Cell cycle regulation in the liver:
Differential functions of E-type Cyclins E1 and E2 for G1/S-phase transition and endoreplication in mice

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vorgelegt von

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1. Introduction

1.1 The cell cycle

The cell cycle is an ordered set of events eventually culminating in cell growth and division into two daughter cells. In virtually all cells, the cell cycle is composed of four discrete phases, which are the DNA synthesis phase (S phase), the mitotic phase (M phase) and the intermediate gap phases referred to as G1-phase (between M and S phases) and G2-phase (between S and M phases).

During the last decades, a wealth of knowledge has become available giving insight into the molecular mechanisms controlling cell cycle regulation. Cyclins and their partners, the cyclin dependent kinases (CDKs) constitute the basis of these molecular mechanisms (Boonstra, 2003).

In primitive eukaryotes such as yeast only one Cdk molecule is able to regulate all phases of the cell cycle by interaction with different cyclins. In mammals (Fig.1) each phase of the cell cycle (G1, S, G2, M) is characterized by its own set of CdkS and cyclins which were thought to be essential for this process (Kaldis and Aleem, 2005).

The most important mammalian cyclin-CDK complexes known so far are, the G1-cyclins D and E in complex with CDK 4/6 and CDK2, respectively.
(compare Fig.1), and the mitotic cyclins A and B which are associated with 
CDK1 (Nigg, 1995; Sherr, 1995).

1.1.1 Cyclin D as an regulatory protein

The decision of a normal cell to replicate DNA and duplicate itself is 
influenced by extracellular signals such as cytokines and growth factors. Growth 
factors are necessary to initiate and maintain the transition through G1 phase 
eventually leading to DNA synthesis. The point in G1 at which commitment 
occurs and the cell no longer requires growth factors to complete the cell cycle 
has been termed “restriction point” (compare Fig.1). Transition of the restriction 
point was proposed to be determined by accumulation of a functionally short- 
lived (labile) regulatory protein (R-protein) such as cyclin D, whose synthesis is 
regulated by growth factors and accumulates to a critical amount before a cell 
can pass the restriction point and proceed towards DNA synthesis.

D-type cyclins represent a very unique component of the cell cycle 
apparatus. Unlike other cyclins that are periodically induced during the cell cycle 
progression, the level of D-cyclins is controlled by the extracellular mitogen 
environment. For this reason, D-cyclins are believed to serve as “links” between 
the extracellular environment and core cell cycle machinery (Kozar and Sicinski, 
2005). Growth factors regulate cyclin D1 by at least four mechanisms:
  
1. transcriptional induction
2. stabilization of the protein
3. translocation to the nucleus
4. assembly with its catalytic partners, CDK 4 and CDK 6.

After complex formation with CDK4 and CDK6 and subsequent nuclear 
translocation the active cyclin D/CDK kinase complex phosphorlates especially 
retinoblastoma (Rb) proteins (Hinds et al., 1992).

The retinoblastoma family includes three members, Rb/p105, p107 and 
Rb2/p130, respectively, which are collectively referred to as ‘pocket proteins’. 
The unphosphorylated Rb proteins repress gene transcription by binding and 
thereby inhibiting E2F transcription factors. Phosphorylation of Rb is required 
for transition from G1 to S phase as it prevents its binding to E2F, thus 
activating E2F-mediated gene transcription and also relieving transcriptional 
repression(Giacinti and Giordano, 2006). Consequently activation of E2F in G1
allows the transcription of genes required for cell cycle progression (Grana et al., 1998) (Fig.2).

\[
\begin{align*}
\text{Growth Factors} & \rightarrow \text{CDK 4} \rightarrow \text{p}_p\text{Rb} \rightarrow \text{release} \rightarrow \text{E2F} \rightarrow \text{S phase}
\end{align*}
\]

**Fig. 2:** The restriction knot (simplified scheme).

Cyclin D-CDK4/6 phosphorylate Rb, thus activating E2F-mediated transcription and also relieving transcriptional repression. E2F-1 transactivates cyclins E and A. Cyclin E enters into a complex with CDK2 and collaborates with cyclin D-CDKs to complete Rb phosphorylation. This shift from cyclin D-CDK 4 to cyclin E-CDK2 accounts for the loss of dependency on growth factors. Given numerous positive and negative feedback loops, the restriction point is in fact a restriction knot (adopted from Blagosklonny and Pardee, 2002; Saile and Ramadori, 2007).

### 1.1.3 Cyclin E as a „Master Cyclin“

In contrast to growth factor inducible D type cyclins, the expression of E-type cyclins is controlled by an autonomous mechanism and peaks sharply at G1/S border (Koff et al., 1992). After passing the restriction point, the cell cycle becomes substantially less responsive to extracellular factors, which can delay entry into the S-phase or even arrest the cell cycle. The regulation of the cell cycle progression through the restriction point is believed to be the main function of the E-cyclins. E-type cyclins complete phosphorylation of pRB (compare Fig 3), which was initiated by the action of cyclin D-CDK complexes. Since cyclin E/cdk2 complexes themselves also phosphorylate pocket proteins such as Rb and its relatives p130 and p107, the concentration of released E2F increases leading to further amplification of cyclin E transcription. Moreover, cyclin E/cdk2 complexes can phosphorylate E2F and thereby modulate its activity. This constitutes a classical feedback mechanism, conferring cyclin E the ability to stimulate its own transcription (Moroy and Geisen, 2004).
Aside from this specific function as a regulator of S-phase-entry, cyclin E plays a direct role in the initiation of DNA replication, the control of genomic stability, and the centrosome cycle (Moroy and Geisen, 2004).

Repression of the cyclin E gene during G2-M and the early G1 phase of the cell cycle is mediated through the assembly of a multiprotein-complex, containing hypophosphorylated pRB, Histone deacetylase (HDAC), and chromatin remodeling factors of the SWI/SNF family, which are recruited to E2F transcription factors bound to the cyclin E promoter in order to silence cyclin E gene transcription.

The regular cyclin E protein contains a so-called “cyclin-box” which is a domain stretching from amino acid position 129-215 and is conserved to a certain degree among many cyclins. The cyclin-box domain is a structural motif required for cyclin–CDK complex formation. The C-terminal domains of cyclin E contain a conserved TPP motif. CDK2-mediated phosphorylation of this site leads to the dissociation of the complex and targets ubiquitin-mediated proteolysis (Moroy and Geisen, 2004).

---

**Fig. 3:** “R-factor.”

Both D-type cyclins and cyclin E meet some criteria of restriction factors. Phosphorylation of Rb is initiated by CDK4/6-cyclin D, but is completed by CDK2-cyclin E. (adopted from Blagosklonny and Pardee, 2002)

---

**Fig. 4:** Alignment of the cyclin E1 and E2 cyclin-box domain.

The highlighted region indicates conserved amino acid residues necessary for interaction with CDKs
Recently, three research groups independently discovered a second cyclin E family member, cyclin E2 (Gudas et al., 1999; Lauper et al., 1998; Zariwala et al., 1998). The methods of discovery included a homology search using a cyclin box domain to query an expressed sequence tag (EST) database, and a yeast two-hybrid screen using either human p27Kip1 or a catalytically inactive mutant of CDK2 as bait. Both cyclins show significant amino acid similarity (75% within the cyclin box, 47% throughout the entire coding sequence (Fig.4). Despite these similarities, it is still unknown whether the two proteins perform similar functions, or alternatively, control S-phase entry in different ways (Payton and Coats, 2002).

1.1.4 The role of Cyclin A and B as “mitotic cyclins”

Among the cyclin family, cyclin A is especially interesting as it can activate two different cyclin dependent kinases (CDK2 and CDK1/CDC2) and function in both S-phase and mitosis. An embryonic form of cyclin A that is only essential for spermatogenesis is also present in some organisms. In S-phase, phosphorylation of components of the DNA replication machinery such as CDC6 by cyclin A-CDK2 is believed to be important for initiation of DNA replication and to restrict the replication to only one per cell cycle. In mitosis, the precise role of cyclin A is still obscure, but it may contribute to the control of cyclin B stability (Pagano et al., 1992). Cyclin A starts to accumulate during S phase and is abruptly degraded before metaphase. The synthesis of cyclin A is mainly controlled at the transcription level, involving E2F and other transcription factors. Consistent with its role as a key cell cycle regulator, expression of cyclin A is found to be elevated in a variety of tumors (Yam et al., 2002).

Entry into mitosis in eukaryotic cells is controlled by activation of the Serine/Threonine kinase cdc2 which interacts with one of several B-type cyclins.

Protein levels of cyclin B begin to accumulate in late S-phase, peak at the G2/M transition, and decline considerably by the onset of anaphase. The temporal accumulation of cyclin B is required for cells to progress through the G2/M transition and degradation of the protein is a necessary step in cellular exit from mitosis. Spatially, cyclin B translocates into the nucleus in a phosphorylation-dependent manner. Once the activated cyclin B/cdc2 complex moves into the nucleus, it can phosphorylate a variety of substrates, including
1. Introduction

histone H1, microtubule-associated proteins (MAPs), nuclear lamins, and centrosomal proteins. These nuclear phosphorylation events regulate the initiation and progression of mitosis (Porter and Donoghue, 2003).

At mitotic entry, cyclin B1/cdc2 promotes chromosome condensation, nuclear lamina resolution and mitotic spindle assembly, while cyclin B2/cdc2 can induce Golgi disassembly (Yu and Yao, 2008).

1.1.5 Living with or without cyclins and cyclin-dependent kinases

Entry into and progression through the mammalian cell cycle in response to extracellular mitogenic stimuli are presumed to be governed by cyclin-dependent kinases which are regulated by different cyclins. Studies performed over more than one decade have supported the view that these holoenzymes are important, if not essential, for these processes. However, recent experiments, in which the genes encoding all three D-type cyclins, the two E-type cyclins, cyclin D dependent kinases CDK4 and CDK6, or cyclin E-dependent kinase CDK2 have been disrupted in the mouse germ line revealed that most of fetal development occurs normally in their absence. Thus, none of these genes is strictly essential for cell cycle progression (Sherr and Roberts, 2004). The recent findings based on in vivo gene inactivation studies of cyclins and cyclin-dependent kinases are summarized in tables 1 and 2, respectively.
<table>
<thead>
<tr>
<th>Disrupted gene</th>
<th>Survival</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>viable</td>
<td>Small body size, hypoplastic retinopathy, defective breast lobuloalveolar development during pregnancy, and uncharacterized neuropathy with altered clasping reflexes (Fantl et al., 1995; Sicinski et al., 1995)</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>viable</td>
<td>Defective ovarian granulosa cell development and female sterility. Males have hypoplastic testes but are fertile. Abnormal postnatal cerebellar development due to a reduced number of granule neurons and loss of stellate interneurons. Impaired proliferation of peripheral B-lymphocytes (Lam et al., 2000; Sicinski et al., 1996; Solvason et al., 2000)</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>viable</td>
<td>Hypoplastic thymus with loss of T-cell maturation from double-negative (CD4−, CD8−) to double-positive (CD4+, CD8+) cells due to cytokine-independent defects in pre-TCR signaling (Geng et al., 2003)</td>
</tr>
<tr>
<td>Cyclin D2 and D3</td>
<td>Embryonic lethality before E18.5</td>
<td>Death likely is due to severe megaloblastic anemia. Other hematopoietic lineages were not evaluated (Cierny et al., 2002).</td>
</tr>
<tr>
<td>Cyclin D1 and D3</td>
<td>Death at P1, but a few survive up to 2 months</td>
<td>Neuropathy leading to meconium aspiration is cause of early death. Survivors fail to thrive and exhibit hypoplastic retinas (Cierny et al., 2002).</td>
</tr>
<tr>
<td>Cyclin D1 and D2</td>
<td>Viable but die within first three postnatal weeks</td>
<td>Retarded growth and impaired coordination. Inhibited postnatal cerebellar development, and hypoplastic retinas (Cierny et al., 2002).</td>
</tr>
<tr>
<td>Cyclins D1, D2, and D3</td>
<td>Dead by E16.5</td>
<td>Severe hematopoietic deficits affecting number and proliferative capacity of stem cells and multipotential progenitors. Fetal liver lacks progenitors and cannot reconstitute lymphoid or myeloid function after transplantation. Death due to anemia and defects in heart development. MEFs can be propagated in culture but exhibit greatly reduced susceptibility to transformation by oncogenic Ras + Myc, E1A, or DN-p53 (Kozar et al., 2004).</td>
</tr>
<tr>
<td>Cyclin E1</td>
<td>Viable</td>
<td>Overtly normal (Geng et al., 2003)</td>
</tr>
<tr>
<td>Cyclin E2</td>
<td>Viable</td>
<td>Hypoplastic testes, reduced sperm count and male infertility (Geng et al., 2003).</td>
</tr>
<tr>
<td>Cyclins E1 and E2</td>
<td>Embryos die by E11.5 due to failure of endoreduplication of trophoblast giant cells. Tetraploid rescue allows most embryos to develop to term.</td>
<td>Cardiac anomalies of varying severity in rescued embryos. Reduced endoreduplication in megakaryocytes. MEFs proliferate somewhat slowly with an increased G1 phase fraction and undergo senescence; however, quiescent MEFs cannot re-enter the cell cycle due to a failure in loading MCM proteins onto prereplication origins. MEFs resist transformation by oncogenicRas + Myc, E1A, or DN-p53 (Geng et al., 2003).</td>
</tr>
<tr>
<td>Cyclin A1 (only in male germ cells)</td>
<td>Viable</td>
<td>Males are sterile due to a block of spermatogenesis before the first meiotic division, whereas females are normal (Wolgemuth et al., 2004).</td>
</tr>
<tr>
<td>Cyclin A2 (widely expressed)</td>
<td>Embryonic lethality before E5,5</td>
<td>Embryos develop normally until post implantation, around day 5.5. This observation may be explained by persistence of a maternal pool of cyclin A2 protein until at least blastocyst stage (Murphy et al., 1997).</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>Embryonic lethality</td>
<td>(Brandeis et al., 1998)</td>
</tr>
<tr>
<td>Cyclin B2</td>
<td>Viable</td>
<td>Mice are fertile, but have small litter sizes and overall reduced body size (Brandeis et al., 1998).</td>
</tr>
</tbody>
</table>

Tab.1: Phenotypes of mice disrupted for cyclin genes
1. Introduction

Disrupted gene | Survival | Phenotype
---|---|---
Cdk4 | Viable | Small body size. Most males are sterile due to hypoplastic testes and low sperm counts. Female sterility is due to defects in the hypothalamic–pituitary axis, abnormal estrus, and failure of corpus luteum. Abnormal development of pancreatic -islet cells leads to insulin-dependent diabetes within the first 2 mo of life. MEFs can be propagated in culture with decreased ability to enter the cell cycle from quiescence; they express aberrantly high levels of p21C1p1 and resist transformation by oncogenic Ras + DN-p53 (Rane et al., 1999).

Cdk6 | Viable | Thymic and splenic hypoplasia, and mild defects in hematopoiesis. T-lymphocytes exhibit delayed S-phase entry (Malumbres et al., 2004).

Cdk2 | Viable | Meiotic failure, gonadal hypertrophy, and male and female sterility. MEFs can proliferate and undergo senescence and spontaneous immortalization; quiescent MEFs exhibit delayed entry into S phase and/or decreased ability to re-enter the cell cycle. MEFs can be transformed with oncogenic Ras + E1A, but not as efficiently as wild-type cells (Ortega et al., 2003).

