

***Progression of chemically induced Hepatocellular
Carcinoma in mice is suppressed by Caspase-8
through non apoptotic mechanisms***

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Abbreviations

ABC	Avidin-Biotin Complex
AIF	Apoptosis-inducing Factor
ALT	Alanine-aminotransferase
APAF-1	Apoptosis activating Factor-1
APC/C	Anaphase promoting complex/cyclosome
APS	Ammoniumperoxodisulfat
AST	Aspartat-Aminotransferase
ATP	Adenosintrisphosphat
BSA	Bovine Serum Albumin
°C	Degree Celsius
CAD	caspase-activated DNase
Casp8	Caspase-8
Casp8 ^{ff}	Caspase-8 wild type
Casp8 ^{Δhepa}	Caspase-8 knockout
CD	Cluster of differentiation
cDNA	Complementary DNA
CDI	Cyclin dependent kinase inhibitor
CDK	Cyclin dependent kinase
Cip	Cdk interacting protein
CK	Cytokeratin
Da	Dalton
DAB	3,3'-diamino benzidine
DAPI	4,6-diamino-2-phenylindole
DEN	Diethylnitrosamine
DEVD	Caspase 3 specific substrate
DMBT 1	Deleted in Malignant Brain Tumors 1
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylendiamintetraacetic acid
EGTA	Ethylene glycole tetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	hour
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HE	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase

HrtA2	High temperature requirement A 2
I κ B	See Nfkbib
ICAD	Inhibitor of caspase-activated DNase
IKK	I κ B kinase complex factors
IL	Interleukin
i.p.	intraperitoneally
JNK	c-Jun N-terminal kinase
k	kilo
KCl	Potassium chloride
Kip	Kinase inhibitory Protein
L	liter
μ -	micro (10^{-6})
m-	mili (10^{-3})
m	meter
M	molar
min	minute
MOMP	Mitochondrial outer membrane permeabilization
MOPS	Morpholinopropansulfon
MPS	Mononucleous phagocytes system
MPT	Mitochondrial permeability transition
mRNA	Messenger RNA
NaF	Sodium fluoride
NASH	Non-alcoholic steatohepatitis
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
Nfkbib	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
NK	Natural killer cells
NKT	Natural killer-like T-cells
OD	Optical density
PBS	Phosphate buffered saline
PBST	PBS-Tween
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PH	Partial hepatectomy
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
RIP1	Ribosome inactivating protein 1
RNA	Ribonucleic acid
rpm	Rotations per Minute
RT	Room temperature

s	second
SDS	Sodium dodecyl sulphate
SMAC	Second activator of mitochondrial apoptosis
TE	Tris-EDTA-Buffer
TAE	Tris Acetate EDTA Buffer
TBE	Tris Borate EDTA Buffer
TBS	Tris buffered saline
TEMED	N',N,N',N'-Tetramethyldiamine
TGF-beta	Transforming growth factor beta
TNF	Tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
Tris	2-Amino-2(hydroxymethyl)-1,3-propandiol
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tween 20	Polyoxyethylensorbitanmonolaureat
UV	ultraviolet
V	Volt
v/v	Volume per Volume
WT	Wild type

1 Introduction

1.1 The liver - an important gland of the gastrointestinal tract

1.1.1 The liver's most important task is its major risk: detoxification

Beside the pancreas, the liver is the most important gland of the gastrointestinal tract and with a weight of 1.4 to 1.8 kg the biggest gland of the human body. As a central metabolic organ, it is supplied with aliment of the digestive system, which is processed into several nutrients like glycogen, plasma proteins and fatty acids. In addition to its functions in metabolism, biosynthesis, excretion and secretion the liver is essentially involved in detoxification of absorbed substances (Malhi et al. 2006). Accordingly, the liver is continuously exposed to toxic noxae resulting in a high risk of hepatocytes for undergoing cell death (MLP Duale Reihe, Anatomie, 2007). As a marker for liver damage, activation of Alanine-Aminotransferase (ALT) and Aspartat-Aminotransferase (AST) can be determined. While ALT is a very specific liver enzyme, AST levels can also be additionally elevated in heart, kidney and skeletal muscle tissue. However, increased AST values also serve as an indicator for severe injury due to its main location in mitochondria (Malhi et al. 2010).

1.1.2 Induction of liver injury triggers compensatory hepatic regeneration

Different cell types are located in the liver. Hepatocytes are the parenchymal cells of the liver and constitute the largest hepatic cell population. Hepatocytes are polyhedral cells with a diameter of 20 – 30 µm consisting of two to four nuclei. Hepatocytes have two poles: the apical one, which forms the gale capillary side, and the basolaterale surface, which turns either to different hepatocytes or to the “space of Disse” which is a with blood-plasma filled space between the discontinuous endothelium and the hepatocytes (Lüllmann Rauch 2003). Under healthy conditions, only 0.001 – 0.01 % of hepatocytes proliferate at any given time (Diehl & Rai 1996, Michalopoulos & DeFrances 1997). In cases of liver injury such as surgical partial hepatectomy (PH) or toxic cell death, hepatocytes transiently leave their state of quiescence and start to

proliferate until the original liver mass is restored. This unique compensatory capacity of the liver is actually not a true regeneration as the liver morphology is not fully restored, but rather reflects a compensatory hyperplasia (Koniaris et al. 2003). In addition, after acute liver injury, as triggered for example by toxic substances, hepatic stem cells are sometimes involved in tissue repair (Michalopoulos 2007). These stem cells are termed Oval cells and are located in a single layered epithelium surrounding the Canals of Hering which is a continuation of gale fluid leading Canaliculi biliferi. Moreover, two other important cell types are located in the liver: Kupffer-cells are defined as the resident macrophages in the liver. They belong to the mononucleous phagocytes system (MPS) which is associated to the sinusendothelial cells and have the ability to phagocytize bacteria, cell debris or aged erythrocytes. Ito-cells are fat-saving hepatic stellate cells situated in the Space of Disse. These cells can be activated upon liver injury and transactivate into collagen-producing myofibroblasts, which is the basis for formation of liver fibrosis and cirrhosis (MLP Duale Reihe, Anatomie 2007).

1.2 Apoptosis and necrosis in the liver – different triggers, but similar outcomes

Two mechanisms, apoptosis and necrosis, are frequently involved in acute and chronic liver injury. While necrosis has classically been viewed as an accidental and un-regulated form of cell death, apoptosis is a precisely regulated and genetically determined process that can be induced via death receptor-mediated, extrinsic pathways or through intrinsic mechanisms activated by intracellular stress. Due to its strict genetic regulation, apoptosis has been assigned a function for the directed elimination of dysfunctional or even dangerous cells (Liedtke 2011).

1.2.1 Extrinsic and intrinsic apoptosis signaling pathways

Apoptosis is a process of tightly coordinated cell death which is morphologically characterized by cell membrane blebbing, shrinkage, chromatin condensation and nuclear fragmentation. As a result of apoptosis apoptotic bodies are formed. These cellular leftovers are subsequently phagocytised by

Kupffer cells (Canbay et al. 2003a, Canbay et al. 2003b, Johansson et al. 2003).

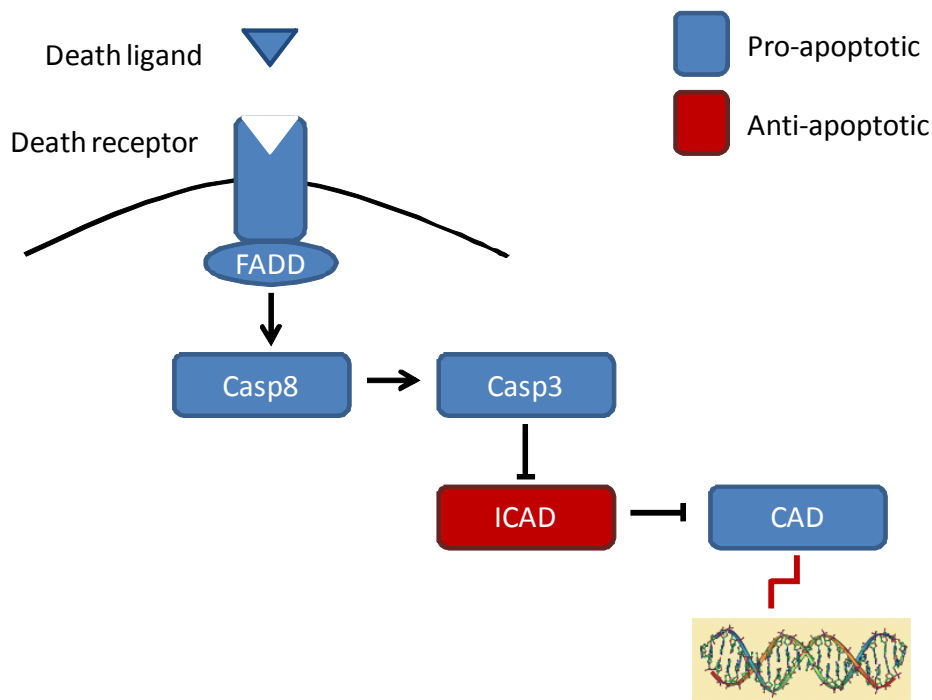


Figure 1.1: Extrinsic pathway of apoptosis. In extrinsic apoptosis, the signal is mediated through external death ligands and ligand-receptor interaction resulting in activation of Casp8 and subsequent proteolytic activation of effector caspases such as Caspase 3 (Casp3). In turn, Casp3 degrades several proteins that are important for cell survival. As an example, the Inhibitor of caspase-activated DNase (ICAD) is shown which is converted to caspase-activated DNase (CAD) by Casp3 allowing efficient DNA degradation.

