EBSS positive	Gibco
Fetal Calf Serum (FCS)	PAA
PBS pH 7,4, 1x and 10x	Gibco
Penicillin/Streptomycin (P/S)	Gibco

2.1.5 Instruments and equipments

Name of product	Company
7300 Real Time PCR System with Sequence Detection Software Version 1.3.1.	Applied Biosystems
Blotting Trans-Blot™ Cell	BioRad
Cell culture incubator	Heraeus
Centrifuge 5415D	Eppendorf
Cooling centrifuge 5417R	Eppendorf
Cryostat HM 550	Microm
ELISA Reader µQuant +KC4 Software	BIO-TEK
LAS mini 4000	Fuji
Overhead shaker REAX 2	Heidolph
Perfusion pump	Ismatec
pH Meter PB-11	Sartorius
Power supply PowerPac™ HC	BioRad
Roller Mixer SRT 9	Stuart
Rotating incubator	Bachofar
SDS PAGE electrophoresis system	PeQLab
Shaking incubator Unimax 1010	Heidolph
Thermocycler T3000 and Tpersonal	Biometra
Thermomixer 5436	Eppendorf
Water bath Haake W13	Fisons

2.1.6 Consumables

Name of product	Company
96 well Microplates for RT-PCR	Star LAB
96 wells Microplates for protein measurements	Greiner
96 wells Microplates for RNA measurements (UV-STAR)	Greiner
Catheter with injection port (Biovalve® 20G; 1,0x32mm)	Vygon
Disposable scalpels sterile No. 11	Feather
Eppendorf tubes 1ml, 1,5 and 2,0ml	Eppendorf
ETHIBOND* EXCEL 2-0 P6937H, non-absorbable suture	ETHICON
Hematocrit capillary	Brand
Heparine (Liquemin®)	Roche
Injection needles (Sterican®; Gr.17 24Gx25mm; 0,4x12mm)	Braun
Isoflurane (Forene®)	Abbott
Ketamine (Ketamin®)	Albrecht
Micro tube (1,1 ml Z-Gel)	Sarstedt
Multiply-PCR Strips	Sarstedt
Nitrocellulose membrane Protran®	Whatman
Super Frost slides	Roth
Surgical instruments	Aesculap
Syringe Omnican®	Braun
VICRYL* 4-0 V7980E, absorbable suture	ETHICON
X ray films	Amersham
Xylazin (Rompun®)	Bayer

2.2 Methods

2.2.1 Animal experiments

2.2.1.1 Housing and breeding of mice

Animals were housed under standardized conditions in the “Institut für Versuchstierkunde“ of the University Hospital of the RWTH Aachen. A 12-hours light/dark cycle, controlled temperature of 20 – 24°C and humidity of 50 – 60% were maintained. Mice were kept in macrolon cages with free access to food and water. All animal experiments were carried out according to the German legal requirements and animal protection law and approved by the government of the state North Rhine-Westphalia (LANUV, AZ 8.87-50.10.35.08.284).

2.2.1.2 Knockout mouse strains

Mice with a floxed allele of *Cdk2* (*Cdk2^{flf}*) (Ortega et al. 2003) were obtained from M. Barbacid (CNIO, Madrid, Spain). The generation and characterization of Cyclin E1 (*CcnE1*) knockout mice has been described in previous studies (Geng et al. 2003; Geng et al. 2007; Nevzorova et al. 2009). To generate hepatocyte-specific *Cdk2* knockout (*Cdk2^{Δhepa}*) mice, *Cdk2^{flf}* animals were crossed with transgenic *alfp-cre* mice expressing cre-recombinase under control of albumin promoter – and α -fetoprotein enhancer elements in a C57BL/6 background (Kellendonk et al. 2000).

These mice were further crossed with *CcnE1^{-/-}* mice to obtain *Cdk2^{Δhepa}CcnE1^{-/-}* double knockout (DKO) mice. *Cdk2^{flf}* littermates were used as wildtype (WT) controls.

2.2.1.3 Partial hepatectomy (PH)

For liver regeneration studies 2/3 PH was performed as described earlier (Nevzorova et al. 2009). Briefly, male mice between 6-8 weeks of age were anesthetized by intraperitoneal (*i.p.*) injection of 0.3 ml Ketamin/Rompun anaesthetic per 20 g body weight. The abdominal cavity was opened with a midline laparotomy. The right lateral, the left lateral and left anterior liver lobes were removed. Afterwards peritoneum and dermis were closed separately. For Roscovitine treatment 50 mg/kg Roscovitine or vehicle (DMSO) were delivered by *i.p.* injections 28 hours (h) and 40 h after PH. To determine the S-phase progression 300 μ l Bromodeoxyuridine (3 mg/ml)

Material and Methods

was given by *i.p.* injection 2 h before sacrificing. For different indicated time points after PH, at least 5 mice per group were sacrificed by cervical dislocation.

Anaesthetic

Substance	Volume
Ketamine	450 μ l
Rompun	50 μ l
0.9% NaCl	add 5 ml

2.2.1.4 Isolation and cell culture of primary hepatocytes

Primary mouse hepatocytes were isolated from 6-8 week old mice by collagenase perfusion. Mice were anesthetized by *i.p.* injection of 0.3 ml Ketamin/Rompun anaesthetic per 20 g body weight and followed by anti-coagulation treatment with 300 μ l (50 I.E.) heparin solution. After opening the abdominal cavity with a caudocranial cut in the *linea alba*, the *vena portae* was cannulated with an intravenous catheter (Biovalve 20G). Immediately after cutting the *vena cava caudalis* the liver was rinsed with EBSS negative buffer including 250 μ l EGTA solution (100 mM, pH 8.3) in a flow rate of 6 ml/min until the hepatic vascular system was clarified from blood. The perfusion was continued with 30 ml EBSS positive buffer. Ultimately, the liver was perfused with 50 ml EBSS positive buffer containing 30 mg collagenase type II and 2 mg trypsin inhibitor. Afterwards the abrogated liver was withdrawn and hepatocytes were released in ice cold DMEM medium. After filtering through sterile gossamer, cell suspension was centrifuged at 50 g at 4°C for 3 minutes to sediment hepatocytes from other cell populations and cell residues. The suspension was discarded and the pellet was washed again with DMEM medium. Finally the washed pellet was resuspended in DMEM medium containing 10% fetal calf serum (FCS) and penicillin/streptomycin (100 U/ml penicillin and 0.1 mg/ml streptomycin). Cell viability was determined microscopically by using trypan blue. Live cells were plated on collagen type I coated petri dishes at a density of 2×10^5 /cm². Cells were incubated at 37°C with 5% CO₂ supply. After 4 h incubation the culture medium was changed and non-adhered cells were washed away. Culture medium was changed every day.

To stimulate cell proliferation, cells were given 10 ng/ml EGF and 2×10^{-2} IU/ml insulin every day. To inhibit cell proliferation, Roscovitine was used in cell culture at a concentration of 15 μ g/ml. The use of the recombinant adenovirus over-expressing CcnE1 (adv-CcnE1) in liver and hepatocytes has been described previously (Nevzorova et al. 2009).

2.2.2 DNA and mRNA analysis

2.2.2.1 Genotyping of genetically modified mice with PCR

To determine the genotype of mice, DNA from tail biopsies was isolated and PCR was performed. For DNA isolation, tail biopsies from young mice were lysed in 200 µl NID lysis buffer and 1 µl Proteinase K at 56°C overnight on a thermo-mixer. Proteinase K was inactivated at 95°C for 10 minutes. After mixing vigorously the lysate was centrifuged for 10 minutes with maximum speed. For PCR 2 µl of the supernatant containing DNA was used as template.

NID lysis buffer

Substance	Stock concentration	Volume	Final concentration
Tris pH 8.3	1 M	10 ml	10 mM
KCl	1 M	50 ml	50 mM
MgCl ₂	1 M	2 ml	2 mM
NP-40	-	4.5	0.45%
Tween 20	-	4.5	0.45%
Gelatine type III	10 mg/ml	10 ml	0.1 mg/l
H ₂ O		add 1l	

PCR

Reagent	Concentration	Volume
Taq master Mix ¹	2x	12.5 ml
sense Primer	10 µM	1 µl
antisense ² Primer	10 µM	1 µl
DNA	-	2 µl
H ₂ O	-	7.5 µl

¹ Red Taq master mix for Cre, Cyclin E1, Cyclin E2; HotstarTaq master mix for Cdk2

² For detection of Cyclin E1 and E2 deletion in Cyclin E1 or E2 constitutive knock mice a third antisense primer was used.

Target gene	Sequence in 5' – 3' orientation
Cre	CCACGACCAAGTGACAGCAAT TTCGGATCATCAGCTACACCA
CcnE1	CGCCATGGTTATCCGGGAGATGG CGCATACTGAGACACAGACT GATCTCTCGTGGGATCATTG
CcnE2	GGTTCTCCCATTAGAGCACAG GCTATAGCAGTTGTTTCTGTTTG GATCTCTCGTGGGATCATTG
Cdk2	CAAGTTGACGGGAGAAGTTGTG GAAGACCCTCCAGGTGAATGAA

Material and Methods

Cre PCR program

Step	Temperature	Duration	Cycle
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	15 s	35
Annealing	55°C	45 s	
Elongation	72°C	45 s	
Final Elongation	72°C	5 min	1
Conservation	4°C	∞	

CcnE1 (for both constitutive and conditional mice) and CcnE2 PCR program

Step	Temperature	Duration	Cycle
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	1 min	37
Annealing	60°C	1 min	
Elongation	72°C	1 min	
Final Elongation	72°C	3 min	1
Conservation	4°C	∞	

Cdk2 PCR program

Step	Temperature	Duration	Cycle
Initial Denaturation	95°C	15 min	1
Denaturation	94°C	15 s	32
Annealing	53°C	30 s	
Elongation	72°C	30 s	
Final Elongation	72°C	2 min	1
Conservation	4°C	∞	

PCR to determine deletion efficiency of Cdk2 gene

Step	Temperature	Duration	Cycle
Initial Denaturation	95°C	5 min	1
Denaturation	98°C	20 s	37
Annealing	60°C	35 s	
Elongation	72°C	1 min	
Final Elongation	72°C	5 min	1
Conservation	4°C	∞	

2.2.2.2 Isolation of mRNA from liver tissue

From deep frozen liver tissue a small piece (about 50 mg) was transferred into 1 ml peqGOLD-RNAPure®. After homogenization with an Ultra-Thurax® the lysate was incubated for 5 minutes at room temperature. Afterwards 200 µl (1/5 volume) chloroform was added and the mixture was agitated thoroughly for 15 seconds. After incubation for 10 minutes at room temperature the lysate was centrifuged at 12000x g

at 4°C. The upper phase containing mRNA (about 500 µl) was transferred carefully to a new tube and precipitated by adding 500 ml (1 volume) 2-propanol. After incubation for 10 minutes at room temperature mRNA was pelleted by centrifugation for 10 minutes at 12000x g at 4°C. The supernatant was discarded and the pellet was washed twice with 1 ml 70% ethanol. The pellet was dried on ice and then dissolved in appropriate volume of RNase-free water (about 50 µl). For long term storage mRNA was kept at -80°C.

2.2.2.3 Isolation of mRNA from cell culture

Cells (primary hepatocytes or Hepa1-6 hepatoma cells) were washed with cold PBS 3 times and collected to an eppendorf tube. After centrifugation at 1500x g and 4°C supernatant was discarded and 500 µl QIAzol[®] lysis reagent were added. The tube containing the homogenate was incubated at room temperature for 5 minutes, and then 200 µl chloroform were added. After shaking vigorously for 15 seconds the tube was incubated again at room temperature for 3 minutes. Afterwards the tube was centrifuged at 12000x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new collection tube. One volume of 70% ethanol was added and thoroughly mixed by vortex. Without delay the mixture including any precipitate was pipetted into an RNeasy Mini Spin Column in a 2 ml collection tube. The tube with column inside was centrifuged at 12000 rpm for 30 seconds at room temperature. The flow-through was discarded. 350 µl Buffer RW1 were pipetted into the RNeasy mini column and centrifuged for 30 seconds at 12000 rpm. DNA was digested by incubating with Dnase I solution for 15 minutes at room temperature. Columns were washed once again with 350 µl buffer RWI and then 2 times with buffer RPE. To elute mRNA, 30-50 µl RNase-free water was added directly onto column membrane and centrifuged for 1 minute at 12000 rpm.

2.2.2.4 Determination of nucleic acid concentration

Isolated nucleic acid was diluted 1:50 in *aqua bidest.* (ddH₂O) and its extinction was determined using ELISA Reader µQuant at 260 nm (E_{260}). RNA concentration was calculated according to the formula (RNA concentration = $40 \times E_{260} / 1000$ [µg/µl]), while the calculation of DNA concentration was based on the formula (DNA concentration = $50 E_{260} / 1000$ [µg/µl]). The purity of nucleic acid was determined with

Material and Methods

an additional measurement of the optical density at 280 nm (E_{280}); a ratio of E_{260} / E_{280} between 1.8 and 2.1 reflects a suitable purity.

2.2.2.5 Reverse Transcription (RT)-PCR

To converse single-stranded mRNA into complementary DNA (cDNA) for further analysis of gene expression, reverse transcription was performed by using Omniscript RT Kit according to manufacturer's recommendations.

Component	Volume / reaction
10x Buffer RT	2 μ l
dNTP mix (5 mM each)	2 μ l
Oligo-dT primer 10 μ M	2 μ l
Omniscript Reverse Transcriptase	1 μ l
Template RNA	2 μ g
ddH ₂ O	add 20 μ l

Temperature	Time
37°C	60 min
95°C	5 min

2.2.2.6 Quantitative real-time PCR

To analyze the transcriptional expression level of cell cycle relevant genes, real-time PCR was performed by using a 7300 Real-Time PCR System with SDS software (version 1.3.1) and a SYBR Green PCR Kit (Invitrogen). The $2^{-\Delta\Delta C_t}$ method was used to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen 2001). For this purpose the gene expression of GAPDH was used as internal control.

Component	Volume / reaction
SYBR Green	12.5 μ l
Primer sense (10 nM)	1 μ l
Primer antisense (10 nM)	1 μ l
ddH ₂ O	5.5 μ l
cDNA	5 μ l

Material and Methods

The following primers were used to detect transcriptional expression level of target genes as indicated.

Gene	Sense primer (5' - 3')	Antisense primer (5' - 3')
7SL	GTGTCCGCACTAAGTTCGG	TATTCACAGGCGCGATCC
AFP	CCCACTTCCAGCACTGCCTGCGG	GGCTGCAGCAGCCTGAGAGTC
Albumin	TGTCCCCAAAGAGTTTAAAGCTG	TCTTAATCTGCTTCTCCTTCTCTGG
CcnD1	AAGCATGCACAGACCTTTGTGG	TTCAGGCCTTGCATCGCAGC
CcnE1	TCCACGCATGCTGAATTATC	TTGCAAGACCCAGATGAAGA
CcnE2	AAAAAGTCTTGGGCAAGGTAAG	GCATTCTGACCTGGAACCAC
CcnA2	GTGGTGATTCAAACCTGCCA	AGAGTGTGAAGATGCCCTGG
CD133	ACCTCACCATCCCGCATCT	GAAAAGTTGCTCTGCGAACC
CK19	GGGGGTTCAGTACGCATTGG	GAGGACGAGGTCACGAAGC
E2F1	AGAGTGAGCAGCAGCTGGAT	GGTCCTGGCAGGTCACATAG
GAPDH	TGTTGAAGTCACAGGAGACAACCT	AACCTGCCAAGTATGATGACATCA
Sox9	TGCCCATGCCCGTGCGCGTCAA	CGCTCCGCCTCCTCCACGAAGGGTCT

2.2.2.7 Analysis of DNA content by fluorescence activated cell sorting (FACS)

Cultured hepatocytes were washed with cold PBS 3 times and then detached with Accutase. Cell suspension was centrifuged at 50 g for 5 minutes at 4°C. The pellet was washed again with cold PBS twice and then fixed with 4% PFA for 20 minutes at room temperature. After washing twice with cold PBS cells were permeabilized in Nicoletti buffer for 15 minutes at room temperature. Cell suspension was centrifuged and washed with cold PBS. Permeabilized cells were incubated with PI/RNase solution for DNA staining. Samples were measured using a BD Canto flow cytometer. Data analysis was performed with FlowJo software version 7.6.

2.2.2.8 Fluorescence *in situ* hybridization (FISH)

Pre-hybridization and hybridization: Primary hepatocytes were cultured on cover slips (described in 2.2.1.4). Cover slips were washed with cold PBS 3 times and then fixed with 3:1 methanol:acetic acid for 5 minutes at room temperature and allowed to dry overnight. For aging, cover slips were baked for 5 minutes at 95°C and followed by incubation in 2xSSC for 30 minutes at 37°C. Cover slips were treated with 1% pepsin/0.01M HCl for 13 minutes at 37°C. After rinsing in 1xPBS for 5 minutes at room temperature, cover slips were fixed in 2.5% formaldehyde/0.45% MgCl₂/PBS for 5 minutes at room temperature. Cover slips were again rinsed in 1xPBS/2mM glycine (pH8.5) for 5 minutes at room temperature and then hydrated by 2 minutes washes in 70%, 80%, 90% and 100% ethanol series. Once dried, 5 µl probes (Mouse & Rat

IDetect™ Chromosome Paint probe) were added to cover slips. Thereafter cover slips were sealed by rubber cement and incubated at 69°C for 2 minutes and at 37°C overnight.

Post-hybridization: After incubation overnight cover slips were shortly put in 2x SSC/0.15% NP-40 and further washed in 0.4x SSC/0.3% NP-40 at 73°C for 2 minutes. Cover slips were washed again in 2x SSC/0.15% NP-40 for 1 minute at room temperature and mounted with DAPI mounting medium. Samples were analyzed using fluorescence microscopy.

2.2.3 Protein analysis

2.2.3.1 Protein isolation and quantification

Total proteins from frozen liver tissues or cell pellets were extracted with ice cold NP40-Buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40 and 50 mM NaF freshly supplemented with protease inhibitors (Complete Mini, Roche), phosphatase inhibitors (PhosSTOP, Roche) and 1 mM DTT.

Nuclear proteins from frozen liver tissues were prepared by using 2 different lysis buffers Buffer A (10 mM HEPES pH 7.6, 10 mM KCl, 2 mM MgCl₂ and 0.1 mM EDTA and Buffer C (50 mM HEPES pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA and 10% Glycerol). Both buffers were freshly supplemented with Complete Mini, PhosSTOP and 1 mM DTT. Proteins from cytosol were extracted with Buffer A including 0.5% NP-40. After centrifugation the supernatants were used for cytosolic protein lysates. The pellet was extracted by Buffer C to get nuclear protein lysates.

For isolation of chromatin fractions from primary hepatocytes, cells were incubated in ice cold CSK buffer (20 mM HEPES-KOH pH7.6, 40 mM potassium glutamate, 1 mM MgCl₂, 1 mM EGTA and 300 mM sucrose freshly supplemented with Complete Mini, PhosSTOP and 1 mM DTT) with 0.1% Triton X-100 for 20 minutes. Extracts were centrifuged at 1500x g for 3 minutes and supernatants (soluble fractions) were collected. The pellets were extracted with CSK buffer without Triton X-100 and these insoluble fractions were used as chromatin fractions (modified from Geng 2003 and Okuno 2001).

Protein concentrations were determined by BIO-RAD protein assay (BIO-RAD) according to manufacturer's instruction. BSA solutions with different concentrations (1, 2, 4, 6, 8 µg/µl) were used as protein standard. The absorbance of protein lysates and

protein standard was measured at 595 nm and protein concentration was calculated according to the BSA standard curve. Protein concentrations in insoluble chromatin fractions could not be measured reliably due to insoluble flocculent. Instead, in proportion to the protein concentration of soluble fractions the equalization was performed by adding CSK buffer.

2.2.3.2 Western Blot

Samples containing equal amount (50 µg) of protein were combined with 6x Laemmli sample buffer and boiled at 95°C for 5 minutes. Samples were separated by sodium dodecyl sulfate polyarylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred from gel to cellulose membrane (Whatman® Protran®) using a wet blotting system (BIORAD). Membranes were blocked for 1 h in blocking buffer (2.5% Dry-Milk in TBS-Tween) and then probed with appropriate antibodies in blocking buffer overnight at 4°C. After incubation membranes were washed with TBS-Tween 4 times for 5 minutes and then incubated with a HRP-linked secondary antibody for 1 hour in room temperature. Washing of membranes was performed 4 times for 5 minutes in TBS-Tween and followed by incubating with ECL western blotting detection reagent. Proteins with ECL signals were detected by a digital detection system (FULJIFILM). GAPDH was used as loading control. TATA binding protein (TBP, abcam) was used as loading control for chromatin fractions and nuclear proteins.

2.2.3.3 Immunoprecipitation and *in vitro* kinase assay

Samples containing equal amount (500 µg) of protein were subjected to immunoprecipitation *via* incubating with 1 µg antibody at 4°C for 4 h and then conjugated to protein A/G PLUS agarose beads (Santa Cruz) for 2 h. The conjugated beads were collected by centrifugation at 2000 rpm and 4°C for 3 minutes and washed 3 times with NP-40 buffer and once with wash buffer. After washing beads were incubated with 30 µl kinase assay buffer containing substrate (Histone H1 or recombinant Rb), ATP and ³²P-γATP at 30°C. Reaction was stopped by adding 6x SDS loading buffer and boiled at 95°C for 5 minutes. Samples were separated by SDS-PAGE using 10% Gel and analyzed by autoradiography.

Material and Methods

Buffer for Histone H1 kinase assay:

Wash buffer (15 ml)

Components	Volume	Final concentration
0.5 M Tris-HCl (pH 7.4)	1.5 ml	50 mM
1 M MgCl ₂	225 µl	15 mM
1 M KCl	150 µl	10 mM
0.1 M EGTA	150 µl	1 mM
1 M DTT	15 µl	1 mM
ddH ₂ O	add 15 ml	

Histone H1 kinase assay buffer (240µl)

Components	Volume	Final concentration
Wash buffer	227 µl	
10 mM ATP	1.2 µl	50 µM
10 µCi/µl ³² P-γATP	6 µl	0.25 µCi/µl
20 µg/µl Histone H1	6 µl	0.5 µg/µl

Buffer for Rb kinase assay:

Wash buffer (15 ml)

Components	Volume	Final concentration
0.5 M HEPES (pH 7.4)	1.5 ml	50 mM
1 M MgCl ₂	225 µl	15 mM
1 M KCl	150 µl	10 mM
0.1 M EGTA	150 µl	1 mM
PhosSTOP tablet	1 piece	
ddH ₂ O	add 15 ml	

Histone H1 kinase assay buffer (240µl)

Components	Volume	Final concentration
Wash buffer	223 µl	
10 mM ATP	1.2 µl	50 µM
10 µCi/µl ³² P-γATP	8 µl	10 µCi/30µl
0.5 µg/µl Histone H1	8 µl	0.5 µg/30µl

2.2.4 Immunofluorescence

2.2.4.1 Quantification of BrdU incorporation by immunofluorescence microscopy

BrdU (5-bromo-2-deoxyuridine) incorporation and subsequent staining was used to quantify the number of cells in S-phase *in vivo* and *in vitro*. For *in vivo* labeling, BrdU was injected *i.p.* (30 µg / g body weight) 2 h before animals were sacrificed. Pieces of liver tissues were frozen in Tissue-Tek at -80°C. For staining, 5 µm cryosections on Superfrost slides were made and then air-dried at room temperature for 1.5-2 h and fixed with pre-chilled acetone at -20°C for 10 minutes. After rehydration in 1x PBS for 5 minutes, slides were treated with 2 M HCl for 30 minutes and then neutralized with 0.1 M sodium borate (pH 8.0) for 9 minutes. Slides were washed in tap water for 2 minutes 4 times and in PBS for 4 minutes 3 times. After washing, samples were incubated with anti-BrdU mouse monoclonal antibody (GE Healthcare, 1:4000 dilution) at 4°C overnight. On the second day, slides were washed in PBS for 4 minutes 3 times and then incubated with anti-mouse Alexa 488 antibody for 1 h at room temperature in the dark. Samples were then washed in PBS for 15 minutes, mounted with DAPI containing mounting medium (Vector Laboratories) and covered with glass coverslips.