CDK4 and CDK6 | Progressive embryonic lethality from E14.5 | Small embryos. Partial failure of hematopoiesis results from reduced multipotential progenitors and multilineage deficits, including severe megaloblastic anemia. MEFs proliferate with increased generation time and reduced S-phase fraction. Some D-type cyclins associate with and activate Cdk2. MEFs resist transformation (Malumbres et al., 2004).

CDK2 and CDK6 | Viable | Phenotype identical to Cdk2 and Cdk6 single mutants (Malumbres et al., 2004)

CDK2 and CDK4 | Embryos die by E15 | Cardiovascular defects (Santamaria et al., 2007).

CDK4, CDK2, CDK6 | Embryonic lethality before E15.5 | The livers of live TKO embryos at E12.5–E13.5 showed a threefold reduction in cellularity, undergo substantial apoptosis at E13.5– E14.5, as determined by active caspase 3 immunoreactivity. The hearts of TKO embryos showed thinner ventricular walls owing to a decrease in the number of proliferating cardiomyocytes (Santamaria et al., 2007).

p21 | Viable | Mice are more susceptible to chemically induced skin carcinoma. Female have decreased viability and develop a syndrome similar to human lupus because of increased T-lymphocyte proliferation

p27 | Viable | Mice grow 20-40% large, because of alteration in the balance between proliferation and withdrawal from the cell cycle at the critical period of development. Female are infertile

Tab.2: Phenotypes of mice with disrupted CDK genes
1.2 Liver regeneration

The liver is an important organ within the body that has a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. The liver produces a large number of proteins including albumin, acute-phase proteins, enzymes and cofactors. Importantly, the liver is the main detoxifying organ of the body which removes wastes and xenobiotics by metabolic conversation and biliary excretion.

The main cell types of the liver are:
- **Parenchymal cells** or hepatocytes (80% of liver cells) which carry out most of the liver function.
- **Non-parenchimal cells** (20% of liver cells) which can be roughly subdivided into:
  - Endothelial cells, which line the intrahepatic vessels and provide the large surface area for nutrient absorption
  - Kupffer cells, which are located in sinusoids and play essential roles in phagocytosis of foreign particles and infecting organisms and the production of cytokines
  - Hepatic stellate cells – cells located in the portal field and around the pericentral area, are the principal fibrogenic cell types in the liver (Dudas et al., 2003).
The proliferative index of adult hepatocytes in the healthy liver is extremely low, with less than 1 per 1000 hepatocytes undergoing DNA synthesis at any point of time. Quiescent hepatocytes become proliferative and replicate to restore the liver functional capacity as well as its mass, in the case of tissue resection (Fig.5). This amazing regenerative capacity of the liver is most clearly demonstrated by the two-thirds partial hepatectomy (PH) model in rodents, which was pioneered by Higgins and Anderson in 1931 (Higgins and Anderson, 1931). In this model, 70% of the liver is surgically removed. The residual liver lobes restore the original liver mass within approximately one week after surgery, though the resected lobes never grow back. In this experimental system, liver regeneration does not require the recruitment of liver stem cells or progenitor cells, but involves replication of the mature functioning liver cells (Taub, 2004).

The events of the liver regeneration can be divided into three phases (compare Fig.6):

2. **Initiation/priming**
3. **proliferation**
4. **Inhibition**

However, there is no distinct border between these processes, as all phase are closely linked and share several mechanisms.
1.2.1 Initiation of liver regeneration

During initiation, hepatocytes are primed for subsequent replication. Initiation factors include the cytokines Tumor Necrosis Factor Alpha (TNF) and interleukin 6 (IL-6).

TNF is a critical cytokine in the initiation of the regenerative process. Although TNF signals through both type 1 and type 2 receptors (TNFR-1 and TNFR-2) only signaling through TNFR-1 is required for liver regeneration after partial hepatectomy. This signaling pathway always follows the sequence:

\[
\text{TNF} \Rightarrow \text{TNFR1} \Rightarrow \text{NF-κB} \Rightarrow \text{IL6} \Rightarrow \text{STAT3}
\]

The transcriptional factor NF-κB controls the expression of cytokine-encoding genes, regulates the proliferatory response and acts anti-apoptotic in the liver during the regenerative process. NF-κB is present as an inactive form in the cytoplasm, where it forms complexes with an inhibitor known as I-κB. Activation of NF-κB involves its dissociation from the inhibitor (Fausto, 2000). After release from I-κB, NF-κB translocates to the nucleus, where it binds to its recognition sequence and activates target genes.

TNF also triggers the expression of IL-6, which is an important inducer of transcriptional factors and is released by hepatic stellate cells and Kupffer cells. During liver regeneration, IL6 activates two main cascades through the gp130-IL-6R complex- the STAT3 and MAPK signaling pathways (Black et al., 2004).

Are there other priming factors? Subsequent to priming/initiation, several immediate early-phase genes related to hepatocytes proliferation are induced.
within 2 hours. The almost immediate activation of these genes is the first step in a cascade of events that leads to DNA synthesis.

Detailed studies of the immediate early response genes revealed that more than 70 genes are activated during the first few hours after PH. The most important between them are c-fos, c-jun and c-myc (Morello et al., 1990).

C-Jun and c-fos transcript levels increase in the liver almost immediately after PH and return to normal levels by 2h. This is followed by an elevation of the c-myc mRNA level which is highest at 2h but decreases to the basal level at 4h. C-Jun is a critical component of the early proliferative response and induces the G0 to G1 transition via cyclin D (Zimmermann, 2004), while c-Myc expression results in the increased formation of new cyclin E/Cdk2 complexes and also prevents their association with of inhibitor p27 (Obaya et al., 1999).

1.2.2 The proliferation phase

Progression of primed/competent hepatocytes through G1 and subsequent replicative cycling is dependent on Hepatocyte Growth Factor (HGF) and Transforming Growth Factor (TGF-alpha) signaling after which the proliferation process seems to proceed autonomously under the control of cyclins and cyclin-dependent kinases (Taub, 2004; Zimmermann, 2004).

HGF is synthesized by non-parenchymal cells such as Kupffer cells, endothelial cells and particularly Ito cells and therefore affects hepatocytes in a paracrine manner. HGF is a potent stimulator of DNA synthesis in hepatocytes. Plasma concentration of HGF increase during the first 3 hours post PH. HGF mRNA in the liver increases between 12 and 24 hours after operation. Elevation of HGF mRNA after PH is also presented in the lung, spleen and kidney. Thus the effect of HGF in the liver could be exerted also by the factor containing in the blood (autocrine).

The production of TGF-α by hepatocytes is the critical step that leads the cells towards DNA synthesis, whereas gene expression changes observed before this point might constitute a priming state in which hepatocytes prepare for DNA synthesis but are not committed to enter into it. Increased levels of TGFα were observed within 8h after PH with a peak of expression at 24h, followed by a decline and subsequent smaller elevation with a peak at 72h.
These changes in the TGFα gene expression parallel the kinetics of DNA synthesis.

<table>
<thead>
<tr>
<th>Immediate early genes (c-fos, c-jun, jun B, c-myc)</th>
<th>Delayed Genes (bclx)</th>
<th>Cell Cycle Genes (p53, p21)</th>
<th>DNA Replication and Mitosis (cyclin D1, cyclin E, B, ras)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>4h</td>
<td>8h</td>
<td>20h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G1</td>
<td></td>
</tr>
<tr>
<td>G0</td>
<td></td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 7:** Sequence of gene activation in the regenerating liver after PH. Adopted from Fausto, (2000)

Expression of immediate early genes and transcription-factors activation is followed by expression of cell-cycle related genes. They include both inducers and inhibitors of the cycle. In mice, at the time between 30h and 36h post PH, most of the hepatocytes cross the G1/S phase boundary (Satyanarayana et al., 2004). A peak of DNA synthesis is observed at 40h and mitosis occurs few hours later. This process requires a tight coordination of several pathways.

Cyclin D1/Cdk4 or Cdk6 are activated in mid-G1, while cyclin E/Cdk2 control G1/S phase transition. Progression of DNA synthesis is assured by cyclin A/Cdk2.

Induction of cyclin E and Cdk2 occurs at time points near the G1/S boundary in the regenerating mouse hepatocytes. Furthermore, a subcellular rearrangement of cyclins and Cdkks is observed in the remnant liver with cyclin E and Cdk2 moving from the cytosol into the nucleus. The cyclin E/Cdk2 complex acts as a histone H1 kinase and the peak of its activity closely correlates with the peak of DNA synthesis in the regenerating hepatocytes. It is suggested that cyclin E and Cdk2 are important in orchestrating the cell cycle in the regenerating liver, but the exact role of the active cyclinE/Cdk2 complex remains to be defined (Fausto, 2001; Starkel et al., 2005).
1. Introduction

1.2.3 Termination of liver regeneration

Subsequent to the expansion phase the growth response must finally be terminated. Major factors involved in the termination response comprise TGF-beta and the activins. The mRNA levels of TGFβ and activin are low or undetectable in normal liver but increase significantly a few hours after PH. It has been suggested that there is a balance between stimulators (TGFα and HGF) and inhibitors (TGFβ and activin) of DNA synthesis during liver regeneration in a way that the balance favors DNA synthesis induction during the first hours after PH but gradually shifts toward inhibition in subsequent days. (Fausto et al., 1995; Michalopoulos, 1990; Steer, 1995; Taub, 2004).

1.3 Liver Fibrosis

Liver fibrosis is defined as an excessive deposition of extracellular matrix. It is the main complication of chronic liver damage and its endpoint - the liver cirrhosis, is responsible for impressive morbidity and mortality.

The most important role in the injury response and fibrogenesis in the liver is played by a mesenchymal cell type known as the hepatic stellate cell.

Different types of liver injury lead to activation of cells of the immune system in the liver such as Kupffer cells and monocytes (Fig.8). Any activation of these cells leads to secretion of cytokines, among others TGF-β. TGF-β in turn leads to an activation of quiescent hepatic stellate cells. This activation involves changes of cell morphology but also of the protein expression pattern as activated stellate cells express α-smooth-muscle-actin and collagen thereby mainly contributing to the production of extracellular matrix. In progressing fibrosis, the architecture of the liver is gradually disrupted. Destruction of the structure of the sinusoids impairs the liver function and eventually leads to cirrhosis and its complications including portal hypertension and liver failure (Weiler-Normann et al., 2007).
Fig. 8: Different types of liver injury lead to activation of immune cells in the liver such as Kupffer cells (KC), followed by secretion of cytokines, among others TGF-β.

TGF-β will lead to compensatory proliferation of hepatocytes and an activation of resting hepatic stellate cells (HSC), which results in secretion of extracellular matrix.

<table>
<thead>
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<th>marker</th>
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<th>portal smooth muscle cells</th>
<th>activated HSC</th>
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<td>inducible</td>
<td>?</td>
<td>+++</td>
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<tr>
<td>collagen synthesis</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+++</td>
</tr>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>desmin</td>
<td>+ or -</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GFAP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Tab. 3: Comparison of expression profiles of mesenchymal subpopulations in the liver (Saile and Ramadori, 2007)
+ - strong expression, positive staining
- - no expression, negative staining
+/− - variable expression level
+++ - very strong expression
?- uncertain data
1. Introduction

1.4 Aim of the study

It has been recently demonstrated that cyclin E1 and E2 are dispensable for continuously dividing cells, but are essential for both endoreplication and cell cycle re-entry of quiescent cells. The aim of this study was to explore the role of E-type cyclins during liver regeneration and fibrogenesis by using constitutive knockout mice for cyclin E1 and cyclin E2, respectively.

During liver regeneration hepatocytes are activated and have to leave their quiescent state in order to restore liver mass. Therefore the cell cycle regulation during the regeneration process is an essential mechanism. From previous works (Geng et al., 2003) it is known that E-type cyclins are critical for cell cycle re-entry from quiescence and therefore play an essential role during G0/G1-S phase transition at least in murine embryonic fibroblasts.

The first specific aim of this study was to characterize constitutive cyclin E1 and cyclin E2 knockout mice in the partial hepatectomy model in order to investigate the impact of cyclin E1 and E2 deletion on hepatocyte proliferation during liver regeneration.

The hallmark of liver fibrosis is an increase of amount of extracellular matrix. Many cell types are involved in that process - hepatocytes, hepatic stellate cells, immune cells. All of these cells are activated by fibrogenic extracellular signals and at least some of these cell types such as hepatic stellate cells and hepatocytes have to leave their quiescent state during early fibrogenesis. Therefore, the second aim was to investigate the relevance of E-type cyclins in murine experimental models of liver fibrosis.
## 2. Material and methods

### 2.1 Materials

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</tr>
<tr>
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<td>Invitrogen</td>
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## 2. Material and methods

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<td>anti Histone H3 antibody</td>
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<td>anti-PCNA antibody</td>
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## Instruments and equipment

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2. Material and methods

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<td>Water bath Haake W13</td>
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**General materials**

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<tr>
<td>96 well Microplates for RT-PCR</td>
<td>Star</td>
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<tr>
<td>96 wells Micrplates for protein measuring</td>
<td>Greiner</td>
</tr>
<tr>
<td>96 wells Micrplates for RNA measuring</td>
<td>Greiner</td>
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2. Material and methods

### Material for animal experiments

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<td>Ethibond® Excel plated, laminated, not absorbable; 6/0 metric 0,7</td>
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<td>Altromin</td>
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<tr>
<td>Hematocrit capillary</td>
<td>Brand</td>
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<td>Heparine (Liquemin®)</td>
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<td>Ethicon</td>
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<td>Xylazin (Rompun®)</td>
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### 2.2 Methods

#### 2.2.1 Housing and breeding of mice

All animals used for experiments were maintained in the animal facility of the University Hospital Aachen in a temperature controlled room with 12-hour light/dark cycle according to the German legal requirements. For our study we used previously described constitutive knock-out mice for cyclin E1 (E1\(^{-/-}\)) and cyclin E2 (E2\(^{-/-}\)) on a 129/Ola background (Geng et al., 2003) of male gender. As controls, we used wildtype (WT) littermates derived from heterozygous breeding couples.
2. Material and methods

2.2.2 Partial hepatectomy (PH) and tissue sampling

For liver regeneration studies, midioventral laparotomy with two third partial hepatectomy was performed according to the method of Higgins and Anderson (Higgins and Anderson, 1931). Five to eight week-old males (E1\(^{+/−}\), E2\(^{+/−}\) and WT) were anesthetized intraperitonealy with a combination of 10\% Ketamin and 2\% Xylazinhydrochlorid in 0.9\% NaCl. A midline lapartomy was done. The major liver lobes were identified - anatomically, the median lobe, with its two central portions together with the left lateral lobe, forms somewhat of a unit which lends itself to surgical removal. First these liver lobes were ligated with the silk tie and then ligated tissues were resected. The abdomen was closed with two layers. After operation animals immediately got access to water and food. Five to ten mice from each group were sacrificed before or at different times after PH. The remnant liver was extracted for the weight measurement and further molecular and histological analysis. A portion of liver was immediately snap frozen in liquid nitrogen for protein and RNA isolation, whereas another portion was frozen in Tissue-Tek or stored in 4\% paraformaldehyde (PFA) before paraffin embedding.

As a model of fatal hepatic failure we performed 90\% hepatectomy with a subset of animals as described recently (Makino et al., 2005). The surgery procedure was similar to the 2/3 hepatectomy. However, in these experiments all liver lobes except the caudate were resected.