In contrast to necrosis, apoptosis is triggered by specific stimuli such as the TNF superfamily of death ligands (e.g. TNF, Fas, TRAIL). Two apoptotic signaling pathways have been defined depending on the inducing stimuli: in the extrinsic signaling pathway, apoptosis is induced by external signals such as the death ligands Fas, TRAIL or TNF (Malhi et al. 2006). Binding of these ligands to their cognate death receptors leads to the induction of cysteine-dependent aspartate specific proteases (caspases) (Omata et al. 2007). The apical caspase to be activated is the initiator caspase, Caspase-8 (Casp8). However, besides its relevance for inducing apoptosis, Casp8 may also regulate several other cellular functions such as proliferation, differentiation and NF- κ B-activation (Maelfait & Beyaert 2008). Recent data also suggested a novel function of Casp8 for the prevention of an alternative mode of cell death termed necroptosis (Liedtke et al. 2011, Oberst & Green 2011) which could

contribute to tumor suppressive properties. Nevertheless, the main function of initiator caspases such as Casp8 is the activation of downstream executor caspases such as Caspase 3, 6 and 7. These executor caspases eventually induce the actual process of cell death by proteolytic degradation of proteins involved in maintenance of cell architecture, homeostasis, survival and DNA stability (e.g. ICAD) (Taylor et al. 2008).

Alternatively, apoptosis can be induced through intrinsic mechanisms. Intracellular stress (e.g. DNA-damage) may induce pro-apoptotic members of the Bcl-2 protein family (e. g. Bax and Noxa) resulting in mitochondrial outer membrane permeabilization (MOMP). Several activators of intrinsic apoptosis such as cytochrome c, *second activator of mitochondrial apoptosis* (SMAC), endonuclease G, high temperature requirement A2 (HrtA2) and apoptosis-inducing factor (AIF) are released into the cytosol (Rasola & Bernardi 2007). Cytochrome c release into the cytosol promotes formation of a protein complex termed apoptosome consisting of Caspase 9 and apoptosis activating factor-1 (APAF-1) (Danial & Korsmeyer 2004, Green & Kroemer 2004). This complex eventually activates Caspase 9 and subsequently results in the activation of executor caspases and cell death.

It has been proposed that the main function of apoptosis might be the elimination of injured cells. For instance, gamma irradiation can lead to DNA damage, genetic instability and thus to malignant transformation of cells. Several mechanism exist to repair DNA damage (e.g. activation of tumor suppressor protein p53 (Selivanova & Wiman 1995) or p27 (Qin & Tang 2002)). However, if the DNA repair machinery fails, the injured cells can alternatively be driven into p53-dependent, intrinsic apoptosis (Cheah & Looi 2001, Wang et al. 2003). Therefore, p53 has been termed “guardian of the genome” (Lane 1992). Accordingly, p53 mutations are frequently associated with cancer and are considered a predisposition factor of hepatocarcinogenesis (Hagiwara et al. 2006).

1.2.2 Mechanisms and morphology of necrotic cell death

In contrast to apoptosis, functions and pathways related to necrosis are less understood. Necrosis is the result of oncosis, which is characterized by

cellular swelling and cell blebbing eventually leading to membrane breakdown (Lemasters et al. 1981). Necrosis is a consequence of metabolic injury, such as acute drug-induced hepatotoxicity or ischemia, resulting in ATP depletion (Malhi et al. 2006). This leads to a metastable state including mitochondrial depolarization, lysosomal breakdown and failure of ionic pumps (Gores et al. 1989) which results in cell dissolving and finally in cell death. In contrast to apoptosis, the dead cells and debris resulting from necrosis are not phagocytised by macrophages, but rather induce an inflammatory response e.g., by stimulating Toll-like receptors (TLR) (Naugler & Karin 2008). It has been suggested that necrosis-induced inflammation could mechanistically link necrosis with tumor growth (Vakkila & Lotze 2004) by inducing pro-inflammatory cytokines such as interleukin-6 (IL-6) or TNF, which also play a crucial role in tumorigenesis (Naugler & Karin 2008). Interestingly, several triggers such as oxidative stress can induce both modes of cell death, e.g., apoptosis and necrosis, respectively (Malhi & Gores 2008). However, the molecular switch between these two cell death pathways is largely unknown so far.

1.3 The experimental animal model of Diethylnitrosamine-induced tumorigenesis

Diethylnitrosamine (DEN) belongs to the substance group of the nitrosamines and has been characterized as a carcinogenic reagent. DEN is metabolized predominantly in the liver into an alkylating agent inducing DNA mutations (Verna et al. 1996) and hepatocyte death (Farber & Gerson 1984). Briefly, DEN is hydroxylated by cytochrome P450, an enzyme with the highest activity in the centrilobular hepatocytes, into α -hydroxynitrosamine (Verna et al. 1996). The acetaldehyde residue is cleaved and an electrophilic ethyldiazonium ion is formed. This ion is the trigger for DNA damage by reacting with DNA-bases (Heindryckx et al. 2009). Treatment of mice with DEN for several weeks rapidly induces tumorigenesis (Bralet et al. 2002), and causes formation of hepatocellular carcinoma (HCC) with high incidence in male mice (90 - 100 %) and to a much lesser extend (10 – 30 %) in female mice (Naugler et al. 2007). Several studies indicated that DEN induces liver cancer in a dose-dependent manner (Williams et al. 2000). Initially, DEN has been described as an agent solely inducing HCC. However, more recent studies indicate that DEN may also

induce pro-inflammatory pathways in the liver. In this context, Kupffer cells are activated on the basis of the DEN-induced hepatocellular damage. These cells produce hepatomitogens such as IL-6 promoting compensatory proliferation of surviving hepatocytes (Naugler et al. 2007, Maeda et al. 2005). Moreover, recent studies showed that DEN also triggers chromosomal instability – and down-regulation of tumor suppressor genes such as Runx3 and NrOb2/Shp, which potentially further triggers tumor initiation (Aleksic et al. 2011).

1.4 Mechanisms of Hepatocarcinogenesis

Hepatocarcinogenesis proceeds through a multi-step process that can best be monitored by histological analysis. Basically, two models have been postulated to explain the origin of hepatic cancer cells: Multiple evidences suggested that genetic or epigenetic modification of mature hepatocytes may lead to malignant transformation and subsequent development of liver cancer. Alternatively, some evidence exists that HCC may also originate from cancer stem cells (Mishra et al. 2009). These two hypotheses are supported by two common theories: Knudson's two hit hypothesis and the cancer stem cell theory. In 1953, Knudson's hypothesis postulated that a first pro-tumorigenic hit (e.g. a mutation) can result in formation of dysplastic nodules. These are characterized by abnormal cytological features including clear cell changes and nuclear crowding. A second hit may then lead to marked genomic instability with loss of the tumor suppressor gene p53 defining the time point of malignant transformation into a HCC cell. During hepatocarcinogenesis, dysplastic tissue first forms well differentiated tumors, which can be modulated into a moderately differentiated or a poorly differentiated HCC. The latter one represents the most malignant form of primary liver cancer. With the loss of differentiation, the potential of invading the surrounding fibrous stroma and vessels as well as the capacity to form metastasis increase (Farazi & DePinho 2006).

A proto-typical example for the development of HCC is the chronic viral hepatitis induced by HBV- or HCV-infection. Viral hepatitis typically results in chronic hepatic cell death (apoptosis or necrosis) and subsequent continuous cycles of compensatory proliferation. As a negative side effect of chronic liver injury and compensatory proliferation, the hepatic tissue repair ultimately results in formation of liver fibrosis and cirrhosis. At this stage, hyperplastic

nodules can consist of focal regenerating hepatocytes with normal cytological features, but are meant as a potential first step towards HCC. Continuous liver regeneration can result in genetic or epigenetic alterations such as activation of oncogenes or inactivation of tumor suppressors which represents Knudson's second hit (Spangenberg et al. 2009).