For *in vitro* labeling primary hepatocytes, which were cultured on collagen-coated coverslips, were incubated with 20 µM BrdU for 2 h before fixation. Labeled cells were washed 3 times with cold PBS and fixed with pre-chilled acetone at -20°C for 10 minutes. The subsequent steps were as described for the *in vivo* BrdU staining protocol.

2.2.4.2 β -catenin staining

Cryosections of liver tissue were cut at 5 µm thickness and fixed in ice cold methanol for 20 min. After washing 3 times for 5 min in PBS, slides were first blocked with 5% goat serum in PBS (blocking buffer) and then incubated with a fluorescein conjugated anti- β -catenin antibody (1:1000 dilution in blocking buffer) at 4°C overnight. On the following day, slides were washed in PBS for 5 min 3 times and then mounted with DAPI containing mounting medium and covered with glass coverslips for microscopical visualization.

2.2.4.3 Ki-67 and HNF4 α co-staining

Cryosections of liver tissue were cut at 5 μ m thickness and fixed in 4% PFA for 15 min at RT. After washing 3 times for 5 min in PBS, slides were first blocked in blocking buffer (5% donkey serum and 0.3% Triton X 100 in PBS) and then incubated with rat-anti-mouse Ki-67 antibody and goat-anti-mouse HNF4 α antibody (both 1:500 dilution in blocking buffer) at 4°C overnight. On the following day, slides were washed in PBS for 10 min 3 times and then incubated with Alexa 546 donkey anti-goat antibody and Alexa 488 donkey anti-rat antibody (both 1:200 dilution in blocking buffer) for 1 h at RT. Thereafter slides were washed for 10 min 3 times in PBS and then mounted with DAPI containing mounting medium and covered with glass coverslips for microscopical visualization.

2.2.5 Electrophoretic mobility shift assay (EMSA)

EMSA was performed to analyze the promoter regulation of the *cyclin A2* gene. The method is based on the fact that labeled double-stranded oligonucleotides (probe) migrate faster through a non-denaturing polyacrylamide gel compared to specific protein-probe complexes indicating DNA-protein interactions.

For the labeling of the probe, equal amounts of complementary single-stranded oligonucleotides (50 μ g each) were added with 26 μ l 10x annealing buffer (200 mM Tris-HCl, pH 7.6; 100 mM MgCl₂; 500 mM NaCl) and H₂O to a final volume of 260 μ l. The mixture was incubated for 5 minutes at 95°C and annealed by cooling down to room temperature overnight. Prepared double-stranded probes were labeled with ³²P- γ ATP by incubating with the bacteriophage T4 polynucleotide kinase for 1 h. The labeling reaction was stopped by adding 2 μ l EDTA (0.5 M, pH 8.0). The labeled probe was centrifuged in a Nick-spin-column for 2 minutes to remove non-bound ³²P- γ ATP. The radioactivity of probe was measured in a β -scintillation counter.

For protein and probe interaction, in each reaction 5 μ g nuclear protein extracts were mixed with 4 μ l 5x binding buffer, 2 μ l 10x proteinase inhibitor cocktail, 1 μ l Poly dI:dC (1 μ g/ μ l), 1 μ l BSA (10 μ g/ μ l), ³²P labeled probe (30000 cpm per reaction) and H₂O up to a final volume of 20 μ l. In a control reaction protein was not added to the free probe. The reaction mixtures were incubated for 15 minutes at room temperature and further 15 minutes on ice. For super-shift-assays 1 μ l antibody was added to a

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reaction mixture, which was incubated for another 30 minutes on ice. The reaction was stopped with 2 µl loading buffer containing 20% Ficoll. Samples were loaded on a 6% non-denaturing polyacrylamide gel and separated at 300 volt and 4°C for approximately 3 h. Afterwards the gel was fixed in 10% Acetic Acid; 20% Methanol for 30 minutes and dried under vacuum at 80°C. At last the gel was exposed on x-ray film at -80°C and analyzed by autoradiography.

Oligonucleotide sequences used for EMSA

Name	Sense (5' - 3')	Antisense (5' - 3')
cycAwt	TTCAATAGTCGCGGGATACTT	AAGTATCCCGCGACTACTATTGAA

Reaction mix for ³²P labeling

Components	Volume
125 ng Oligonucleotide	in 3.3 µl
10x T4-kinase buffer	2.0 µl
T4-kinase	1.0 µl
10 µCi/µl ³² P-γATP	4 µl
ddH ₂ O	9.7 µl

10x proteinase inhibitor cocktail

Components	Volume	Final concentration
Aprotinin solution	200 µl	
0.1 M Pefabloc	50 µl	50 mM
1 M DTT	20 µl	20 mM
ddH ₂ O	add 1 ml	

5x binding buffer

Components
125 mM HEPES, pH 7.6
25 mM MgCl ₂
170mM KCl

5x TBE buffer

Components
45 mM Tris-base
440 mM Boric acid
1 mM EDTA, pH 8.0

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6% EMSA gel

Components	Volume
5x TBE	2.5 ml
30% acrylamide	10 ml
10% APS	350 μ l
TEMED	50 μ l
ddH ₂ O	add 50 ml

0.25x running buffer

Components	Volume
5x TBE	75 ml
ddH ₂ O	1425 ml

3. Results

3.1 Hepatocyte-specific ablation of Cdk2 does not affect liver regeneration after PH

3.1.1 Analysis of G1/S transition and S-phase progression in Cdk2^{Δhepa} mice

To study the proliferative capacity of Cdk2-deficient hepatocytes, hepatocyte-specific conditional Cdk2 knockout mice (Cdk2^{Δhepa}) were generated using the albumin-Cre/loxP system.

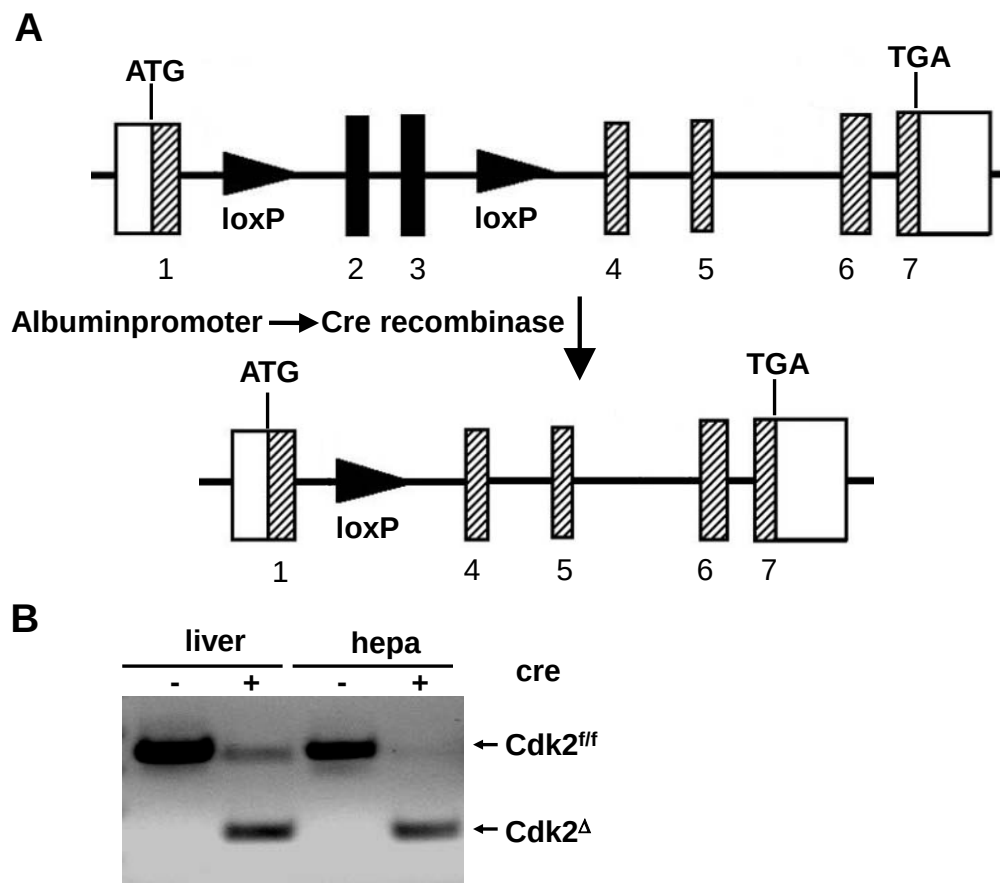


Figure 3.1: Generation of hepatocyte-specific Cdk2 deletion in liver. (A) Gene-targeting strategy for creating hepatocyte-specific deletion of Cdk2. LoxP recombination sites were integrated in the introns 1 and 3 of the genomic Cdk2 locus (Ortega et al. 2003). Expression of cre-recombinase results in deletion of exons 2 and 3. **(B)** Cdk2 deletion efficiency in liver and isolated primary hepatocytes was measured by PCR with primers located in exons 1 and 4. The upper band specific for the wildtype locus is 465 base pairs (bp) in size and the lower band (255 bp) indicates Cdk2 knockout.

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Cdk2^{loxP/loxP} animals with two loxP sites flanking coding exons 2 and 3 were crossed with transgenic animals expressing cre-recombinase under the control of an Albumin/ α -fetoprotein promoter/enhancer (Alfp-Cre) construct. In liver parenchymal cells the expression of albumin activates cre-recombinase, which excises exons 2 and 3 from the Cdk2 gene (Figure 3.1 A). Excision of exons 2 and 3 in the Cdk2 locus causes a frame shift in the open reading frame of transcribed mRNA resulting in premature termination of protein translation. The truncated residual short polypeptide lacks Cdk2 function (Ortega et al. 2003). RT-PCR was performed to determine the Cdk2 deletion efficiency in liver and isolated primary hepatocytes. Nearly complete Cdk2 deletion was shown by RT-PCR with freshly isolated primary hepatocytes, while a slight residual signal for Cdk2 was still detectable in liver samples which can be explained by Cdk2 expression in non-parenchymal cells (Figure 3.1 B).

To determine the effect of Cdk2 deletion in hepatocytes during liver regeneration, 2/3 partial hepatectomy was performed in 7-8 weeks old Cdk2 ^{Δ hepa} mice and Cdk2^{ff} control animals. At different time points after PH, the mice were sacrificed and frozen cryosections were subjected to BrdU staining in order to determine the percentage of cells undergoing DNA-synthesis. Both groups showed identical kinetics of S-phase progression in the time course following PH (Figure 3.2 A-B). DNA synthesis started approximately 36 h after surgery and reached peak levels 48 h post PH. No significant differences between Cdk2^{ff} and Cdk2 ^{Δ hepa} mice were observed. In line with these findings, there was also no difference in the liver weight/body weight ratio between Cdk2 ^{Δ hepa} and Cdk2^{ff} animals at all investigated time points after PH (Figure 3.2C). These results indicate that Cdk2-deficient hepatocytes exhibit full potential to regenerate presumably through yet unknown compensatory proliferative mechanisms following liver dissection.

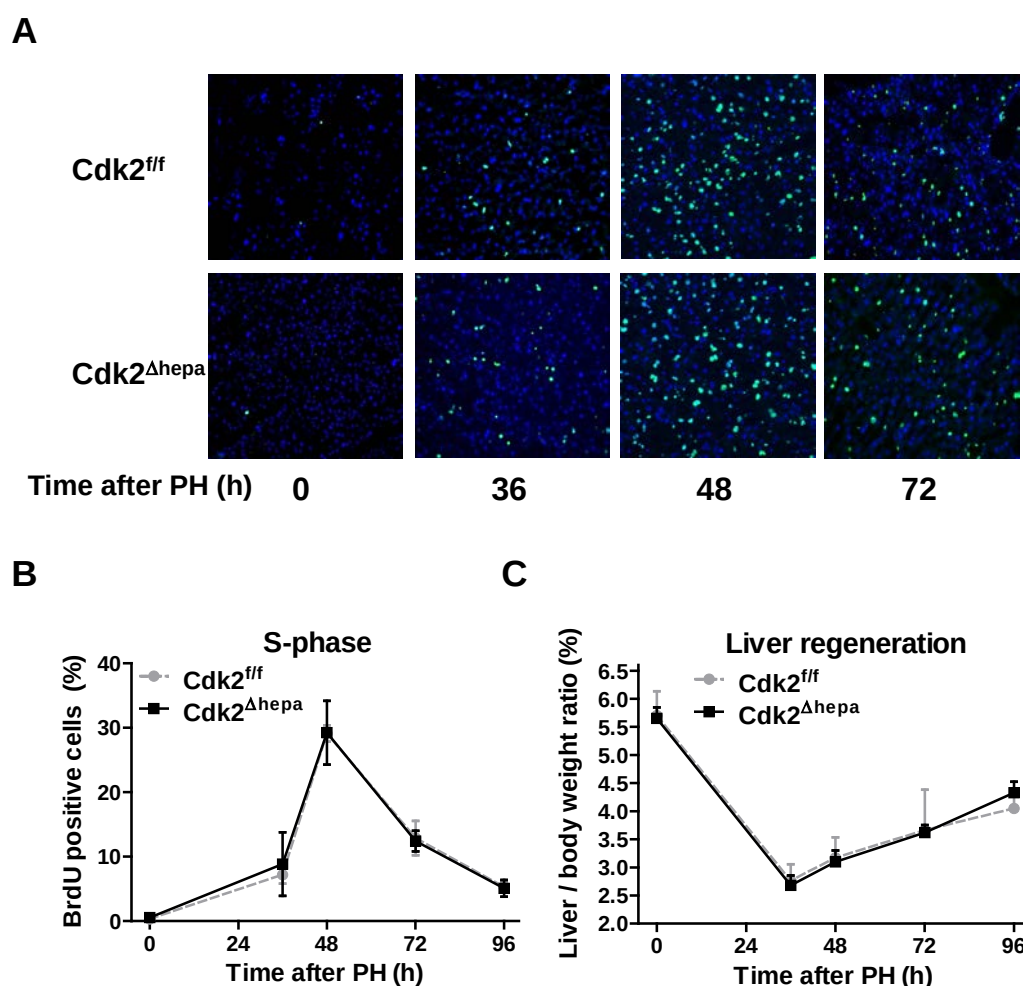


Figure 3.2: Hepatocyte-specific ablation of Cdk2 does not affect liver regeneration after PH. Cdk2^{f/f} and Cdk2^{Δhepa} mice were subjected to PH and sacrificed at indicated time points. Two hours before killing, all mice were injected with BrdU. **(A)** Immunofluorescence staining for BrdU incorporation on liver cryosections. BrdU positive signals located in nuclei were stained green by Alexa 488. Total nuclei were stained blue with DAPI. **(B)** Quantification of BrdU-positive hepatocytes in Cdk2^{f/f} and Cdk2^{Δhepa} mice. Data is calculated as percentage of BrdU-positive nuclei per magnification field. For each mouse at least 5-10 representative magnification fields were counted. Each value represents the mean of 5-10 mice. **(C)** Liver weight/body weight index (%) at indicated time points after PH. Data is presented as the average ± standard deviation (S.D.).

3.1.2 Gene expression and protein regulation in Cdk2^{Δhepa} mice during liver regeneration

The previous experiment revealed that livers with Cdk2 deficiency exhibited normal restoration of both mass and function after PH. This was unexpected as Cdk2 is believed to be an important factor for DNA replication. However, the underlying

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mechanisms for compensating the loss of Cdk2 at a molecular level remained unknown. Thus, the effect of Cdk2 deficiency on gene expression and protein regulation of other cell cycle related genes involved in G1/S-phase transition in quiescent liver as well as in regenerating liver was further investigated by qPCR and western blot analysis. Interestingly, basal gene expression of CcnD1 was approximately 2.5 fold increased in Cdk2^{Δhepa} livers and CcnE1 expression was also slightly but significantly elevated in these mice. In contrast, basal gene expression of the second E-type cyclin (CcnE2) was not enhanced in Cdk2^{Δhepa} livers (Figure 3.3 A). In accordance with higher CcnD1 mRNA levels, western blot analysis confirmed weak basal expression of CcnD1 protein in quiescent liver but also in primary hepatocytes of Cdk2^{Δhepa} mice after prolonged exposition (Figure 3.3 B). Of notice, expression of cell cycle inhibitors such as p16, p21 and p27 was not affected by the loss of Cdk2 in quiescent Cdk2^{Δhepa} livers. These data suggest that quiescent Cdk2-deficient hepatocytes are unexpectedly predisposed to G0/G1-S-phase transition *via* aberrantly higher CcnD1 and CcnE1 expression.

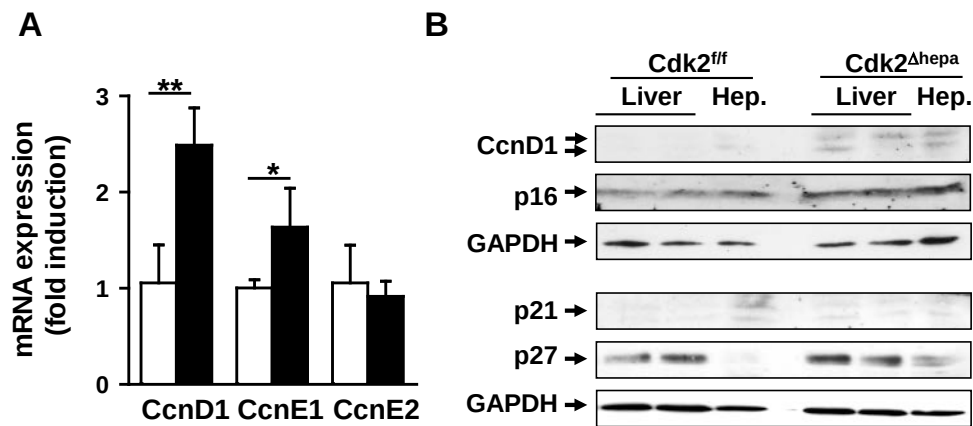


Figure 3.3: Depletion of Cdk2 results in basal over-expression of CcnD1 and CcnE1. Livers and primary hepatocytes of untreated Cdk2^{ff} and Cdk2^{Δhepa} mice were investigated for gene- and protein expression of indicated cell cycle mediators. **(A)** qPCR analysis of CcnD1, CcnE1 and CcnE2 in livers from 7-8 weeks old Cdk2^{ff} and Cdk2^{Δhepa} mice. **(B)** Western blot analysis of CcnD1, p16, p21 and p27 protein expression. Two independent livers per group and cell lysates from primary hepatocytes were used. The expression level of GAPDH is presented as internal loading control. Data is presented as the average $\pm$ S.D. *: $p < 0.05$; **: $p < 0.01$.

To further analyze the consequences of Cdk2-deficiency on cell cycle signaling during liver regeneration, expression profiles of CcnE1, CcnE2 and its transcriptional activator E2F1 were determined in Cdk2^{ff} and Cdk2^{Δhepa} mice at important time points after PH. In accordance with its slightly increased basal expression, CcnE1 showed

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an accelerated induction and premature down-regulation in $Cdk2^{\Delta hepa}$ livers compared to WT controls (Figure 3.4 A). In contrast, CcnE2 and E2F1 were similarly induced in $Cdk2^{\Delta hepa}$ and $Cdk2^{ff}$ mice, but also showed earlier down-regulation in liver of $Cdk2^{\Delta hepa}$ mice (Figure 3.4 B-C). These data suggest that accelerated induction of CcnE1 is largely independent of its prototypical transcriptional activator E2F1.

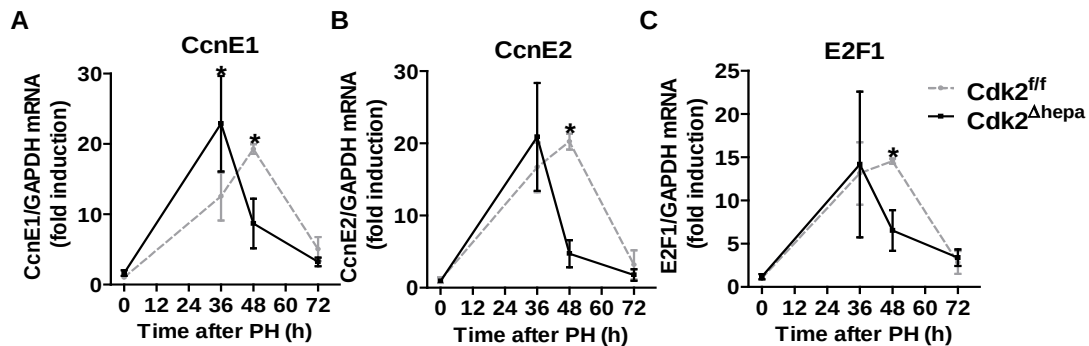


Figure 3.4: Depletion of Cdk2 results in earlier induction of CcnE1 and premature down-regulation of CcnE1, CcnE2 and E2F1. (A-C) Gene expression profiles of CcnE1, CcnE2 and E2F1 were determined in $Cdk2^{ff}$ and $Cdk2^{\Delta hepa}$ mice at 0-96 h post PH by qPCR. For each time point, 3-5 animals were analyzed. Data is presented as the average $\pm$ S.D. *: p < 0.05.

Consistent with these data, western blot analysis for CcnE1 expression revealed accelerated induction in $Cdk2^{\Delta hepa}$ livers as well, which was first detectable already 36 h post PH (Figure 3.5). Furthermore, slightly increased CcnD1 gene expression in $Cdk2^{\Delta hepa}$ mice 48 and 72 h after PH could be detected on the protein level correlating with slightly elevated protein levels of Cdk4 and Cdk6. Of notice, protein expression of CcnA2 and Cdk1 - indicative of S-phase progression and G2/M-transition - was similar in both groups (Figure 3.5). Proliferating cell nuclear antigen (PCNA) is a nuclear protein involved in initiating DNA synthesis (Theocharis et al. 1994). Interestingly, western blot analysis revealed overall stronger up-regulation of PCNA between 36-72 h after PH in $Cdk2^{\Delta hepa}$ mice indicating that Cdk2 depletion does not inhibit, but rather supports G1/S-phase initiation (Figure 3.5).