2.2.3. Isolation and analysis of DNA

2.2.3.1. Isolation of genomic DNA from tail biopsies

To genotype mice by PCR, DNA was isolated from murine tail tips of around 0.3 cm in length. The tissue was lysed for at least 3 hour at 56°C in 500 μl of lysis buffer containing 20 μl of proteinase K (15,6 mg/ml). The samples were centrifuged for 10 minutes at 14000 rpm. 500 μl of 2-propanol were added to the achieved supernatant for precipitation. After mixing thoroughly centrifugation was performed for another 10 minutes. Supernatant was discarded and the nucleic acid containing pellet was washed twice in 70\% ethanol for removal of salts by mixing and subsequent centrifugation at 14000 rpm for 2 minutes. The pellet was dried at 37°C and resuspended in 200 μl 1M RNAse/TE. Storage was carried out at 4°C.
2. Material and methods

**Lysis buffer:**

25 ml 1M Tris-HCl, pH 8.0 = 50 mM Tris  
100 ml 0.5M EDTA, pH 8.0 = 100 mM  
50 ml 1M NaCl = 100 mM  
25 ml 20% SDS = 1%  
300 ml H₂O

2.2.3.2. Genotyping

The genotypes of genetically modified mice were determined by PCR analysis from mouse tail biopsies according to standard procedures.

A PCR specifically detecting the gene disruption of cyclin E1 was performed using the following thermocycling program:

<table>
<thead>
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<th>Procedure</th>
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</tr>
<tr>
<td>Conservation</td>
<td>4°C</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

The primers were used are:

**3N3** (5'-GAT CTC TCG TGG GAT CAT TG -3),  
**11-2** (5'-CGC ATA CTG AGA CAC AGA CT -3),  
**E11-A** (5'-CGC CAT GGT TAT CCG GGA GAT GG-3).

The knock-out (KO) band is about 300 bp and the wild type (Wt) band is about 180 bp.

The knockout of cyclin E2 was identified with the same PCR program using the following primers:

**3N3** (5'-GATCTC TCG TGG GAT CAT TG -3),  
**E2G** (5'-GGT TCT CCC ATT TAG AGC ACA G-3),  
**E2L** (5'-GCT ATA GCA GTT GTT TCT GTG TG-3).

The KO band is about 300 bp and WT band is about 400 bp.
2. Material and methods

**PCR Reaction Mix:**

2 µl DNA template (100 ng)
1 µl Sense Primer 1 (10 pmol/µl)
1 µl Sense Primer 2 (10 pmol/µl)
1 µl Antisense Primer (10 pmol/µl)
12.5 µl Ready Mix™ retdag PCR reaction mix with mgCl₂
7.5 µl H₂O
25 µl final volume

2.2.4 Isolation and analysis of RNA

2.2.4.1 Isolation of RNA from liver tissue

From deep frozen liver tissue around 50-100 mg was cut off on dry ice and 1 ml of *peqGOLD-RNAPure®* was added. After homogenization using an Ultra-Torrax the lysate was incubated for 5 minutes at room temperature. After adding 200 µl of chloroform under an extractor hood the sample was vortexed thoroughly for 15 seconds and again incubated at room temperature for 10 minutes. Due to centrifugation at 12000 x g for 5 minutes the lysate formed up three phases. The liquid upper phase contains the disassociated RNA, whereas the interphase and the lower phase contain impurities of DNA and protein. Avoiding any contact with the interphase, the upper phase was transferred carefully to a fresh reaction tube. After addition of 500 µl 2-propanol samples were incubated for 10 minutes at room temperature. Precipitated RNA was then isolated by centrifugation for 10 minutes at 12000 x g and 4°C. After discarding the supernatant the pellet was washed twice with 1 ml 70% ethanol. For washing, centrifugation was performed at 12000 x g at 4°C for 10 minutes. Subsequently the pellet was dried for 30 min on ice and dissolved in 40-80 µl RNAsae-free dH₂O. Storage of isolated RNA was performed at -80°C.

2.2.4.2 Determination of RNA concentration

Purified RNA was diluted 1:50 in RNAsae-free dH₂O to measure optical density at 260nm (OD₂₆₀). The RNA concentration was calculated according to the formula: \( 1 \text{OD}_{260} = 40 \mu \text{g RNA/ml} \). The purity of RNA was analyzed with additional determination of the extinction at 280nm. A ratio of E₂₆₀/E₂₈₀ between 1.9 and 2.1 accounts for pure RNA.
2. Material and methods

2.2.4.3 Reverse Transcription (RT-PCR)

Reverse transcription is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) using the enzyme reverse transcriptase. In combination with real time PCR this method is applied for profiling mRNA gene expression.

Reverse transcription (RT) reactions were performed with an Omniscript RT Kit and OligodT primers (Qiagen, Hilden, Germany) according to the manufacturers instructions using the following parameters:

**Reaction Mix:**
- Template RNA 2 μg
- 10x BufferRT (Omniscript RT Kit; Omniscript RT Kit ) 2,0 μl
- dNTP Mix (5 mM each dNTP;Omniscript RT Kit ) 2,0 μl
- Oligo-dT primer (10 μM;Omniscript RT Kit ) 2,0 μl
- Omniscript Reverse Transcriptase 1,0 μl
- RNase –free water variable
- Total volume 20,0 μl

**Thermocycling program:**
- 60min – 37°C
- 5 min - 93°C

2.2.4.4 Quantitative real-time PCR

Relative gene-expression of cell cycle relevant genes (cyclin E1, E2, A2, D1, B1, p21 and p27) was measured via real-time PCR using a 7300 Real Time PCR System with SDS software 1.3.1 and a SYBR Green PCR Kit (Invitrogen).

**Reaction Mix:**
- 5μl cDNA template
- 2 μl sense primer (100μM)
- 2 μl antisense primer (100μM)
- 12,5 μl SYBR® GreenER qPCR SuperMix
- 3,5 μl H₂O
- 25 μl final volume

Measuring was normalized using GAPDH expression as internal standard.
PCR was performed for 40 cycles with annealing at 58°C and elongation at 60°C using the following primers:

<table>
<thead>
<tr>
<th>Gene</th>
<th>sense primer (5’-3’)</th>
<th>antisense primer (5’-3’)</th>
</tr>
</thead>
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<tr>
<td>cyclin D1</td>
<td>AAGCATGCAAGACATTTTGTGG</td>
<td>TTCAGGCCCTTGCATCGCAGC</td>
</tr>
<tr>
<td>cyclin B1</td>
<td>CTGCACTTCCTCCGATAGGC</td>
<td>AAAATGCACCATGCTGAGTCCTCC</td>
</tr>
<tr>
<td>cyclin A2</td>
<td>CAACAGTAATCAAGTTCCCTTTACC</td>
<td>CATCTATTCAAATCAGTTTCTCC</td>
</tr>
<tr>
<td>cyclin E1</td>
<td>ACAGCAGGTCTTGGCGAGGATCC</td>
<td>CTGAACAGGACACACCATGAG</td>
</tr>
<tr>
<td>cyclin E2</td>
<td>CATGGAAGATAGACACAAATATCC</td>
<td>CATATAATGACTCCATTACACACTG</td>
</tr>
<tr>
<td>p21:</td>
<td>TTGCACTCTGAGTCTGGAGC</td>
<td>TCTGCGCTGGAGGAGTAGTGAGA</td>
</tr>
<tr>
<td>p27:</td>
<td>GACAATCGGCAGGCTCGGTTAGC</td>
<td>TCTGCTCTGTTGCGCCCCCTTTT</td>
</tr>
<tr>
<td>collagen 1</td>
<td>TCACTGCAAGCAGCCGGTTG</td>
<td>GATGACTGTCTGGCCGCAAGTTT</td>
</tr>
<tr>
<td>αSMA:</td>
<td>AAAACACGAGGAACGAAATCAGA</td>
<td>TCAGCGCCTCAGTTTCT</td>
</tr>
<tr>
<td>GAPDH:</td>
<td>TGTTGAAGTCAGGCCAGACACCT</td>
<td>AACTGCAAGTATGAGCATCA</td>
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</table>

Quantitative real-time PCR for cyclin E1 and E2 was performed accordingly using standards containing defined numbers of cyclin E1, E2 and GAPDH cDNA molecules in a range of 10^8 -10^1. Expression was calculated as number of target molecules/10^6 GAPDH molecules.

2.2.5. Isolation and analysis of proteins

2.2.5.1 Protein isolation and quantification

Liver tissues were homogenized in 500μl of lysis buffer containing 50mM Tris-HCl; 150mM NaCl; 0.5% NP40; 0.1M NaVO₃; 1M DTT; 100mM PMSF and protease inhibitors (Complete MINI®, Roche).

Protein concentrations were determined using a Bio-Rad Protein Assay according to manufacturer’s instructions. Different concentrations of BSA (2, 4, 6, 8 and 10 mg/ml) were used as standards. 2 μl of protein sample was added to 998 μl of the 1:5 diluted dye reagent. The absorbance was measured at 595 nm, which allowed calculation of protein concentration deduced from a BSA standard curve.
2. Material and methods

2.2.5.2 Western blot

Samples containing 30μg of protein and loading dye were heated for 5 minutes at 95°C and were then applied to sodium dodecyl sulfide polyacrylamide gel electrophoresis. Separating was performed between 50 and 120 V in Rotiphorese 1x SDS page running buffer.

Composition of separating gel

<table>
<thead>
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<th>Compounds</th>
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<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>4.6 ml</td>
<td>4.0 ml</td>
<td>3.28 ml</td>
</tr>
<tr>
<td>Tris M 1.5 pH 8.8</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Acrylamid</td>
<td>2.7 ml</td>
<td>3.3 ml</td>
<td>4.06 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
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<tr>
<td>10% APS</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6.0 μl</td>
<td>7.5 μl</td>
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Composition of collection gel

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<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>3.1 ml</td>
</tr>
<tr>
<td>Tris M 1.5 pH 8.8</td>
<td>1.25 ml</td>
</tr>
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<td>10% SDS</td>
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<td>10% APS</td>
<td>25μl</td>
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<tr>
<td>TEMED</td>
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</table>

After electrophoresis, proteins were transferred to a Nitrocellulose Membrane (PROTRAN ©) using a wet western blotting system (BioRad) for 2 hours at 300 mA. Membranes were blocked for 30 min in 5% Dry-Milk/TBS-Tween and incubated with primary antibodies in 5% Dry-Milk/TBS-Tween overnight at +4°C. Antibodies for cyclin E, B1, D1 and A were diluted 1:500; the GAPDH antibody was used at a 1:10000 dilution. Washing of membranes was performed three times for 10 min in TBS-Tween followed by incubation with a secondary antibody (anti-rabbit HRP-linked IgG) diluted 1:5000 in 5% Dry-Milk;TBS-Tween; 2% BSA or anti-mouse Ig-HRP for 1 hour, followed by washing in TBS-Tween. The antigen-antibody complexes were visualized by using ECL Western blotting Substrate (Pierce).
2. Material and methods

2.2.5.3 Immunoprecipitations and in vitro Kinase assays

A total of 500 μg of liver protein extracts were subjected to immunoprecipitation using 1μg of cdk2 antibody at +4°C overnight and then conjugated to Protein-A agarose beads. Immunoprecipitated complexes were collected by centrifugation, washed 2 times with NP40 buffer and once with Histone-washing buffer. After final centrifugation, pellet was incubated for 30 min at 37°C with 2μg recombinant Histone H1, 100mM ATP and 7 μl of 32P-γATP (10μCi/μl). Reaction was stopped by adding SDS loading buffer (10% glycerol, 1% SDS, 25M Tris-HCL). Samples were separated on 10% SDS-PAGE-gels and analyzed by autoradiography.

For Cyclin A depletion experiments, a total of 500 μg of liver protein extracts were first subjected to immunoprecipitation using 1μg of cyclin A antibody for 2h at +4°C and then conjugated to Protein-A agarose beads for 2h. After 2min of gentle centrifugation, the supernatant containing cyclin A-depleted liver proteins was collected and subjected to immunoprecipitation with cdk2 antibody (2h, at +4°C). Kinase assays with immunoprecipitated complexes were carried out as described before.

Histone washing buffer:
2,5 ml Tris-HCl pH7,5 = 25mM
7,7 ml 1M NaCl = 70 mM
2 M MgCl$_2$ = 10mM
200 1μl 1M DTT = 1mM

SDS loading buffer:
15ml 100% Glycerol = 50% Glycerol
3g SDS = 5% SDS
7,5 ml 0,5 M Tris-HCL, pH 6,8 = 125mM
2. Material and methods

2.2.6 Work with primary cells

2.2.6.1 Isolation of hepatocytes

Primary mouse hepatocytes were isolated by a two-step collagenase perfusion technique. Mice were anesthetized intraperitoneally (i.p.) with a combination of 10% Ketamin – 2% Xylazinhydrochlorid in 0.9% NaCl. 40 IE Heparin in 300µl 0.9% NaCl were injected i.p. prior to liver perfusion to avoid blood coagulation. Midline laparotomy was performed, the vena cava inferior was cannulated and the portal vein was cut immediately. Mouse liver was retrograde perfused with the warmed up solution at 6 ml/min with 40 ml Hepatocyte buffer (HB) in the presence of 1 mM EDTA followed by second perfusion of HB containing 2mM CaCl₂, and Liberase 3™ (14 mg/l) (0.7mg in 50ml of HB), (Roche). After perfusion, the liver was carefully extracted, minced in 10 ml of HepatoZyme-SFM medium (Invitrogen), filtered through 70µm cell strainer (BD Bioscience) and immediately stored on ice.

**Hepatocytes buffer (pH 7.4)**

- 0.15 M NaCl
- 6 mM KCl
- 5mM glucose
- 0.02 M Hepes
- 2.5 M NaHCO₃

2.2.6.2 Analysis of DNA content by Fluorescence Activated Cell Sorting (FACS)

Freshly isolated primary hepatocytes were centrifuged at 50g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was washed twice with PBS at 50g. Hepatocytes were resuspended in Nicoletti buffer at a cell density of 10⁶ cells/ml. Probes were measured using a FACS Canto flow cytometer in the PerCP-Cy5-5 channel. For each measurement, a minimum of 10⁵ cells were analyzed. Analysis was performed with FlowJo Software 7.1.2. Ploidy fractions were counted in percentages excluding the apoptotic cell population.

**Nicoletti buffer (pH 7.4)**

- 0.1% Sodium citrate
- 0.1% Triton X-100;
- 50 µg/ml Propidium iodide
2.2.6.3 Fluorescent In Situ Hybridization (FISH)

Primary mouse hepatocytes were isolated 96 hours post PH and immediately transferred to glass slides using a cytospin centrifuge. Slides were shortly dried at room temperature and then fixed in methanol/acetic acid (3:1) for 10 min, and treated with RNase (1mg/mL) for 1 h at 37°C in a humidified chamber. The samples were dehydrated in an ethanol series (70%, 80%, 90%, 100%) and air dried followed by incubation in 0.01M HCl containing 5% pepsin. Slides were fixed in 4% PFA, dehydrated in an ethanol series (70%, 80%, 90%, 100%), air dried and subjected to aging at 65°C for 60 min. For denaturation of chromosomal DNA, slides were incubated in 70% formamide/2X SSC at 70°C for 5 min, dehydrated by serial ethanol washing for 2 min each in 70%, 80%, 90%, 100% ethanol and air dried. A probe corresponding to the murine Y chromosome was prepared according to the manufacturer’s supplementary protocol. 15 μl of Cy3 labelled probe was applied to each slides under cover slips. The hybridization was performed overnight at 37°C in a humidified chamber. Post hybridization washings were carried out at 58°C in 2xSSC, followed by Stringency Wash Solution (deionised Formamide/1*SSC = 1:1) and 1xSSC; each washing step was performed twice for 5 min. After that slides were immersed in Detergent Wash solution (0,05% Tween20 in 4*SSC) for 4 min and mounted with Vectashield containing DAPI. Cells were examined with a Carl Zeiss fluorescence microscope using a Ds Red 43 filter. Three hundred to 1000 nuclei of each mouse strain were analyzed and the percentage of nuclei containing 2, 4 or more than 4 signals was determined.