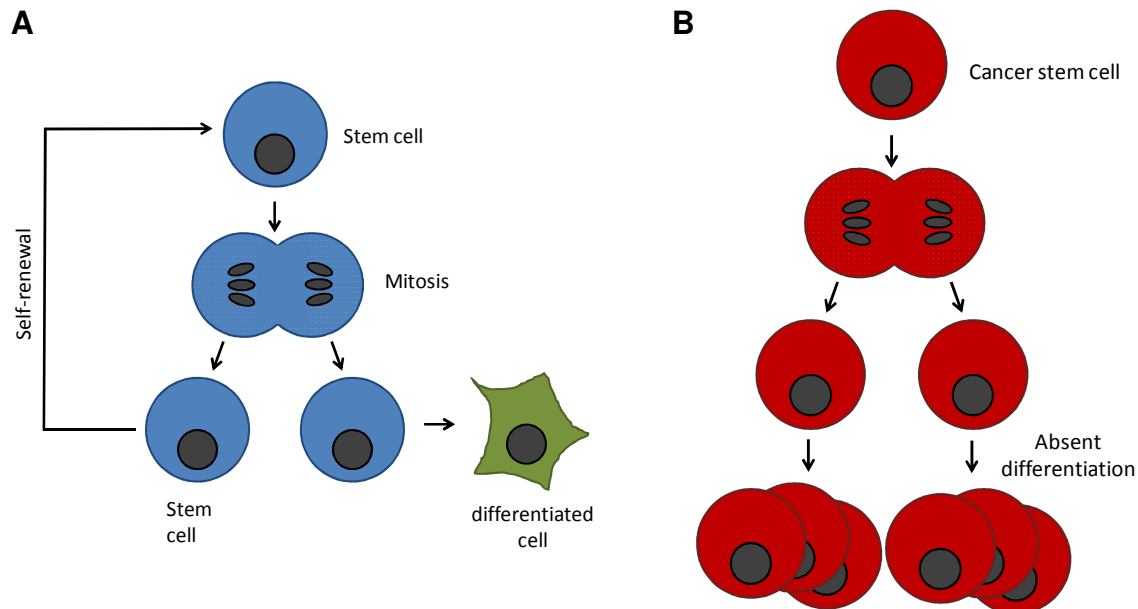


Figure 1.2: A. Normal stem cell pathway. B. Cancer stem cell theory. In healthy conditions, pluripotent cells (blue) divide into two daughter cells. One stays pluripotent, whereas the other one differentiates into a specific cell (green). The cancer stem cell theory postulates a deregulation of pluripotent cells: Differentiation fails and aberrant stem cells (red) do not stop to proliferate.

In contrast to the 2-hit hypothesis, the cancer stem cell theory postulates that tumors originate from the transformation of progenitor cells or tissue stem cells (Gupta et al. 2009). Stem cells show two special features: they have the ability for self-renewal and the capacity to differentiate into specific cells of the tissue of origin (Trosko & Chang 1989). Stem cells are immortal until they differentiate. Under normal conditions, an activated stem cell divides into two daughter cells with one differentiating into a specialized cell, while the other one remains pluripotent (Figure 1.2 A).

Proliferation of the stem cell pool without differentiation may increase the potential of malignant degeneration (Figure 1.2 B). It has been postulated that tumor initiation prevents the differentiation of stem cells and could therefore be the first step in tumorigenesis (Potter 1978, Trosko & Chang 1980).

Several modes of acute liver injury, such as aflatoxin intoxication or DEN-treatment may not only lead to hepatocytes proliferation, but also to activation of liver stem cells (Michalopoulos 2007). Interestingly, 28 - 50 % of patients with HCC contain cells expressing markers of hepatic progenitor cells (oval cells) such as CD133+ or cytokeratin 19 (CK19) (Roskams 1998, Roskams et al. 2006, Zhou et al. 2007). CD133+, also known as Prominin 1, is a five-transmembrane cell surface glycoprotein (Miraglia et al. 1997, Yin et al. 1997, Shmelkov et al. 2005). CD133+ positive stem cells seem to be involved in tumorigenesis in brain, prostate, and colon cancer (Singh et al. 2003, Singh et al. 2004, Hemmati et al. 2003, Richardson et al. 2004, O'brien et al. 2007, Ricci-Vitiani et al. 2007). Recently, it has been suggested that CD133+ positive stem cells isolated from bone marrow are able to repopulate up to 10 % of the normal liver when transplanted (Gehling et al. 2005, am Esch et al. 2005). Moreover, several studies suggested that CD133+ positive cells isolated from HCC tissue showed significant tumorigenic capacity (Ma et al. 2007, Yin et al. 2007). Using human HCC cell lines, it was demonstrated that CD133+ expression defines an aggressive cancer stem cell phenotype. In the adult murine liver, CD133+ expression identifies a population of oval cells with the gene expression profile and function of primitive, bipotent liver stem cells (Ma et al. 2007, Yin et al. 2007, Fujii et al. 2010, Tsuchiya et al. 2009).

1.4.1 DMBT1 – a multi-functional protein with a putative role for oval cell differentiation in the liver

Very recent data indicated a mechanistical link between a population of liver stem cells (Oval cells), hepatitis B virus-related liver diseases and de-regulation of a poorly investigated protein called Deleted in Malignant Brain Tumors 1 (DMBT1) (Deng et al. 2012). DMBT1 is de-regulated in several cancers (Mori et al. 1999, Takeshita et al. 1999, Wu et al. 1999, Mollenhauer et al. 2004) including medullablastoma (Mollenhauer et al. 1997). In addition, two further functions of DMBT1 have been discussed: First, DMBT1 affects innate immunity and mucosal protection (Tynan et al. 2005) by interacting with pathogens (e.g. streptococci, helicobacter, influenza viruses) and mucosal defense proteins (e.g. IgA and surfactant proteins) (Ligtenberg et al. 2007). More importantly, DMBT1 seems to be involved in epithelial and stem cell

differentiation (Tynan et al. 2005). In line with these conclusions, DMBT1 seems to have a direct effect on differentiation of oval cells into hepatocytes (Deng et al. 2012).

1.4.2 Tumor development involves de-regulation of cell cycle regulators

The current accepted hypothesis postulates that tumors arise through a multistep process involving (i) generation of somatic mutations and (ii) de-regulation of tumor suppressor genes and oncogenes (Fearon & Dang 1999). Many tumor suppressor genes encode proteins known to limit cell cycle activity. For instance, proteins of the INK4 (Inhibitor of Cdk4) family and the cip/kip family (CDK interacting protein/Kinase inhibitory protein) including p57, p21 and p27 inhibit cyclin-dependent kinases (CDKs) which are essential for cell proliferation (Malumbres & Barbacid 2005). Accordingly, due to their inhibiting function, CDK-inhibitors (CDIs) may act as tumor suppressors and a loss-of-function mutation of CDIs could lead to an up-regulation of CDKs, which may endanger for a higher risk of tumorigenesis. Besides its function in halting cellular proliferation, p21 also promotes differentiation and cellular senescence (Abbas & Dutta 2009). Moreover, it mediates p53-dependent G1 growth arrest (Deng et al. 1995, Brugarolas et al. 1995) and interacts with Proliferation Cell Nuclear Antigen (PCNA) which plays an important role in S phase DNA replication and DNA damage repair. Of notice, increased p21 expression correlates significantly with incidence in patients with HCV-associated chronic liver disease. Moreover, cytoplasmic p21 is associated with HCCs, especially in moderately and poorly differentiated tumors (Abbas & Dutta 2009).

In comparison, a second cell cycle inhibitor, termed p27, is highly expressed in early stages of HCC (Yachida et al. 2008), presumably as a protective cell mechanism. However, p27 is usually down-regulated in HCC with severe tumor grade or recurrence of tumors (Tannapfel et al. 2000, Fiorentino et al. 2000, Armengol et al. 2003). Of interest, p27 is inhibited by RIP1 (the death domain-containing kinase receptor-interacting protein 1) (Liang & Slingerland 2003) which in turn can be proteolytically degraded by Casp8 (Vandenabeele et al. 2006). Recent data indicate that absence of Casp8 leads to an accumulation of RIP1 and may result in necrosis-like cell death

(necroptosis, Liedtke et al. 2011). Moreover, recent data suggested non-apoptotic functions of Casp8 eventually affecting cell cycle regulation, although the precise mechanisms are poorly understood (Maelfait & Beyaert 2008).

Proto-oncogenes are cell cycle promoting proteins, e.g. mitogens or transcription factors, which are also expressed in healthy cells. However, if proto-oncogenes are de-regulated, they may convert into active oncogenes and trigger increased proliferation and tumorigenesis. In this context it is controversially discussed if the pro-survival transcription factor NF- κ B might be a proto-oncogene. In its inactive form, the NF- κ B subunits (e.g. p50, p65) are sequestered into the cytoplasm. Activation of the I κ B kinase complex (IKK), consisting of the three subunits IKK α , IKK β and IKK γ /NEMO, eventually leads to nuclear translocation of p50 and p65 and subsequent activation of target gene transcription (Leeman & Gilmore 2008). NF- κ B is involved in many processes such as cell survival, inflammation (Sarkar et al. 2008), cancer cell invasion and angiogenesis (Helbig et al. 2003, Huang et al. 2001, Samant et al. 2007). Increased levels of NF- κ B are detected especially in poorly differentiated cancers (Karin 2006). However, mice lacking NEMO in hepatocytes are unable to activate NF- κ B in the liver and develop spontaneous liver apoptosis and hepatocellular carcinoma (Luedde et al. 2005). Thus, it is still under debate if NF- κ B is rather a proto-oncogene or a tumor suppressor (Liedtke & Trautwein 2012).

2 Aim of the present study

Caspase-8 (Casp8) is the most apical caspase in the extrinsic apoptosis pathway. In the past years the functions of Casp8 for suppression of hepatocarcinogenesis have been controversially discussed. On the one hand, Casp8 is frequently down-regulated or absent in murine HCCs (Liedtke et al. 2005) hinting at a tumor suppressive property of this protease. On the other hand, Casp8 was shown to be essential for spontaneous hepatocarcinogenesis in mice lacking the NF- κ B essential modulator (NEMO) suggesting that Casp8 could act as a tumor promoter in the liver in an inflammatory environment (Liedtke et al. 2011). The aim of the present study was the investigation of tumor suppressive and oncogenic properties of Casp8 in the diethylnitrosamine (DEN) model of chemically-induced acute liver injury and hepatocarcinogenesis, respectively. It was hypothesized that loss of Casp8 would enhance either tumor initiation or tumor progression in the liver.