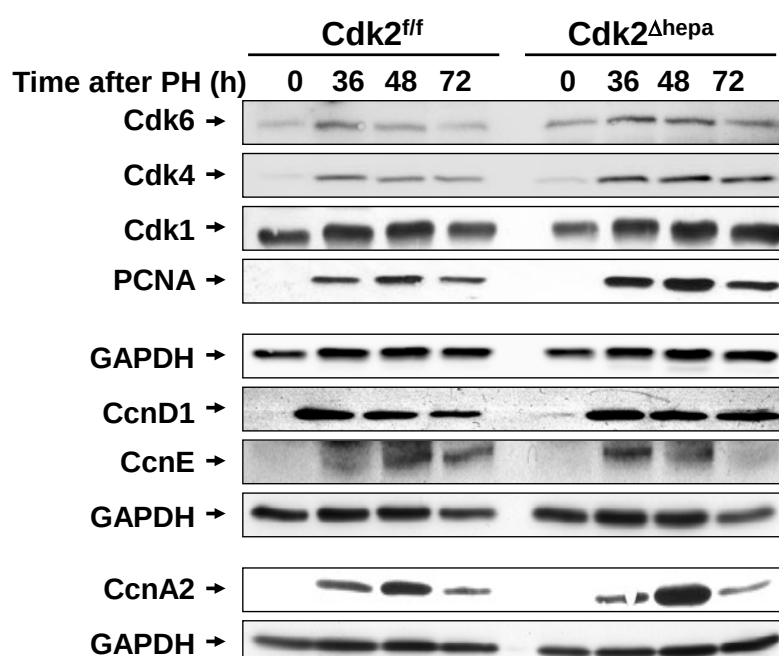


Figure 3.5: Depletion of Cdk2 results in moderately elevated protein levels of interphase cyclins (CcnD1 and CcnE1), Cdks (Cdk4 and Cdk6) and PCNA. Protein expression profiles of CcnD1, CcnE, CcnA2, Cdk4, Cdk6 and Cdk1 in *Cdk2^{f/f}* and *Cdk2^{Δhepa}* mice at 0-72 h post PH are shown. GAPDH expression was used as internal loading control.

3.1.3 Identification of compensatory kinase activities in absence of Cdk2

The canonical role of CcnE involves association with Cdk2 and formation of an active kinase complex capable of phosphorylating substrates. As a central regulator of G1/S transition, the Cdk2/CcnE complexes play multiple roles on cell cycle events including DNA replication and centrosome duplication. One key event of G1/S transition is phosphorylation of the pocket protein Rb via Cdk2/CcnE complexes. Rb phosphorylation was first detectable 36 h after PH and reached the peak level 48 h after surgery in both *Cdk2^{f/f}* and *Cdk2^{Δhepa}* animals (Figure 3.6 A). These similar expression profiles in both groups were unexpected and hint at the existence of an additional kinase activity (or activities) in *Cdk2^{Δhepa}* mice which can compensate loss of Cdk2. To identify this kinase (or these kinases), kinase activities of known G1 and S-phase Cdk/cyclin complexes involved in cell cycle regulation were systematically examined in quiescent (0 h) and regenerating livers (36-48 h after PH, Figure 3.6 B). As expected, Cdk2-related kinase activity could not be detected at all in *Cdk2^{Δhepa}* mice. In contrast, the experiment revealed an elevated kinase activity of CcnD1-related complexes (CcnD1/Cdk4 and CcnD1/Cdk6) 36 h after PH in *Cdk2^{Δhepa}*

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mice, which correlates with slightly enhanced gene expression of CcnD1, Cdk4 and Cdk6 (compare with figure 3.5). Furthermore, Cdk1 exhibited enhanced activity 48 h after PH in Cdk2^{Δhepa} mice compared to controls, which indicates that loss of Cdk2 at this time point could be compensated by Cdk1. One canonical binding partner of both Cdk1 and Cdk2 is CcnA2. However, *in vitro* kinase assays revealed that CcnA2-related kinase activities were substantially reduced in Cdk2^{Δhepa} mice (Figure 3.6 B). Thus, it is unlikely that Cdk1/CcnA2 alone compensates for loss of Cdk2.

Earlier studies demonstrated that CcnE1 can also associate with Cdk1 in absence of Cdk2 in embryo, spleen and thymus (Aleem et al. 2005; Santamaria et al. 2007). However, the present results clearly showed that none of the E-type cyclins form any compensatory kinase activity during liver regeneration in Cdk2^{Δhepa} mice. Taken together, this experiment demonstrated that lack of Cdk2 kinase is very likely compensated by CcnD1/Cdk4/Cdk6 at 36 h and substituted by Cdk1 at 48 h after PH.

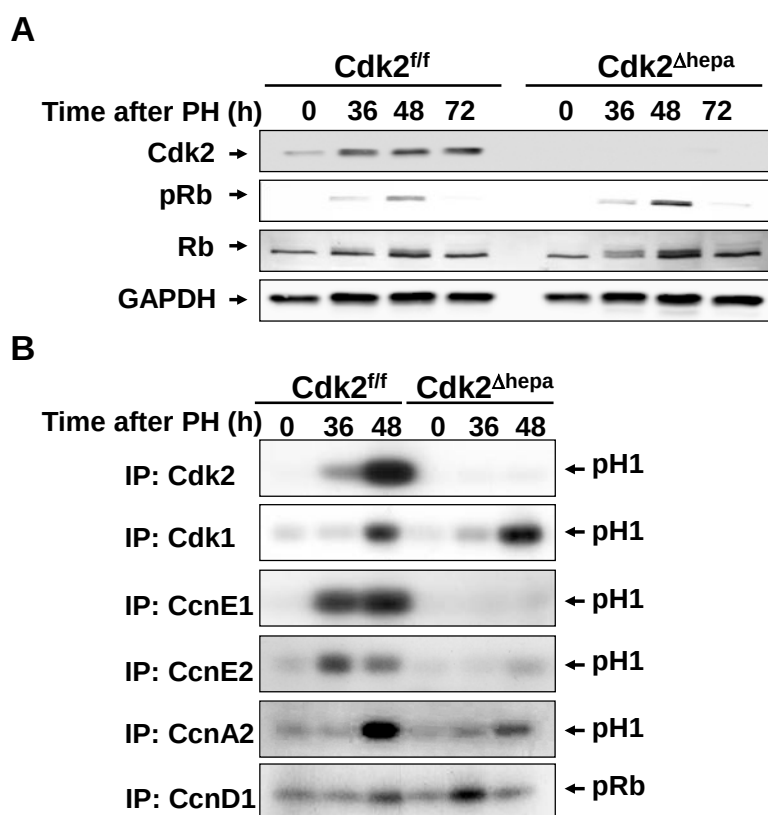


Figure 3.6: Cdk2 deficiency in liver is compensated by alternative Cdk/cyclin complexes. (A) Phosphorylation of Rb in Cdk2^{ff} and Cdk2^{Δhepa} mice at 0-96 h post PH was determined by western blot analysis. (B) Cdk2^{ff} and Cdk2^{Δhepa} mice were subjected to PH. At time points after PH as indicated, liver extracts were subjected to immunoprecipitation (IP) with antibodies directed against Cdks and cyclins. Precipitated Cdk/cyclin complexes were used for *in vitro* kinase assays using recombinant histone H1 (H1) or Rb as substrate. Phosphorylated proteins are highlighted by arrows.

3.2 Hepatocyte proliferation and liver regeneration can be inhibited by application of the Cdk-inhibitor Roscovitine

The previous data revealed that genetic loss of Cdk2 in hepatocytes was not sufficient to block the proliferation of hepatocytes after PH. With regard to the anticipated important role of Cdk2 for the cell cycle, these findings were largely unexpected and thus should be further investigated using an independent pharmacological approach.

3.2.1 Roscovitine effectively blocks DNA-synthesis during liver regeneration

The low molecular inhibitor Roscovitine predominantly inhibits Cdk2 but also Cdk1 and to a lesser extend Cdk7 and Cdk9 (Nutley et al. 2005). To determine the inhibitory effect of Roscovitine on DNA-synthesis during liver regeneration, mice were injected with Roscovitine 2 times after PH according to the scheme in Figure 3.7 A.

Roscovitine treatment significantly diminished DNA-synthesis 48 h after PH in WT mice (Figure 3.7 B-C). However, Roscovitine-treated mice rather showed a delayed proliferation peak than a complete proliferation block, since a significantly higher percentage of BrdU positive cells was observed 72 h after PH in Roscovitine-treated mice indicating that the effect of Roscovitine was reversible. Roscovitine completely blocked both Cdk2 and Cdk1 kinase activities in regenerating mice 48 h after PH (Figure 3.7 D). Delayed DNA-synthesis in Roscovitine-treated mice appearing 72 h post-surgery correlated with full restoration of Cdk1 kinase activity and slight activation of Cdk2.

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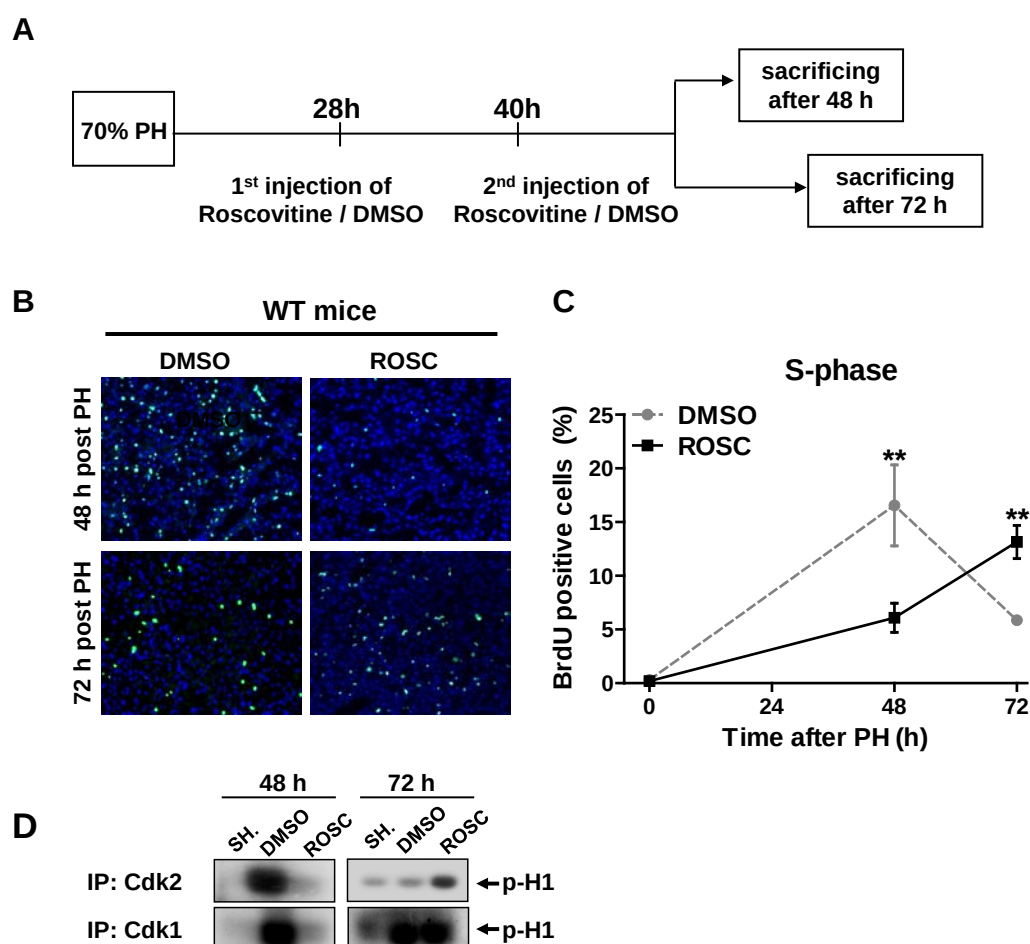


Figure 3.7: Roscovitine effectively blocks DNA-synthesis in the regenerating liver. (A) Experimental setting: WT mice were subjected to PH. Twenty-eight and 40 h after surgery, Roscovitine (or DMSO as solvent control) was administered *i.p.* at a concentration of 50 mg/kg. Mice were sacrificed 48 h and 72 h after PH, respectively. (B) Immunofluorescence staining for BrdU-positive hepatocytes (stained in green) on liver cryosections at 48 and 72 h after PH in WT mice treated with DMSO or Roscovitine. (C) Quantification of BrdU-positive hepatocytes in DMSO or Roscovitine-treated mice. Data is calculated as percentage of BrdU-positive nuclei per magnification field. Each value represents the mean of 3-5 mice. For each mouse at least 5-10 representative magnification fields were counted. **: $p < 0.01$. (D) Native Cdk2 and Cdk1 complexes were immunoprecipitated (IP) with indicated antibodies from WT livers at 48 and 72h after PH and Roscovitine/DMSO treatment and then subjected to histone H1 kinase assays. Phosphorylation of H1 (p-H1) is highlighted by arrows.

To further dissect the contribution of Cdk2 and Cdk1 for S-phase progression in the liver, the same experimental settings (PH and Roscovitine treatment) were applied to Cdk2^{Δhepa} mice. Interestingly, Roscovitine application inhibited DNA-synthesis in WT and Cdk2^{Δhepa} animals to the same extend (Figure 3.8 A). This strongly suggests that the inhibitory effect of Roscovitine on DNA-synthesis is independent of Cdk2 and rather mediated *via* blocking both Cdk2 and Cdk1.

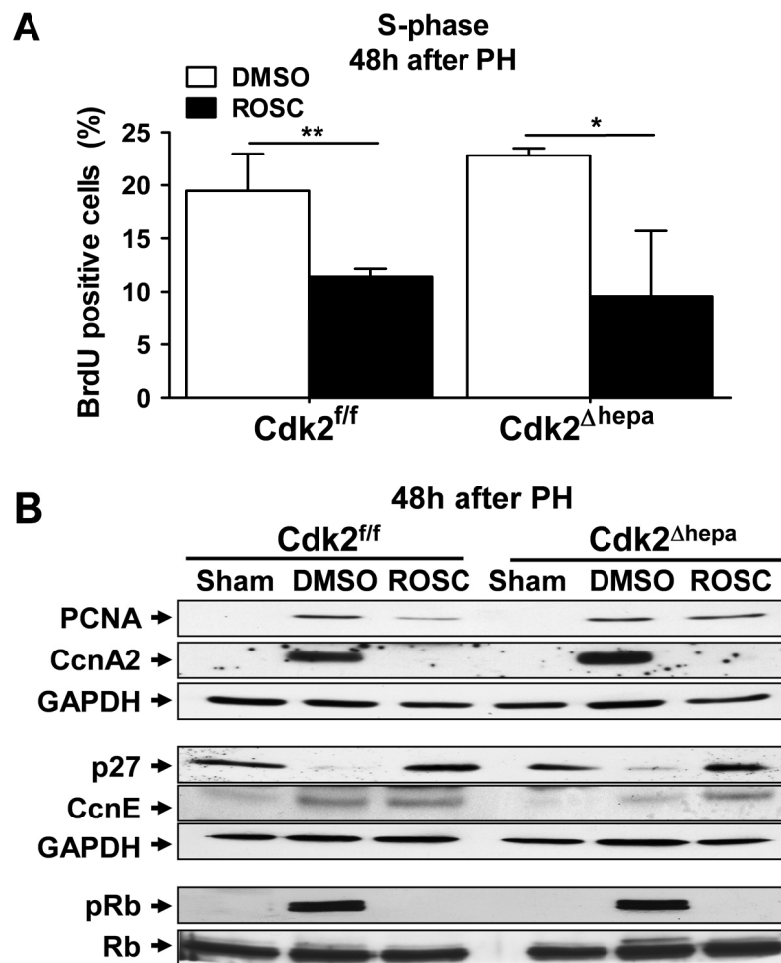


Figure 3.8: Aberrant cell cycle signalling by Roscovitine in liver regeneration is similar in presence and absence of Cdk2. $Cdk2^{f/f}$ and $Cdk2^{\Delta hepa}$ mice were subjected to PH or sham operation and treated twice with DMSO or Roscovitine (ROSC) 28 h and 40 h after surgery. Animals were sacrificed 48 h post PH and analyzed for proliferation and cell cycle markers. **(A)** Quantification of BrdU-positive hepatocytes in liver cryosections. Each value represents the mean of 3-5 mice. **(B)** Representative western blots analyzing protein expression of selected cell cycle regulators.

Following Roscovitine treatment gene expression of CcnA2, which is responsible for S-phase progression, was completely blocked (Figure 3.8 B). Consistently, phosphorylation of Rb was also completely inhibited by Roscovitine treatment in WT and $Cdk2^{\Delta hepa}$ mice. The latter finding (lack of Rb-phosphorylation in Roscovitine-treated $Cdk2^{\Delta hepa}$ mice) suggests an essential role of Cdk1 for compensating the loss of Cdk2 activity, since $Cdk2^{\Delta hepa}$ mice showed normal Rb-phosphorylation and induction of Cdk1 activity 48 h after PH (Figure 3.6 A-B). Roscovitine also induced the kinase inhibitor p27 thereby further supporting cell cycle arrest in the liver. In contrast, CcnE protein expression was not inhibited by

Roscovitrine (Figure 3.8 B). In Cdk2^{Δhepa} mice even slightly elevated protein levels of CcnE were observed. Taken together, these data suggest that Roscovitrine inhibits DNA-synthesis in an E2F-Rb dependent manner by blocking Cdk2 and Cdk1.

3.2.2 Over-expression of CcnE1 rescued CcnA2 gene expression and Rb phosphorylation in Roscovitrine treated primary hepatocytes

The previous data indicates that Roscovitrine inhibits DNA-synthesis of the proliferating liver *via* blocking both Cdk2 and Cdk1 activities. Moreover, genetic inactivation of CcnE2 results in over-expression of CcnE1 and accelerated S-phase initiation after PH (Nevzorova et al. 2009). Due to this over-expression of CcnE1, Roscovitrine treatment induced slight reduction of DNA-synthesis after PH in CcnE2^{-/-} mice rather than blocked strongly S-phase progression as shown in WT mice (Nevzorova, personal communication). With regard to tumorigenesis, CcnE1 over-expression was frequently observed in cancer cells and could contribute to carcinogenesis (Hwang and Clurman 2005). Thus, it is of high interest to test if over-expression of CcnE1 expression can partially rescue hepatocyte proliferation after Roscovitrine-mediated inhibition of Cdk1 and Cdk2. Using an adenoviral approach CcnE1 was over-expressed in mitogen stimulated primary WT hepatocytes (Figure 3.9 A). Thereafter hepatocytes were transiently treated with Roscovitrine. After two days, control cells entered S-phase as indicated by onset of CcnA2 expression and Rb phosphorylation, which was strongly inhibited by Roscovitrine treatment (Figure 3.9 B). However, adenoviral over-expression of CcnE1 partially rescued the Roscovitrine-mediated S-phase arrest demonstrating that CcnE1 over-expression facilitates G1/S transition even when the most, if not all, kinase activities of Cdk1 and Cdk2 are inhibited.

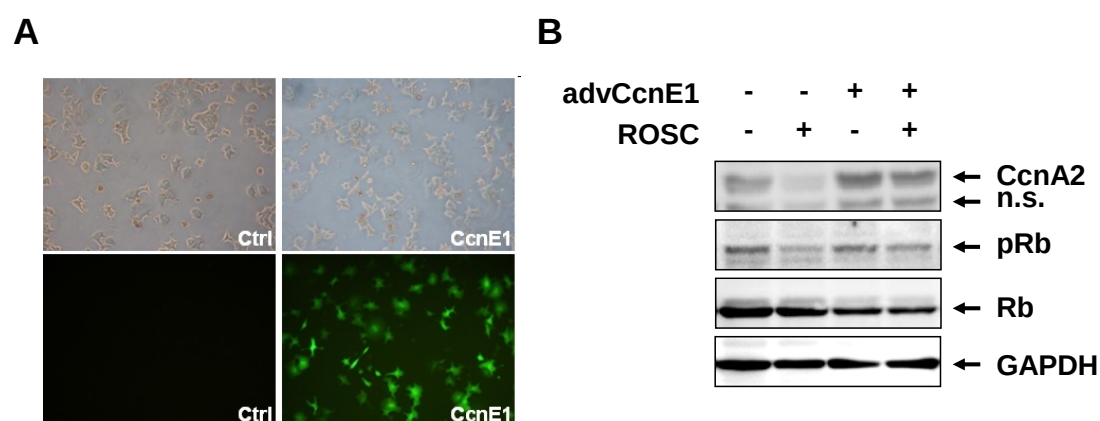


Figure 3.9: Adenoviral CcnE1 over-expression can partially rescue the Roscovitine-mediated cell cycle inhibition. (A) Primary hepatocytes from WT mice were isolated and transduced with a luciferase control adenovirus (Ctrl) or a bicistronic adenovirus expressing CcnE1 and GFP (CcnE1). Adv-CcnE1 transduction efficiency was determined by fluorescence microscopy 24 h post transfection. Infected hepatocytes appear in green; total number of plated cell was in parallel visualized by light microscopy. (B) Primary hepatocytes were stimulated with mitogen EGF and insulin 16 h after transfection. 24 h after virus infection, cells were transiently stimulated with Roscovitine or DMSO for 4 h and harvested 48 h after initial adenoviral infection. As marker for S-phase progression the expression of CcnA2 and phosphorylated Rb (pRb) was determined by western blot analysis. n.s.: non-specific signal.

3.3 CcnE1 plays an essential role for MCM2 loading on pre-RC and CcnA2 regulation in Cdk2^{Δhepa} mice

3.3.1 Cdk2-deficiency in hepatocytes is associated with premature pre-RC formation involving CcnE1

The previous experiments identified potential compensatory Cdk/cyclin complexes, which revealed enhanced activity during liver regeneration in absence of Cdk2. However, it was completely unexpected that CcnE1 was over-expressed in Cdk2^{Δhepa} liver at early liver regeneration (36 h post PH, Figure 3.4 A), but did not contribute to any non-canonical Cdk kinase activity (Figure 3.6 B). This was especially surprising as E-cyclins are essential for G0/G1-S-phase transition at least in embryonic fibroblasts (Geng et al. 2003). Additional evidence suggests a non-canonical function of E-cyclins in formation of the pre-replicative (pre-RC) complex, which is kinase-independent (Geng et al. 2007).