10xSSC pH7.2

Natrium Chloride  87,65g
Natrium Citrat 44,1g
H₂O 1000 ml

2.2.7 Quantification of liver proliferation and immunohistochemistry

2.2.7.1 PCNA Staining

5 μm cryosections were fixed in 4% Formaldehyde/PBS, followed by washing with PBS for 5 min. Slides were incubated in methanol containing 3% H₂O₂ for 10 min and then washed in PBS 5 min. Sodium Citrat (10mM; pH 6.0)
was preheated in a microwave oven for 5 min at 800 W, then slides were
immersing in hot Sodium Citrat for 15 min in the microwave oven at 240 W. The
slides were cooled down to room temperature and washed three times in TBS-
Tween for 3 min. After that, incubation with an anti-PCNA antibody (Dianova) at
a dilution of 1:200 was performed at +4°C overnight. The slides were washed
three times in PBS for 3 min and incubate for 30 min at room temperature with a
secondary anti-mouse antibody (goat anti-mouse IgG2aHRP Sc-2061) at a
dilution of 1:100. Washing was performed 3 x 3 min in PBS, followed by staining
with DAB solution and counterstaining with haematoxylin. The reaction was
stopped by washing into distilled water for 5 min. PCNA positive cells were
calculated as a percentage to all nuclei per field at 20x magnification.

2.2.7.2 BrdU Labeling

For \textit{in vivo} labeling experiments, 5-2-deoxyuridine (BrdU) was injected i.p.
(30 µg/g body weight) 2 hours before animals were sacrificed. Liver tissues
were immediately frozen in Tissue-Tek.

\textbf{Protocol1 (bright field microscopy):} 5 µm cryosections were fixed in ice-
cold acetone-methanol (50%-50%) for 10 min and then washed 5 min in TBS-
Tween (pH 7.6) two times. The slides were dehydrated in 70% ethanol for 30
min and dried for 30 min at room temperature. The block of endogenous
peroxidase activity was done by incubating in methanol/3% H2O2 in for 10 min,
followed by washing 2x5 min in TBS-Tween. Denaturation was made by
immersing slides for 30 min in 70°C Formamide/NaOH, washing by 5 min TBS-
Tween at 70°C, and then immersing in 0.15 M 6ml Natriumcitrat in 100 ml
Formamid during 15 min. The reaction was stopped by washing slides in ice-
cold TBS-Tween (2 times 7 min). A second fixation was performed in PBS/3%
formaldehyde for 30 min. After that slides were washed in TBS-Tween 2 times
for 5 min and then immersed in 25% Glutaraldehyde. Washing was made in
TBS-Tween 2x5 min. Slides were incubated in a light-protected wet chamber
overnight at +4°C with a primary anti BrdU antibody (\textbf{Amersham Bioscience}).
The next day, washing was performed four times for 4 min in TBS-Tween,
followed by incubation with a secondary anti-mouse antibody 1:100 (goat anti-
mouse IgG2aHRP) for 30 min at room temperature. Samples were washed 4 x 4
min with TBS-Tween and slides were shortly incubated with DAB solution
(SIGMA FAST), followed by immersing in Haematoxylin solution (10 min) and
washing in distilled water (5 min). Samples were analyzed in a light microscope at 20 x magnification. The BrdU index was calculated as percentage of BrdU positive cell to all nuclei per field.

Protocol 2 (fluorescence microscopy): Cryosections (5 µm) were air-dried and fixed with ice-cold acetone for 15 min. After rehydration with 1xPBS for 5 min, samples were treated with 2 N HCl for 30 min and neutralized with 0.1 M sodium borate (pH 8.0) for 10 min. Samples were washed 3 times with PBS. Slides were incubated in dark wet chamber overnight at +4°C with a primary anti-BrdU antibody (1:40). Washing was performed 3 times for 10 min in TBS-Tween, followed by 1 h incubation with secondary anti-mouse antibody (Alexa Fluor 488). Slides were washed twice in TBS-Tween and mounted with Vectashield containing DAPI. Sections were analyzed with a fluorescence microscope.

2.2.7.3 Measurement of histone H3 phosphorylation.

Frozen 5 µm liver sections were fixed in ice cold Acetone-Methanol (50%/50%) for 10 min, dried and then rinsed in PBS-Tween for 10 min. Slides were blocked with goat serum for 45 min. Incubation with anti-Histone H3 antibody was done at +4°C at a dilution of 1:200 overnight, followed by 3 x 10 min washing in PBS-Tween. Slides were incubated with fluorescence-labeled secondary anti-rabbit-antibody (AlexaFluor 488) for 1 hour in a dark humidified chamber at room temperature. Slides were washed twice in PBS-Tween and mounted with Vectashield containing DAPI.

2.2.7.4 Histological analysis and morphometry

Hepatic tissues were fixed in 4% PFA immediately after extraction, embedded in paraffin, sectioned and first fixed in acetone for 10 minutes. For staining nuclei of liver cells, sections were immersed in Mayer's hematoxylin solution for 8 minutes and then thoroughly rinsed in running tap water for 10 minutes. Counterstaining was performed with 1% eosin solution for 1 minute. Afterwards, dehydration was carried out in first 70% and then in absolute alcohol for 2 minutes each. Slides were subsequently immersed in Rotihistol for 2 minutes and cover with cover glass.
The histological analysis was kindly performed by Prof. Dr. Nikolaus Gassler (Institute of Pathology, University Hospital Aachen). For morphometric analysis, from two DAPI-stained independent tissue sections per mouse, 10 magnification areas were randomly selected and subjected to computer-aided morphometry using an eclipse 80i fluorescence microscope in combination with image quantifying software NIS-Elements BR2.30 (Nikon).

### 2.2.8 Generation and *in vivo* use of recombinant cyclin E2 adenovirus (adv-CcnE2)

The plasmids pΔE1sp1A (Bett et al., 1994) and pJM17 (McGrory et al., 1988) used for construction of the adenoviral vector were obtained from Microbix Biosystems Inc. (Toronto, Ontario, Canada). A 2.2 kB DNA fragment containing the 1.4 kB open reading frame of the murine cyclin E2 gene under control of the cmv-promoter was cloned into the EcoRI and BglII sites of pΔE1sp1A, respectively, resulting in pΔE1sp1A-CMV-CcnE2. The integrity of cloning boundaries were verified by restriction analysis and sequencing with flanking primers 5' - GCGTAACCGAGTAAGAATTTG - 3' and 5' – GGCGACCATCAATGCTGGAG - 3', respectively. Integration of vector sequences from pΔE1sp1A-CMV-CcnE2 into the adenoviral backbone vector pJM17 was performed by *in vitro* homologous recombination in the human embryo kidney cell line 293 in cooperation with Prof. Dr. Weiskirchen (Institute of Clinical Chemistry and Pathobiochemistry, University Hospital Aachen) as described before (Weiskirchen et al., 2000). Successful generation of recombinant viral particles were visualized by viral foci formation. After total infection viral particles were released from cells by three rounds of a freeze-thaw cycle. Viral particles were separated from cell debris by centrifugation at 3000 rpm for 10 minutes. To generate higher titer viral stocks, 293 cells were re-infected at a multiplicity of infection (MOI) of 1 and grown for 3-4 days, at which time amplified viruses were harvested, concentrated through standard CsCl gradient centrifugation and subsequently purified using the BD Adeno-X™ Purification Filter system (BD Biosciences, Clontech, Palo Alto, CA) according to the manufacturer’s instructions.

For *in vivo* application of adv-CcnE2, a virus suspension containing 1.2 X 10^{10} plaque forming units (pfu) were injected i.v. via tail vein into mice 24 hours
before hepatectomy. As control, an adenovirus expressing cmv-luciferase (adv-Luc) was used.

2.2.9 Induction of Liver Fibrosis with CCl₄

Hepatic fibrosis in mice was induced by intraperitoneal injections with 0.6 ml/kg body weight of CCl₄ dissolved in sunflower oil, 2 times per week for up to 4 weeks.

2.2.10 Sirius red staining

Liver tissues were fixed in formalin (3.5%) and embedded in paraffin. Liver sections of 5 µm thickness were incubated in a solution containing 0.1% Sirius red and 0.1% Pikrinacid (pH 2.0) for 1 hour. Then sections were treated with 0.01M HCl for 2 minutes, washed in H₂O, fixed in an ascending sequence of ethanol and covered.
3. Results

3.1 Cyclin E1 – and cyclin E2 deficient mice have different kinetics of S-phase initiation after partial hepatectomy

In the adult liver, hepatocytes are normally quiescent, highly differentiated cells. However, after a regenerative stimulus such as 70% partial hepatectomy (PH) they rapidly switch to the proliferative state and re-enter the cell cycle. To study the role of cyclin E1 and E2 during liver regeneration in mice, PH was performed with E1⁻/⁻, E2⁻/⁻ and WT mice and the livers were analyzed for cell cycle progression of hepatocytes in a time frame of 24-168 hours after surgery. For each time point a minimum of five animals per group was investigated. Of notice, all animals survived the surgical intervention.

The Proliferating Cell Nuclear Antigen (PCNA) is a processivity factor for replicative DNA polymerases (Maga and Hubscher, 2003) serving as an essential factor for initiation of DNA synthesis. Therefore, first G1-S phase cell cycle progression was studied by analyzing nuclear PCNA expression in histological cryosections of liver tissues at different time points after PH. In WT animals, PCNA expression in the liver started 36 h after surgery and peaked at 40 h after PH followed by a continuous decrease of PCNA positive hepatocytes to almost baseline level until 168 h (Fig. 8).

In comparison, E1⁻/⁻ mice showed only a slight delay of 4 h in the onset of PCNA expression (Fig. 8 A, B) and had an expression maximum 48 h after surgery (Fig. 8 B). However, between 48-96 h post PH the total number of PCNA positive hepatocytes was significantly higher compared to the WT control.

In striking contrast to these findings the PCNA expression pattern in E2⁻/⁻ mice was completely different showing three times more PCNA-positive nuclei already 36 h after surgery (38% compared to 13% in WT animals) with no obvious expression peak but a continuous high expression level up to 72h after surgery (Fig. 8 A, C).
3. Results

Fig. 8: Quantification of G1-S phase transition

Immunohistochemical staining for PCNA on liver cryosections at 36 h and 72 h after PH. White arrows indicate examples of PCNA-positive nuclei (A). Quantification of staining by calculating the percentage of hepatocytes showing PCNA in liver tissues of WT compared to E2−/− mice (B) or WT compared to E1−/− mice (C) at time 0h to 168 h after PH. Each value represents the mean of 5-10 mice. (*) p < 0.05; (#) p < 0.05.

This initial analysis indicated that E1−/− and E2−/− mice have different kinetics in G1-S phase transition. To link this result to the function of either E-type cyclin, the activity of the cdk2 kinase complex was analysed by measuring in vitro histone H1 phosphorylation. Cyclin E/Cdk2 kinase complexes have been
described as the main regulators for G1-S phase transition (Pines, 1995) and histone H1 serves as a prominent substrate of this kinase showing maximal phosphorylation at the peak of DNA synthesis in the regenerating hepatocytes (Jaumot et al., 1999).

In WT mice histone H1 phosphorylation was evident exclusively between 40 h and 48 h after PH (Fig 9, upper panel) which is consistent with previous studies (Dierssen et al., 2008). In contrast, E1\(^{-}\) mice showed stronger and prolonged cdk2 activation until 72 h post PH compared to control animals (Fig. 9, medium panel). In coincidence with the PCNA expression pattern, E2\(^{-}\) mice showed a strong induction of cdk2 activity already after 36 h up to 72 h (Fig. 9, lower panel). In addition, cdk2 kinase activity was also evident in E2\(^{-}\) mice 96 h after PH although to a lesser extend.

Fig.9: Determination of cdk2 kinase activity

A total 500µg of protein extract from mouse livers of WT, E1\(^{+}\) and E2\(^{+}\) mice harvested between 0 h and 168 h post PH were subjected to immunoprecipitation using an anti-cdk2 antibody and histone H1 kinase assay were performed with the immune complexes. The signals indicating phosphorylated histone H1 are highlighted by arrows.

However, cdk2 kinase activity can be mediated via cyclin E/cdk2 or cyclin A/cdk2. In further experiments these two complexes were dissected by first depleting cyclin A/cdk2 activities using an anti-cyclin A antibody and subsequent analysis of the remaining, cyclin A-free protein extracts by histone H1 kinase assays (Fig. 10). In E1\(^{+}\) mice, depletion of cyclin A resulted in a substantial reduction of cdk2 kinase activity both 40 h and 72 h after PH, indicating that cyclin E1 deficiency is predominantly substituted by cyclin A. In
3. Results

Contrast, deprivation of cyclin A did not affect total cdk2 activity in E2−/− animals 72 h post PH and only to a minor extent at 40 h after surgery. This suggests that the prolonged S-phase and kinase activity seen in E2−/− mice is mediated by cyclin E1/cdk2.

**Fig. 10:** Dissection of cyclin E/cdk2- and cyclin A/cdk2 kinase activities. Protein extracts from WT, E1−/− and E2−/− mice after PH were depleted for cyclin A/cdk2 complexes by immunoprecipitation with an anti-cyclin A antibody where indicated and subjected to histone H1 kinase assays in comparison to untreated extracts representing total cdk2 activity.

RB phosphorylation through cyclin E/cdk2 kinase is a key event for G1/S-phase transition eventually leading to release and activation of E2F transcription factors. E2F may further activate E- and A-cyclins as well as its own transcription thereby initiating a positive feedback loop. Therefore, RB phosphorylation and E2F gene expression was analyzed in WT, E1−/− and E2−/− mice.

In WT mice, slight RB phosphorylation was evident 36 h after PH but hyper-phosphorylation of RB was restricted to 48 h after surgery (Fig. 11 A). Interestingly, E1−/− mice displayed a similar RB phosphorylation pattern but showed stronger RB phosphorylation at 48 h post PH (Fig. 1E) which correlated with increased PCNA expression. However, these animals also show a delayed onset of E2F1 expression (Fig. 11B) indicating that not a lack of cdk2 kinase
activity, but absence of E2F1 may contribute to delayed S-phase onset in E1\(^{-/-}\) mice.

**Fig. 11:** Analysis of RB phosphorylation and E2F1 expression.

**(A)** Whole liver cell extracts were investigated for RB phosphorylation. Two distinct signals for phospho-RB representing phosphorylated and hyper-phosphorylated RB were detected and highlighted by arrows. The expression levels of total RB and GAPDH are presented as internal loading controls. **(B)** Kinetics of E2F1 gene expression 0-48 h post PH measured by quantitative real-time PCR. The average of five independent experiments is shown.