To test this hypothesis, two experimental models should be applied to mice lacking Casp8 specifically in hepatocytes: In the first model, DEN should be applied at low dose to induce HCC. The specific aim of this experiment was to evaluate if Casp8-deficient mice would reveal aberrant tumor incidence or tumor progression. Moreover, the expression levels of prominent oncogenes and tumor suppressors were supposed to be analyzed in Casp8-deficient tumor tissues. In the second model, DEN should be applied at high dose to induce acute liver injury and compensatory liver regeneration with the aim to determine, if Casp8 would be involved in the immediate response towards genotoxic liver injury and its postulated function for elimination of premalignant hepatocytes.

It was anticipated that these investigations may help to evaluate a therapeutically benefit of caspase inhibitors in patients suffering from hepatitis. Recent studies showed that caspase-inhibitors reduced hepatocytes apoptosis, liver injury, hepatic inflammation and hepatic fibrosis (Canbay et al. 2003). The present study may clarify if these benefits in treatment of liver disease are accompanied by an increased risk of hepatocarcinogenesis.

3 Material and Methods

3.1 Material

3.1.1 Chemicals

Reagent	Manufacturer
Acetic acid (glacial) 100 % (CH_3COOH)	Merck, Darmstadt
Acetone ($\text{C}_3\text{H}_6\text{O}$)	VWR, Darmstadt
Agarose (Electrophoresis Grade)	Serva, Heidelberg
Ammoniumpersulfate (APS)	Roth, Karlsruhe
Bromophenol Blue	Bio-Rad, Munich
BSA (Bovine Serum Albumin)	Sigma, Steinheim
Calciumchloride (CaCl_2)	Sigma, Steinheim
CHAPS ($\text{C}_{32}\text{H}_{58}\text{N}_2\text{O}_7\text{S}$)	Sigma, Steinheim
Chloroform (CHCl_3)	AppliChem, Darmstadt
Diethylether	Baker Chemicals, Griesheim
DTT (Dithiothreitol) ($\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$)	Sigma, Steinheim
EDTA (Ethylene Diamine Tetraacetate)	AppliChem, Darmstadt
EGTA (Ethylene glycol tetraacetic acid)	AppliChem, Darmstadt
Eosin	Sigma, Steinheim
Ethanol ($\text{C}_2\text{H}_5\text{OH}$)	AppliChem, Darmstadt
Ethidium bromide ($\text{C}_{21}\text{H}_{20}\text{BrN}_3$)	Invitrogen, Karlsruhe
Formamide	AppliChem, Darmstadt
Formaldehyde 37 % (CH_2O)	Roth, Karlsruhe
Glucose ($\text{C}_6\text{H}_{12}\text{O}_6$)	Merck, Darmstadt
Glutaraldehyde ($\text{C}_5\text{H}_8\text{O}_2$)	Roth, Karlsruhe
Glycerol gelatin	Sigma, Steinheim
Glycine ($\text{C}_3\text{H}_5(\text{OH})_3$)	AppliChem, Darmstadt
Goat Serum	Promocell, Heidelberg
Hematoxylin	Sigma, Steinheim
HEPES ($\text{C}_6\text{H}_{18}\text{N}_2\text{O}_4\text{S}$)	Roth, Karlsruhe
Hydrochloric acid (HCl)	Merck, Darmstadt
Isopropyl alcohol ($\text{C}_3\text{H}_8\text{O}$)	AppliChem, Darmstadt
Magnesium chloride (MgCl_2)	Merck, Darmstadt
β -Mercaptoethanol	AppliChem, Darmstadt
Methanol (CH_4O)	VWR, Darmstadt
MOPS (3-[N-Morpholino] propanesulfonic acid)	Sigma, Steinheim
Nonidet P – 40	AppliChem, Darmstadt
Paraformaldehyde (CH_2O) _n	Roth, Karlsruhe
Phenylmethylsulfonyl fluoride (PMSF)	Roth, Karlsruhe
Ponceau S	Sigma, Steinheim
Potassium chloride (KCl)	Merck, Darmstadt
Salicylic acid	Sigma, Steinheim
Sodium carbonate (Na_2CO_3)	Merck, Darmstadt
Sodium hydrogencarbonate (NaHCO_3)	Merck, Darmstadt
SDS (Sodium Dodecylsulfate) $\text{NaC}_{12}\text{H}_{25}\text{SO}_4$	Roth, Karlsruhe
Sodium fluoride (NaF)	Sigma, Steinheim
Sodium vanadate (Na_3VO_4)	Sigma, Steinheim
Sulfosalicylic acid	Merck, Darmstadt

Trichloroacetic acid ($C_2HCl_3O_2$)	Merck, Darmstadt
Tri-sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2HCl$)	Roth, Karlsruhe
TEMED (Tetramethylethylenediamine)	Bio-Rad, Munich
Tris ($C_4H_{11}NO_3$)	AppliChem, Darmstadt
Triton X-100	Roth, Karlsruhe
Trypan blue stain 0.4%	Gibco
TWEEN-20	Sigma, Steinheim
Xylene	VWR, Darmstadt

3.1.1.1 Radiochemicals

Product	Manufacturer
$[^{32}P]$ - γ -ATP (10mCi/ml)	Hartmann Analytic

3.1.2 Instruments and Equipment

Product	Manufacturer
Wallac β -scintillation counter	Perkin Elmer, Jügesheim
Botting Trans-Blot TM Cell	BioRad, Munich
Bright Field Microscope DM1000	Leica, Wetzlar
Centrifuge Tabletop 5417	Eppendorf, Hamburg
Cryostat HM 550	MicromThermo Scientific, Dreieich
Cold light source KL 1500 LCD	Schott AG, Mainz
DISKUS Z16 APO	Leica, Wetzlar
ELISA Reader μ Quant +KC4 Software	BIO-TEK, Bad Friedrichshall
FUJI FILM LAS-4000	FUJI FILM Europe, Düsseldorf
Gel chamber Subcell GT	Biorad, Munich
Gel chamber	Peqlab, Erlangen
Microcentrifuge IR 220 VAC	Roth, Karlsruhe
pH Meter PB-11	Sartorius, Goettingen
7300 Real Time PCR System	Applied Biosystems, Darmstadt
+ Sequence Detection Software Version 1.3.1.	Heidolph, Nürnberg
Shaking incubator Unimax 1010	Biometra, Göttingen
Thermocycler T3000 and Tpersonal	Eppendorf, Hamburg
Thermomixer 5436	Heidolph, Nürnberg
Vortex Reax top	

3.1.3 General material

Product	Manufacturer
Cryotubes (1,5 ml)	BD, Erlangen
Hybond N membranes	Amersham Pharmacia Biotec Europe, Nümbrecht
Nitrocellulose membrane Protran [®]	Whatman, Dassel
X ray films	Amersham Pharmacia Biotec Europe, Nümbrecht

3.1.4 Material for animal experiments

Product	Manufacturer
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General chow for rodents
Hematocrit capillary

Injection needles Sterican®
Micro tube (1,1 ml Z-Gel)
Sodium chloride 0.9 % (NaCl)
Sterile gossamer (Sterilkompresse®)
Surgical instruments
Syringe Omnican®

Altromin, Lage
Hirschmann Laborgeräte,
Eberstadt
Braun, Melsungen
Sarstedt, Nümbrecht
Delta Select, Dreieich
Beese, Barsbüttel
FST, Heidelberg
Braun, Melsungen

3.1.5 Antibodies

3.1.5.1 Primary antibodies

Product	Manufacturer
c-Jun (N)	Santa Cruz Biotechnology, Inc.
CD 11b	BD Pharmingen
CD 45	ebiosciences
CK 19	Santa Cruz Biotechnology, Inc.
DMBT1	Gift from J. Mollenhauer, Copenhagen
F4/80	AbD Serotec
GAPDH	AbD Serotec
Ki67	Dianova GmbH
JNK1 (FL)	Santa Cruz Biotechnology, Inc.
p21	Santa Cruz Biotechnology, Inc.
NF-κB p65	Santa Cruz Biotechnology, Inc.
Phospho-c-Jun (Ser73)	Cell Signaling Technology
Phospho-Histone H3 (Ser10)	Cell Signaling Technology
NF-κB p65 (A)	Santa Cruz Biotechnology, Inc.
NF-κB p50 (NLS) x for EMSA	Santa Cruz Biotechnology, Inc.
NF-κB p65 (A) x for EMSA	Santa Cruz Biotechnology, Inc.