Therefore, in the present study it was hypothesized that CcnE1 holds a Cdk2-independent function during liver regeneration in absence of Cdk2 involving pre-RC complex formation. To address this hypothesis, the expression and

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localization of replication-related factors such as PCNA, CcnE1, MCM2 and Cdt1 was further investigated in mitogen-stimulated primary hepatocytes *in vitro*. In comparison to WT cells, PCNA expression was induced one day earlier (day 2 after plating) in Cdk2-deficient hepatocytes, which was correlated with accelerated onset of CcnE1 expression. In addition, peak expression of MCM2 appeared also earlier in Cdk2^{Δhepa} cells (Figure 3.10 A). In line with these findings in Cdk2^{Δhepa} hepatocytes, the amount of chromatin-bound CcnE1 was also increased in these cells after two days of culturing. Higher CcnE1 level in chromatin-bound fraction correlated with increased MCM2-loading on chromatin in Cdk2^{Δhepa} hepatocytes (Figure 3.10 B). Moreover, in the absence of Cdk2, CcnE1 constitutively interacted with Cdt1 as demonstrated by co-immunoprecipitation experiments (Figure 3.10 C). Physical interaction of CcnE1 with Cdt1 might facilitate the loading of MCM2 at Pre-RC (Geng et al. 2007). These data suggests that up-regulation of CcnE1 in Cdk2-deficient cells results in earlier pre-RC formation.

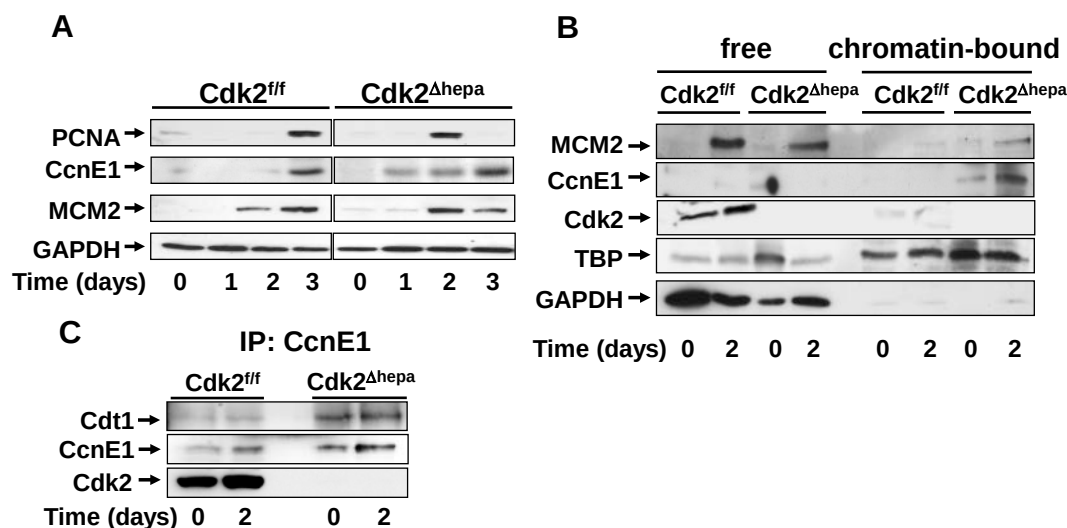


Figure 3.10: CcnE1 is overexpressed in Cdk2-deficient primary hepatocytes and shows accelerated co-localization with MCM2 and Cdt1 at chromatin. Primary mouse hepatocytes from Cdk2^{fl/fl} and Cdk2^{Δhepa} mice were isolated and cultured for up to 3 days in the presence of the mitogenic stimuli EGF and insulin. (A) Western blots showed protein expression of PCNA, CcnE1 and MCM2. GAPDH expression was determined as internal loading control. (B) The expression and cellular localization of MCM2, CcnE1 and Cdk2 were analyzed by western blot using fractionated cell extracts representing whole free cytoplasmic protein proteins (free) or the chromatin-bound proteins, respectively. TATA binding protein (TBP) and GAPDH represent loading controls for chromatin-bound and free fractions, respectively. (C) CcnE1 complexes were isolated from total proteins by immunoprecipitation (IP: CcnE1) and probed for Cdt1, CcnE1 and Cdk2 by western blot analysis.

3.3.2 Additional depletion of CcnE1 in Cdk2^{Δhepa} hepatocytes markedly reduces MCM2 loading

The data from Cdk2^{Δhepa} mice emphasize an essential kinase-independent role of CcnE1 in hepatocyte proliferation in a Cdk2 deficient background, especially in the loading of MCM2 complexes at pre-RC. This leads to the hypothesis that hepatocytes with deletion of both Cdk2 and CcnE1 genes might have a defect in MCM2 loading, which could impair DNA-synthesis. To test this hypothesis, Cdk2^{Δhepa}CcnE1^{-/-} double knockout mice were generated by crossing Cdk2^{Δhepa} mice with mice constitutively lacking CcnE1 (CcnE1^{-/-}). The latter ones were recently shown to have only a minor effect on hepatocyte proliferation after PH (Nevzorova et al. 2009). Briefly, CcnE1^{-/-} mice showed a slight delay in S-phase entry but otherwise normal DNA synthesis and liver regeneration after partial hepatectomy.

Cdk2^{Δhepa}CcnE1^{-/-} mice did not show any abnormality during embryonic and postnatal development (data not shown). Primary hepatocytes of 7–8 weeks old Cdk2^{Δhepa}CcnE1^{-/-} mice were isolated and cultured in the presence of mitogenic stimulation. After two days culturing, a strong reduction of MCM2 level in both the chromatin fraction and the free fraction was observed compared to WT or Cdk2^{Δhepa} hepatocytes (Figure 3.11). Collectively, these data indicated that a Cdk2-independent function of CcnE1 is essential for the loading of MCM2 at chromatin at least in the absence of Cdk2.

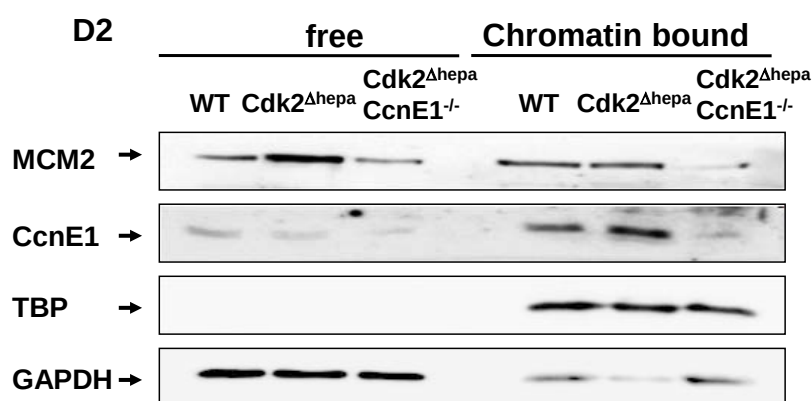


Figure 3.11: Combined depletion of Cdk2 and CcnE1 in hepatocytes results in reduced MCM2 loading on chromatin. Primary mouse hepatocytes from WT, Cdk2^{Δhepa} and Cdk2^{Δhepa}CcnE1^{-/-} mice were isolated and cultured for 2 days in the presence of the mitogenic stimuli EGF and insulin. The expression and cellular localization of MCM2 and CcnE1 were analyzed by western blot using fractionated cell extracts representing whole cytoplasmic protein (free) or the chromatin-bound proteins, respectively. TATA binding protein (TBP) and GAPDH represent loading controls for chromatin- and cytoplasmic fractions, respectively.

3.3.3 CcnE1 is essential for driving CcnA2 gene expression in Cdk2-deficient hepatocytes

To further characterize the consequences of combined Cdk2 and CcnE1 ablation for DNA synthesis and hepatocyte proliferation, the expression of CcnA2 (indicative of S-phase progression) was determined. Results from qRT-PCR and western blot analysis clearly demonstrated that additional depletion of CcnE1 in Cdk2^{Δhepa} cells substantially inhibited CcnA2 gene expression (Figure 3.12 A-B). Thus, in absence of Cdk2, expression of CcnE1 is imperative for CcnA2 gene and protein expression in hepatocytes.

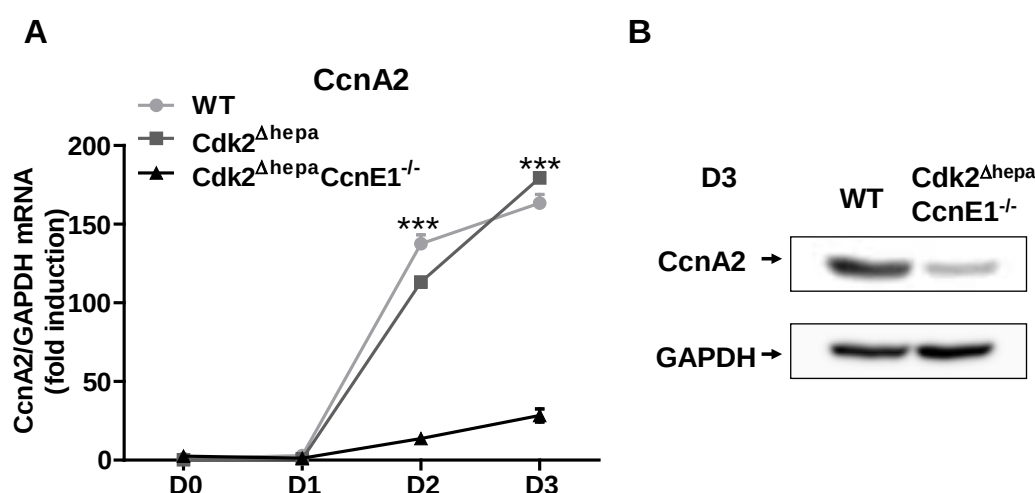


Figure 3.12: Combined depletion of Cdk2 and CcnE1 results in strongly reduced CcnA2 expression. Primary mouse hepatocytes from WT, Cdk2^{Δhepa} and Cdk2^{Δhepa} CcnE1^{-/-} mice were isolated and cultured for up to 3 days in the presence of the mitogenic stimuli EGF and insulin. **(A)** qPCR analysis of CcnA2 gene expression. Data is calculated as fold induction in comparison to untreated WT cells and normalized using GAPDH expression as internal standard. Data is presented as the average $\pm$ S.D. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. **(B)** Western blot analysis of CcnA2 protein expression in WT and Cdk2^{Δhepa} CcnE1^{-/-} hepatocytes three days after plating (D3).

During G1/S transition, onset of CcnE expression is followed by induction of CcnA2 gene expression in an E2F-dependent manner. Previous studies demonstrated that the Cdk2/CcnE complex can directly associate with E2F/p107 transcription factors bound to an E2F binding site located in the CcnA2 promoter resulting in transactivation of CcnA2 gene expression (Schulze et al. 1995; Zerfass-Thome et al. 1997). To further analyze the function of CcnE1 on CcnA2 promoter regulation, EMSA analysis was performed using nuclear proteins from WT

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and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ hepatocytes and a double-stranded probe containing a documented E2F binding site derived from the WT murine *CcnA2* promoter. The experiment revealed two protein/DNA complexes (I and II) with low mobility in WT samples which accumulated with time and *CcnA2* expression (Figure 3.13; also compare Figure 3.12 for *CcnA2* expression profile). In sharp contrast, complex I and II were barely detectable in proteins from $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ hepatocytes at any time point investigated. These data suggests that simultaneous loss of *CcnE1* and *Cdk2* inhibits the association of a trans-activating E2F protein complex at the *CcnA2* promoter.

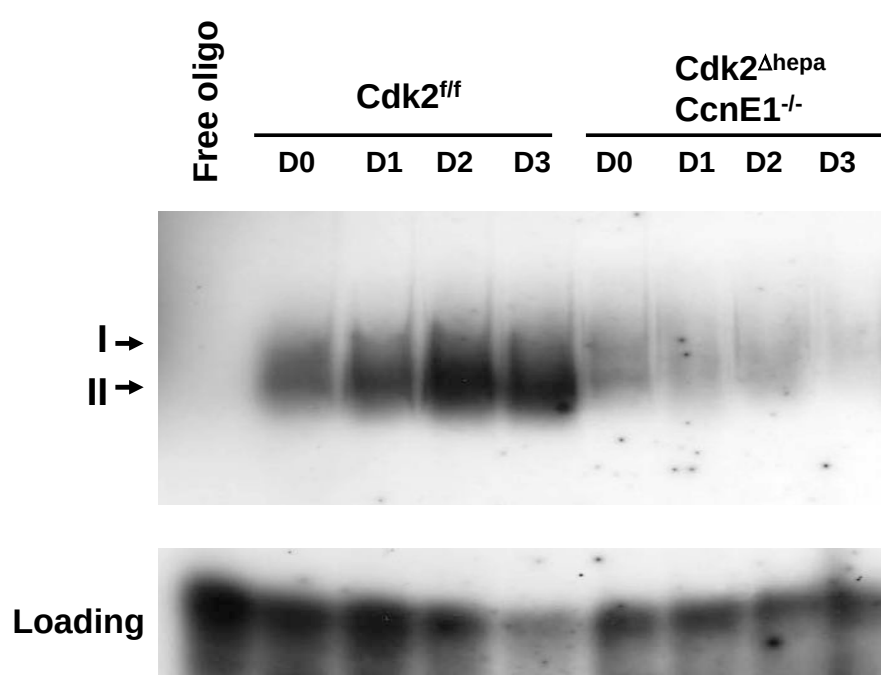


Figure 3.13: Combined depletion of *Cdk2* and *CcnE1* results in reduced protein binding to *CcnA2* promoter. Primary hepatocytes from WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were isolated and cultured for up to 3 days (D0 – D3) in the presence of the mitogenic stimuli EGF and insulin. At time points indicated, nuclear proteins were isolated from hepatocytes. 5 μg nuclear proteins per sample were subjected to EMSA. In the first lane (free oligo) the oligonucleotide was loaded without adding protein. The signal specific for unbound probe with high mobility is shown as a loading control. I: complex I binding to probe; II: complex II binding to probe.

3.3.4 Combined deletion of *Cdk2* and *CcnE1* impairs DNA-synthesis and proliferation of primary hepatocytes

CcnA2 promotes S-phase progression and S/G2-M phase transition by association with either *Cdk2* or *Cdk1*, respectively (Chibazakura et al. ; Copeland et al. ; Sherr and Roberts 2004). Thus, impaired *CcnA2* gene expression as well as the

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defect in MCM2 loading at the pre-RC, which was observed in $Cdk2^{\Delta hepa}CcnE1^{-/-}$ hepatocytes could affect S-phase entry and subsequent DNA synthesis. To test this hypothesis, DNA-synthesis in primary hepatocytes from $Cdk2^{\Delta hepa}CcnE1^{-/-}$ mice and WT controls was determined by *in vitro* BrdU incorporation assay. After one day of culturing (D1), no BrdU positive cells were observed in $Cdk2^{\Delta hepa}CcnE1^{-/-}$ cells, while very few BrdU positive cells were detectable in WT and $Cdk2^{\Delta hepa}$ hepatocytes. In the subsequent two days of cell culture WT or $Cdk2^{\Delta hepa}$ hepatocytes showed a substantial induction of DNA synthesis which peaked at day 3 (D3) after plating, while $Cdk2^{\Delta hepa}CcnE1^{-/-}$ hepatocytes had strongly reduced S-phase reflecting approximately 50% BrdU incorporation compared to controls at day 2 and only 25% of controls at day 3 after plating (Figure 3.14 A-B).

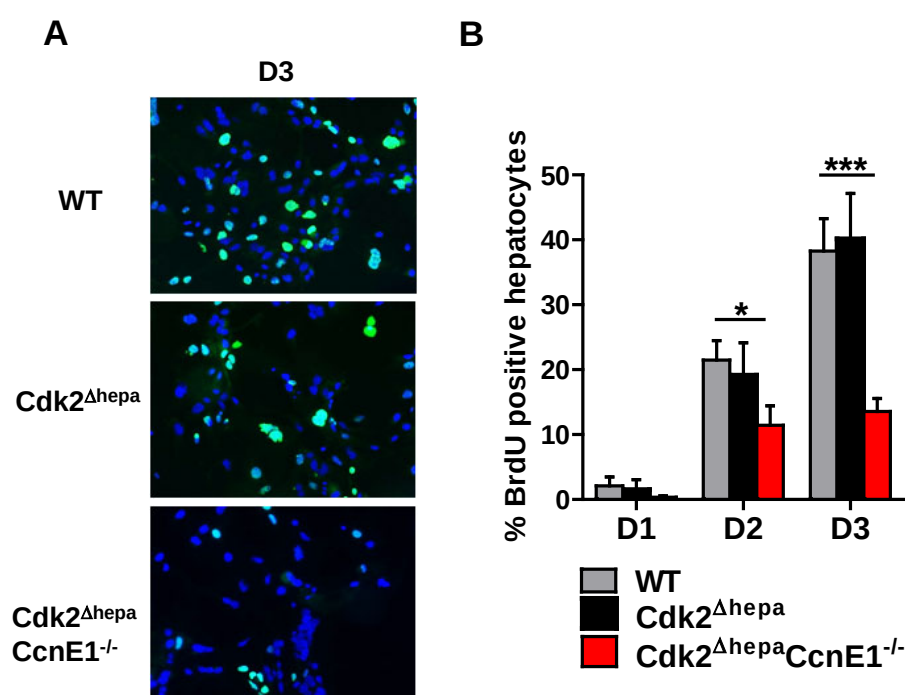


Figure 3.14: Combined depletion of Cdk2 and CcnE1 results in impaired DNA-synthesis. Primary hepatocytes from WT, $Cdk2^{\Delta hepa}$ and $Cdk2^{\Delta hepa}CcnE1^{-/-}$ mice were isolated and cultured for up to 3 days on collagen coated cover slips. Two hours before cells were harvested, 20 μ M BrdU were supplied to cell medium. **(A)** Immunofluorescence staining for BrdU incorporation. Nuclei with BrdU incorporation in newly synthesized DNA are stained in green. Total nuclei are counter-stained with DAPI (blue). **(B)** Quantification of BrdU-positive hepatocytes. Data is calculated as percentage of BrdU-positive nuclei per magnification field. Data is presented as the average $\pm$ S.D. *: $p < 0.05$; ***: $p < 0.001$.

These data clearly demonstrated that hepatocytes without CcnE1 and Cdk2 and subsequent low levels of CcnA2 have a severe defect in DNA-replication. In addition, light microscopy analysis demonstrated that $Cdk2^{\Delta hepa}CcnE1^{-/-}$ hepatocytes displayed

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massive reduction of cell density over time indicating severely impaired viability and induction of cell death (Figure 3.15 A-B).

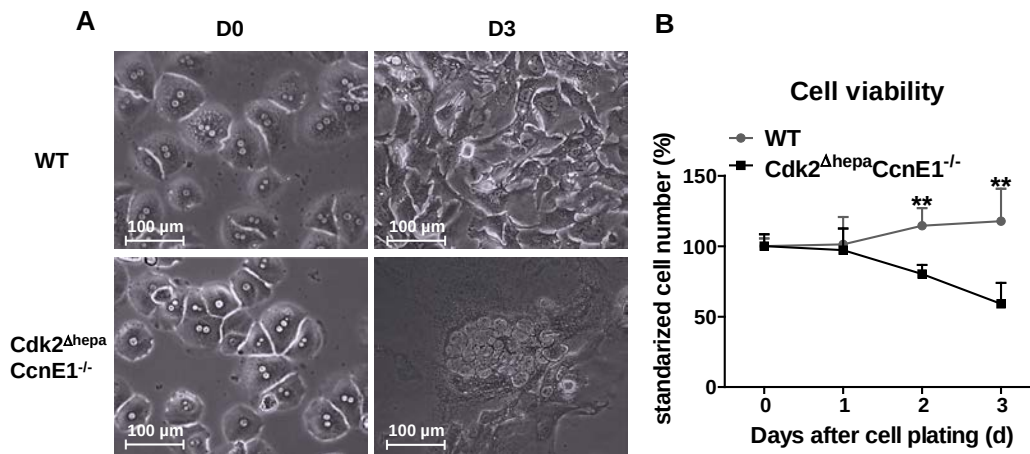


Figure 3.15: Hepatocytes lacking Cdk2 and CcnE1 display severely impaired viability. Primary hepatocytes from WT and Cdk2^{Δhepa}CcnE1^{-/-} mice were isolated and cultured for up to 3 days in the presence of mitogenic stimuli (EGF, insulin). **(A)** Microscopic images of cultured hepatocytes. D0 is defined as the day following cell plating; D3: hepatocytes three days after plating. **(B)** Quantification of hepatocytes per magnification field. For each mouse at least 10 representative magnification fields were counted. Cell numbers were normalized to the average cell numbers determined at D0. Data is presented as the mean ± standard deviation. **: p < 0.01.

To confirm the finding that Cdk2^{Δhepa}CcnE1^{-/-} hepatocytes have reduced DNA-synthesis and increased cell death, flow cytometry of propidium iodide stained primary hepatocytes was performed to determine the DNA content. In sharp contrast to WT hepatocytes, a large population (approximately 50%) of Cdk2^{Δhepa}CcnE1^{-/-} cells showed a DNA content which was lower than typically found in diploid resting cells (2n). This cell population is usually referred to as sub-G1 population and reflects mostly apoptotic cells with DNA degradation. Moreover, the WT cells contained approximately 2 times more diploid hepatocytes (2n) and 3 times more tetraploid hepatocytes (4n) compared to Cdk2^{Δhepa}CcnE1^{-/-} cells (Figure 3.16 A-B). The difference in cell populations having octoploidy (8n) and hyperploidy (>8n) was not significant between two groups. These data clearly confirmed that Cdk2/CcnE1-deficient hepatocytes are almost completely devoid of DNA replication and highly prone to apoptosis.

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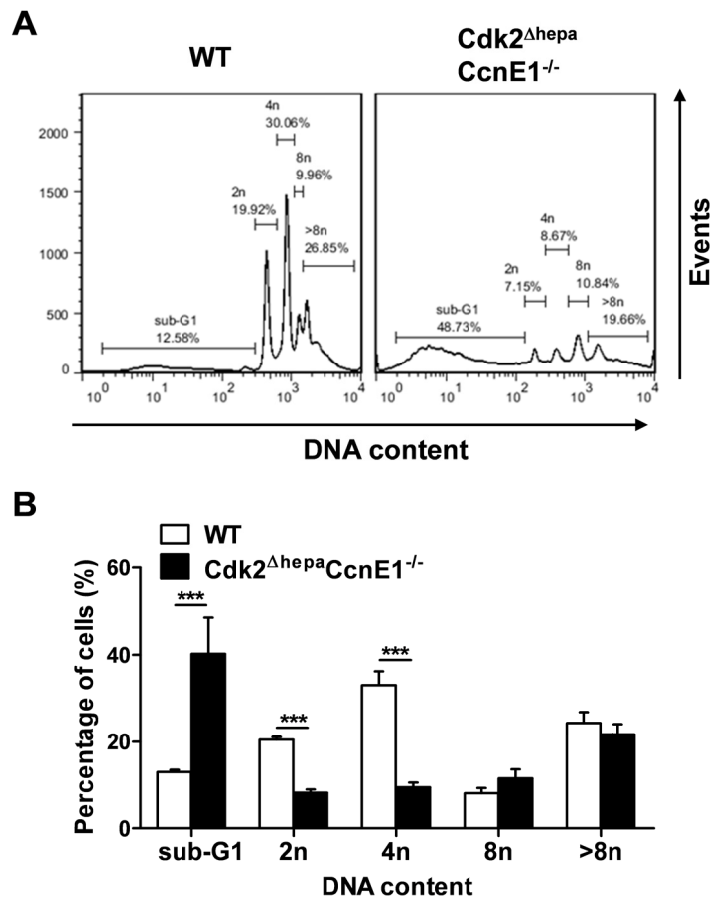


Figure 3.16: Primary hepatocytes lacking Cdk2 and CcnE1 are devoid of DNA synthesis and prone to apoptosis during cultivation. Primary mouse hepatocytes from WT and $Cdk2^{\Delta hepa} CcnE1^{-/-}$ mice were isolated and cultured for up to 3 days in the presence of mitogenic stimuli (EGF, insulin). Hepatocytes were detached from cell culture plates using Accutase treatment after three days of culturing. Nuclei of hepatocytes were extracted and stained with propidium iodide (PI). DNA-content of PI stained hepatocytes was determined by flow cytometry analysis. **(A)** Representative histograms after flow cytometry for WT and $Cdk2^{\Delta hepa} CcnE1^{-/-}$ hepatocytes show the distribution of apoptotic cell fractions (sub-G1), diploid (2n), tetraploid (4n), octoploid (8n) and high polyploid (>8n) cells. X-axis: PI intensity; y-axis: number of events. **(B)** Quantification of different cell fractions. Data is presented as the mean $\pm$ standard deviation. ***: $p < 0.001$.