In contrast, E2\(^{-/-}\) mice displayed strong RB hyper-phosphorylation beginning 24 h post PH (Fig. 11 A) and average cdk2 kinase activity appeared to be significantly stronger compared to WT or E1\(^{-/-}\) mice. In parallel, E2F1 expression was slightly prolonged compared to controls suggesting that earlier onset of S-phase in E2\(^{-/-}\) livers might be directly regulated by accelerated cdk2 kinase formation, whereas the prolonged S-Phase could in addition be attributed to prolonged E2F expression.
3.2 Altered S-phase initiation in E1−/− and E2−/− mice correlates with aberrant expression of cyclin E1, cyclin E2 and p27

In liver regeneration after PH, the checkpoint cyclin D1 is directly activated by mitogenic factors such as NF-κB and Jun/AP1 (Guttridge et al., 1999; Schwabe et al., 2003) eventually leading to activation of Cyclin E/cdk2 and subsequent G1/S-phase transition. In order to check, whether the priming phase of liver regeneration in hepatocytes is already different in WT-, E1−/− - and E2−/− mice, cyclin D1 expression after PH in these animals was analysed (Fig. 12). In all animals investigated, first appearance of cyclin D1 expression was at 36 h after PH irrespective of the cyclin E genotype and the expression profile was similar in all three groups demonstrating that the differences seen in S-phase initiation are cyclin D-independent.

![Western Blot](image)

**Fig. 12:** Liver protein extract (30 µg) of were isolated at indicated time points after PH from WT, E1−/− and E2−/− mice and subjected to Western blot analyzes using antibodies for detection of cyclin D1. GAPDH expression was used as loading control.

Next, the expression pattern of E-cyclins in all mouse strains after PH was analysed. In WT mice, cyclin E1 protein expression was limited to a time frame of 40h-48h following PH with the maximum 48h after surgery (Fig 13, upper panel). In contrast, E2−/− mice displayed almost continuous cyclin E1 expression from 24h-96h, with strongest expression from 24h-72h after PH. This indicates that cyclin E2 depletion may lead to prolonged cyclin E1 expression in the course of liver regeneration which was also clearly correlated with the prolonged cyclin E/cdk2 kinase activity in E2−/− mice (Fig. 9).
3. Results

Fig. 13: Liver protein extract (30 µg) were isolated at indicated time points after PH from WT, E1−/− and E2−/− mice and subjected to Western blot analyzes using antibodies for detection of cyclin E1. GAPDH expression was used as loading control.

Among many antibodies tested, no commercial antibody was available specifically detecting the murine cyclin E2 protein in immunoblots. Thus, cyclin E1 and E2 expression was measured on the mRNA level in all mouse strains by quantitative real-time PCR.

In WT mice following PH an almost identical mRNA expression pattern of cyclin E1 and E2 was observed with a single peak of approximately 25fold expression 40 h after surgery compared to untreated animals (compare Fig. 14 A and B). Hence, maximum expression of cyclin E1/E2 mRNA correlates with the maximum of cyclin E1 protein expression and may serve as an additional marker to study the regulation of E-cyclins.
Fig. 14: Relative real-time-PCR analysis of the indicated genes measured in the liver at various time points after PH in E1<sup>−/−</sup>, E2<sup>−/−</sup> and control mice (WT). The expression levels at all time points were normalized to GAPDH expression and calculated as fold induction in comparison to untreated animals of the same cohort. (A) mRNA expression profile of cyclin E2 in E1<sup>−/−</sup> mice and WT controls. (B) Gene expression profile of cyclin E1 in E2<sup>−/−</sup> - and WT mice.

In order to quantitatively compare the expression levels of cyclin E1 and cyclin E2, an absolute measurement of cyclin E1 and E2 using standard curves for both molecules was also performed (Fig. 15). Interestingly, already the basal expression of cyclin E2 in untreated mice was approximately 10fold higher compared to cyclin E1 expression and in the course of liver regeneration the number of cyclin E2 mRNA molecules always exceeds cyclin E1 between 2-10 fold.
Fig. 15: Absolute measurement of cyclin E1 and E2 mRNA level in WT mice. The absolute number of cyclin E1 and E2 molecules was measured using standard curves and normalization to GAPDH expression as indicated on the y-axis.

Next, cyclin E2 mRNA expression in WT and E1\(-/-\) mice was compared (Fig. 14 A). Interestingly, significantly lower levels of cyclin E2 in E1\(-/-\) mice were detected for all investigated time points in comparison to WT animals. This implicates that cyclin E1 may positively regulate cyclin E2 gene expression in liver regeneration. Of notice, E1\(-/-\) mice do not show any induction of cyclin E2 gene expression within the first 36 hours after PH which fits to the delayed expression of PCNA shown in Fig. 8 B.

In contrast, E2\(-/-\) mice expressed more cyclin E1 m-RNA compared to WT controls especially between 40 h and 72 h after PH (Fig. 14 B). Of notice, this is consistent with the time frame of increased cdk2 kinase activity in these animals (compare Fig. 9) thereby implicating that increased cyclin E1 expression in E2\(-/-\) mice contributes to prolonged cdk2 kinase activity and enhanced S-phase initiation.

Many studies demonstrated that the activity of cyclin E/cdk2 complexes might be negatively regulated by CDK-inhibitory proteins of the Cip/Kip family such as p21 and p27 (Albrecht et al., 1998). Therefore p21 and p27 protein expression were investigated in the course of liver regeneration in WT-, E1\(-/-\) - and E2\(-/-\) mice.

During liver regeneration in WT mice following PH, p27 is continuously expressed and only slightly up-regulated at the peak of DNA synthesis (Fig. 16, upper panel), which is consistent with earlier studies. (Albrecht et al., 1998). A
similar expression profile was also found in E1⁻/⁻ animals, although a significant
down-regulation 72 h after PH was evident correlating with prolonged cdk2
kinase activity at this time point. Importantly, depletion of Cyclin E2 resulted in a
strong down-regulation of p27 expression in both quiescent livers and
throughout the whole time course of liver regeneration (Fig. 16, upper panel),
thereby potentially contributing to accelerated and prolonged S-phase
progression.

In WT mice, p21 is tightly regulated during liver regeneration with two
peaks at 36 h and 72 h, respectively (Fig. 16, middle panel). Although E1⁻/⁻ and
E2⁻/⁻ mice also showed a regulated p21 expression, no p21 was detected 72 h
after PH in both strains which correlated with absence of p27 and exceeding
cdk2 activity at this time point.

![Fig. 16: Whole liver cell extracts were investigated for the expression of cell cycle
inhibitors p27 and p21. The expression levels of GAPDH are presented as internal
loading controls.](image)
3.3 Kinetics of DNA synthesis differs in E2\textsuperscript{-/-} and E2\textsuperscript{-/-} mice

All previous experiments indicated that depletion of cyclin E1 delays S-phase entry as measured by onset of PCNA expression and cdk2 kinase activity, whereas cyclin E2 ablation accelerates G1/S-phase transition. Next, S-phase progression in E1\textsuperscript{-/-} and E2\textsuperscript{-/-} mice was studied by measuring \textit{in vivo} incorporation of the thymidine analogue Bromodeoxyuridine (BrdU) into DNA following PH.

In WT mice, the first hepatocytes showing BrdU incorporation were detected 36 h after PH (Fig. 17, A left panel and B). Maximum numbers of hepatocytes in S-phase were found between 40 h and 48 h (26% and 21%, respectively) after surgery (Fig. 17 B). This was correlated with a maximum gene expression of the S-phase marker cyclin A2 at 40 h after PH (Fig. 18 A) and detectable cyclin A2 protein expression between 40 h and 48 h (Fig. 18 B). At later time points (72 h-168 h) the number of BrdU positive cells continuously decreased, and cyclin A expression was no longer detectable, indicating termination of S-phase.

Hepatocytes of E1\textsuperscript{-/-} mice showed retarded S-phase progression starting 40 h after PH (Fig. 17, A medium panel and B), which was consistent with a shift of the S-phase peak to 48 h but a significantly higher level of BrdU positive cells at that time point. Cyclin A2 mRNA expression was detectable from 40 h-72 h after PH in these animals (Fig. 18 A). Most strikingly, maximum gene expression was found not until 72 h and consistent with that, E1\textsuperscript{-/-} mice displayed a prolonged cyclin A2 protein expression from 40 h-72 h compared to the WT group (Fig. 18 B). Altogether these findings show that cyclin E1\textsuperscript{-/-} mice perform a slightly delayed S-phase progression at the beginning of liver regeneration which is then compensated by a prolonged S-phase driven by an extended cyclin A expression.
In contrast E2⁻/⁻ mice showed a completely different pattern of S-phase progression during liver regeneration. At the time point of S-phase entry (36 h post PH) E2⁻/⁻ mice already have significantly more hepatocytes in S-phase (12.5%) compared to the WT group (6.7%, Fig. 17 A and C). Further analysis revealed that for the majority of time points investigated (except 84 h and 168 h post PH) the livers of E2⁻/⁻ mice display a higher number of hepatocytes in S-phase compared to WT animals indicating excessive DNA-synthesis in these mice.

Cyclin A gene expression was prolonged and in overall higher as in controls (Fig. 18 A). Of notice, Cyclin A2 protein expression in E2⁻/⁻ mice peaked at the maximum of S-phase progression as expected (40 h-48 h). However, a basal Cyclin A2 expression was detected at all time points in these mice.
animals, which was not observed in WT or E1^{-/-} mice and might contribute to the strong DNA synthesis in E2^{-/-} mice (Fig. 18 B).

![Graph showing gene expression and protein levels](image)

**Fig. 18:** Protein extracts and mRNA were isolated at indicated time points after PH from livers of E1^{-/-}, E2^{-/-} and WT mice. (A) Analysis of cyclin A2 gene expression was performed via relative real-time PCR. The expression values were normalized to GAPDH expression and calculated as fold induction in comparison to untreated controls at 0 h. (B) Western blot analysis of cyclin A expression in E1^{-/-}, E2^{-/-} and WT control mice at indicated time points after PH. As internal loading control, GAPDH expression is shown.

### 3.3 Ectopic over-expression of cyclin E1 or E2 changes the cell cycle progression in the regenerating liver

Previous data indicated that cyclin E2 might also have antagonist properties for cell cycle progression. To support this hypothesis, a recombinant adenovirus expressing murine cyclin E2 under the control of the CMV-promoter
(adv-CcnE2) was generated. As control, an adenovirus expressing luciferase was used (adv-Luc). WT - and E2^{-/-} mice were injected with these adenoviruses followed by PH 24 h after virus application. E2^{-/-} mice were sacrificed 36 h after PH (strongest difference in S-phase compared to WT), whereas WT mice were investigated at the peak of S-phase (48 h after surgery). Serum transaminases demonstrated only mild toxicity after adenovirus delivery to the liver (data not shown). Semi-quantitative RT-PCR revealed cyclin E2 expression in adenovirus-treated E2^{-/-} mice following PH indicating adenoviral transduction (Fig. 19A). In adv-CcnE2 transduced WT mice, cyclin E2 signals were slightly stronger compared to control animals 48 h after PH (Fig. 19B).

**Fig. 19:** Semi-quantitative reverse-transcription PCR for cyclin E2 from total RNA of (A) E2^{-/-} mice and (B) WT controls. Cyclin E2 expression in adv-CcnE2 transduced animals is indicated by an arrow. GAPDH expression is shown as internal control.

**Fig. 20** Quantitative measurement of cyclin E2 cDNA molecules in WT – and E2^{-/-} mice following adv-CcnE2 transduction and PH. Cyclin E2 gene expression was calculated as number of molecules/10^6 GAPDH.
Absolute quantification revealed that adenovirus delivery induced an approximately 2-fold increase of cyclin E2 mRNA expression in WT mice after PH. In E2⁻/⁻ mice, adv-CcnE2 induced cyclin E2 expression was comparable to WT baseline levels (Fig. 20). This is explained by moderate gene delivery via adv-CcnE2 showing a gradient with most efficient levels in close proximity to portal venules and less evident in peripheral regions (Fig. 21).

![Fig. 21: Determination of adv-CcnE2 transduction efficiency. Liver cryosections of non-transduced (ctrl) or adv-CcnE2 injected (CcnE2) WT mice were sacrificed 48 h after PH and stained with an antibody directed against the adenoviral E1A protein and subjected to immunofluorescence microscopy. Adenoviral transduced hepatocytes are indicated by green staining.](image)

The impact of ectopic cyclin E2 expression was determined by measuring BrdU incorporation. As expected, adv-Luc treated E2⁻/⁻ mice showed stronger S-phase activity compared to WT controls, but similar BrdU incorporation compared to non-transduced E2⁻/⁻ animals (Fig. 22 A) proving that mild virus transduction per se did not affect cell cycle progression. However, adv-CcnE2 delivery significantly reduced the number of E2⁻/⁻ hepatocytes in S-phase from 10.8% to 3.8% supporting the hypothesis that cyclin E2 has antagonistic effects on S-phase entry. Accordingly, adenoviral cyclin E2 expression reduced S-phase activity in WT mice from 19.5% to 10.6% (Fig. 22 B). Of notice, in periportal hepatocytes with highest adenovirus transduction BrdU incorporation was minimal (see Fig. 21).
In a second approach, WT mice were transduced with adv-CcnE1 and analyzed before- and after PH (strongest effect of Cyclin E2 depletion on DNA synthesis compared to WT). As control, an adenovirus expressing luciferase was used (adv-Luc). WT mice were injected into tail vein with adenoviruses and subjected to PH 24 h after virus application.

Relative RT-PCR revealed that in quiescent livers, adenoviral Cyclin E1 delivery already resulted in a 10fold up-regulation of Cyclin E1 mRNA expression which further increased during liver regeneration (Fig. 23 A). The influence of ectopic cyclin E1 over-expression on hepatocytes proliferation in WT animals was identified by BrdU staining (Fig. 23 B).
3. Results

A.

**adv-CcnE1: CcnE1 expression**

36 h PH

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**B.**

**Fig. 23:** Ectopic over-expression of Cyclin E1 in hepatocytes after PH.

(A). WT mice were transduced with adv-CcnE1 (Cyclin E1) or adv-Luc (Luc) where indicated. Cyclin E1 cDNA was measured in virus-transduced and mock-treated mice before – and 36 h after PH.

(B). Determination of BrdU incorporation following adenoviral over-expression of Cyclin E2 in WT - and E2\(^{-}\) mice (green staining) or Cyclin E1 in WT animals (red staining) for time points post PH as indicated. Total nuclei are stained in blue with DAPI. As control, animals were transduced with adv-Luc (Luc). For each group a minimum of three animals was analyzed and quantitative data of five cryosections per animal is shown in the right part of the panel. (*) p < 0.01; (#) p < 0.05.

In consistency with previous data, adv-Luc treated mice 36h after PH shown only slight increase of proliferation (3.1% BrdU-positive cells) (Fig. 23 B).

In sharp contrast, ectopic over-expression of Cyclin E1 in WT animals resulted in substantial higher DNA-synthesis (8.9% BrdU-positive cells, Fig 23B).
3. Results

Taken together these data indicate that over-expression of Cyclin E1 induce S-phase progression in WT hepatocytes after PH and reflects the accelerated onset of S-phase found in E2\textsuperscript{−/−} animals.