3.1.5.2 Secondary HRP-labeled antibodies

Product	Manufacturer
anti-mouse IgG, HRP-linked	Cell Signaling Technology

3.1.5.3 Secondary fluorochrome-conjugated antibodies for fluorescence microscopy

Product	Manufacturer
Alexa 488 donkey anti-mouse	Invitrogen
Alexa 488 rabbit anti-rat	Invitrogen
Alexa 546 goat anti-rabbit	Invitrogen
Alexa 546 rabbit anti-goat	Invitrogen

3.1.6 Standard buffers

Product	Manufacturer
Phosphate buffered saline (PBS)	PAA, Cölbe
Restore Western Blot Stripping Buffer	Thermo Scientific, Bonn
Roti Block 10x	Roth, Karlsruhe
Rotiphorese 10x SDS PAGE	Roth, Karlsruhe
TAE 50x	AppliChem, Darmstadt

3.1.7 Analytical chemicals, reagents, enzymes and kits

Product	Manufacturer
Acrylamide Solution 30 %	Biorad, Munich
BIO-RAD Protein Assay	Bio-Rad, Munich
Block and Sample	Promega, Mannheim
Complete Mini® (protease inhibitors)	Roche, Mannheim
Covering solution for cryosections (Tissue-Tek®)	Dako, Hamburg
Diaminobenzidine tablets (DAB)	Sigma, Steinheim
Dako Pen	Dako, Hamburg
DNA marker 1kb plus Ladder®	Invitrogen, Karlsruhe
ECL-Western-Blot-analysis Solution®	Pierce, Bonn
HotStarTaq™ Master Mix	Qiagen, Hilden
Omniscript RT Kit	Qiagen, Hilden
Oligo dT primer	Qiagen, Hilden
Pefabloc SC Protease Inhibitor	Roth, Karlsruhe
Peq Gold RNA Pure™	PeqLab, Erlangen
Peroxidase Blocking Solution	Dako, Hamburg
Primer	MWG Biotech AG, Ebersberg
Proteinase K Solution	AppliChem, Darmstadt
Protran Membrane	Whatman, Dassel
ReadyMix™ redtaq™ PCR reaction mix with MgCl ₂	Sigma, Steinheim
Red Taq	Thermo Scientific
Restore Western Blot Stripping Buffer	Sigma, Steinheim
RNAse	Roth, Karlsruhe
Rotiblock	Roth, Karlsruhe
Roti Histokit	Roth, Karlsruhe
Rotiphorese 10x SDS Page	Roth, Karlsruhe
SYBR® GreenER™ qPCR SuperMix	Invitrogen, Karlsruhe
TAE Puffer 50x	AppliChem, Darmstadt
Trypsin Inhibitor	Sigma, Steinheim
TUNEL Kit	Roche, Mannheim
Ultraspec II RNA	Biotech Laboratories, Houston, USA
Vectashield Mounting Medium with Dapi	Dako, Hamburg
Vectastain ABC Kit	Dako, Hamburg

3.2 Methods

3.2.1 Animal maintenance and treatments

All mouse strains were initially bred and maintained in a specific pathogen-free facility of the University Hospital Aachen in a temperature-controlled room with 12 hours light/dark cycles and free access to standard food and water. All experiments were performed in accordance with the German legislation on protection of animals and approved by the government of the state North Rhine-Westphalia (LANUV, AZ 8.87-50.10.35.08.284). The generation and breeding of hepatocyte-specific Caspase-8 knockout mice (Casp8^{Δhepa}) and the use of appropriate control mice (Casp8^{f/f}) has been described recently (Liedtke et al. 2011). For genotyping of genetically modified mice, tail biopsies were taken after weaning and ear marking and subjected to genomic DNA extraction and subsequent PCR analysis according to standard procedures (for primer sequences see 3.2.5).

To induce hepatocellular carcinoma (HCC), a single dose of DEN (25 mg/ kg body weight) was injected intraperitoneally (i.p.) into male mice at two weeks of age. After 24 or 40 weeks on normal chow, mice were sacrificed and livers were analyzed for macroscopic, histologic and molecular signs of tumor formation. Immediately after sacrifice, visible liver tumors were counted and the size was measured and documented using a Z16 APO macroscope (Leica) with DISCUS software (Hilgers, Königswinter, Germany).

To induce acute liver damage, 6 - 8 week old male animals were injected with DEN at a dose of 200 mg/ kg body weight i.p. Livers were removed 24 - 96 hours after treatment and subjected to molecular and histological analysis.

3.2.2 Blood sampling and serology

Retro-orbital sampling was performed in mice under transient anesthesia using isoflurane by penetrating the retro-orbital sinus with a glass capillary. Blood samples were collected in a serum tube and centrifuged at 12000 rpm for 10 minutes. Serum was transferred into a fresh tube and stored at -20 °C.

Serumtransaminases (Alanine-Aminotransferase (ALT); Aspartate-Aminotransferase (AST)) are specific markers for liver damage. In this study, aminotransferases were analyzed from murine blood. Subsequently, measurements were performed in the clinical routine laboratories of the Institute of Clinical Chemistry and Pathobiochemistry, UK Aachen.

3.2.3 *Extraction and sectioning of complete livers*

Mice were sacrificed by cervical dislocation to remove the full liver. The abdominal cavity was cut open along the *Linea alba*. Two relaxation cuts were placed along the costal arch at the lateral abdominal walls and the liver was removed by dissecting the ligaments. Subsequently, after removing the gall bladder, equal parts of liver tissue were either fixed in 4 % formaldehyde/PBS for paraffin embedding, snap frozen in liquid nitrogen and stored at -80 °C or embedded in Tissue-Tek and stored at -80 °C.

Using the liver paraffin blocks serial whole organ 4 µm sections were performed and subsequently stained with Hematoxylin and Eosin (HE) or subjected to immunohistological stainings following routine protocols.

3.2.4 *RNA extraction, cDNA preparation, Real-Time PCR*

RNA was extracted from mouse livers using peqGOLD RNA Pure™ (PqLab) following manufacturer's instructions. Briefly, a piece of liver tissue was minced in peqGOLD RNA with an electric homogenizer. Lysates were incubated at room temperature for five minutes. After addition of 0.2 volumes of chloroform to the supernatants followed by centrifugation at 12000 g, the upper phases were incubated 10 minutes with isopropanol and RNA was pelleted by centrifugation at 12000 g and 4 °C. The pellets were washed two times with 1 ml of 70 % ethanol, air dried for five minutes and eluted in RNase-free water. RNA was quantified by spectrophotometry at an optical density (OD) of 260 nm using dH₂O as blank. 1 OD₂₆₀ corresponds to 40 µg RNA/ ml.

For gene expression analysis, single stranded mRNA was converted into complementary DNA (cDNA) by reverse transcription (RT). RT was performed using an Omniscript kit (Quagen) according to the manufacturer's protocol. The reaction was composed as subsequently listed in a total volume of 20 µl and

incubated in a PCR thermocycler at 37 °C for 60 minutes followed by a termination at 95 °C for five minutes. Subsequently, the reaction was cooled to 4 °C. After RT, samples were diluted 1:200 with purified water and used for quantitative real-time PCR or alternatively stored at -20 °C.

<i>Reverse transcription reaction mix</i>	<i>Volume / Reaction</i>
10x Buffer RT	2 µl
dNTP mix	2 µl
Oligo-dT primer 10 µM	2 µl
Omniscript Reverse Transcriptase	1 µl
template RNA (1 µg/µl)	2 µl
dH ₂ O add	20 µl

Quantitative real-time PCR was performed using the 7300 Real TimePCR System with SDS software 1.3.1 (Applied Biosystems) and a SYBR Green PCR Kit (Invitrogen). Reactions were performed in duplicate in a total volume of 25 µl with 40 cycles and a combined annealing/elongation at 60 °C for one minute. Details for the reaction mix are given below:

<i>Real-time PCR reaction mix</i>	<i>Volume / Reaction</i>
cDNA	5 µl
primer sense (20 pmol)	2 µl
primer antisense (20 pmol)	2 µl
Sybr green master mix	12.5 µl
dH ₂ O add	25 µl

After PCR reaction, baselines and threshold values were determined using AB 7300 System SDS software. Relative mRNA expression values were calculated according to the $\Delta\Delta CT$ approximation method as reported (Pfaffl 2001) in comparison to a calibrator probe, which was derived from an untreated control. Primer sequences used for RT-PCR analysis are given in table 3.1.

Table 3.1: Primer sequences used for quantitative real-time PCR

<i>gene</i>	<i>orientation</i>	<i>sequence in 5'-3' orientation</i>
AFP	sense	AGC AAA GCT GCG CTC TCT AC
	antisense	AGG GGC TTT CCT CGT GTA AC
Bax	sense	CGA GCT GAT CAG AAC CAT CA
	antisense	GGT CCC GAA GTA GGA GAG GA
CD 133	sense	CAA ACC CAT GGC CAC CGC GA
	antisense	CAC CGT GGC TTT CCC TAT GCC G
DMBT 1	sense	AAT CCC CCA GCA AAG AAA AG
	antisense	AGG AAG ACT CCG CCA AAG AT
DR5	sense	GAG GCT GTG AAC GGG ATC C
	antisense	GTC GTT TCC GTT TAC CGG AAC C
E2F1	sense	AGA GTG AGC AGC AGC TGG AT
	antisense	GGT CCT GGC AGG TCA CAT AG
F4/80	sense	CTT TGG CTA TGG GCT TCC ATC
	antisense	GCA AGG AGG ACA GAG TTT ATC GTG
Fas-L	sense	GTT TTC TGA GCC GAC CTT TG
	antisense	TCA TTG CAC TGG AGG TAT GC
Fas-R	sense	TCC AGA AGG ACC TTG GAA AA
	antisense	CCT CTC TTC ATG GCT GGA AC
GAPDH	sense	TGT TGA AGT CAC AGG AGA CAA CCT
	antisense	AAC CTG CCA AGT ATG ATG ACA TCA
I κ B- α	sense	CCA AGT ACC CGG ATA CAG CA
	antisense	CTG GCC TCC AAA CAC ACA GT
IL-6	sense	CTT CCA TCC AGT TGC CTT CTT
	antisense	AAT TAA GCC TCC GAC TTG TGA AG
TNF	sense	AGC ACA GAA AGC ATG ATC CG
	antisense	CCC GAA GTT CAG TAG ACA GAA GAG
p21	sense	TTG CAC TCT GGT GTC TGA GC
	antisense	TCT GCG CTT GGA GTG ATA GA
p27	sense	GAC AAT CAG GCT GGG TTA GC
	antisense	TCT GTT CTG TTG GCC CTT TT
TRAIL	sense	CCA ACG AGA TGA AGC AGC
	antisense	CCA TCA GTG GAG TCC CAG
TRAIL-R	sense	CCG ACA GAC ATC TAG CAC GA
	antisense	TTA CCG GAA CCA GCA ACT TC