Interestingly, microscopic analysis also revealed that besides strongly reduced cell number, surviving $Cdk2^{\Delta hepa} CcnE1^{-/-}$ hepatocytes frequently form multinuclear giant cells after three days of culturing (Figure 3.15 A). The best known mechanism explaining polynuclear hepatocytes is endomitosis - a process involving DNA replication without subsequent cell division. However, it was unlikely that these cells were generated by excessive endomitosis as the previous experiments clearly demonstrated that hepatocytes lacking Cdk2 and CcnE1 had strongly reduced DNA replication. Therefore it was hypothesized that polynuclear giant $Cdk2^{\Delta hepa} CcnE1^{-/-}$

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hepatocytes might originate from cell fusion. To test this possibility, $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ hepatocytes were isolated from both female and male mice, mixed in a 1:1 ratio, and cultured for up to 3 days on cover slips. Each remaining hepatocyte was then examined for presence of Y-chromosomes by Fluorescence *in situ* Hybridization (FISH). In fact, many polynuclear giant cells containing both Y-chromosome-positive nuclei (derived from male hepatocytes) and Y-chromosome-negative nuclei (derived from female hepatocytes) were detected (Figure 3.17). This observation suggests that $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ hepatocytes tend to cell fusion with adjacent cells under *in vitro* conditions. However, the physiological relevance of the cell fusion mechanism in this scenario is still unknown and will not be further investigated as a part of this study.

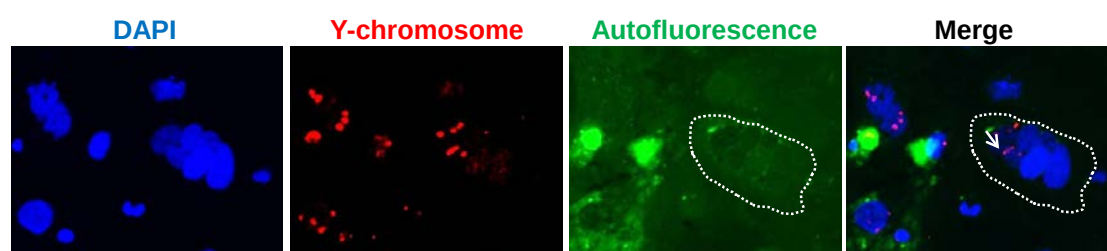


Figure 3.17: Primary hepatocytes lacking Cdk2 and CcnE1 frequently undergo cell fusion resulting in polynuclear giant cells. Primary hepatocytes from female and male $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were mixed in a 1:1 ratio and cultured for up to 3 days on collagen coated cover slips. Y-chromosomes are stained by specific Y-chromosome painting probe (red signals). Nuclei are stained blue by DAPI. Green autofluorescence of hepatocytes is used to identify individual single cells.

3.4 Combined ablation of Cdk2 and CcnE1 *in vivo* impairs liver regeneration after PH

3.4.1 Analysis of G1/S transition and S-phase progression in livers of $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice after PH

The previous *in vitro* experiments clearly demonstrated that an additional deletion of CcnE1 in $Cdk2^{\Delta\text{hepa}}$ mice prevents MCM2 loading at pre-replication complexes and inhibits CcnA2 transcription in *ex vivo* isolated hepatocytes leading to impaired DNA-synthesis and cell viability. Therefore it is of high interest to investigate if proliferation of hepatocytes lacking CcnE1 and Cdk2 is also prevented in the liver regeneration model. Thus, 7-8 weeks old $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice as well as WT controls were subjected to 70% partial hepatectomy. BrdU staining revealed that livers of $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice showed dramatically reduced DNA synthesis 40-48 h after

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PH (Figure 3.18 A-B) which is in good agreement with the previous findings *in vitro*.

As a consequence of reduced DNA-synthesis, $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice showed a significantly reduced liver weight/body weight ratio 7 days after surgery which demonstrates that liver regeneration is inefficient after simultaneous loss of CcnE1 and Cdk2 (Figure 3.18 C). However all $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice survived the surgery and were viable for at least one month after PH (data not shown).

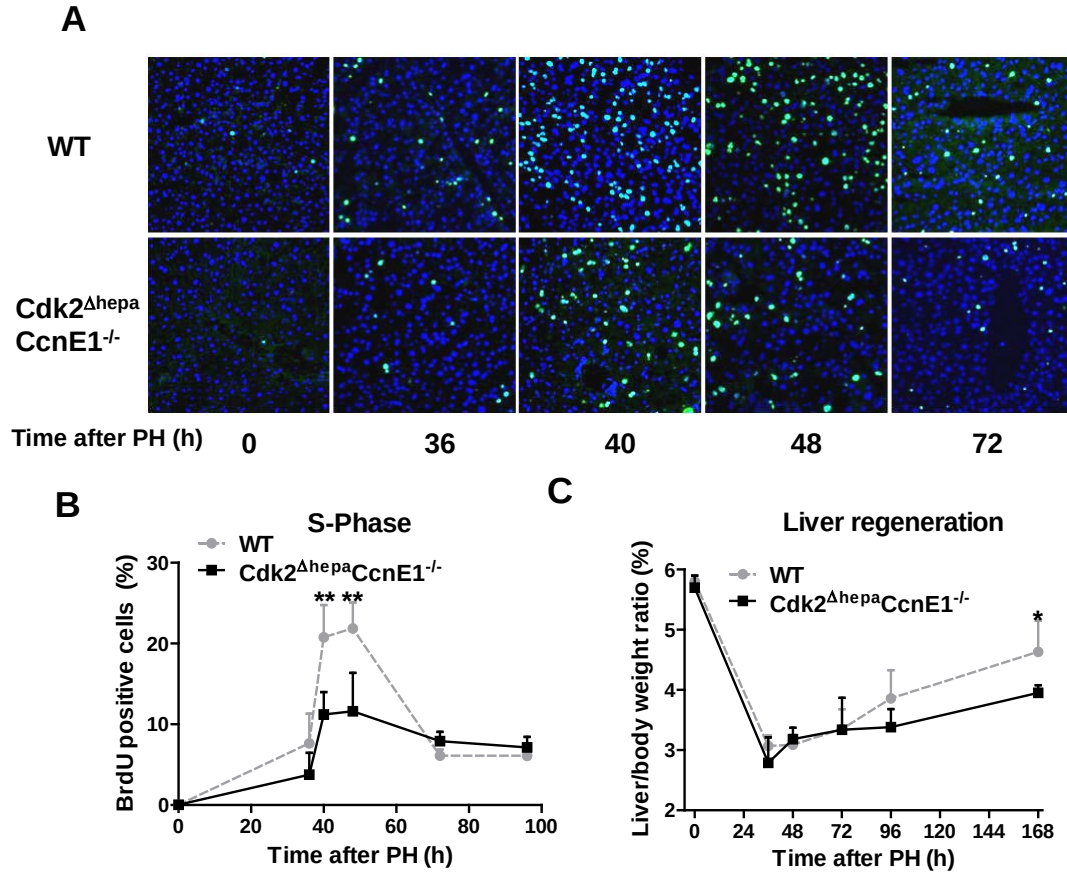


Figure 3.18: Simultaneous genetic ablation of Cdk2 and CcnE1 in hepatocytes substantially impairs DNA-synthesis of hepatocytes and liver regeneration after PH. WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were subjected to PH. Remnant livers were analyzed at 0-168 h after surgery. **(A)** Immunofluorescence staining for BrdU incorporation on liver cryosections. BrdU positive signals located in nuclei are stained in green. Total nuclei are counter-stained with DAPI (blue). **(B)** Quantification of BrdU-positive hepatocytes. Data is calculated as percentage of BrdU-positive nuclei per magnification field. For each mouse at least 5-10 representative magnification fields were counted. Each value represents the mean of 5-10 mice. **(C)** Liver weight/body weight index (%) at indicated time points after PH. Data is presented as the mean $\pm$ standard deviation.

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Alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) are liver enzymes, which are activated upon liver damage and are thus established serum markers for the quantification of liver injury. To test if impaired liver regeneration in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice resulted in enhanced liver damage, AST and ALT values were measured at different time points after PH. However, in comparison to controls no elevation in transaminase activity was detected in serum of $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice 40-48 h post-surgery (Figure 3.19 A) as well as 7 days after PH. This observation was in part unexpected and is in contradiction to the poor viability of *ex vivo* isolated $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ hepatocytes (compare Figure 3.15).

In line with this finding, $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ livers did not show aberrantly enhanced apoptosis 40 h and 48 h after PH as evidenced by TUNEL analysis (Figure 3.19 C). Instead, both groups revealed only few apoptotic liver cells after PH suggesting that apoptosis plays only a minor role in the progression phase of liver regeneration.

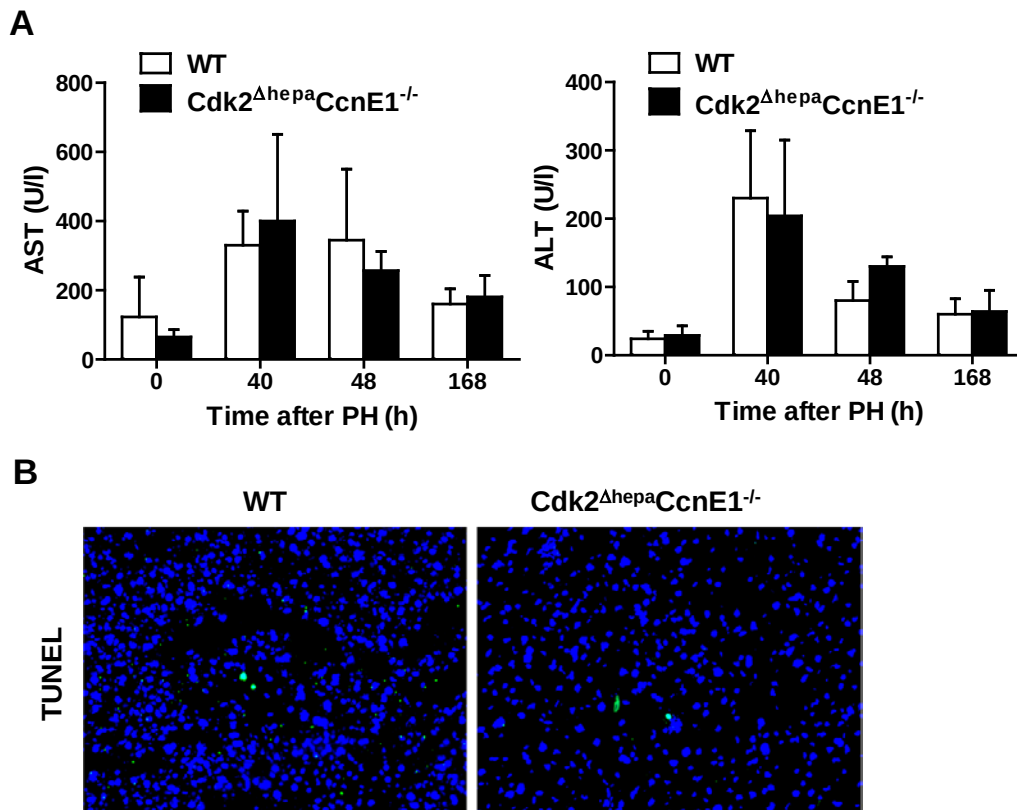


Figure 3.19: Liver damage after PH is not aggravated by concomitant ablation of *CcnE1* and *Cdk2*. WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were subjected to PH. (A) AST level and (B) ALT level in serum were measured at time points indicated. Each bar represents an average value of 5 animals. Data is presented as the mean $\pm$ standard deviation. (C) Representative images of TUNEL staining on liver cryosections from WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice 48 h after PH. Nuclei are stained blue by DAPI and apoptotic cells are stained green.

3.4.2 Inhibition of DNA-synthesis in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice results in increased cell size and reduced cell number after liver restoration

The previous data showed that in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice DNA-synthesis was dramatically blocked and this further resulted in substantially impaired liver mass restoration. However, all $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice survived the liver resection without showing any phenotypic abnormality or enhanced liver damage (compare Figure 3.19). These discordant observations raised the question how these mice maintained proper liver function with impaired DNA-synthesis and subsequent propagation of residual hepatocytes. To address this question, liver structure and morphology of hepatocytes in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were investigated in detail 7 days after PH by morphometric analysis (Figure 3.20 A-B).

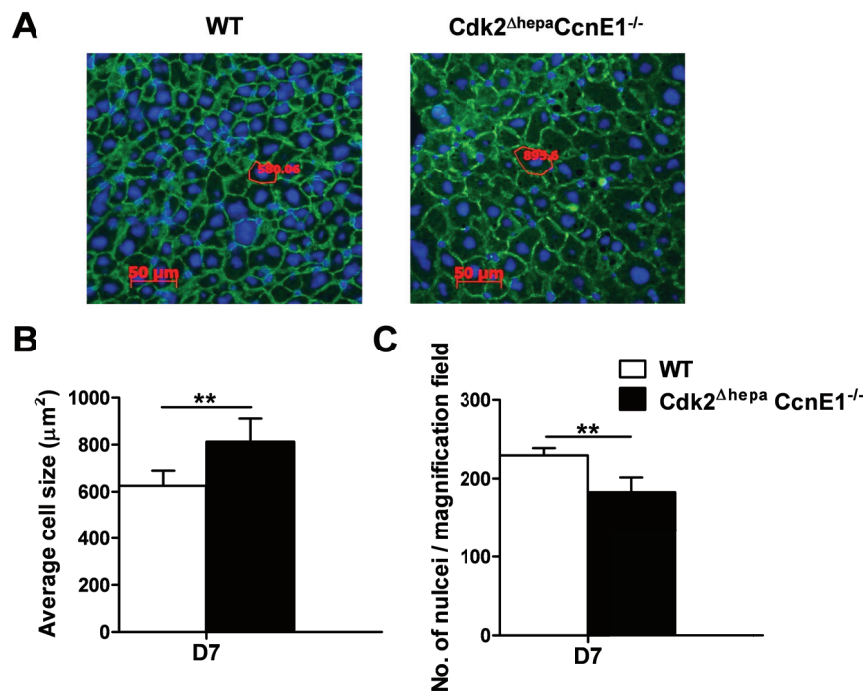


Figure 3.20: Combined depletion of CcnE1 and Cdk2 results in enlarged cell size and declined cell number after liver regeneration. WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were subjected to PH. Mice were sacrificed 7 days (D7) after PH. **(A)** Cryosections of liver tissue were stained with a fluorescein-conjugated β -catenin antibody to visualize the cell boundaries. Cell areas were quantified using AxioVision software (Rel. 4.6). Representative cells from both groups are highlighted in red and their respective size is given in μm^2 **(B)** Quantification of average cell size. A total of 3 mice per group were analyzed and 200 cells per liver section were measured. **(C)** Liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Samples were subjected to HE staining. Nuclei number per magnification field was counted. For each mouse at least 5-10 representative magnification fields were counted. Each value represents the mean of 3 mice. Data is presented as the average $\pm$ standard deviation. **: $p < 0.01$.

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In comparison to WT hepatocytes, cells lacking CcnE1 and Cdk2 showed an overall enlargement in cell size, which was statistically significant. In addition, Cdk2^{Δhepa}CcnE1^{-/-} livers showed a lower density of nuclei after liver regeneration. These data suggests that in Cdk2^{Δhepa}CcnE1^{-/-} livers normal liver function is restored at least in part by hypertrophy of hepatocytes and to a lesser extent by cell division hinting at an alternative mechanism for liver regeneration.

Hypertrophic enlargement of cells is closely correlated with activity of RNA polymerase III, which produces transfer RNAs, 5S ribosomal RNA and several other un-translated RNAs thereby enhancing translational capacity (Goodfellow and White 2007). To further examine the hypertrophic status of Cdk2^{Δhepa}CcnE1^{-/-} cells at the molecular level, gene expression of 7SL ribosomal RNA (White 2004), which is a product of RNA polymerase III, was determined (Figure 3.21). Interestingly, basal 7SL expression in Cdk2^{Δhepa}CcnE1^{-/-} mice was already two times higher compared to WT animals. Seven days after surgery (termination of liver regeneration in WT mice), expression level of 7SL in Cdk2^{Δhepa}CcnE1^{-/-} liver was still two times higher than in WT liver indicating that combined loss of Cdk2 and CcnE1 constitutively triggers a stronger metabolic capacity in the liver.

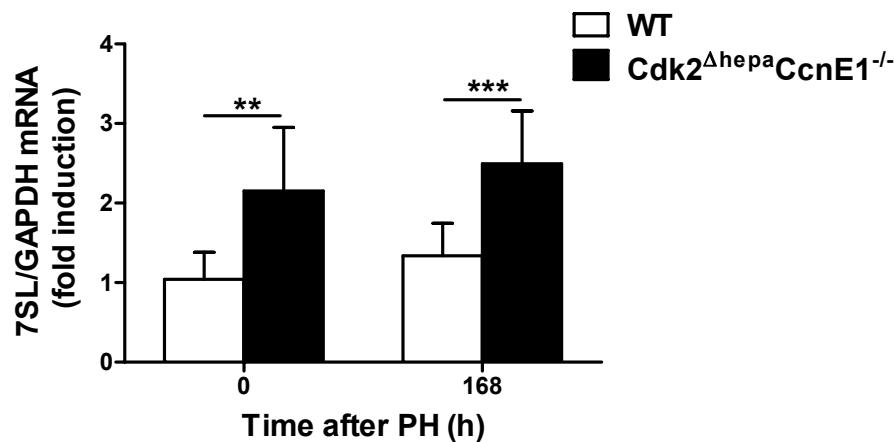


Figure 3.21: Hypertrophy of Cdk2^{Δhepa}CcnE1^{-/-} liver is associated with high activity of mRNA polymerase III. Gene expression of 7SL in untreated liver and regenerated liver 168 h post PH from WT and Cdk2^{Δhepa}CcnE1^{-/-} mice. For each group, 5 animals (7-9 weeks old) were analyzed. Data is presented as the mean ± standard deviation. **: p<0.01; ***: p<0.001.

3.4.3 Molecular analysis of cell cycle signaling in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice after PH

In order to understand the underlying molecular mechanisms resulting in partial inhibition of DNA-synthesis and impaired liver regeneration in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice, the expression of several cell cycle regulators was systematically investigated at the protein level. Liver samples of hepatectomized mice at different investigated time points were subjected to western blot analysis (Figure 3.22). As expected, CcnE1 was not detected at all in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver. In accordance with the data obtained from *in vitro* experiments (Figure 3.12), the CcnA2 protein expression was also strongly reduced in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice compared to WT animals 48 h after PH, which reflects the peak of DNA-synthesis in WT liver. Moreover, CcnB1 protein expression, which is essential for mitosis, was induced 48 h after PH in WT controls but barely detectable in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver. This implies that inhibition of DNA-synthesis could also indirectly block mitosis. Of notice, PCNA was normally regulated post PH in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver suggesting that PCNA expression is regulated upstream of Cdk2/CcnE1 and thus not affected by the deletion of Cdk2 or CcnE1.

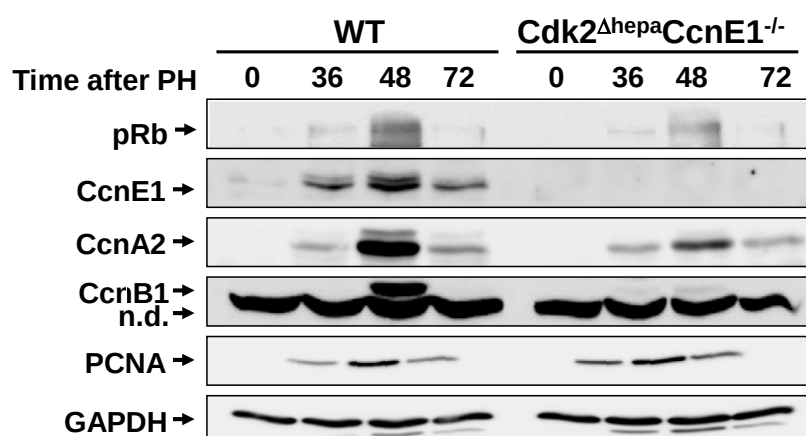


Figure 3.22: Combined depletion of CcnE1 and Cdk2 inhibits protein expression of CcnA2 and CcnB1 and prevents Rb phosphorylation. Protein expression profiles of phospho-Rb, CcnE1, CcnA2, CcnB1 and PCNA in WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice at 0-72 h post PH. GAPDH was used as loading control.

The previous data demonstrated that in absence of Cdk2 alone Rb is still completely phosphorylated following PH presumably via a CcnD1 related kinase activity 36 h post PH and by Cdk1 kinase activity (e.g. Cdk1/CcnA2 kinase) 48 h post PH (compare Figure 3.6). In $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver, CcnA2 expression was strongly

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reduced. Accordingly, CcnA2 kinase activity was also substantially impaired 48 h after PH (Figure 3.23). Concomitantly with this, the kinase activity of Cdk1 was also markedly reduced 48 h after PH supporting the hypothesis that Cdk1/CcnA2 complex may compensate the loss of Cdk2 in Cdk2^{Δhepa} liver 48 h after PH which is no longer possible in Cdk2^{Δhepa}CcnE1^{-/-} liver due to impaired CcnA2 expression. In line with this data Rb phosphorylation was also reduced 48 h after PH (Figure 3.22). Systematic examination of kinase activities also revealed that Rb phosphorylation 36 h after PH was most likely executed by CcnD related kinase complexes in Cdk2^{Δhepa}CcnE1^{-/-} liver, which was comparable to data in Cdk2^{Δhepa} mice after PH. Taken together, in Cdk2^{Δhepa}CcnE1^{-/-} liver the loss of Cdk2 kinase activity is not fully compensated by another kinase presumably due to lack of sufficient CcnA2 expression.