3.4 Increased DNA-synthesis in E2\textsuperscript{−/−} mice results in augmented liver mass and improved survival in a model of fatal liver failure

Liver regeneration following PH in mice is characterized by 1-2 rounds of DNA replication in the remnant hepatocytes and compensatory hyperplasia of the remaining liver usually leading to full restoration of the original liver mass approximately 168 h after surgery (Fausto et al., 2006).

To investigate the physiological consequences of the earlier onset of S-phase entry and amplified DNA synthesis, the efficiency of liver regeneration in WT, E1\textsuperscript{−/−} and E2\textsuperscript{−/−} mice was compared by determining the liver weight/body weight ratio of the remnant liver at different time points after liver resection. Of notice, untreated E2\textsuperscript{−/−} mice displayed a slightly reduced relative liver mass, but 24 h after PH any differences in the liver weight index between the three groups was not found (Fig. 24). However, seven days after PH the liver mass in E2\textsuperscript{−/−} animals was 44% higher compared to WT mice (5.2% of body weight compared to 3.6% in WT animals, Fig. 24).

**Fig. 24:** At indicated time points after PH, the remnant livers were extracted and the liver weight index was calculated as the liver weight/body weight ratio in percent.
This was accompanied by a macroscopically visible increase of liver size (Fig. 25) suggesting that cyclin E2 depletion leads to de-regulated hepatocyte growth resulting in hepatomegaly. However, two weeks and four weeks after PH the liver weight index in these animals did not further increase (data not shown) indicating that the liver regeneration process in E2^/- mice is also terminated although at a later time point.

**Fig. 25:** Representative livers from WT, E1^+- and E2^/- mice 168 h after PH are shown. Regenerated livers from E2^/- mice show macroscopically visible increase in size and mass.

To further analyze the effects of accelerated onset of S-phase in E2^/- mice, the model of fatal hepatic failure following 90% hepatectomy was applied to these animals. During this procedure, 100% of WT animals died within 24 h due to acute liver failure (Fig. 26). In contrast, at this time point 80% of the E2^/- mice were still alive and the majority of the animals died not before 40 h after liver resection although eventually none of the mice survived this treatment. This argues that the accelerated and stronger start of DNA synthesis in E2^/- mice might result in a better prognosis after fatal liver injury probably due to improved metabolic capacity in the remnant liver.
3. Results

Fig. 26: Depletion of cyclin E2 provides improved survival after 90% hepatectomy. Kaplan-Meyer survival curve shows time-dependent survival of WT mice (broken line, n=5) and E2−/− mice (solid line, n=5).

3.5 Amplified DNA synthesis in E2−/− mice is not correlated with increased mitotic activity

The next aim was to understand the cellular mechanisms underlying the observed hepatomegaly in E2−/− mice following PH. Increased DNA synthesis might directly result in stronger mitotic activity of hepatocytes and in order to test this hypothesis, the number of mitotic cells in the course of liver regeneration was first investigated by measuring histone H3 phosphorylation in situ. Histone H3 phosphorylation at serine 10 is closely correlated with mitotic chromosome condensation and serves as a marker for early mitosis (Hans and Dimitrov, 2001).

As expected, histone H3 phosphorylation was undetectable in quiescent livers of WT, E1−/− and E2−/− mice. In WT animals the first appearance of mitotic cells was observed 36 h after PH and mitosis reached its maximum 48 h after surgery (Fig. 27 A, B). Of notice, in these animals a second peak of H3 phosphorylation was detected at 84 h after PH (Fig. 27 A, B) which is consistent with the finding, that normal hepatocytes undergo two rounds of mitosis following PH (Trembley et al., 1996). Accordingly, the gene expression profile of cyclin B1, which served as an additional mitosis marker, was also biphasic with expression maxima at 48 h and 84 h after PH (Fig. 28 A) and the corresponding protein expression was evident at comparable time points (Fig. 28 B).
3. Results

Fig. 27: Liver cryosections from WT, E1$^{-/-}$ and E2$^{-/-}$ mice at time points after PH as indicated were analysed for phosphorylation of histone H3 at Ser10 using immunofluorescence microscopy. (A) Representative images at relevant time points are shown. Total nuclei are counterstained with DAPI (blue); green nuclei indicate H3 phosphorylation. (B, C) Calculation of histone H3-positive nuclei (presented in percentage) in liver sections following PH from WT and E1$^{-/-}$ mice (B) and WT and E2$^{-/-}$ mice (C). Each value represents the mean of 5-10 animals.

E1$^{-/-}$ mice revealed a strong mitotic activity between 40 h and 48 h after surgery, but showed significantly reduced mitosis at all other time points compared to WT controls (Fig. 27 B). In particular, these animals lacked a second wave of mitosis as also evidenced by diminished cyclin B mRNA - and protein expression (Fig. 28 A-B) indicating that cyclin E1 ablation negatively affects hepatocyte mitosis.

Surprisingly, no significant difference of the H3 phosphorylation pattern was detected in E2$^{-/-}$ mice compared to controls except that also E2$^{-/-}$ mice lacked the second round of mitotic activity which was found in WT mice (Fig. 27 A, C). Cyclin B protein expression was detectable between 36 h-48 h at a level comparable to controls (Fig. 28 A-B). These findings were completely unexpected and suggest that the accelerated and amplified DNA synthesis found in E2$^{-/-}$ mice does not initiate significantly more mitosis in the course of liver regeneration.
3. Results

Fig. 28: (A) Kinetics of cyclin B mRNA expression in response to PH at indicated time points in E1<sup>−/−</sup>, E2<sup>−/−</sup> and WT mice. The expression values were normalized to GAPDH expression and calculated as fold induction in comparison to untreated controls at 0 h. (B) Analysis of cyclin B protein expression in E1<sup>−/−</sup>, E2<sup>−/−</sup> and control mice (WT) between 0 h and 168 h after PH. GAPDH expression is indicated as internal standard.

### 3.6 Cyclin E1 and cyclin E2 provide complementary functions for endoreplication in hepatocytes after PH

E2<sup>−/−</sup> mice did not show increased mitotic activity after PH although they performed stronger DNA synthesis and developed hepatomegaly. To explain this unexpected phenomenon, the replicated DNA in the regenerating hepatocytes was tracked down by FACS analysis in subsequent experiments.

Following PH in WT, E1<sup>+/+</sup> and E2<sup>−/−</sup> animals, primary hepatocytes were isolated from these mice at 0 h (untreated controls), 48 h (maximum of S-
3. Results

phase), 72 h (late regeneration phase) and 96 h (termination of liver regeneration) and then subjected to FACS analysis after propidium iodid staining. The cell populations were analysed by their DNA content and classified as diploid (2n), tetraploid (4n) and higher polyploid (>4n) hepatocytes (Fig. 29 A-D).

In untreated mice (Fig. 29 A) no major differences were found in the distribution of polyploid hepatocytes in all three groups and in all livers the majority of hepatocytes turned out to be higher than 4n, which indicated that E-cyclins are not essential for promoting polyploidy in the course of liver development. At 48 h after PH first differences in the DNA distribution were observed between WT mice on the one hand and E1−/− and E2−/− mice on the other hand (Fig. 29 B). However, as these mice are still in S-phase progression, this rather reflects differences in DNA synthesis efficiency between the three strains. During the late phase of liver regeneration (72 h) the distribution of >4n hepatocytes in WT - and E2−/− mice was similar (approximately 58%), whereas in E1−/− mice the number of >4n hepatocytes was strongly reduced to 23% (Fig. 29 C) suggesting an impact of cyclin E1 depletion on DNA synthesis or endoreplication.

Of special interest was the analysis of hepatocytes 96 h after PH when liver regeneration and S-phase were thought to be terminated. In WT controls, only a minor subpopulation of hepatocytes (24%) turned out to be diploid after termination of liver regeneration, whereas the majority of cells were tetraploid (40%) or >4n (36%). This result was not unexpected as hepatocytes usually maintain high polyploidy due to their strong metabolic activity (Gupta, 2000) and it has been reported earlier that the polyploidy further increases after PH (Sigal et al., 1999). However, in E1−/− mice a different distribution was found as the majority of hepatocytes (45%) was shown to be diploid whereas only a small subpopulation (25%) was more than tetraploid (Fig. 29 D, E).
3. Results

Fig. 29: Primary hepatocytes from E1<sup>−/−</sup>, E2<sup>−/−</sup> and WT mice were isolated before and at different time points after PH. DNA content of propidium iodide (PI) stained hepatocytes was determined by FACS analysis. The diagrams show the distribution of diploid (2n), tetraploid (4n) and hyperploid (>4n) hepatocytes from three independent experiments as percentage of viable cells. (A) Quiescent hepatocytes from untreated mice. (B) Hepatocytes 48 h post PH (C) 72 h post PH; (D) 96 h hours post PH. (E) Representative FACS histograms for WT, E1<sup>−/−</sup> and E2<sup>−/−</sup> hepatocytes isolated 96 h after PH. On the x-axis the intensity of PI per cell is shown; the y-axis indicates the number of cells showing a distinct PI intensity; gates were set for 2n, 4n and more than 4n hepatocytes excluding the apoptotic cell fraction.

In complete contrast to these findings, E2<sup>−/−</sup> hepatocytes were highly polyploid 96 h after PH with more than 60% of cells showing a ploidy >4n (Fig. 29 D, E, right panel).

The conclusion from this data is that E1<sup>−/−</sup> hepatocytes have a defect in endoreplication during liver regeneration, whereas the excessive synthesized DNA found in E2<sup>−/−</sup> livers following PH could be tracked down in highly polyploid hepatocytes.
3. Results

With FACS analysis, entire hepatocytes were sorted and investigated. Therefore, with this method binuclear cells (which may develop from nuclear division without cytokinesis) cannot be differentiated from mononuclear cells of higher ploidy (as a result of endoreplication). To further investigate, if E1\(^-\) and E2\(^-\) hepatocytes are really affected in endoreplication we next isolated primary hepatocytes from male mice 96 h after PH and subjected them to FISH analysis using a specific probe for the Y chromosome.

![Fig. 30](image)

**Fig. 30**: Hepatocyte ploidy in nuclei of primary hepatocytes 96 h after partial hepatectomy was measured by FISH technique. Males WT, E1\(^-\) and E2\(^-\) mice were subjected to PH. 96 h after surgery, primary hepatocytes were isolated and probed for the Y chromosome (Cy3, red signals). (A) WT hepatocytes. (B) E1\(^-\) hepatocytes. (C) E2\(^-\) hepatocytes. (D) Quantification of polyploidy in primary hepatocytes from WT, E1\(^-\) and E2\(^-\) mice 96 h post PH. For each group, a minimum of 1000 nuclei were analyzed for the number of Y chromosomes. The data was calculated as the percentage of 2n, 4n and >4n nuclei per population. Significance was calculated according to student’s t-test.

In WT cells, the majority of nuclei (approximately 70%) turned out to be diploid (Fig. 30 A, D) and only minor subpopulations (26.2%) were tetraploid or octoploid and higher (7%). This is not necessarily in contrast to our FACS data (Fig. 29 E) but reflects the fact, that WT hepatocytes are frequently binuclear (Mossin et al., 1994; Wheatley, 1972).
Most strikingly, 90% of nuclei from E1⁻/⁻ hepatocytes were only diploid (Fig. 30 B, D) supporting our hypothesis that E1⁻/⁻ hepatocytes are defect for efficient endoreplication. On the contrary, E2⁻/⁻ hepatocytes showed an equal distribution of diploid, tetraploid and higher polyploid nuclei (Fig. 30 C, D) eventually reflecting overall higher polyploidy in E2⁻/⁻ cells. This data demonstrates that cyclin E1 is critical for restoring polyploidy after liver regeneration, whereas cyclin E2 is antagonizing this effect.

In addition, the FISH experiment revealed a direct correlation between the ploidy of a nucleus and its respective size revealing predominantly large nuclei in polyploid E2⁻/⁻ hepatocytes (Fig. 30 C) which might contribute to increased liver mass and hepatomegaly as found in these animals.
3.7 Role of E-type cyclins in liver fibrogenesis

3.7.1 Over-expression of c-myc in hepatocytes induces liver fibrosis in a cyclin E1 dependent manner

Hepatocyte specific alb-myc transgenic animals (alb-myc\textsuperscript{tg}) have been intensivly investigated for their potential to develop hepatocellular carcinomas (Calvisi and Thorgeirsson, 2005). However, the early molecular and cellular changes in the respecting livers leading to inaccurate gene regulation or pathological stages such as liver fibrosis and cirrhosis have not been unveiled. Therefore, as a part of this study, early and time dependent molecular events in alb-myc transgenic livers were investigated in order to specifically address changes in gene regulation related to cyclin E1 and E2.

In alb-myc\textsuperscript{tg} mice (Fig. 31 A) an age dependent increase of the cyclin E1 mRNA expression was detected, which indicate a correlation of cyclin E1 levels and the liver pathogenesis as old mice are highly predisposed to HCC development. In contrast, cyclin E2 expression was down-regulated with an age-dependent tendency (Fig. 31 B).

![Fig. 31: Time dependent expression of cyclin E1 (A) and E2 (B) by quantative real time PCR. Kinetics of cyclin E1 and E2 mRNA expression in alb-myc\textsuperscript{tg} mice at the age of 9, 20 and 58 weeks. The expression values were normalized to GAPDH expression and calculated as fold induction in comparison to Wt controls at 0 h.](image-url)
3. Results

The human pathogenesis leading to the development of hepatocellular carcinoma is mostly induced by a chronic liver injury and inflammation and progresses from liver fibrosis to cirrhosis further resulting in tumor formation. To test, whether HCC development in alb-myc\textsuperscript{tg} mice is also preceded by liver fibrosis and if this might be dependent on Cyclin E1 or E2 expression, Cryosections from 40 week old WT, alb-myc\textsuperscript{tg}, or E1\textsuperscript{-/-} alb-myc\textsuperscript{tg} and E2\textsuperscript{-/-} alb-myc\textsuperscript{tg} mice, respectively, were stained with an collagen I antibody and analysed using immunofluorescent microscopy (Fig. 32). As expected, in WT mice collagen I expression was limited to liver vessels. Interestingly, in alb-myc\textsuperscript{tg} mice increased collagen deposition in the liver with fiber formation was observed. When Cyclin E1 was depleted in these animals, collagen deposition was reduced to almost WT background levels, whereas knocking down Cyclin E2 in alb-myc\textsuperscript{tg} mice resulted in even stronger collagen expression and fiber formation (Fig. 32).

![Image](image.png)

**Fig. 32:** Representative collagen I immunofluorescence at the age of 40 weeks in WT, alb-myc\textsuperscript{tg}, E1\textsuperscript{-/-} alb-myc\textsuperscript{tg}, E2\textsuperscript{-/-} alb-myc\textsuperscript{tg} mice. Fibrillar collagen deposition (red fibers) is detected in alb-myc\textsuperscript{tg} mice, significantly reduced in E1\textsuperscript{-/-} mice and strongly increased in E2\textsuperscript{-/-}.