3.2.5 Isolation and analysis of genomic DNA

A tail tip biopsy was incubated in 700 μ l of lysis buffer (50 mM Tris-HCl pH 8, 100 mM EDTA pH 8, 100 mM NaCl, 1 % SDS), supplemented with 35 μ l proteinase K and incubated at 37 °C, 350 rpm on a thermo mixer overnight. The solution was centrifuged at 5000 g and 4 °C for 5 minutes, supernatant containing DNA was transferred in a clean microcentrifuge tube and precipitated with 800 μ l isopropanol. After centrifugation at 13000 g (4 °C for 10 minutes), the supernatant was discarded and pellet was washed twice with 1 ml of cold 70 % ethanol. Pellet was dissolved in 50 μ l TE buffer (10 mM Tris, pH 7.5 with 1 mM EDTA) and concentration was determined by measuring OD at 260 nm. A

260/280 ratio ≥ 1.8 was considered high purity. For subsequent PCR applications, 1 μ l of this DNA was used per 25 μ l reaction.

For determining the Casp8 status of mice, three different PCR reactions per sample specific for cre-recombinase, the WT and the floxed Casp8 allele, respectively, were performed using the primer pairs as listed in table 3.2.

Table 3.2: Primer sequences used for genotyping of Casp8 ^{Δ hepa} and Casp8^{ff} mice

Allele	orientation	sequence in 5'-3' orientation
Cre transgene	sense	CCA CGA CCA AGT GACA GCA AT
	antisense	TTC GGA TCA TCA GCT ACA CCA
WT Casp8 allele	sense	CAT ACT GGT TGA GAA CAA GAC CTG G
	antisense	GCA GAG GTG ACA AGA GGC CAC TG
Floxed Casp8 allele	sense	AAC TTC GGC CGG CCA ATA ACT TCG
	antisense	AGC AGA AAA ACT TGA AGA AAC TTG G

PCR was performed using Red Taq Mastermix (Sigma) according to manufacturer's instructions with 35 cycles and an annealing temperature of 56 °C.

3.2.6 Protein extraction and analysis

3.2.6.1 Isolation of whole cell protein from liver tissue

For protein extractions, approximately 1 mg of frozen liver tissue was homogenized on ice in 500 μ l NP40 buffer. The homogenate was centrifuged for 10 minutes at 12.000 rpm and 4 °C. The pellet was resuspended in NP40 protein extraction buffer, then snap frozen and warmed up few times to break the cell membrane and finally centrifuged 10 minutes at 12.000 rpm, 4 °C. Aliquots of the supernatants containing whole cell proteins were frozen at -80 °C.

NP40-Buffer	final concentration
Tris HCl 7.5 pH (1 M)	50 mM
NaCl (5 M)	150 mM
Nonidet P-40	0.5 %
Sodium Fluoride	50 mM

<i>freshly added:</i>	
Sodium Vanadate (100 mM)	1 mM
DTT (1 M)	1 mM
PMSF (100 mM)	1 mM
1 Complete Mini® tablet per 10 ml buffer	
1 PhosSTOP tablet per 10 ml buffer	

3.2.6.2 Isolation of nuclear extracts

A piece of frozen liver was minced in buffer A and incubated for 10 minutes at 4 °C. After addition of 10% of NP-40, the tube was mixed and incubated at 4 °C for 1 minute. Nuclei were pelleted for 1 minute at 10000 rpm and 4 °C and washed with 1 ml of buffer A 2 - 3 times. The pellet was then resuspended in buffer C and incubated for 30 minutes on ice, mixed gently, and spun down 10 minutes at 10000 rpm (4 °C). The supernatant contained the nuclear proteins and was stored at -80 °C before use in EMSA analysis. Composition of buffers A and C was as follows:

Buffer A		Buffer C	
HEPES pH 7.6	10 mM	HEPES pH 7.8	50 mM
MgCl ₂	2 mM	KCl	50 mM
KCl	10 mM	NaCl	300 mM
EDTA	0.1 mM	EDTA	0.1 mM
+ Protease inhibitors		Glycerol	10%
		+ Protease inhibitors	

3.2.6.3 Measurement of protein concentration

The concentration of whole protein extracts was determined using BIO-RAD Protein Assay reagent. This technique is based on the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. According to manufacturer's protocol, proteins and BSA standards with pre-adjusted concentrations were measured in a spectrophotometer at OD₅₉₅. Protein concentrations of samples were then calculated by comparison with the OD₅₉₅ values of the BSA standard curve.

Concentrations of nuclear protein extracts were measured using BIO-RAD Dc Protein Assay providing higher sensitivity for low protein concentrations. According to manufacturer's instructions, protein and standard were measured in a spectrophotometer at OD₇₅₀. The OD₇₅₀ of each sample was compared to a standard curve prepared with BSA.

3.2.7 Western Blot

Protein samples were separated electrophoretically on 10 %, 12 % or 15 % denaturing SDS-polyacrylamide gels or on pre-cast 4 – 12 % polyacrylamide gels (Invitrogen) according to standard procedures. Gels and buffer were prepared as follows (Laemmli, 1970):

5x Protein loading buffer		1x SDS Running buffer	
Tris-HCl pH 6.8	125 mM	Tris-Base	1.25 M
Glycerol	50%	Glycine	2 M
SDS	5%	SDS	1%
Bromphenol blue	0.025%		
β-Mercaptoethanol 50 µl per 1 ml loading buffer			

Reagent	Resolving Gel			Stacking Gel
Percent	10%	12.5%	15%	5%
dH ₂ O	20 ml	16.4 ml	12.3 ml	13 ml
1.5M Tris-HCl, pH 8.8	12.5 ml	12.5 ml	12.5 ml	-
0.5M Tris-HCl, pH 6.8	-	-	-	6.25 ml
30% Acrylamide	16.5 ml	20.3 ml	24.35 ml	5 ml
10 % SDS	500 µl	500 µl	500 µl	250 µl
10 % APS	500 µl	500 µl	500 µl	500 µl
100 % TEMED	37.5 µl	37.5 µl	37.5 µl	37.5 µl

20x MOPS running buffer (500 ml)		
Reagent	Mass (gram)	Final concentration (1x)
MOPS	104.6	50 mM
Tris base	60.6	50 mM
SDS	10.0	3.5 mM
EDTA	3.0	1 mM

10x Transfer Buffer		
Reagent	Mass (gram)	
Tris	24.4	
Glycine	112.6	
H ₂ O add	10.0	
For 1x Transfer Buffer:		
Reagent	Volume	Final concentration (1x)
10x Transfer Buffer	100 ml	10 %
Methanol	200 ml	20 %
dH ₂ O add	1 l	

Briefly, 5x Protein loading buffer was added to each sample in appropriate volumes and samples were boiled at 96 °C for 5 minutes before loading on the gel. Separation was performed in 1x SDS running buffer at 50 - 80 V overnight (freshly prepared gels) or in MOPS running buffer at 120 V for approximately 2 hours (Invitrogen pre-cast gel system). For immunological detection, the separated proteins were transferred from the gel to a nitrocellulose membrane (Protran, Whatman) using a wet blotting chamber (BIO-RAD) in transfer buffer according to standard procedures. Successful protein transfer and equal loading was confirmed by Ponceau Red staining.

Subsequently, the blotted membrane was blocked for 1.5 hours in PBS-Tween (Phosphate buffered saline, 0.05 % TWEEN 100) (PBST) supplemented with 5 % non-fat dry milk or alternatively in 1x Roti-block (Roth) to block non-specific antibody-binding sites. The membrane was shortly washed and then incubated overnight at 4 °C in PBS with primary antibody at appropriate concentrations. The membrane was washed 3 x 5 minutes in PBST and then incubated with a secondary horseradish-peroxidase conjugated antibody diluted 1:5000 in PBST for 1 hour at RT. After incubation, the membrane was washed 3 x 5 minutes in PBST and then incubated in ECL Substrate (Pierce) for 5 min. The membrane was exposed to x-ray film (hyperfilm, Amersham) or a digital detection system (LAS, FUJIFILM).

3.2.8 Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic Mobility Shift Assay (EMSA) was used to determine the DNA binding activity of the transcription factor NF- κ B. The principle of this assay is based on the fact that complexes of proteins (e.g. transcription factors) with DNA (e.g. transcription factor binding sites) migrate more slowly in a non-denaturing polyacrylamide gel compared to free DNA. Briefly, [32 P] end-labeled DNA fragments containing a NF- κ B consensus binding site were incubated under native conditions with nuclear protein extracts potentially containing activated transcription factors. Binding of activated NF- κ B to its consensus sequence resulted in formation of slow migrating complexes, which were resolved and visualized in non-denaturing polyacrylamide gel electrophoresis. Detection of NF- κ B in these complexes was carried out by addition of an antibody specific for the NF- κ B subunit p65 resulting in formation of an antibody/NF- κ B/DNA super-complex with further retarded migration properties (supershift).