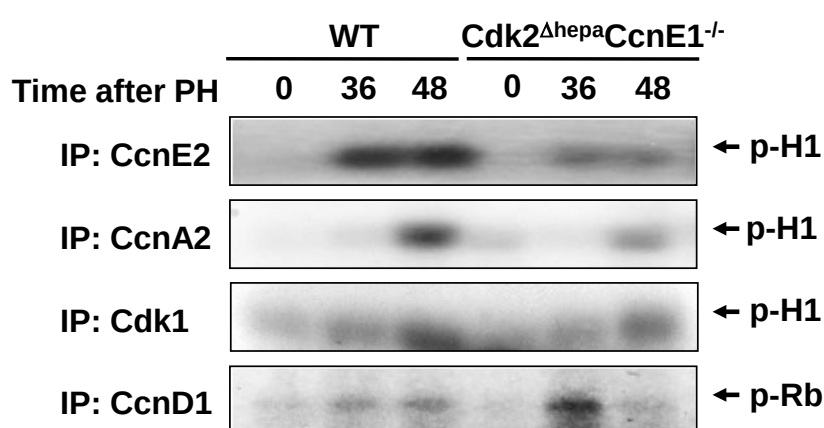


Figure 3.23: Additional deletion of CcnE1 in a Cdk2-deficient background represses the compensatory Cdk1/CcnA2 kinase activity. Whole liver cell extracts were collected from WT and Cdk2^{Δhepa}CcnE1^{-/-} mice at indicated time points after PH. Native Cdk/cyclin kinase complexes were immunoprecipitated (IP) with antibodies as indicated and subjected to *in vitro* kinase assays using either recombinant Rb or histone H1 as substrate. Signals indicating phosphorylation are highlighted by arrows.

3.4.4 Contribution of non-parenchyma cells to liver regeneration

The use of Cdk2^{Δhepa}CcnE1^{-/-} mice implicates that Cdk2 is only deleted in liver parenchyma cells, while CcnE1 is constitutively depleted. Therefore, in the previous experiments it could not be excluded that the residual cell proliferation observed in Cdk2^{Δhepa}CcnE1^{-/-} mice after PH was performed by Cdk2 proficient cells such as hepatic progenitor cells (also termed oval cells) or hepatic immune cells which proliferated or infiltrated the liver as an immediate response towards acute surgical liver injury.

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To address this very important question, the level of residual Cdk2 gene expression in the regenerating liver of $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice was determined at different time points after PH using a semi-quantitative rtPCR approach (Figure 3.24 A-B). While the Cdk2 deletion efficiency in total liver of untreated $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice was approximately 95%, Cdk2 re-expression in liver of hepatectomized animals gradually increased until 96 h after PH. At this time point, Cdk2 deletion efficiency was reduced to 70%. However, 7 days after surgery, the Cdk2 deletion efficiency was again almost complete reflecting the level of untreated mice (Figure 3.24 B). These data demonstrate that liver regeneration and thus residual hepatocyte proliferation in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice is associated with a transient repopulation of the liver with Cdk2-expressing cells of yet unknown identity.

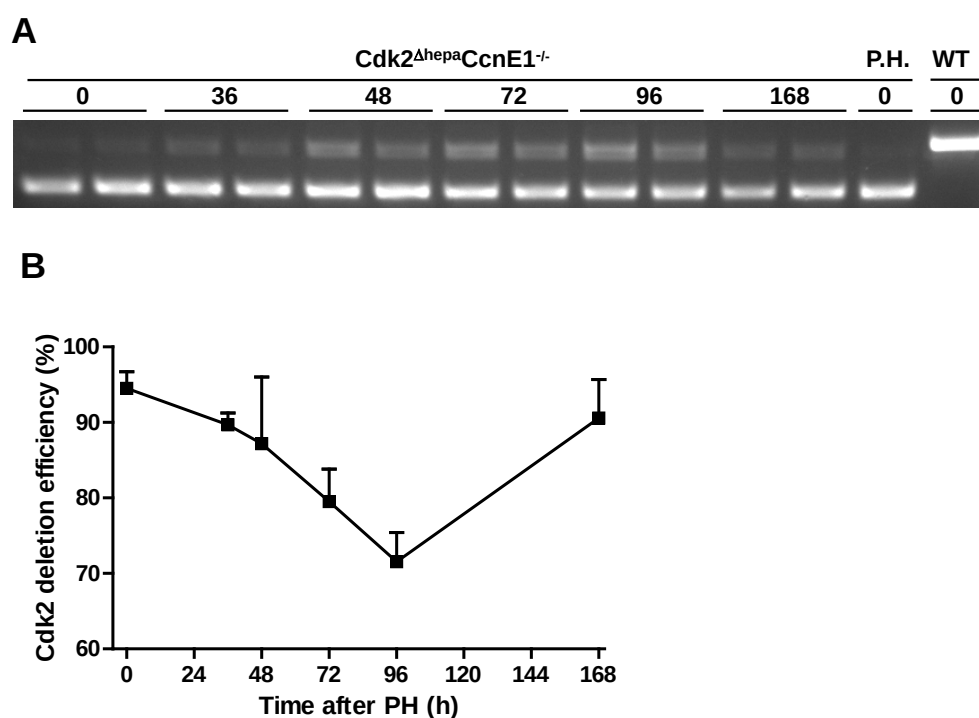


Figure 3.24: Transient Cdk2 re-expression in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver after PH. WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were subjected to PH. At time points indicated, mRNA of liver samples was isolated and reverse transcribed to cDNA. **(A)** Semi-quantitative rtPCR for measuring Cdk2 deletion efficiency was performed with cDNA as template using primers locating in exon 1 and exon 4 of the Cdk2 gene respectively. The upper band specifically indicates WT Cdk2 gene expression while the lower band indicates a truncated Cdk2 cDNA lacking exon 2. P.H.: primary hepatocytes derived from $Cdk2^{\Delta\text{hepa}}$ mice were used as positive control for complete Cdk2 deletion. **(B)** Quantification of deletion efficiency. Signal intensity of WT and knock out bands was digitally quantified using ImageJ software (<http://rsbweb.nih.gov/ij/download.html>). Deletion efficiency was calculated as the contribution of the lower band to total signal intensity in percent. Each value represents an average of 3 independent liver samples. Data is presented as the mean $\pm$ standard deviation.

Results

Two major cell populations were considered to be most likely involved in Cdk2 re-expression in $Cdk2^{\Delta hepa}CcnE1^{-/-}$ mice: immune cells or liver progenitor cells. Immune cell infiltration is a well characterized phenomenon after PH (Dong et al. 2007) and in $Cdk2^{\Delta hepa}CcnE1^{-/-}$ mice these cells would be Cdk2 proficient. Liver progenitor cells could also contribute to liver regeneration. However, the precise contribution of oval cells for liver regeneration is still controversially discussed (Duncan et al. 2009; Malato et al. 2011).

To further test the hypothesis that progenitor cells such as oval cells were involved in liver regeneration of $Cdk2^{\Delta hepa}CcnE1^{-/-}$ mice, expression of potential markers for progenitor cells e.g. cytokeratin 19 (CK19), Prominin/CD133, α -fetoprotein (AFP) and Sry (sex determining region Y)-box 9 (Sox9) was measured using qRT-PCR (Alison et al. 2008; Dorrell et al. 2011).

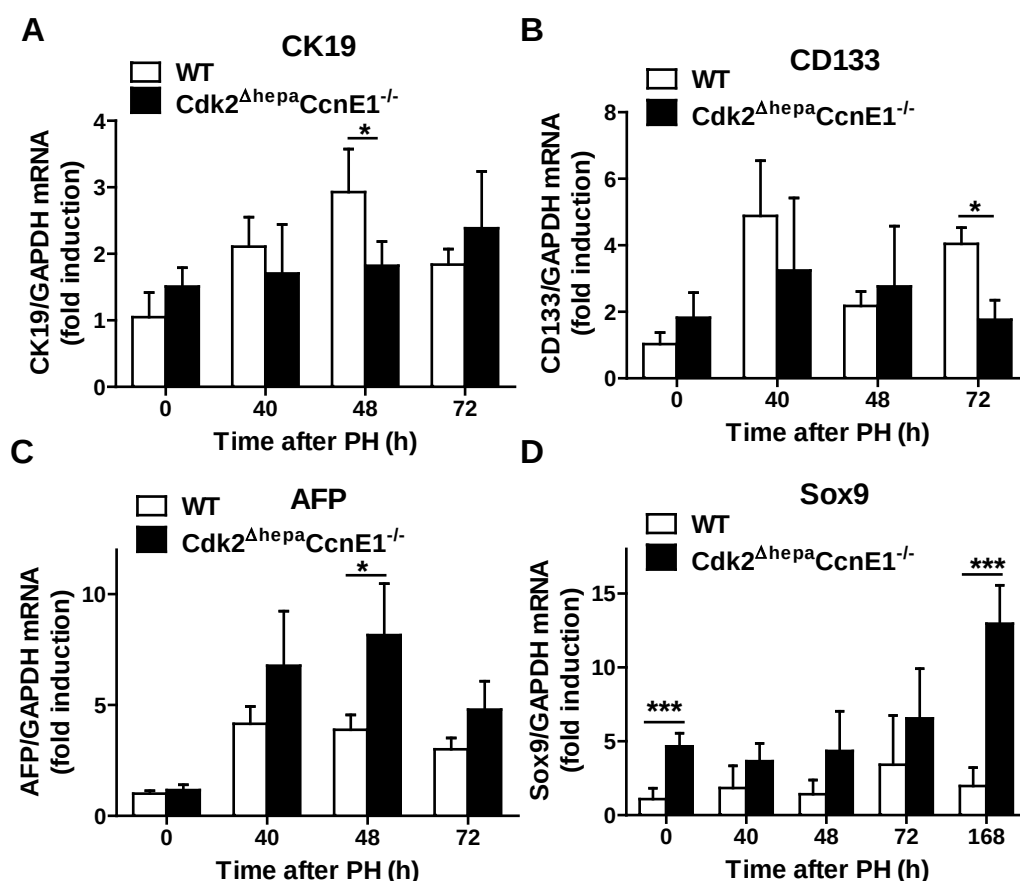


Figure 3.25: Gene expression of potential markers for liver progenitor cells (A-C) Gene expression profile of CK19, CD133 and AFP in WT and $Cdk2^{\Delta hepa}CcnE1^{-/-}$ mice at 0-72 h post PH was determined by qPCR. **(D)** Gene expression profile of Sox9 in WT and $Cdk2^{\Delta hepa}CcnE1^{-/-}$ mice at 0-168 h post PH was determined by qPCR. For each group, 3-5 animals (7-9 weeks old) were analyzed. Data is presented as the mean $\pm$ standard deviation. *: $p < 0.05$; ***: $p < 0.001$.

Results

Gene expression analysis of these examined progenitor cell markers displayed a moderate induction of all factors in WT liver after PH (Figure 3.25 A-D) suggesting that activation of progenitor cells marginally contributes to normal liver regeneration. CK19 and CD133 showed significantly higher expression in WT liver especially 48 h and 72 h after PH (Figure 3.25 A-B). In contrast, AFP showed significantly higher expression in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver 48 hour after PH (Figure 3.25 C). The timely overlap between Cdk2 re-expression and enhanced AFP induction hints at the possibility that an AFP-expressing cell population could contribute to liver regeneration in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver. Interestingly, Sox9 was substantially higher expressed in untreated liver of $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice compared to WT animals (Figure 3.25 D). After PH, the expression level of Sox9 fluctuated slightly in WT and $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver until 72 h and was overall higher in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver. Most strikingly, Sox9 expression reached the peak value after 7 days liver reconstitution in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice indicating a repopulation of Sox9-expressing cells, which was not observed at all in WT animals. These measurements provided evidence of progenitor cells for liver regeneration in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice. However, a clear identification of a certain progenitor cell population will need further investigation.

To further characterize the cell population that is still capable of proliferating in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver, co-staining experiments were performed using Ki-67 as a general proliferation marker and Hepatocyte Nuclear Factor 4 alpha (HNF4 α) as a marker for hepatocytes. Of note, it has been suggested that HNF4 α might also be involved in differentiation of oval cells into hepatocytes and thus is not a very pure hepatocyte marker (Nagy et al. 1994; Li et al. 2006; Hay et al. 2008).

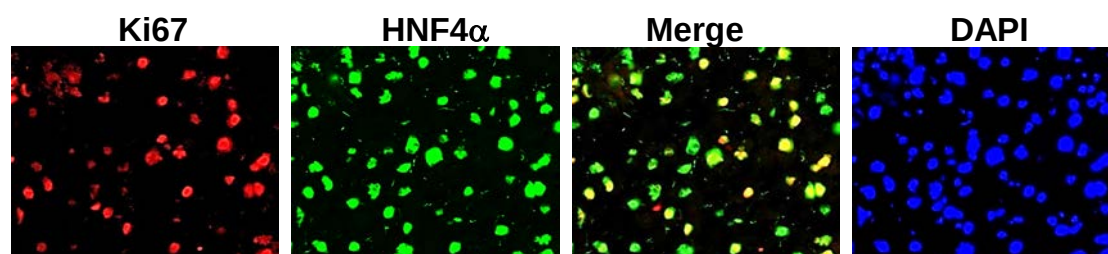


Figure 3.26: Proliferating liver cells in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice after PH are hepatocyte-like cells and express HNF4 α . $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice were subjected to PH and then sacrificed 40 h after PH. Liver sections were co-stained with fluorescence-labelled antibodies directed against Ki-67 (red) and HNF4 α (green). Nuclei are stained blue by DAPI. Co-expression of Ki-67 and HNF4 α is indicated in yellow.

Results

Representative staining images are shown in Figure 3.26, which clearly demonstrate that the vast majority of Ki-67 positive cells also express HNF4 α implicating that proliferating cells in Cdk2 $^{\Delta\text{hepa}}$ CcnE1 $^{-/-}$ liver after PH are mostly hepatocytes.

3.5 Genetic dissection of the complete Cdk2/CcnE complexes in the liver regeneration model

3.5.1 CcnE2 is dispensable for residual liver regeneration in murine liver lacking Cdk2 and CcnE1

The previous results hint at an essential role of CcnE1 during liver regeneration if Cdk2 is no longer available (i.e. in Cdk2 $^{\Delta\text{hepa}}$ mice). However, the obvious question whether and how CcnE2 (the closest homologue of CcnE1) contributes to compensatory liver regeneration in Cdk2 $^{\Delta\text{hepa}}$ mice, was not answered so far.

To address this problem, Cdk2 $^{\Delta\text{hepa}}$ CcnE1 $^{\Delta\text{hepa}}$ CcnE2 $^{-/-}$ mice were generated as part of this study and subjected to PH. Interestingly, hepatocytes lacking the complete Cdk2/CcnE complex displayed substantial reduction of DNA synthesis 40 h and 48 h after PH, which was very similar to the observations previously described for Cdk2 $^{\Delta\text{hepa}}$ CcnE1 $^{-/-}$ mice (Figure 3.27 A medium panel and Figure 3.18). These unexpected data demonstrates that CcnE2 does not contribute to residual hepatocyte proliferation in Cdk2 $^{\Delta\text{hepa}}$ CcnE1 $^{-/-}$ mice.

To further support this finding, Cdk2 $^{\Delta\text{hepa}}$ CcnE2 $^{-/-}$ mice were generated and also tested for efficient liver regeneration. Surprisingly, combined deficiency for Cdk2 and CcnE2 allowed normal DNA synthesis and liver regeneration, which was not significantly different in comparison to WT controls (Figure 3.27 A-B). Accordingly, CcnE2 is not essential for normal S-phase progression in Cdk2 $^{\Delta\text{hepa}}$ mice.

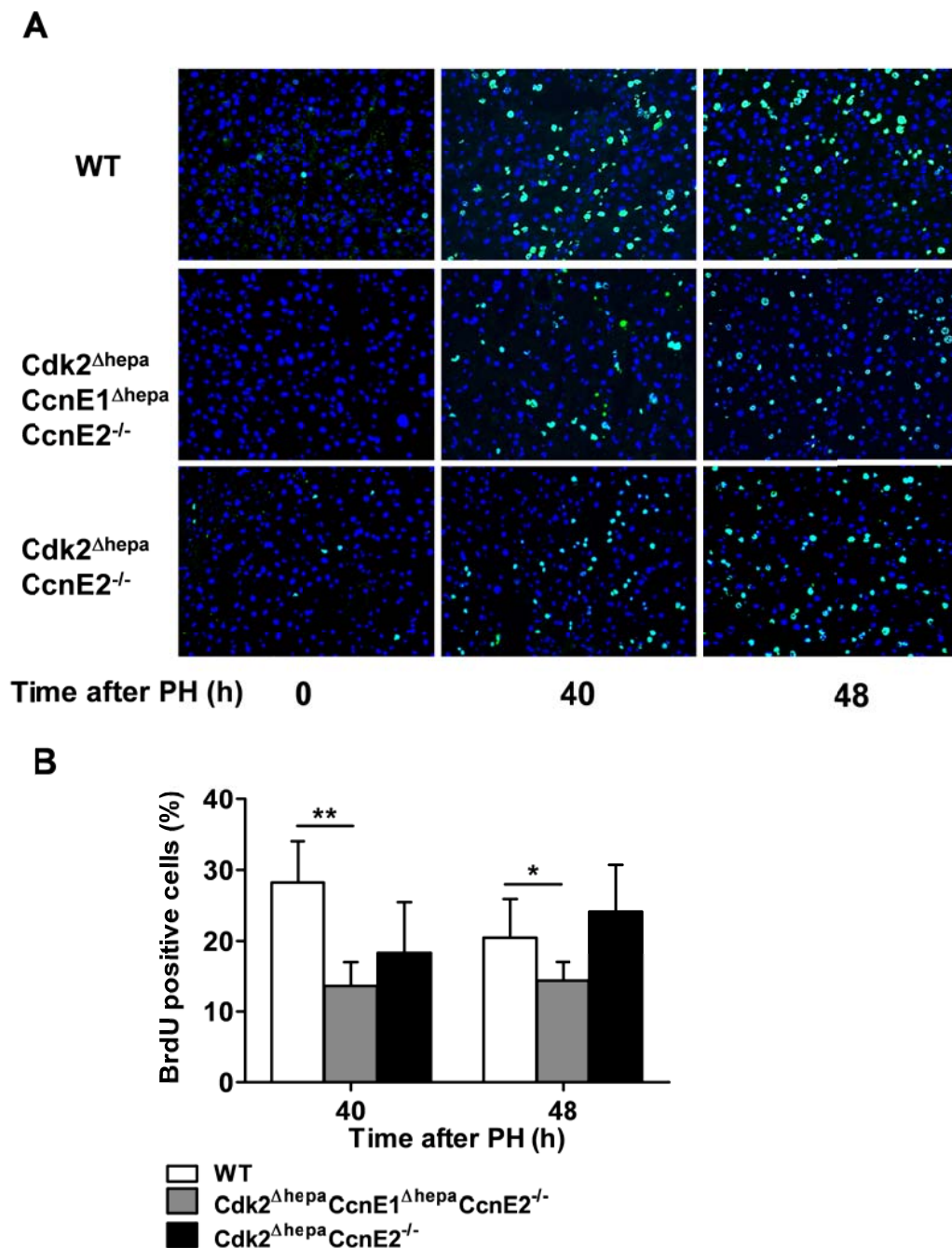


Figure 3.27: CcnE2 is dispensable for DNA-synthesis after PH in Cdk2-deficient liver. WT, $Cdk2^{\Delta hepa} CcnE1^{\Delta hepa} CcnE2^{-/-}$ and $Cdk2^{\Delta hepa} CcnE2^{-/-}$ mice were subjected to PH and sacrificed 40 and 48 hours after resection. Two hours before killing, all mice were injected with BrdU. (A) Immunofluorescence staining for BrdU incorporation on liver cryosections was performed and percentage of BrdU positive hepatocytes was quantified. (B) Quantification of BrdU-positive hepatocytes. Data is calculated as percentage of BrdU-positive nuclei per magnification field. Each value represents the mean of 5-10 mice. For each mouse at least 5-10 representative magnification fields were counted. Data is presented as the mean $\pm$ standard deviation.

3.5.2 Concomitant ablation of both E-type cyclins mimics the phenotype of $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice and substantially inhibits liver regeneration

E-type cyclins were once believed to be crucial to drive cell entry into S-phase. Surprisingly, E-type cyclins were shown to be dispensable for a large number of cell types during mouse development. However, for some other cell types E-type cyclins are critically required. They are for example indispensable for endoreplication of trophoblast giant cells and megakaryocytes as well as cardiac development. Furthermore, E-type cyclins are absolutely essential for re-entry of murine embryonic fibroblasts into S-phase from quiescence induced by serum starvation (Geng et al. 2003).

Based on recent reports, one working hypothesis of the present study was that combined ablation of CcnE1 and CcnE2 in hepatocytes should completely block cell proliferation and subsequent liver regeneration due to inability of hepatocytes to exit the G0 phase.

In order to test this hypothesis, constitutive $CcnE2^{-/-}$ mice were crossed with conditional, hepatocyte-specific CcnE1 knockout mice under control of an Alfp-Cre transgene to generate $CcnE1^{\Delta\text{hepa}}CcnE2^{-/-}$ mice. In subsequent experiments, 7-8 weeks old $CcnE1^{\Delta\text{hepa}}CcnE2^{-/-}$ mice as well as matching WT controls were subjected to 70% partial hepatectomy. BrdU staining revealed that both livers of WT and $CcnE1^{\Delta\text{hepa}}CcnE2^{-/-}$ animals started DNA-synthesis 36 h after PH. In agreement with the previous experiments, WT mice reached peak of DNA-synthesis between 40-48 h after PH. In contrast $CcnE1^{\Delta\text{hepa}}CcnE2^{-/-}$ mice showed extensively inhibited DNA-synthesis at these time points (Figure 3.28 A-B). These data suggested that the onset and termination of S-phase is not affected by combined loss of both E-type cyclins. However, E-type cyclins are essential for efficient DNA-synthesis.