These findings were further investigated by performing western blot analysis of collagen I protein expression from livers of WT, alb-myc\textsuperscript{tg}, E1\textsuperscript{-/-} alb-myc\textsuperscript{tg} and E2\textsuperscript{-/-} alb-myc\textsuperscript{tg} mice at the age of 20 and 40 weeks, respectively (Fig. 33), thereby confirming that collagen I expression in alb-myc\textsuperscript{tg} mice is clearly increased in comparison to WT animals, which is dependent on cyclin E1 expression and antagonized by cyclin E2.
c-myc transgenic mice

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![Fig. 33: Western blot analysis of collagen I expression in alb-myc<sup>tg</sup>, alb-myc<sup>tg</sup>/ E1<sup>−/−</sup>, alb-myc<sup>tg</sup>/ E2<sup>−/−</sup> and WT control mice at the age of 20 and 40 weeks, respectively. As internal loading control, GAPDH expression is shown.](image)

These results indicated for the first time, that over-expression of c-myc in the liver is not only related to hepatocarcinogenesis, but also to fibrogenesis in a cyclin E1/E2 dependent manner leading to the current hypothesis that c-myc and cyclin E1 are pro-fibrotic factors whereas cyclin E2 potentially antagonizes fibrosis.

Liver fibrogenesis requires chronic liver injury. To further elucidate the mechanisms leading to c-myc dependent fibrogenesis, TUNEL assays and phospho-histone H3 stainings were performed in liver cryosections from alb-myc<sup>tg</sup> mice at different age between 9-40 weeks. One representative example is shown in Fig. 34. Hepatocyte-specific c-myc-transgenic mice showed spontaneous apoptosis with a frequency of approximately 1-2% of all hepatocytes independent of age (Fig. 34A), which was correlated with a compensatory proliferation of the same frequency as shown by increased number of phospho-histone H3 positive hepatocytes indicating mitosis (Fig. 34B).

This continuous apoptosis and subsequent compensatory proliferation in alb-myc<sup>tg</sup> reflects a scenario which is very similar to a mild chronic liver injury and might contribute to the liver fibrosis observed in these animals.
3. Results

3.7.2 Cyclin E1 is an essential pro-fibrotic factor in the liver

The previous results indicated that Cyclin E1 expression is related to liver fibrogenesis in alb-myc<sup>tg</sup> mice which is potentially antagonized by cyclin E2. However, as over-expression of c-myc in the liver is not an established animal model for liver fibrosis so far, the further aim of this study was to evaluate the pro-fibrotic function of cyclin E1 in a well accepted model of liver fibrosis. Therefore, cyclin E1<sup>-/-</sup> and E2<sup>-/-</sup> mice were further characterized in the animal model of carbon tetrachloride (CCL<sub>4</sub>)-induced liver fibrosis.

WT, E1<sup>-/-</sup> and E2<sup>-/-</sup> mice were treated with CCl<sub>4</sub> (0.6 ml/kg body weight in olive oil i.p., twice per week). After 4 weeks of treatment, mice were sacrificed and the progression of liver fibrosis was quantified. As shown in Picrosirius red-stained sections, repeated injections of CCL<sub>4</sub> for 4 weeks induced prominent
fibrosis associated with septum formation in WT animals (Fig. 35 A), whereas in E1⁻/⁻ mice, the extend of fibrosis was considerably reduced (Fig. 35 B). Of notice, there was no significant difference in septum formation between E2⁻/⁻ and WT mice after 4 weeks of CCL₄ application.

![Fig. 35: Picrosirius-red staining of CCL₄ (4 weeks) induced hepatic fibrosis in WT (A) E1⁻/⁻ (B) E2⁻/⁻ (C) mice.](image)

To explore the mechanism underlying the inhibition of liver fibrosis in E1⁻/⁻ mice, additional markers of liver fibrogenesis were investigated. Expression of alpha-smooth muscle actin (α-SMA) is a marker for activated, proliferating hepatic stellate cells (HSC), which was investigated by immunostaining in cryosections from WT and E1⁻/⁻ mice following 4 weeks of CCL₄ application (Fig. 36 A-B). This experiment demonstrated that the number of α-SMA positive cells in E1⁻/⁻ mice was significantly reduced compared to corresponding wild-type controls 4 weeks after CCL₄ treatment.

Moreover, quantitative real-time PCR analysis of collagen I expression revealed that – as expected, in WT animals following CCl₄ treatment the collagen expression was strongly increased (20 times fold induction in
3. Results

Comparison to untreated WT controls). In sharp contrast, E1<sup>-/-</sup> mice showed only a slight increase of collagen I expression (2 times higher compared to background expression, **Fig. 36C**) further demonstrating that inhibition of cyclin E1 protects from liver fibrosis.

**Fig. 36:** Deletion of cyclin E1 reduces HSC proliferation. (A) α-SMA positive HSCs were detected by Immunohistochemistry, (B) RT-PCR analysis of collagen I mRNA expression in response to CCL4 application (4 weeks) in E1<sup>-/-</sup> and WT mice. The expression values were normalized to GAPDH expression and calculated as fold induction in comparison to untreated controls.

Concomitantly with fibrosis progression in WT mice, the level of cyclin E1 mRNA was slightly up-regulated in these animals 4 weeks after CCl4 treatment compared to untreated controls (**Fig. 37A**). As expected, no cyclin E1 expression was detected in E1<sup>-/-</sup> mice, which served as a negative control (**Fig. 37A**).

In contrast, the expression of cyclin E2 was not changed in the WT group following CCl4 treatment (**Fig. 37B**). However, in E1<sup>-/-</sup> mice 4 weeks after CCL4 administration, the expression of cyclin E2 was 2 times higher compared to WT controls (**Fig. 37B**) further supporting the idea, that cyclin E2 counteracts cyclin E1 and might have anti-fibrotic functions.
3. Results

![Graph](image)

**Fig. 37:** Up-regulation of cyclin E1 level in WT mice after 4 weeks of CCL4 treatment. (A) Induction of cyclin E2 in E-/- mice after 4 weeks of CCL4 treatment. (B) Significance was calculated according to student’s t-test (p<0.05).

3.7.3 Inhibition of Cyclin E2 accelerates the onset of liver fibrogenesis

The previous experiments (see chapter 3.7.2) demonstrated that depletion of cyclin E2 had no significant effect on the stage of fibrosis after 4 weeks of CCL4 treatment (compare Fig. 35 A, C). However, during liver regeneration the inactivation of cyclin E2 had the most striking effects during the earliest steps of cell cycle initiation (compare Fig. 8, 17). Therefore, it was also investigated whether depletion of cyclin E2 might have an effect on the earliest steps of liver fibrogenesis. Accordingly, WT and E2-/- mice were treated with CCl4 (0.6 ml/kg body weight in olive oil i.p., twice per week) for a short time span. Two weeks after CCl4 treatment mice were sacrificed and progression of liver fibrosis was analyzed by measuring sirius red staining of tissue sections and collagen I and α-sma mRNA expression, respectively.

The sirius-red staining revealed that two weeks of CCl4 treatment is not sufficient to induce relevant fibrosis or fiber formation in WT mice. In sharp contrast, E2-/- mice already showed clear signs of liver fibrosis with first septum formation (Fig. 38 B). This finding was supported by increased expression of both collagen I and α-sma RNA in these animals compared to the WT control group (Fig 39) suggesting that cyclin E2 depletion results in an earlier onset of liver fibrosis.
3. Results

**Fig. 38:** Depletion of cyclin E2 accelerates early stage liver fibrosis after 2 weeks of CCL4 treatment. Picrosirius-red staining in WT (A), E2⁻/⁻ (B) mice.

**Fig. 39:** Changes in expression profile of collagen I (A) and α-SMA (B) m-RNA after 2 weeks of CCL4 treatment in E2⁻/⁻ and WT mice. The expression values were normalized to GAPDH expression and calculated as fold induction in comparison to untreated controls. Significance was calculated according to student’s t-test.
4. Discussion

4.1 Role of E-type cyclin during liver regeneration

The expression of E-type cyclins sharply peaks at the G1/S restriction point of the cell cycle (Koff et al., 1992) and the canonical model of cell cycle regulation implicated that cyclin E/cdk2 kinase activity might be essential for S-phase entry and subsequent cell cycle progression. Therefore, recent studies demonstrating normal development and cell cycle progression in mice either deficient for cyclin E1 or cyclin E2 were unexpected and implicated redundant mechanisms of cell cycle progression in higher eukaryotes (Geng et al., 2003). However, studies in E1−/−E2−/− embryos revealed an essential function of E-type cyclins in endoreplication of normally highly polyploid trophoblast giant cells (Geng et al., 2003; Parisi et al., 2003). Moreover, E1−/−E2−/− primary embryonic fibroblasts failed to re-enter the cell cycle after cell cycle arrest demonstrating an essential role of E-type cyclins for S-phase entry of quiescent cells.

Liver regeneration following 2/3 liver resection in mice is a powerful experimental model for the study of the G0/G1-S-phase transition of predominantly quiescent hepatocytes. In the priming phase of liver regeneration, cytokines such as TNF, IL-6 and TGFβ drive the activation of mitogens and growth factors, which directly activate cyclin D/CDK4 and cyclin D/CDK6 complexes (Taub, 2004). It was shown that active cyclin D/cdk4/6 kinases may phosphorylate the retinoblastoma protein Rb resulting in release of E2F transcription factors, subsequent transcription of cyclin E and formation of active cyclin E/cdk2 kinase which is believed to eventually drives the hepatocytes from late G1-phase into initiation of DNA synthesis.

As the need of E-cyclins for S-phase entry has to be re-defined, the ultimate aim of this study was to re-evaluate the role of E-type cyclins for the G0/G1-S-phase transition during liver regeneration using the previously described constitutive knockout mice for cyclin E1 and cyclin E2.

_E1−/− mice display only marginal differences in cell cycle progression and show normal liver regeneration_

The present study revealed only a minimal effect of cyclin E1 depletion on the course of liver regeneration following PH. Although E1−/− mice displayed a
slight delay of S-phase entry, this effect was compensated by reinforced DNA synthesis at later time points eventually leading to normal liver mass restoration and liver function. This observation is in agreement with earlier studies showing that cyclin E1 is not essential for cell proliferation per se. Accordingly, it has to be postulated that other cyclins may compensate for loss of cyclin E1 function and potential candidates are cyclin E2 and cyclin A2. Studies directly addressing the function of cyclin E revealed that both cyclins may activate cdk2 (Lauper et al., 1998) and are synchronously expressed during mouse development (Geng et al., 2001) implicating that cyclin E1 and cyclin E2 may provide redundant functions.

Although the current study also shows an exactly identical expression profile of cyclin E1 and E2 during liver regeneration in WT mice, it seems to be rather unlikely that cyclin E2 predominantly compensates for loss of cyclin E1. First, cyclin E2 contributes only little to kinase activity in E1−/− mice during liver regeneration. Even more important, cyclin E2 gene expression is strongly down-regulated in E1−/− mice implicating that cyclin E1 positively controls cyclin E2 presumably via E2F transcription factors. It can be proposed that A-cyclins compensate for loss of cyclin E1 as the prolonged cyclin A protein expression as found in E1+/− mice perfectly matched with the obvious contribution of cyclin A for cdk2 kinase activity in these animals.

*Ablation of cyclin E2 up-regulates cyclin E1 expression and triggers excessive DNA synthesis during liver regeneration*

Based on the initial hypothesis that cyclin E1 and E2 may share redundant functions, present results using E2+/− mice were unexpected. After PH, E2+/− mice showed accelerated S-phase entry, persistent and strong DNA synthesis eventually leading to abnormal liver regeneration and hepatomegaly. This was associated with strong cdk2 kinase activity, higher and prolonged cyclin E1 and cyclin A2 expression and down-regulation of p27.

These data lead to the hypothesis that cyclin E2 is able to negatively control DNA synthesis by negatively regulating cyclin E1 expression thereby restricting cdk2 kinase activity. To test this hypothesis an adenovirus expressing cyclin E2 been constructed. Expression levels of extrinsic cyclin E2 were in the physiological range. Interestingly, in both WT and E2+/− mice the adv-CcnE2 construct was able to block DNA synthesis. Therefore these results further
support initial findings and strengthened the hypothesis that at least in the liver cyclin E2 negatively controls DNA synthesis and thus has antagonistic functions compared to cyclin E1.

Functionally, inhibition of DNA synthesis by cyclin E2 might be achieved on different levels. The experiments performed in the three mouse strains revealed that in E2-/- mice gene-expression and protein levels of cyclin E1 and A2 are strongly up-regulated, which correlates with increased DNA synthesis. This suggests a regulatory crosstalk between cyclins E1, E2 and A2 at the level of gene expression, where cyclin E2 is a negative regulator of cyclin E1 and A2. This concept is supported by the excessive cyclin E2/E1 mRNA expression ratio in WT mice which is strongest in quiescent livers (10:1) and weakest at the proliferation maximum (2:1). Hence, cyclin E2-/- livers express increased levels of cyclin E1 leading to stronger cyclin E1/cdk2 activity eventually explaining excessive S-phase progression.

Therefore these results clearly suggest that cyclin E2 provides inhibitory functions on cyclin E1 and cell cycle progression in vivo although it cannot be exclude that these effects are limited to the regenerating liver.

In this context antagonizing functions have recently also been described for the D-type cyclins D2 and D3 (Rojas et al., 2007). Hence, cyclin D2 and D3 seem to play opposite roles in a model of tumor development with the ability of cyclin D3 to inhibit cyclin D2 expression which eventually leads to reduced cell growth in terms of tumor development. Consequently, the existence of several D- or E-type cyclins might not be relevant for functional redundancy but might be rather necessary to serve regulatory functions for ensuring proper cyclin activity.

**Pathophysiological consequences of dysregulated cyclin E activities**

Normal liver regeneration in E1-/- mice and full liver mass restoration occurred within the normal time frame of seven days, although this study indicated that liver regeneration in E1-/- mice was associated with slightly reduced mitosis. However, restoration of liver mass following PH can even occur without hepatocyte proliferation by hepatocyte hypertrophy (Minamishima et al., 2002). In conclusion, liver regeneration in E1-/- mice is the combined result of the slightly reduced hepatocyte proliferation and some hypertrophy.
In contrast aberrant liver regeneration was found in E2\(^{-/-}\) mice associated with development of hepatomegaly within seven days. However, eventually liver regeneration was also terminated in E2\(^{-/-}\) animals without further increase in liver mass two weeks and four weeks after PH, respectively. Hepatomegaly in E2\(^{-/-}\) mice was related to excessive DNA synthesis, but not increased hepatocyte proliferation suggesting that liver mass was due to aberrant polyploidy. No indication was found that this phenotype might contribute to development of liver disease or impaired liver function. However, it can be suggested that E2\(^{-/-}\) mice may develop a more severe phenotype in a model of chronic liver injury and hepatitis leading to continuous compensatory hepatocyte regeneration probably related to stepwise increase of liver mass and hyperploidy thereby potentially sensitizing the liver to tumor development. Further investigations in more clinical related models will be necessary to investigate this aspect.

However, depletion of cyclin E2 clearly had some benefits in a model of hepatic liver failure following 90% hepatectomy. WT mice died within 24h following 90% hepatectomy as reported earlier (Makino et al., 2005) from acute liver injury associated with hypoglycaemia, substantially increased blood ammonia levels and encephalopathy (Soto-Gutierrez et al., 2006) contributing to the fact that the liver is a detoxificating organ. Interestingly, E2\(^{-/-}\) mice had an improved survival after 90% hepatectomy and perhaps the accelerated onset of DNA synthesis is responsible for this effect eventually leading to faster expression of metabolic enzymes and detoxification. This could be of potential interest for future therapeutic approaches e.g. by using cyclin E2 deficient hepatocytes for cell transplantation or bioartificial liver supports.