3.2.8.1 Generation of the double-strand DNA probe

Equal amounts of complementary DNA single strands (each 50 μ g) encoding NF- κ B binding sites (sense: AGTTGAGGGGACTTTCCCAGGC; antisense: GCCTGGGAAAGTCCCCTCAACT) were added to 26 μ l 10x annealing buffer and H₂O at a final volume of 260 μ l. After denaturing the probe for 5 minutes at 95 $^{\circ}$ C, the single stranded DNAs were allowed to anneal by slowly cooling down to room temperature overnight. The resulting double stranded probe could be stored at 4 $^{\circ}$ C.

10x Annealing buffer	final concentration
Tris/ HCl 1 M, 7.6 pH	200 mM
MgCl ₂ , 1 M	100 mM
NaCl, 5 M	500 mM

3.2.8.2 Radioactive end-labeling of double-strand DNA probe

The double-stranded DNA probe was radioactive end-labeled at its 5'-hydroxyl group by incubating 125 ng of double-stranded probe with 0.4 mCi [32 P]- γ -ATP and 1 unit T4 polynucleotide kinase (NEB) in 20 μ l of T4-Kinase

buffer for 1 hour at 37 °C. Labeled probes were purified from free [³²P]-γ-ATP molecules using Sephadex G-50 Quick-spin-columns according to manufacturer's instructions (Amersham). Efficiency of the labeling and the radioactivity was determined using a β-scintillation counter.

3.2.8.3 Gel shift analysis

EMSA reactions were carried out in a total volume of 20 µl. For each reaction 5 µg of nuclear protein extract was mixed with 4 µl 5x binding buffer (1 M HEPES, 0.5 M MgCl₂, 1 M KCl), 2 µl 10x proteinase inhibitor cocktail (1 M DTT, 0.1 M Pefablock, 200 µl Aprotinin), 1 µl Poly dl:dC (1 µg/ µl), 1 µl BSA (10 µg/ µl in dH₂O) and P³²-labelled oligonucleotide (30000 cpm per reaction). As control, a reaction mix without protein (free probe) was applied. The samples were incubated 30 minutes on ice. For super-shift-assays, 1 µl of specific p65 antibody (Santa Cruz) was added to a sample and incubated for another 30 minutes on ice. The reaction was stopped with 2 µl loading buffer containing 20 % Ficoll. Samples were loaded on a non-denaturing polyacrylamide gel (5 %) and separated at 300 volt and 4 °C for approximately 3 hours in 0.25 X TBE running buffer (22.5 mM Tris, 22.25 mM H₃BO₃, 0.5 mM EDTA). The gel was fixed for 30 minutes in 20 % methanol/10 % acetic acid, transferred to a Whatman filter, dried under vacuum and exposed at -80 °C on x-ray film.

3.2.9 Measurement of enzymatic Caspase-3 activity

Caspase-3 is a key effector caspase involved in extrinsic and intrinsic apoptosis. Enzymatic caspase-3 activity was determined using an artificial Caspase-3 specific substrate (DEVD) linked to a fluorochrome (AFC). Active Caspase-3 within protein lysates specifically cleaved the substrate DEVD between D and AFC, thus releasing free AFC which was quantified by UV spectrofluorometry. Therefore, AFC-mediated fluorescence was proportional to Caspase-3 activity. For isolation of native protein extracts, 1 mg of frozen liver tissue was homogenized on ice in 500 µl of lysis buffer containing 10 mM HEPES pH 7.4, 0.1 % CHAPS, 2 mM EDTA, 5 mM DTT, 1 mM PEFA-Block and protease inhibitors (Complete Mini®, Roche). The homogenate was centrifuged for 10 minutes at 12.000 rpm and 4 °C. Supernatant was transferred into new tube and protein concentration was determined. Samples were shock frozen in

liquid nitrogen and stored at -80 °C. For each reaction, 12.5 µl protein extract (typically 1 µg/ µl) were mixed with 487.5 µl reaction buffer containing 10 mM PIPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 25 µM DEVD-AFC. The reaction mix was pipetted in duplicate (200 µl/well) into a 96-well-cellstar plate and incubated at 37 °C with shaking for 1 hour. Subsequently, AFC-release was measured in a fluorospectrometer (Biotec) with an extinction-wavelength of 390 nm and an emission-wavelength of 510 nm after 1 and 2 hours, respectively. The specific alteration of fluorescence per hour was determined and calculated as fold induction in comparison to untreated control samples.

3.2.10 p65 - Immunohistochemistry

Paraffin embedded sections were deparaffinised and rehydrated with xylene and decreasing percentages of ethanol. Antigen retrieval was performed by boiling sections in sodium citrate buffer for 6 minutes in a microwave. After cooling for 15 minutes, sections were rinsed in running tap water and endogenous peroxidases were quenched for 15 minutes. Sections were washed under running water, and subsequently washed for 5 minutes in PBS. Following blocking with biotin blocking reagent for 15 minutes and with Avidin D blocking reagent for 15 minutes, sections were blocked in PBT (PBS, 0.1 % BSA, 0.2 % TritonX-100) and then incubated overnight with p65-antibody (Santa Cruz) at 4 °C in a humid atmosphere. Slides were washed twice with PBS and secondary antibody (anti-mouse, HRP linked (Cell Signaling Technology)) was applied for 1 hour at room temperature in a humidifying box. In parallel, the HRP-ABC solution (VECTORSTAIN ABC reagent: 7,5 µl solution A and 7,5 µl solution B in 500 µl 1% BSA/PBS) was prepared and incubated at least 30 minutes at room temperature before it was applied to the slides. Sections were rinsed twice in PBS and HRP-ABC solution was applied for 1 hour at room temperature in a humidified box. After washing twice in PBS, the signal was developed with DAB and counterstained with hematoxylin. Dehydration was performed with increasing percentages of ethanol and xylene, mounted with Roti-histokit and visualized by bright field microscopy or alternatively by using a Leica Z16 APO macroscope with DISCUS software (Fa. Carl H. Hilgers).

3.2.11 Immunofluorescence

Cryosections (5 μm) from mouse livers were fixed with 4 % paraformaldehyde for 15 minutes and rehydrated with PBS for 15 minutes. All subsequent steps were carried out at room temperature unless otherwise indicated in a humid light tight box to prevent drying and fading. PBS containing 5 % goat serum and 0.3 % Triton was used for blocking for one hour, followed by an overnight incubation with primary antibody in blocking solution at optimized dilutions at 4 °C. Slides were washed with PBS for 15 minutes and incubated with secondary, fluorochrome-conjugated antibody, diluted 1:200, in PBS for one hour at RT. Nuclei were counterstained with DAPI in mounting medium. Expression was visualized and documented by fluorescence microscopy (Zeiss).

For monitoring CK-19 expression, cryosections were fixed with Acetone for 10 minutes at -20 °C. Tissue sections were blocked with PBS containing 5 % goat serum and 0.3 % Triton for one hour at RT, followed by probing with a CK-19 antibody (diluted 1:200 in TBST) over night at RT in a humidified chamber. Slides were washed with TBST and probed with a fluorochrome-conjugated secondary antibody (diluted 1:200 in TBST, 1 % goat serum). Sections were incubated for 60 minutes, washed three times in TBST and mounted with mounting medium containing DAPI.

TUNEL (TdT-mediated dUTP nick end labeling) is an established method for *in situ* labeling of DNA strand breaks that occur early during apoptosis. Terminal deoxynucleotidyl transferase (TdT) is used to introduce labeled nucleotides into partially degraded DNA. For TUNEL analysis, an *In Situ* Cell Death Detection Kit (Roche) was used. Liver cryosections were fixed with 4 % paraformaldehyde for 20 minutes and rehydrated with PBS for 30 minutes. Sections were treated with 3 % H_2O_2 in methanol for 10 minutes, and then permeabilized with 0.1 % Triton, 0.1 % Na-citrate for 2 minutes at 4 °C. Slides were washed with PBS and stained with TUNEL mix according to manufacturer's instructions (Roche) in a humidified chamber overnight at 4 °C. After washing with PBS stained sections were mounted with DAPI mounting medium and analyzed by fluorescence microscopy.

4 Results

4.1 Experimental design of the study

Resistance to apoptosis is a hallmark of cancer. As Caspase-8 (Casp8) is the apical caspase in the extrinsic (e.g. death-receptor-mediated) apoptotic pathway, genetic inactivation of Casp8 in hepatocytes (Casp8^{Δhepa}) was expected to completely block extrinsic apoptosis in parenchymal cells of the liver.

To investigate the effects of Casp8-deficiency for development of liver cancer, the well-established Diethylnitrosamine (DEN) model of chemically induced hepatocarcinogenesis was applied (Bralet et al. 2002). Basically, two different experimental approaches were used to determine the role of Casp8 for HCC development.

In a high dose DEN-model, 6-8 week old male Casp8^{Δhepa} mice and Casp8^{f/f} controls were given 200mg/ kg DEN to induce an acute injury and genotoxic damage (Janani et al. 2008). In these mice, the mode of liver injury and the kinetics of liver regeneration were studied for up to 96 hours to determine potential functions of Casp8 for elimination of premalignant hepatocytes and cell proliferation.

In a low dose DEN-model, two weeks old Casp8^{Δhepa} and Casp8^{f/f} mice were injected with a single dose of 25 mg /kg DEN which usually triggers hepatocarcinogenesis (Diwan & Meier 1976). Cohort of mice were sacrificed 24 weeks and 40 weeks after DEN treatment and analyzed for markers of tumor development in the liver. These studies included macroscopic inspections (determination of tumor size and number), histological analyzes and molecular expression studies on cell cycle related genes, oncogenes and pro-apoptotic factors.