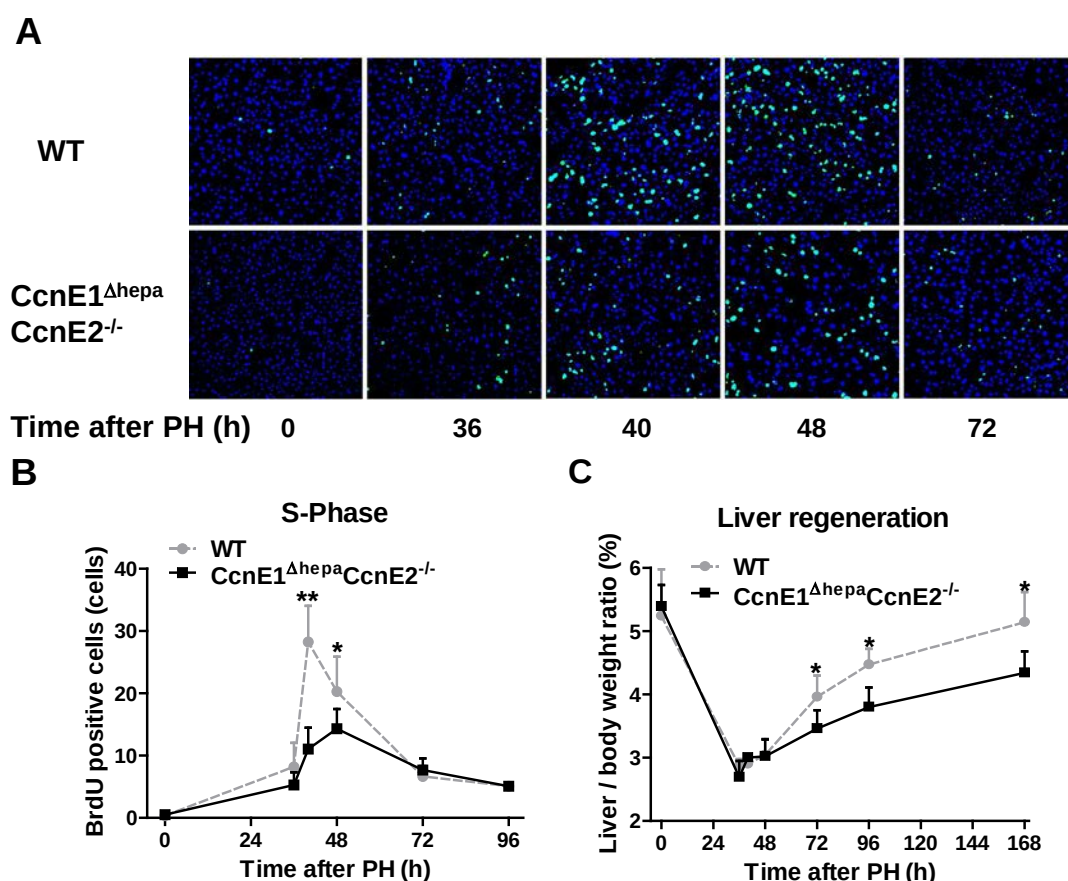


Figure 3.28: Ablation of CcnE1 and CcnE2 in hepatocytes substantially impairs DNA-synthesis of hepatocytes and liver regeneration after PH. WT and $CcnE1^{\Delta hepa} CcnE2^{-/-}$ mice were subjected to PH. Remnant livers were analyzed at 0-168 h after surgery. **(A)** Immunofluorescence staining for BrdU incorporation on liver cryosections. BrdU positive signals located in nuclei were stained green by ALEX 488. Nuclei were stained blue by DAPI. **(B)** Quantification of BrdU-positive hepatocytes. Data is calculated as percentage of BrdU-positive nuclei per magnification field. For each mouse at least 5-10 representative magnification fields were counted. Each value represents the mean of 5-10 mice. **(C)** Liver weight/body weight index (%) at indicated time points after PH. Data is presented as the average $\pm$ standard deviation.

In line with these results involving reduced DNA-synthesis, $CcnE1^{\Delta hepa} CcnE2^{-/-}$ mice showed significantly impaired liver mass restoration as evidenced by a reduced liver mass index at 72-168 h post PH (Figure 3.28 C). Based on the fact that deletion of CcnE1 alone marginally impedes liver regeneration after PH, while deletion of CcnE2 alone even enhances liver regeneration, the present results reveal an overlapping function between CcnE1 and CcnE2.

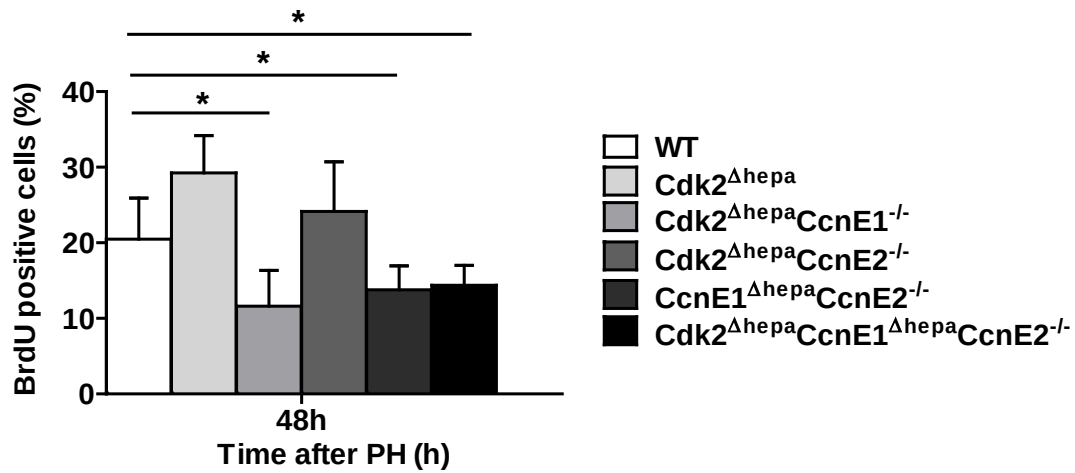


Figure 3.29: Genetic dissection of the Cdk2/CcnE complexes identifies that combined depletion of CcnE1 with CcnE2 or Cdk2 blocks hepatic cell cycle. WT, Cdk2^{Δhepa}, Cdk2^{Δhepa}CcnE1^{-/-}, Cdk2^{Δhepa}CcnE2^{-/-}, CcnE1^{Δhepa}CcnE2^{-/-} and Cdk2^{Δhepa}CcnE1^{Δhepa}CcnE2^{-/-} mice were subjected to PH and sacrificed 48 hours after resection. Two hours before killing, all mice were injected with BrdU. Immunofluorescence staining for BrdU incorporation on liver cryosections was performed and percentage of BrdU positive hepatocytes was quantified. Data is calculated as percentage of BrdU-positive nuclei per magnification field. Each value represents the mean of 5-10 mice. For each mouse at least 5-10 representative magnification fields were counted. Data is presented as the mean ± standard deviation. *: p<0.05.

The key data obtained from the systematic dissection of the Cdk2/CcnE complexes in hepatocytes is summarized in Figure 3.29: Ablation of Cdk2 alone does not affect liver regeneration at all, while combined loss of Cdk2 and CcnE1 resulted in approximately 50% reduction of S-phase during peak interval. An almost identical impairment of liver regeneration was observed, when either both E-cyclins or the complete Cdk2/CcnE complex were abrogated, while concomitant ablation of Cdk2 and CcnE2 had no effect.

4. Discussion

Cell cycle progression in higher eukaryotic cells is governed by specific complex combinations of Cdks and their regulatory subunits cyclins. Cdk2, one of the interphase Cdks (referred to as Cdk4, Cdk6 and Cdk2), orchestrates several cell cycle events such as G1/S transition and progression *via* complex formation with E- and A-type cyclins, respectively (Morgan 1997). Previous *in vitro* studies demonstrated the importance of Cdk2 by showing a series of evidences such as expression of a dominant-negative Cdk2 mutant, over-expression of the Cdk2 inhibitor p27 or microinjection of antibodies against Cdk2, CcnE or CcnA2 arrests cell cycle and blocks DNA replication (Pagano et al. 1992; van den Heuvel and Harlow 1993; Polyak et al. 1994; Ohtsubo et al. 1995). This well accepted function of Cdk2 was challenged *in vivo* by engineering knockout mice constitutively lacking Cdk2. Surprisingly, these mice display overall normal embryogenesis and postnatal development (Berthet et al. 2003; Ortega et al. 2003). Nevertheless, cell type specific functions of Cdk2 in different organs remain still incompletely understood (Berthet and Kaldis 2007). The initial aim of the current work was to examine how far Cdk2 plays a role in liver regeneration and to further investigate the compensatory mechanisms that allow proper liver regeneration in absence of Cdk2 by using the well-established model of partial hepatectomy (PH). This effort was combined with the ultimate aim to substantially block hepatocyte proliferation by inhibiting combinations of Cdk2 and associated cyclins. For this purpose hepatocyte specific Cdk2 conditional knock-out mice were generated by taking advantage of the albumin-Cre/loxP system. Based on data from this mouse model, a crucial kinase-independent role of CcnE1 - but not of CcnE2 - in liver regeneration of Cdk2^{Δhepa} mice was postulated. To test this hypothesis, Cdk2^{Δhepa} mice were crossed with CcnE1^{-/-} mice to generate Cdk2^{Δhepa}CcnE1^{-/-} double knockout mice. These animals revealed a deficiency in DNA replication during liver regeneration associated with a defect in MCM loading and repression of CcnA2 expression. Furthermore, genetic dissection of the complete Cdk2/CcnE complex was conducted by comparing hepatocyte proliferation after PH in mouse mutants harbouring all possible combinations of CcnE1, CcnE2 and Cdk2 knockout alleles. It was shown that only combined depletion of CcnE1 either with CcnE2 or Cdk2 could extensively block DNA synthesis of hepatocytes after PH indicating CcnE1 as a most important subunit of Cdk2/CcnE complexes. The physiological consequences,

underlying compensatory mechanisms and clinical relevance of different knockout mouse models will be discussed below.

4.1 Cdk2 activity is dispensable for liver regeneration

Liver regeneration is an important process for the homeostasis of the normal liver, but also for recovery of liver functions in response to liver injury. However, de-regulated or continuous liver regeneration is also involved in development of liver diseases like hepatocellular carcinoma (HCC) and liver fibrosis (Michalopoulos and DeFrances 1997; Fausto 2001; Michalopoulos 2010). Liver regeneration following 2/3 partial hepatectomy in mice has become a useful model to investigate hepatocyte proliferation *in vivo*. Since the most established functions of Cdk2/cyclin complexes are related to events in G1/S transition and S-phase progression, the current study concentrated on one of the most important stages of liver regeneration, DNA replication, with a special focus on G1/S-phase transition and S-phase progression.

The analysis of cell cycle regulation in Cdk2^{Δhepa} mice revealed that hepatocyte specific deletion of Cdk2 does not affect G1/S transition and S-phase progression after PH at all. After 1 week recovery, liver mass of Cdk2^{Δhepa} mice was restored to the same extent as observed in WT animals. This result is in agreement with a present study using constitutive Cdk2 knockout mice, which also did not reveal significant differences in hepatic S-phase progression after PH between WT and knockout animals (Satyanarayana et al. 2008b). However, another study revealed conflicting results, which showed a strongly reduced percentage of BrdU positive liver cells exactly 42 h after PH in Cdk2 knockout mice (Hanse et al. 2009). Given that both groups used the same Cdk2 knockout mouse strain these contradictory results cannot be explained without knowledge of further experimental details. Nevertheless, the present data clearly support the finding of Satyanarayana et al. that Cdk2 is dispensable for liver regeneration after PH.

4.1.1 Identification of potential compensatory kinase activities in Cdk2-deficient liver

In WT cells, Cdk2 forms kinase complexes with CcnE (i.e. CcnE1 and/or CcnE2) in late G1-phase and completes phosphorylation of Rb together with Cdk4/CcnD and

Discussion

Cdk6/CcnD complexes (Lundberg and Weinberg 1998). Though Cdk4/6/CcnD and Cdk2/CcnE complexes share the task to phosphorylate Rb, there are spatial and temporal differences of phosphorylation between two kinase complexes in WT cells. Temporally, Cdk4/6/CcnD phosphorylates Rb in early G1-phase, while Cdk2/CcnE continues phosphorylation in late G1-phase. Regarding Rb phosphorylation sites, Cdk4/6/CcnD and Cdk2/CcnE complexes show different preferences (Kitagawa et al. 1996; Zarkowska and Mitnacht 1997). It has been postulated that this specificity provided by different kinase complexes temporally regulates Rb phosphorylation (Knudsen and Knudsen 2006).

Following PH, Rb was phosphorylated in Cdk2^{Δhepa} mice to the same extent 36 h and 48 h after surgery as observed in WT mice. At 36 h post-surgery, an elevated CcnD1-related kinase activity was present in Cdk2^{Δhepa} liver suggesting that Cdk4/CcnD1 and/or Cdk6/CcnD1 might compensate for Cdk2-deficiency at this time point. Mice deficient for both Cdk2 and Cdk4 show normal liver regeneration (Barriere et al. 2007). Therefore it is likely that Cdk6 rather than Cdk4 may take over the function of Cdk2 at early regeneration stages after PH. In line with this hypothesis are findings where simultaneous inhibition of Cdk4/Cdk6 using the novel inhibitor Pyridopyrimidine (PD-0332991) blocked hepatocyte proliferation and induced G1/S phase cell cycle arrest in regenerating liver, which also suggests an important function of Cdk6 for liver regeneration (Rivadeneira et al. 2010).

Cdk1 is the only mitotic kinase responsible for cell division in mammalian cells. An augmented kinase activity of the Cdk1 complex was detected at 48 h after PH in Cdk2^{Δhepa} liver suggesting a compensatory function of Cdk1 for the loss of Cdk2 during S-phase progression. In support of this finding, Aleem and colleagues demonstrated in their study that Cdk1 regulates the G1/S phase transition in several tissues (spleen and thymus) of p27^{-/-}Cdk2^{-/-} mice. In this study, the compensatory Cdk1 activity was mediated by CcnE1-Cdk1 interaction in their model (Aleem et al. 2005). However, in the present study the elevated Cdk1 activity in regenerating Cdk2^{Δhepa} liver was not mediated by CcnE, but presumably by CcnA2. Surprisingly, a non-canonical kinase activity of both E-type cyclins with alternative Cdks could not be detected at all. This is likely attributed to the presence of p27, which associates with CcnE and reduces its activity (Berthet et al. 2003; Ortega et al. 2003; Aleem et al. 2005). Another study from Satyanarayana et al. also emphasizes the role of Cdk1 to compensate the loss of Cdk2 *via* premature translocation to the nucleus in Cdk2

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deficient murine embryonic fibroblasts (MEFs). However, in their study the authors did not address the question which cyclin guides the trafficking of Cdk1 into the nucleus. Of note, Cdk1 does not contain a nuclear localization sequence (NLS) and thus can only translocate into the nucleus in association with an NLS-containing cyclin (Moore et al. 1999). So far it is not known if the same mechanism also applies for the proliferating liver. In the present experimental setting, a premature activation of Cdk1 was not observed and an earlier expression of the Cdk1 activator CcnA2 was also not detected in Cdk2^{Δhepa} liver after PH. It is therefore suggested that Cdk2 deficiency is not compensated by premature Cdk1 translocation during liver regeneration. However, this conclusion does not exclude the possibility that the remaining Cdk1 activity is still sufficient to drive cell cycle progression in the liver as it was already shown in embryonic fibroblasts lacking all interphase Cdk2s (Cdk2, Cdk4 and Cdk6) (Santamaria et al. 2007).

In S-phase progression both Cdk2/CcnA2 and Cdk1/CcnA2 contribute to regulation of the DNA replication machinery (Yam et al. 2002). Current work implies that in the absence of Cdk2 more CcnA2 molecules are available for binding to Cdk1 and further enhance Cdk1 activity. The fact that administration of the Cdk1/2 inhibitor Roscovitine strongly reduced the DNA synthesis after PH in both WT and Cdk2^{Δhepa} mice confirms this hypothesis. Consistent with the present results, a recent study using hepatocyte-specific Cdk1 knockout mice interestingly showed that livers lacking Cdk1 have an elevated Cdk2/CcnA2 activity due to enhanced binding of CcnA2 with Cdk2, which is responsible for aberrant DNA re-replication in these mice (Diril et al. 2012). Unexpectedly, the same study also revealed that despite a defect of cell division in Cdk1-deficient liver, liver regeneration was not impaired suggesting that liver regeneration can alternatively be driven by cell growth without cell division.

Taken together, the present results strongly imply that a widespread redundancy exists between interphase Cdk2s during liver regeneration. Multiple combinations of Cdk/cyclin complexes (at least CcnD1-related kinase and Cdk1/CcnA2) contribute to G1/S transition and S-phase progression in absence of Cdk2. Especially Cdk1 has a high compensatory potential to overcome the loss of interphase Cdk2s, which is strongly supported by the Roscovitine experiments (see chapter 3.2.1), where the Roscovitine treatment after PH extensively inhibited hepatocyte proliferation in both WT and Cdk2^{Δhepa} mice.

Besides Rb, Cdk2 has several other targets including proteins involved in centrosome duplication such as nucleophosmin, proteins essential for origin firing

such as Cdc6 and Cdc45, histone biosynthesis such as p220^{NPAT}, DNA replication, its own activator CcnE and its own inhibitor p27^{Kip1} (Hwang and Clurman 2005). One recent study demonstrated that hepatocytes lacking Cdk2 have normal centrosome content and normal centrosome duplication after PH (Hanse et al. 2009). Further investigation is required to identify compensatory mechanisms for phosphorylation of other prototypical substrates in absence of Cdk2.

4.1.2 CcnE1 contributes to compensatory proliferation of Cdk2-deficient hepatocytes in a kinase-independent manner

E-type cyclins (CcnE1 and CcnE2) are considered regulatory subunits of Cdk2 (Morgan 1997). Cyclin E gene expression is tightly modulated in an E2F-mediated manner during cell cycle progression (Le Cam et al. 1999). Transient induction of E-type cyclins in late G1-phase is assumed to provide a threshold for entry into the S-phase (Krude et al. 1997; Geng et al. 2003). Although both CcnE1 and CcnE2 share high structural similarity and are co-expressed in all proliferating cells, CcnE1 has a higher Cdk2 activating capacity than CcnE2 (Lauper et al. 1998; Gudas et al. 1999; Geng et al. 2001). Previous studies from our lab demonstrated that genetic ablation of CcnE2 induces over-expression of CcnE1, which in turn results in accelerated and sustained DNA synthesis after PH due to prolonged Cdk2 activity. After 1 week liver recovery, 45% greater liver mass was observed in CcnE2^{-/-} mice compared with WT animals (Nevzorova et al. 2009). More recently, another study from our lab provides evidence that CcnE1 but not CcnE2 plays a critical role for activation, proliferation and survival of hepatic stellate cells (Nevzorova et al. 2012). Though CcnE1^{-/-} mice did not show any abnormality of liver regeneration except slight delay in S-phase entry after PH, all these evidences point to a more important role of CcnE1 rather than CcnE2 favoring cell survival and proliferation.

Several studies demonstrated that under some circumstances Cdk1 can substitute Cdk2 to form kinase active complexes with CcnE such as in absence of both p27 and Cdk2 or in absence of all interphase Cdks (Aleem et al. 2005; Santamaria et al. 2007). However, current data clearly evidenced that these findings must be cell type specific and do not apply for Cdk2-deficient hepatocytes *in vivo* for two reasons: First, any relevant Cdk1-related kinase activity was not detected at the expression peak of E-type cyclins (36 h post PH). Second, both CcnE1 and CcnE2 related kinase activities were not detected at all investigated time points after PH in

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Cdk2^{Δhepa} mice. Nevertheless, a higher basal expression level of CcnD1 as well as an earlier expression of CcnE1, but not of CcnE2, was observed in Cdk2^{Δhepa} liver after PH. It is therefore speculated that in WT cells a feedback control between activation of Cdk2 and expression of CcnD1 and CcnE1 exists, explaining aberrant expression of these cyclins after loss of Cdk2, which potentially contributes to compensatory effects in a Cdk2-independent manner.

A recent study supporting this hypothesis demonstrated that CcnE1 is loaded onto chromatin of quiescent embryonic fibroblasts in a Cdk2-independent manner, where it facilitates MCM loading during assembly of pre-replication complex *via* physical interaction with Cdt1 (Geng et al. 2007). This kinase-independent function of E-type cyclins seems to be essential for entering S-phase from quiescence (Geng et al. 2003). Quiescent murine embryonic fibroblasts lacking both E-type cyclins therefore fail to re-enter the cell cycle.

To exploit the kinase-independent functions of CcnE1 for proliferation in Cdk2-deficient liver, an *in vitro* model using mitogenic stimulated primary Cdk2^{-/-} hepatocytes was employed. In good agreement with the data from the liver regeneration model, an accelerated induction of CcnE1 was also observed *in vitro*, which was associated with an improved loading of MCM2 on chromatin. A similar phenomenon was observed in another *in vitro* study, where Cdk2 was inhibited by aminothiazole compound 25, which led to an increase in binding of the MCM helicase onto chromatin during S-phase (Zhu et al. 2004). This increased loading of MCM2 onto chromatin in the absence of Cdk2 may favor DNA replication of hepatocytes to compensate the disadvantage caused by the loss of Cdk2 in other cell cycle events. This hypothesis was fully supported by experimental data in the present study demonstrating that Roscovitine induced cell cycle arrest of primary hepatocytes can be partially rescued by adenoviral over-expression of CcnE1 (see chapter 3.2.2). Taken together, the present data obtained so far consider CcnE1 as an important or even essential proliferation factor in absence of Cdk2. It was postulated that in this case mice lacking both Cdk2 and CcnE1 should have a more severe phenotype.

To ultimately proof the hypothesis that non-canonical functions of CcnE1 are indispensable for proliferation of Cdk2-deficient hepatocytes, Cdk2^{Δhepa}CcnE1^{-/-} mice were generated. *In vitro* experiments clearly demonstrated that hepatocytes with combined deletion of Cdk2 and CcnE1 have severely impaired viability in cell culture by displaying dramatically diminished cell proliferation and reduced cell number due

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to cell death. In accordance with these findings, $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver also showed impaired liver regeneration and significantly reduced liver weight / body weight ratio *in vivo* after PH. Of notice, under *in vivo* conditions excessive cell death (as seen in primary hepatocytes) and enhanced liver injury after PH were not observed after concomitant depletion of both $Cdk2$ and $CcnE1$. The improved cell viability of $Cdk2/CcnE1$ knock out hepatocytes in liver may be explained by a protective tissue environment provided through the complex liver structure including immune cells and their production of protective cytokines and mitogens, which cannot be exactly reproduced in a cell culture model (Michalopoulos 1990; Mitaka 1998; Groneberg et al. 2002; Boess et al. 2003). Alternatively, it could be speculated that hepatic progenitor cells, which do not express albumin at this stage and therefore still harbor the *cdk2* gene, differentiate into mature hepatocytes and contribute to restoration of liver mass if $Cdk2^{-/-}CcnE1^{-/-}$ hepatocytes are unable to proliferate. This speculation is supported by two results of the current study: First, it was demonstrated that $Cdk2$ is re-expressed in $Cdk2^{\Delta\text{hepa}}$ liver after PH. The timing of this re-expression (36-96 h post PH) is not completely overlapping with the particular time of immune cell infiltration (Fausto 2006; Dong et al. 2007), pointing to the possibility that indeed a population of $Cdk2$ -proficient stem cells contributes to liver regeneration in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice. Second, expression of the fetal hepatocyte marker alpha-fetoprotein (AFP) is significantly higher in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ liver at the maximum of proliferation (48 h post PH) again hinting at a AFP-positive and thus less differentiated hepatic cell population, which preferentially engrafts the liver in absence of both $Cdk2$ and $CcnE1$. It is concluded that after differentiation of these cells into true hepatocytes, they would start expressing albumin - and thus cre - and consequently induce cre-dependent deletion of the *cdk2* gene. This prediction would perfectly match the observed kinetics of $Cdk2$ re-expression and ablation during liver regeneration. However, to definitely prove that progenitor cells are involved in liver regeneration of $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice, further investigation is required which was beyond the scope of this study.