**Differential roles of cyclin E1 and cyclin E2 for the control of endoreplication in hepatocytes**

Endoreplication results from duplication of genomic DNA without subsequent cell division and is preferentially found in differentiated cells with high metabolic activity (Edgar and Orr-Weaver, 2001) such as salivary glands of *Drosophila melanogaster* (Rudkin, 1972), trophoblast giant cells in mammals (Barlow and Sherman, 1972) and- although to a lesser extend in hepatocytes. In the latter ones, multinucleation due to nuclear division lacking cytokinesis further increases the cellular DNA content (Gerlyng et al., 1993) and it was
shown that partial 2/3 hepatectomy may further increase hepatic polyploidy (Sigal et al., 1999).

Earlier studies demonstrated that E-type cyclins are indispensable for endoreplication in Drosophila salivary gland cells (Su and O'Farrell, 1998), mammalian trophoblast giant cells and megakaryocytes (Geng et al., 2003; Parisi et al., 2003) and it has been postulated that they might be essential for loading the Minichromosome Maintenance (MCM) complex onto DNA replication origins during this process (Su and O'Farrell, 1998). However, in these studies it was not discriminated between cyclin E1 and E2 as they were considered to be functional redundant. Cyclin E1 and E2 mediate non redundant and even antagonizing roles for endoreplication. In this scenario, cyclin E1 turns out to be the key player for mediating endoreplication and consequently, E1−/− hepatocytes show strongly reduced polyploidy following PH. In contrast E2−/− hepatocytes showed the opposing effect with highly increased DNA content of their nuclei. This observation is not contradictory to the recently observed defect for endoreplication in E1−/−E2−/− cells but rather reflects our finding that depletion of cyclin E2 up-regulates cyclin E1 activity thereby enhancing DNA replication.

In conclusion, the evidence that E-type cyclins E1 and E2 perform different, non-redundant functions during liver regeneration and describe for the first time an individual phenotype for E2−/− mice apart from male sterility. As cyclin E2 negatively regulates E1 activity and S-phase progression, presented results may also have some relevance for further understanding of the mechanisms leading to tumorgenesis.

4.2 Role of E-type cyclins during fibrogenesis

Fibrosis, or scarring of the liver, is a wound-healing response to that engages a range of cell types and mediators to encapsulate liver injury. Alcohol consumption, viral hepatitis and non-alcoholic steatohepatitis are 3 major causes of chronic liver injury, leading to liver fibrosis, cirrhosis and liver cancer (Friedman, 2008).

Activation of resident quiescent hepatic stellate cells into proliferative, contractile and fibrogenic cells and their interaction with hepatocytes and Kupffer cells in liver injury remains a dominant theme driving the field. One
important aim of this study was to investigate the contribution of E-type cyclins for proliferation and differentiation of hepatic cells during fibrogenesis.

**Inverse role of cyclin E1 and E2 in c-myc\(^{tg}\) dependent liver fibrogenesis in mice**

The transcription factor c-myc is a prominent oncogene, but also a well-known regulator of cyclin E which is over-expressed during fibrosis, cirrhosis and hepatocarcinogenesis (Obaya et al., 1999).

**In vitro** evidence clearly shows that overexpression of c-myc in transgenic mice induces spontaneous apoptosis but also in parallel constant and spontaneous mitosis. This observation is in agreement with earlier studies that c-myc plays dual roles and induces not only cell cycle but also acts as activator of apoptosis (Prendergast, 1999). The constant balance between apoptosis and cell cycle progression found in c-myc\(^{tg}\) mice which resulted in a continuous compensatory proliferation is comparable to similar effects during mild chronic liver injury. Therefore, compensatory proliferation appears to have a critical role during myc-dependent fibrogenesis.

It has been shown before that c-myc is a positive regulator of G1-specific CDKs and, in particular, of cyclin E/CDK2 complexes (Amati et al., 1998). It correlates with the finding of this present study, where age-dependent up-regulation of cyclin E1 mRNA has been shown. In contrast, the cyclin E2 mRNA expression pattern revealed down-regulation with an age-dependent tendency. These findings are in good agreement with the overall conclusion of this study that cyclin E2 might also mediate cell cycle inhibitory functions. However, under conditions of c-myc overexpression, hepatocytes are involved in the continuous cell cycle progression which correlates with increase of cyclin E1- and in contrast decrease of cyclin E2 expression.

Expression of collagen 1 is a marker of fibrogenesis and has been found in c-myc\(^{tg}\) mice already at the age of 9 weeks. Interestingly, expression of collagen 1 was significantly reduced in E1\(^{-/-}\)myc\(^{tg}\) mice, while analysis of E2\(^{-/-}\)myc\(^{tg}\) mice revealed stronger collagen 1 expression. These findings were confirmed by **in situ** collagen I immunofluorescent staining. These results lead to the working hypothesis that cyclin E1 is a pro-fibrotic factor involved in liver fibrogenesis whereas cyclin E2 potentially antagonized this effect.
The role of E-type cyclins and G1-S phase transition in CCL4 induced hepatic fibrogenesis

Carbon tetrachloride (CCL4) is a hepatotoxin which induces liver fibrosis after repetitive application at a low dose. In the liver, CCl3 radicals are formed, which induce hepatocytes damage and activation of hepatic stellate cells, leading to the production of extracellular matrix (Weiler-Normann et al., 2007).

By using an in vivo model of CCL4-induced liver fibrosis, it was demonstrated in this study that depletion of cyclin E1 leads to the reduction of fibrosis extent. Consistently, a decrease of collagen 1 expression has been shown on the mRNA expression level and by in situ analysis. In good correlation with these findings, the number of αSMA positive cells was significantly reduced in E1−/− mice compared to controls. Of notice, up-regulation of cyclin E1 has been found in WT mice after 4 weeks of CCL4 treatment, which indicates that cyclin E1 is an essential factor for the transition of hepatic cells through the cell cycle during fibrogenesis. All together, these findings define for the first time the importance of tight cell cycle regulation for liver fibrogenesis. In conclusion, depletion of E1 in cyclin E1−/− mice mediates the inhibitory effect on liver fibrogenesis in the CCl4 model.

In contrast, after 4 weeks of CCl4 treatment E2−/− mice displayed strong fibrosis, revealed prominent collagen fibre formation in Sirus red staining and increased levels of α-SMA mRNA. Interestingly, cyclin E1 mRNA levels were 30 times higher in E2−/− mice compared to WT animals. In summary, CCl4 treatment at the absence of cyclin E2 leads to prominent fibrosis similar to effects in WT animals. Most likely, the key player of hepatocytes cell cycle progression during fibrogenesis is cyclin E1.

Liver regeneration in E2−/− mice after PH showed earlier onset of DNA synthesis compared to WT mice. Based on this finding, it was investigated if cyclin E2 depletion might also affect the onset of liver fibrosis. Therefore, a short term (2 weeks) CCl4 administration was performed in E2−/− mice. Interestingly, obvious signs of fibrosis were already found in the E2−/− group while in WT mice only first slight indications of fibrogenesis could be detected. RNA levels of αSMA and collagen 1 in E2−/− mice were also significantly higher compared to the WT group. These findings indicate that depletion of cyclin E2 results in earlier, but not in stronger liver fibrogenesis after CCL4 treatment. It
can be speculated that during liver fibrogenesis both cyclin E1 and cyclin E2 inversely regulate the proliferation of those hepatic cells, which are responsible for formation of extracellular matrix, and the most likely candidates are the hepatic stellate cells.

In conclusion, depletion of cyclin E1 mediates inhibitory effects on liver fibrogenesis in the CCl_4 model, while E2 is antagonizing these affects and acts antifibrotic.
5. References


Boonstra, J. (2003). Progression through the G1-phase of the on-going cell cycle. Journal of cellular biochemistry 90, 244-252.


D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature 384, 470-474.


5. References


6. Summary

E-type cyclins are important regulators for driving quiescent cells into the cell cycle. The aim of the present study was to investigate the relevance of cyclin E1 and E2 for directing quiescent hepatic cells into the cell cycle in vivo.

In the partial hepatectomy (PH) model it was shown that cyclin E1\(^{-/-}\) deletion results in normal liver regeneration with slight delay of G1/S-phase transition but absent endoreplication in hepatocytes. In contrast, cyclin E2\(^{-/-}\) mice showed over-expression of cyclin E1 and prolonged cdk2 kinase activity leading to earlier and sustained DNA synthesis. Higher DNA synthesis in cyclin E2\(^{-/-}\) mice did not result in significant more hepatocyte mitosis and proliferation, but was associated with higher polyploidy in the dividing hepatocytes due to endoreplication. Consistently, cyclin E2\(^{-/-}\) mice showed a 45% higher liver/body weight ratio compared to WT animals after regeneration as a result of excessive polyploidization. In summary, this data suggests a new and unexpected role for cyclin E2 in repressing cyclin E1 function thus explaining increased S-phase entry and excessive endoreplication of cyclin E2\(^{-/-}\) hepatocytes as a result of cyclin E1 over-expression. Moreover, this study demonstrates that cyclin E1 is dispensable for the onset of S-phase, but essential for endoreplication during liver regeneration.

The second aim of this study was to investigate a potential contribution of E-type cyclins for liver fibrogenesis. In this context, over-expression of the proto-oncogene c-myc in murine hepatocytes was shown to serve as an interesting new model for liver fibrosis which is dependent on cyclin E1 and E2. Accordingly, alb-myc\(^{tg}\) mice are prone to spontaneous liver fibrosis which can be inhibited by depletion of cyclin E1, but is even enhanced with a knockout of cyclin E2. In the established animal model of CCl\(_4\) – induced liver fibrosis it was demonstrated that cyclin E1 is an essential pro-fibrotic factor, whereas Cyclin E2 provides inhibitory functions on the early onset of liver fibrogenesis.

In summary, this study shows for the first time a phenotype for cyclin E1 and E2 knockout mice and provides evidence for non-redundant, individual functions of both E-type cyclins during cell cycle progression and endoreplication.
6. Zusammenfassung

E-Cycline sind wichtige Zellzyklusregulatoren und steuern die Transition von ruhenden Zellen in die Zellzyklusprogression. Ziel der vorliegenden Arbeit war es, die Bedeutung der Cycline E1 und E2 für die Zellzyklusaktivierung ruhender Leberzellen *in vivo* zu untersuchen.


Zusammenfassend zeigt diese Arbeit erstmals einen Phänotyp für Cyclin E1- und Cyclin E2 knockout Mäuse und weist nicht-redundante, individuelle Funktionen beider E-Cycline im Rahmen der Zellzyklusprogression und Endoreplikation nach.
### 7. Appendix

#### 7.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>adv</td>
<td>Adenovirus</td>
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<tr>
<td>alb</td>
<td>Albumin</td>
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<td>APS</td>
<td>Ammoniumpersulfate</td>
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<tr>
<td>ATP</td>
<td>Adenosin-Triphosphat</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<td>Cdc2</td>
<td>Cell division cycle 2</td>
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<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Carbon tetrachloride</td>
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<tr>
<td>Cdk</td>
<td>Cyclin-dependent kinases</td>
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<td>cDNA</td>
<td>Copy-DNA</td>
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<tr>
<td>dNTP</td>
<td>Desoxy-Nukleosidtriposphat</td>
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<td>DAPI</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>Dithiotreitol</td>
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<td>E1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>knock-out mice for cyclin E1</td>
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<td>knock-out mice for cyclin E2</td>
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<td>EDTA</td>
<td>N,N,N',N' Ethylenediamintetraacetat</td>
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<tr>
<td>EGTA</td>
<td>Ethylen glycol-bis (2-aminoethyl)- N,N,N',N'- tetraacetate</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EtBr</td>
<td>Ethidiumbromide</td>
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<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorter</td>
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<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<td>Histone 3</td>
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<td>Horseradish peroxidase</td>
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<td>HSC</td>
<td>Hepatic stellate cells</td>
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<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
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<td>HEPES</td>
<td>2-[4-2-hydroxyethyl-1-piperazinyl]-ethansulfon acid</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>Immunoglobulin</td>
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<td>Mouse Embryonic Fibroblast</td>
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<td>Nuclear factor-kappa B</td>
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<td>PH</td>
<td>Partial Hepatectomy</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonylfluorid</td>
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<td>Phosphate buffered saline</td>
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<td>Proliferating Cell Nuclear Antigen</td>
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<td>RT</td>
<td>Reverse Transcription</td>
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<tr>
<td>S-phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfat</td>
</tr>
<tr>
<td>αSMA</td>
<td>alpha Smooth muscle actin</td>
</tr>
<tr>
<td>tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethyldiamin</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
7.2 Publications:

Part of this work has been published in advance as scientific articles, scientific abstracts, posters and oral presentations as indicated below:

Original Papers:


Scientific Talks:


Posters


7.3 Acknowledgements

✓ In the first place I would like to express my sincere gratitude to Dr. Christian Liedtke for his supervision, advice and PATIENT guidance.

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7.4 Erklärung zur Datenaufbewahrung

7.5 Curriculum Vitae

Personal Information:

Name: Yulia Alexandrowna Nevzorova  
Date of birth: 27. Juni 1980 in Arkhangelsk, Russia  
Nationality: Russian  
Marital Status: single  
Current address: Wilhelmstrasse 59  
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Current position:  

since 10/2005  scientific associate at the University Hospital Aachen,  
Department Internal Medicine III (Director: Prof. Dr. C.Trautwein).  
Participant in the Ph.D. program at the University Hospital Aachen, (Medizinischen Fakultät der Rheinisch-Westfälischen Technischen Hochschule Aachen (RWTH).  
Thesis: “Function of Cyclin E1 und E2 in liver regeneration and carcinogenesis”

International experience:  

10/2004-09/2005  Scientific training in basic methods of molecular biology at the  Department of Gastroenterology, Hepatology and Endocrinology, Hanover Medical school, Germany  
09/2004-09/2005  Grant from the German Academic Exchange Program (DAAD) for a scientific training in Germany  
Program: Two weeks – language course of German in University of Applied Sciences, Emden, Germany (Fachhochschule Emden)  
Two months intensive course of German, Bildungsverein Hannover  
Two weeks practical training as doctor assistant, Department of Gastroenterology, Hepatology and
Endocrinology, Hanover Medical school, Germany (MHH)  
(Direktor: Prof. Dr. M.P. Manns)

Education:

07/2004-09/2004  
Resident in Infection diseases, Department of Infection diseases, Northern State Medical University, Arkhangelsk

09/2003-07/2004  
Internship in Infection diseases, Department of Infection diseases, Northern State Medical University, Arkhangelsk

1997-2003  
Study of medicine at the Northern State Medical University, Arkhangelsk, Russia (graduation with honour degree)

1987-1997  
Secondary school Arkhangelsk, Russia ( finishing with Silver medal)

Additional education:

05/2003  
Seminar „Five day Seminar in Basic Epidemiology“, Institute of Community Medicine, University of Tromsø, Norway

Languages:  
English - very good (spoken and written)  
German – satisfactorily (spoken)

Awards and grants

2006: Travel Grant by the GlaxoSmithKline Foundation for the participation at the AASLD Liver Meeting in Boston, MA

2007: Easl Young Investigators’ Bursary

2008: Easl Young Investigators’ Bursary

2008: Travel Grant by the GlaxoSmithKline Foundation for the participation at the AASLD Liver Meeting in San Francisco, CA