Besides expansion of hepatocytes *via* marginal cell division of mature cells (and potentially progenitor cell derived hepatocytes), it was demonstrated that hypertrophic cell growth of hepatocytes also contributed to liver regeneration in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice. This seems to be a common mechanism in liver with cell cycle defects as a recent study using hepatocyte-specific $Cdk1$ knockout mice revealed that hepatocytes lacking $Cdk1$ cannot undergo mitosis but show normal liver regeneration *via*

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hypertrophic growth (Diril et al. 2012). However, the underlying mechanisms responsible for liver hypertrophy are poorly understood so far. Decrypting pathways regulating liver hypertrophy will be of high importance to fully understand liver regeneration.

The DNA-replication defect of Cdk2^{Δhepa}CcnE1^{-/-} hepatocytes, which was observed both *in vivo* and *in vitro* models, can be explained by two factors: strong reduction of MCM loading onto chromatin and low expression of CcnA2 and CcnA2 related kinase activity. The kinetics of pre-RC formation and MCM loading is different in continuously cycling cells (embryonic stem cells, immortal cell lines) compared to usually quiescent cells, such as hepatocytes. During continuous cell cycling, MCM proteins are detectable throughout the cell cycle and binding of MCM to the replication origins occurs immediately after the exit from mitosis (Mendez and Stillman 2000). Accordingly, Cdk2/CcnE is not necessary for pre-RC formation of continuously proliferating cells (Geng et al. 2003; Geng et al. 2007). In contrast, the present data revealed that MCM2 expression is not detectable in quiescent hepatocytes but induced upon mitogenic stimulation. Cdk2 deficient hepatocytes harbour stronger CcnE1 expression compared to WT cells, which may direct MCM and Cdt1 to chromatin in a Cdk2-independent manner potentially facilitating G₁/S-phase transition. Consequently, an additional depletion of CcnE1 in Cdk2^{Δhepa} hepatocytes breaks down this compensatory advantage and leads to the defect in cell proliferation.

Another important factor, which also accounts for impaired DNA replication of Cdk2^{Δhepa}CcnE1^{-/-} hepatocytes, is repression of CcnA2 expression. CcnA2 is essential for regulation and progression through S-phase in virtually all cell types (Chibazakura et al. ; Girard et al. 1991; Erlandsson et al. 2000; Kalaszczynska et al. 2009). During cell cycle progression, CcnA2 gene expression is preceded by activation of E-type cyclins in most if not all cases, at the beginning of S-phase, and expressed throughout S- and G₂-phase. Once induced, CcnA2 activates Cdk2 and Cdk1 *via* complex formation, thereby driving S-phase progression (Yam et al. 2002; Hochegger et al. 2008). *In vitro* studies demonstrated that inhibition of CcnA2 blocked DNA-synthesis as well as G₂/M entry (Girard et al. 1991; Zerfass-Thome et al. 1997; Erlandsson et al. 2000). *In vivo*, CcnA2 knockout mouse embryos died shortly after implantation (Kalaszczynska et al. 2009).

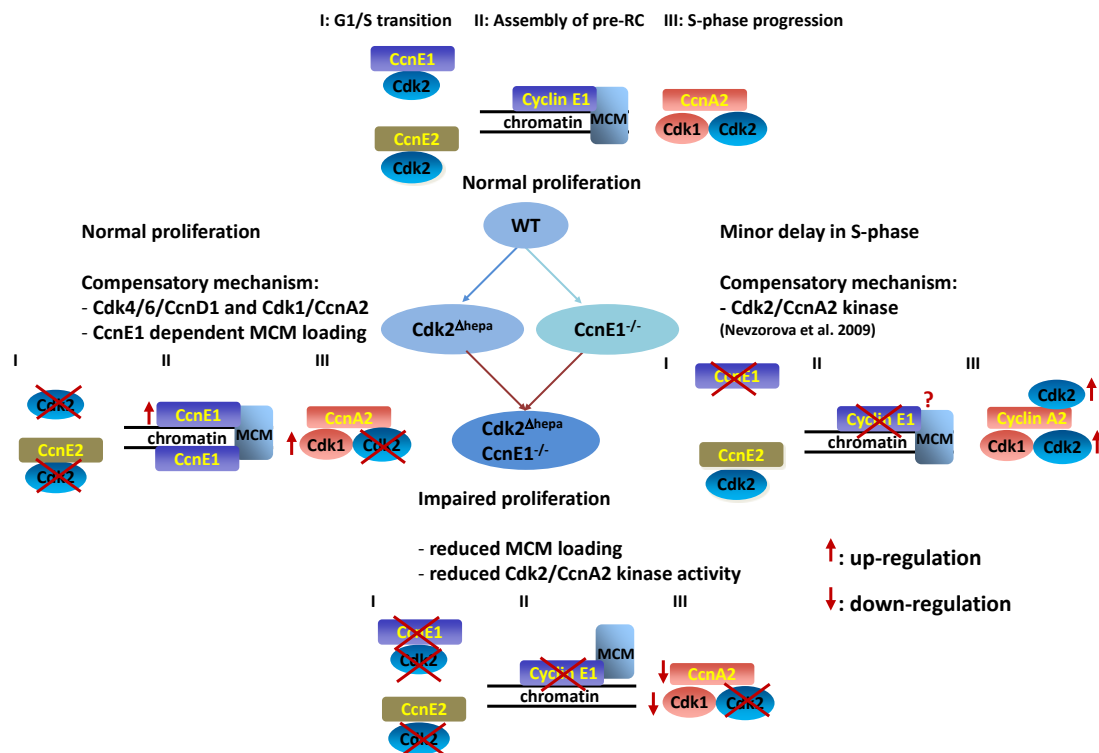


Figure 4.1: Model explaining the phenotype of Cdk2 or CcnE1 knockout mice and Cdk2^{Δhepa}CcnE1^{-/-} double knockout mice in the liver. **Left side:** Deletion of Cdk2 alone does not affect hepatocyte proliferation. Cdk4/6/CcnD1 and Cdk1/CcnA2 activity compensate the loss of Cdk2 activity at early (36 h) and later (48 h) time points after PH, respectively. In addition, accelerated induction of CcnE1 enhances Cdk2-independent MCM loading onto chromatin. **Right side:** Deletion of CcnE1 alone marginally delays S-phase entry of hepatocytes after PH. However, liver regeneration is not impaired. Loss of Cdk2/CcnE1 activity is compensated by Cdk2/CcnA2 activity. **Bottom:** An additional depletion of CcnE1 in Cdk2^{Δhepa} mice abolishes MCM loading, while an additional deletion of Cdk2 in CcnE1^{-/-} mice abrogates the compensatory Cdk2/CcnA2 activity. Altogether, deficiencies in MCM loading and Cdk2/CcnA2 activity reduce DNA-synthesis of hepatocytes and lead to inefficient liver regeneration.

From present data together with previous findings from our lab it is concluded that CcnA2 fulfills essential compensatory functions *via* complex formation with Cdk1 in Cdk2^{Δhepa} mice and with Cdk2 in CcnE1^{-/-} mice, respectively (Figure 4.1). Furthermore, the present data showed that Cdk2^{Δhepa}CcnE1^{-/-} hepatocytes displayed normal CcnD1 expression level but dramatically reduced CcnA2 expression compared to WT cells. Since Cdk2 or CcnE1 single knockout hepatocytes showed normal expression of CcnA2, it seems that Cdk2 and CcnE1 independently regulate transactivation of CcnA2 gene expression. Therefore only combined deletion of both Cdk2 and CcnE1 is sufficient to abrogate CcnA2 gene expression. This idea is in part supported by a previous report, which indicates that Cdk2 and CcnE1 are both required for transcriptional activation of the CcnA2 promoter. Binding of CcnE alone to the CcnA2 promoter is not sufficient for CcnA2 transcription, since a kinase-negative

mutant of Cdk2 abrogates promoter activation of CcnA2 (Schulze et al. 1995; Zerfass-Thome et al. 1997). Accordingly, either Cdk2 kinase activity or CcnE1 is required to initiate CcnA2 expression. In turn, CcnA is absolutely essential for proliferation of embryonic stem cells and hematopoietic cells, while in MEFs E- and A-type cyclins share high functional redundancy. MEFs lacking either E- or A-type cyclins showed normal G1/S transition and S-phase progression, whereas cell cycle was completely extinguished in cells lacking both CcnE and CcnA (Kalaszczyńska et al. 2009). It is concluded that Cdk2^{Δhepa}CcnE1^{-/-} hepatocytes reflect a similar scenario as these cells additionally lack CcnA2 therefore leading to inhibited DNA-replication.

4.2 Genetic dissection of the complete Cdk2/CcnE complexes identifies CcnE1 as the most important component for S-phase transition

To fully understand the role of the Cdk2/CcnE complexes for liver regeneration, all subunits (Cdk2, CcnE1 and CcnE2) were simultaneously deleted in hepatocytes using a genetic approach. Unexpectedly, these mice did not show any developmental abnormality and all survived 70% partial hepatectomy. After PH Cdk2^{Δhepa}CcnE1^{Δhepa}CcnE2^{-/-} mice showed a similar defect in DNA-synthesis as observed in Cdk2^{Δhepa}CcnE1^{-/-} mice indicating that CcnE2 is not contributing to residual liver regeneration at all in Cdk2^{Δhepa}CcnE1^{-/-} mice. This key finding was further confirmed by analyzing Cdk2^{Δhepa}CcnE2^{-/-} mice. In fact these animals displayed full capability of DNA-synthesis after PH identical to WT animals again pointing to a non-essential role of CcnE2 in Cdk2^{Δhepa} mice.

However, CcnE2 is highly important for liver regeneration in absence of CcnE1, since CcnE1^{Δhepa}CcnE2 mice showed severely impaired liver regeneration after PH, which was not the case for CcnE1^{-/-} mice (Nevzorova et al. 2009). This result is in good agreement with earlier *in vitro* studies using CcnE1^{-/-}CcnE2^{-/-} MEFs. Combined genetic ablation of both CcnE1 and CcnE2 completely blocked re-entry of serum starved quiescent MEFs into S-phase (Berthet et al. 2003; Geng et al. 2003; Geng et al. 2007). Unexpectedly, though DNA replication after PH in CcnE1^{Δhepa}CcnE2^{-/-} mice was inhibited by approximately 50% compared to WT animals, a sub-population of hepatocytes lacking CcnE1 and CcnE2 could still re-enter S-phase and proliferate. The possible explanations for these findings have already been discussed in the

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context of liver regeneration in $Cdk2^{\Delta\text{hepa}}CcnE1^{-/-}$ mice (see above) and imply (i) activation of progenitor cells or (ii) partial functional compensation by alternate cyclins such as CcnA2.

In fact previous work provided evidence that CcnA2 can compensate for the loss of E-type cyclins in a kinase-dependent manner (Kalaszczyńska et al. 2009). It is thus highly possible that CcnA2 can also compensate kinase-independent functions of E-type cyclins e.g. facilitate MCM loading. A rescue experiment using over-expression of CcnA2 in $CcnE1^{\Delta\text{hepa}}CcnE2^{-/-}$ hepatocytes in the future could be helpful to prove this hypothesis.

Taken together, genetic dissection of the Cdk2/CcnE complexes identifies CcnE1 as the most important kinase subunit for efficient hepatocyte proliferation. In support of this idea, several lines of evidence shows that CcnE1 plays an important role for carcinogenesis as it is frequently deregulated in many human cancers including hepatocellular carcinoma (Keyomarsi and Herliczek 1997; Spruck et al. 1999; Zschemisch et al. 2006).

Thus, the present study provides a novel therapeutical view to inhibit proliferation of cancer cells by inhibiting CcnE1 together with Cdk2 or both E-type cyclins. The inhibition of Cdk2 alone might not be sufficient to block cell proliferation of certain cancer cells. The current approaches to develop small molecule Cdk-inhibitors might be of limited benefit especially for treating cancer with high expression of CcnE1.

In summary, the present study largely improves the understanding of redundant cell cycle mechanisms in vertebrates and identifies for the first time the minimal requirements necessary for substantial inhibition of DNA synthesis in the liver.

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6. Summary

Complexes of Cdk2 with E-type cyclins (CcnE1 and CcnE2) specifically regulate the onset of DNA replication in previously quiescent mammalian cells. Partial 2/3 hepatectomy (PH) in rodents is an established model to study this process *in vivo* as quiescent hepatocytes synchronously undergo 1-2 cell cycles after surgery. Surprisingly, previous studies from our lab demonstrated that unique depletion of CcnE1 or CcnE2 did not markedly impair hepatocyte proliferation after PH. Thus the initial aim of the present study was to characterize the role of Cdk2 for the hepatic cell cycle. As these analyses revealed that Cdk2 is also fully dispensable for cell cycle progression in hepatocytes, the underlying compensatory mechanisms were evaluated by detailed genetic dissection of the complete Cdk2/CcnE complexes with the ultimate aim to inhibit the hepatic cell cycle by blocking appropriate combinations of Cdk2 and E-cyclins.

Hepatocyte-specific ablation of Cdk2 (Cdk2^{Δhepa}) resulted in accelerated induction and overall stronger expression of CcnE1 after PH. Elevated CcnE1 levels did not contribute to a non-canonical kinase activity but instead performed a kinase-independent function by facilitating the loading of minichromosome maintenance (MCM) proteins on chromatin. Accordingly, hepatocytes lacking both Cdk2 and CcnE1 showed severely impaired loading of MCM and were unable to activate the S-phase mediator CcnA2 which is a prerequisite for S-phase progression. As a consequence, mitogen-primed Cdk2^{-/-}CcnE1^{-/-} hepatocytes displayed severely impaired cell viability and dramatically reduced proliferative capability. *In vivo*, DNA replication and subsequent liver mass reconstitution following PH were substantially – but not completely blocked in Cdk2^{Δhepa}CcnE1^{-/-} mice. Surprisingly, hepatocytes lacking Cdk2 and both E-cyclins did not show further impaired liver regeneration suggesting a negligible role of CcnE2 for cell cycle progression. In fact, Cdk2^{Δhepa}CcnE2^{-/-} mice showed normal S-phase and liver regeneration. However, mice lacking both E-type cyclins exhibited strongly impaired liver regeneration pointing to some functional overlap of CcnE1 and CcnE2.

In summary, the current study identifies for the first time combinations of Cdk2 and/or E-cyclins, which are largely essential for driving the hepatic cell cycle. This contributes to a better understanding of redundant mechanisms in the mammalian cell cycle machinery and may help to inhibit pathological liver cell proliferation e.g. in hepatocellular carcinoma.

7. Zusammenfassung

Komplexe von Cdk2 mit E-Cyclinen (CcnE1 und CcnE2) steuern den Übergang von ruhenden Säugerzellen in die S-Phase des Zellzyklus. Partielle 2/3 Hepatektomie (PH) in Nagern ist ein etabliertes Model, um diesen Prozess *in vivo* zu untersuchen. Nach PH durchlaufen ruhende Hepatozyten sehr synchron 1-2 Zellzyklen. Vorarbeiten zeigten, dass die alleinige Inaktivierung von CcnE1 oder CcnE2 nicht ausreicht, um die Leberregeneration nach PH maßgeblich zu blockieren. Erstes Ziel der Arbeit war es, die Funktion von Cdk2 für den Zellzyklus des Hepatozyten zu charakterisieren. Da diese Analysen frühzeitig zeigten, dass Cdk2 für den Zellzyklus von Hepatozyten nicht benötigt wird, sollten weiterhin die zugrunde liegenden kompensatorischen Mechanismen identifiziert werden mit dem Ziel, durch kombinierte Deletion von Cdk2 und E-Cyclinen den Zellzyklus von Hepatozyten zu hemmen.

Hepatozyten-spezifische Deletion von Cdk2 ($Cdk2^{\Delta hepa}$) führte zu einer beschleunigten Induktion und erhöhten Expression von CcnE1 nach PH. Dies bewirkte jedoch keine zusätzliche Kinaseaktivität, sondern förderte Kinase-unabhängig das Beladen von Replikationsursprüngen mit dem *Minichromosome Maintenance Complex* (MCM). Dementsprechend konnten Hepatozyten mit gleichzeitigem Verlust von Cdk2 und CcnE1 MCM-Komplexe nicht mehr effektiv auf Chromatin laden. Zusätzlich war in diesen Hepatozyten die Aktivierung des S-Phase Mediators CcnA2 fast vollständig inhibiert. Als Folge zeigten Mitogen-induzierte $Cdk2^{-/-}CcnE1^{-/-}$ Hepatozyten ein deutlich vermindertes Überleben und eine erheblich reduzierte DNA-Synthese und Proliferation. *In vivo* war in $Cdk2^{\Delta hepa}CcnE1^{-/-}$ Mäusen die DNA-Synthese sowie die Lebermassenrekonstitution nach PH ebenfalls stark inhibiert. Überraschenderweise bewirkte eine zusätzliche Deletion von CcnE2 in diesen Tieren keine weitere Beeinträchtigung der Hepatozytenproliferation. Daher scheint CcnE2 nur eine geringfügige Bedeutung bei der Leberregeneration zu besitzen. Trotzdem besitzen CcnE1 und CcnE2 eine partielle funktionelle Redundanz, da $CcnE1^{\Delta hepa}CcnE2^{-/-}$ Mäuse ebenfalls eine erheblich verschlechterte Leberregeneration zeigten.

Zusammenfassend identifiziert die vorliegende Arbeit erstmalig Bedingungen, unter denen Cdk2 oder CcnE1 für die Zellzyklusregulation in der Leber essentiell sind. Diese Ergebnisse tragen zu einem deutlich verbesserten Verständnis der redundanten Zellzyklus-Mechanismen in Säugern bei und könnten helfen, die pathologische Leberzellproliferation z.B. beim Hepatozellulären Karzinom, zu hemmen.

8. Appendix

8.1 Publications

Part of this work has been published in advance as scientific abstracts, poster and oral presentations as indicated below.

Oral presentation

Hu W, Nevzorova YA, Haas U, Sicinski P, Barbacid M, Trautwein C, Liedtke C. 2012. Cyclin E1, but not cyclin E2 is required for efficient liver regeneration in Cdk2-deficient liver. *Hepatology*: **56**: Supplement 1: 244A-244A. 63th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, USA.

Hu W, Nevzorova YA, Haas U, Moro N, Sicinski P, Barbacid M, Trautwein C, Liedtke C. 2012. Cdk2-deficient hepatocytes require non-canonical functions of cyclin E1 for efficient liver regeneration in mice. *Journal of hepatology* **56**: S27-S27. 47th Annual Meeting of the European Association for the Study of the Liver (EASL), Barcelona, Spain.

Hu W, Nevzorova YA, Haas U, Barbacid M, Trautwein C, Liedtke C. 2010. The cyclin-dependent kinase 2(Cdk2) is dispensable for liver regeneration in mice and can be substituted by cyclin D-dependent kinase activity and kinase-independent functions of cyclin E. *Hepatology* **52**: 392A-392A. 61st Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, USA.

Poster presentation

Hu W, Nevzorova YA, Haas U, Sicinski P; Barbacid M; Trautwein C; Liedtke C. 2012. Non-Canonical functions of Cyclin E1 for control of hepatic DNA replication and liver regeneration in mice. *Zeitschrift für Gastroenterologie* **50**: P2_09. 28. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber (GASL), Hamburg, Deutschland.

Hu W, Nevzorova YA, Nowak JR, Haas U, Moro N, Geng Y, Sicinski P, Barbacid M, Trautwein C, Liedtke C. 2011. Cyclin E1 and D1 can drive liver regeneration in mice without Cdk2. *Journal of hepatology* **54**: S428-S428. 46th Annual Meeting of the European Association for the Study of the Liver (EASL), Berlin, Germany.

Hu W, Nevzorova YA, Nowak JR, Haas U, Moro N, Geng Y, Sicinski P, Barbacid M, Trautwein C, Liedtke C. 2011. Ablation of Cdk2 uncovers kinase-independent functions of Cyclin E1 for liver regeneration in mice. *Zeitschrift für Gastroenterologie* **49**: P448-66. Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten (DGVS), Leipzig, Deutschland.

Hu W, Nevzorova YA, Moro N, Haas U, Barbacid M, Trautwein C, Liedtke C. 2011. The Cyclin-dependent kinase 2 (Cdk2) is dispensable for liver regeneration in mice. *Zeitschrift für Gastroenterologie* **49**: P2_37. 27. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber (GASL), Regensburg, Deutschland.

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8.3 Curriculum Vitae

Personal Information

Name:	Wei Hu
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Education

11/08 – 11/12	Doctoral study in the department of Medicine III (Director Prof. Trautwein), University Hospital Aachen
10/03 – 02/08	University Bremen Direction: Biology Main subjects: Microbiology, genetics und biochemistry Degree: Diplom (final grade: very good)
04/03 – 09/03	University Bremen Direction: Chemistry
09/02 – 02/03 u. 09/00 – 07/01	East China University of Science and Technology Direction: Chemical technique and technology

Career experience

11/08 – now	Scientific coworker in University Hospital Aachen
10/06 – 02/08	Student Assistant in the “Fraunhofer-Institut für Fertigungstechnik und angewandte Materialforschung (IFAM)” in Bremen

Language Knowledge

Chinese	Native
German	Fluent in writing and speaking
English	Fluent in writing and speaking

Honors and Awards

2012	Young Investigator Travel Award, ORAL presentation at the Liver Meeting 2012 by American Association for the Study of Liver Diseases (AASLD), Boston
2012	Young Investigator Full Bursary, ORAL presentation at the international liver congress by European association for the study of the liver (EASL), Barcelona
2011	Young Investigator Full Bursary, TOP 10% Poster presentation at the international liver congress by European association for the study of the liver (EASL), Berlin
2010	ORAL presentation at the Liver Meeting 2010 by American Association for the Study of Liver Diseases (AASLD), Boston
2004	Nomination for the scholarship of "Studienstiftung des deutschen Volkes"
2001	Second Price of the university year scholarship at University East China University of Science and Technology

8.4 Eidesstattliche Versicherung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Die vorliegende Arbeit wurde zwischen Oktober 2008 und Juni 2012 in der Medizinischen Klinik III des Universitätsklinikums Aachen unter der fachlichen Betreuung von PD. Dr. rer. nat. Christian Liedtke und der Leitung von Prof. Dr. med. Christian Trautwein durchgeführt.

Ich versichere an Eides statt, dass ich diesen Promotionsantrag erstmalig einreiche und keine früheren Versuche einer Promotion unternommen habe.

Aachen, den 15.02.2013

(Wei Hu)