4.2 Loss of Casp8 in hepatocytes does not affect the acute response to DEN-mediated liver injury

In order to determine the role of Casp8 for the acute response towards cancerogenic stimuli, 6 – 8 weeks old male Casp8^{Δhepa} mice and Casp8^{f/f}

controls were injected with 200mg/ kg DEN. Previous reports demonstrated that these high doses of DEN trigger acute liver injury, accumulation of DNA-damage and a compensatory proliferation (Naugler et al. 2007). The DEN-response was monitored over a period of 96 hours with a minimum of three animals per time point and condition. The precise composition of the mouse cohorts used for these experiments is given in table 4.1.

Time point after DEN treatment	Number of animals in control group (Casp8 ^{f/f})	Number of animals in knockout group (Casp8 ^{Δhepa})
0h	9	8
24h	3	3
48h	4	3
72h	3	3
96h	3	3

Table 4.1: Cohort size and time points used for measurements of acute response in the liver following DEN treatment.

Alanine-aminotransferase (ALT) and Aspartat-Aminotransferase (AST) are both liver enzymes used as well established standard indicators for liver injury. ALT is most specific for the liver whereas AST is also found in heart and muscles. During liver injury, the activity of both enzymes in the serum is increased as AST and ALT are released upon liver cell death (Malhi et al. 2010).

Following DEN-treatment, both Casp8^{f/f} and Casp8^{Δhepa} mice showed a strong increase of AST and ALT levels 48 hours after stimulation which was not significantly different between the groups (Figure 4.1). At later time points after treatment (72 - 96 hours post DEN) a tendency towards higher transaminase levels in Casp8^{Δhepa} mice was observed. However, these differences did not reach statistical significance. Thus, genetic inactivation of Casp8 does not protect from overall DEN-induced acute liver injury.

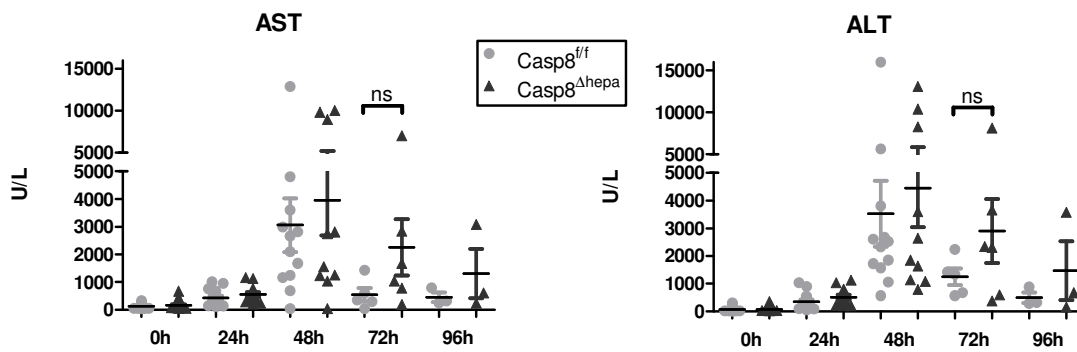


Figure 4.1: Transaminase activation after DEN treatment. *Casp8^{f/f}* and *Casp8^{Δhepa}* mice were subjected to single injection with DEN (200mg/ kg) at the age of 6 - 8 weeks and sacrificed after 24, 48, 72 and 96 hours respectively. Blood was taken every 24 hours and serum was analyzed for AST and ALT activity levels in units per liter (U/L). Data is shown as a scatter plot; each dot represents a serum value of one individual mouse. n.s.: not significant.

The cytokines Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF) are important pro-inflammatory mediators in the liver and are secreted almost immediately after acute liver injury especially from Kupffer cells which are considered the resident macrophages of the liver (Malhi & Gores 2008). It was recently suggested that IL-6 and TNF might be key factors for hepatocarcinogenesis induced by DEN or obesity (Naugler & Karin 2008, Sun & Karin 2008, Park et al. 2010). Therefore, mRNA expression levels of IL-6 and TNF were determined in livers from DEN-treated *Casp8^{f/f}* and *Casp8^{Δhepa}* mice 48 hours after treatment (Figure 4.2) which reflected the state of maximal injury (compare Figure 4.1). In good agreement with previous studies, both IL-6 and TNF were substantially induced by DEN although without significant differences between the groups. This indicates that TNF/IL-6 induction is not dependent on functional Casp8. Of notice, untreated *Casp8^{Δhepa}* mice revealed significantly increased TNF expression levels confirming that loss of Casp8 induces a moderate inflammation in the liver as reported recently (Liedtke et al. 2011).

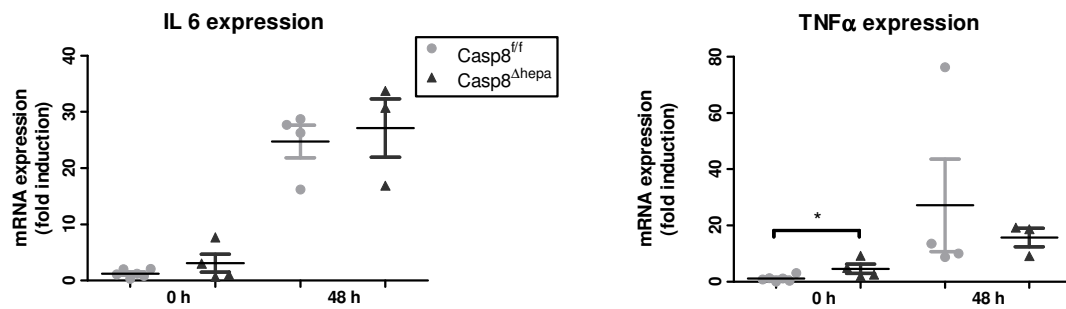


Figure 4.2: Cytokine expression after DEN-mediated liver injury. Casp8^{f/f} and Casp8^{Δhepa} mice were subjected to single injection with DEN (200mg/ kg) at the age of 6 – 8 weeks and sacrificed after 48 hours respectively. Gene expression was normalized via GAPDH expression levels. Data were calculated as fold induction in comparison to untreated Casp8^{f/f} control-mice.

The major known function of Casp8 is the induction of extrinsic pro-apoptotic signals cumulating in cell death. The main focus of this study was to evaluate if Casp8-driven apoptosis might be relevant to prevent liver tumors. Therefore, TUNEL stainings were performed on liver sections from DEN-treated Casp8^{f/f}- and Casp8^{Δhepa}-mice at several time points after stimulation. Surprisingly, at none of the time points investigated, Casp8^{Δhepa} mice showed a decrease of TUNEL positive cells (Figure 4.3 A, B). In contrast, 72 hours after treatment, Casp8^{Δhepa} livers showed even significantly increased TUNEL staining. These unexpected data indicated that Casp8 is not essential for DEN-induced liver cell death and hinted at alternative cell death pathways (e.g. necrosis, intrinsic apoptosis) that might be more relevant in this model.

It has been reported that in some cases TUNEL staining may also label necrotic cells. To clearly distinguish apoptotic cell death from necrosis, the enzymatic activity of caspase-3 was determined after DEN treatment. Caspase-3 is an executioner caspase, which acts downstream of intrinsic and extrinsic apoptotic signaling pathways. Thus, caspase-3 activation is a highly specific indicator of apoptosis. Basically, determination of caspase-3 activity confirmed the data generated by TUNEL analysis. In both Casp8^{f/f} and Casp8^{Δhepa} mice, caspase-3 activity was substantially up-regulated 48 hours after treatment and still elevated 72 hours post DEN (Figure 4.3 C). However, there was not any significant difference between the groups at any investigated time point. These data clearly demonstrated that DEN induces at least some apoptosis which is independent of Casp8 and most likely involves intrinsic signaling pathways.

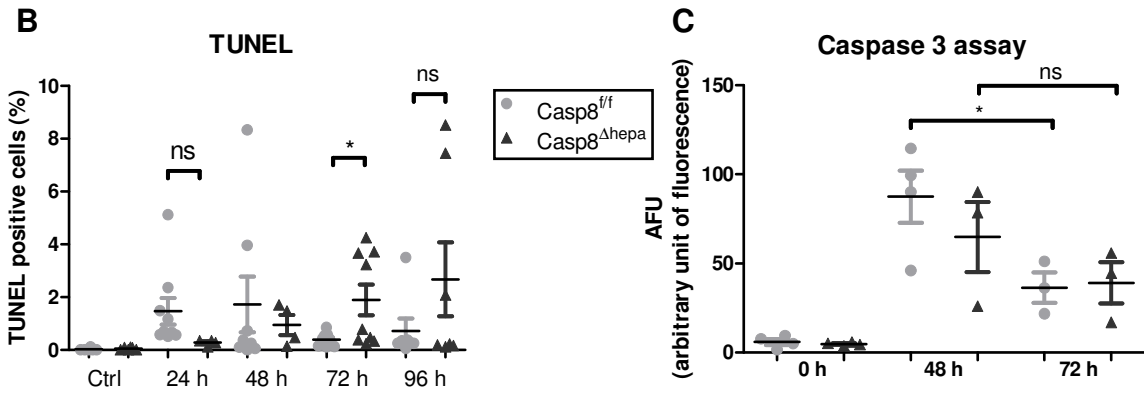
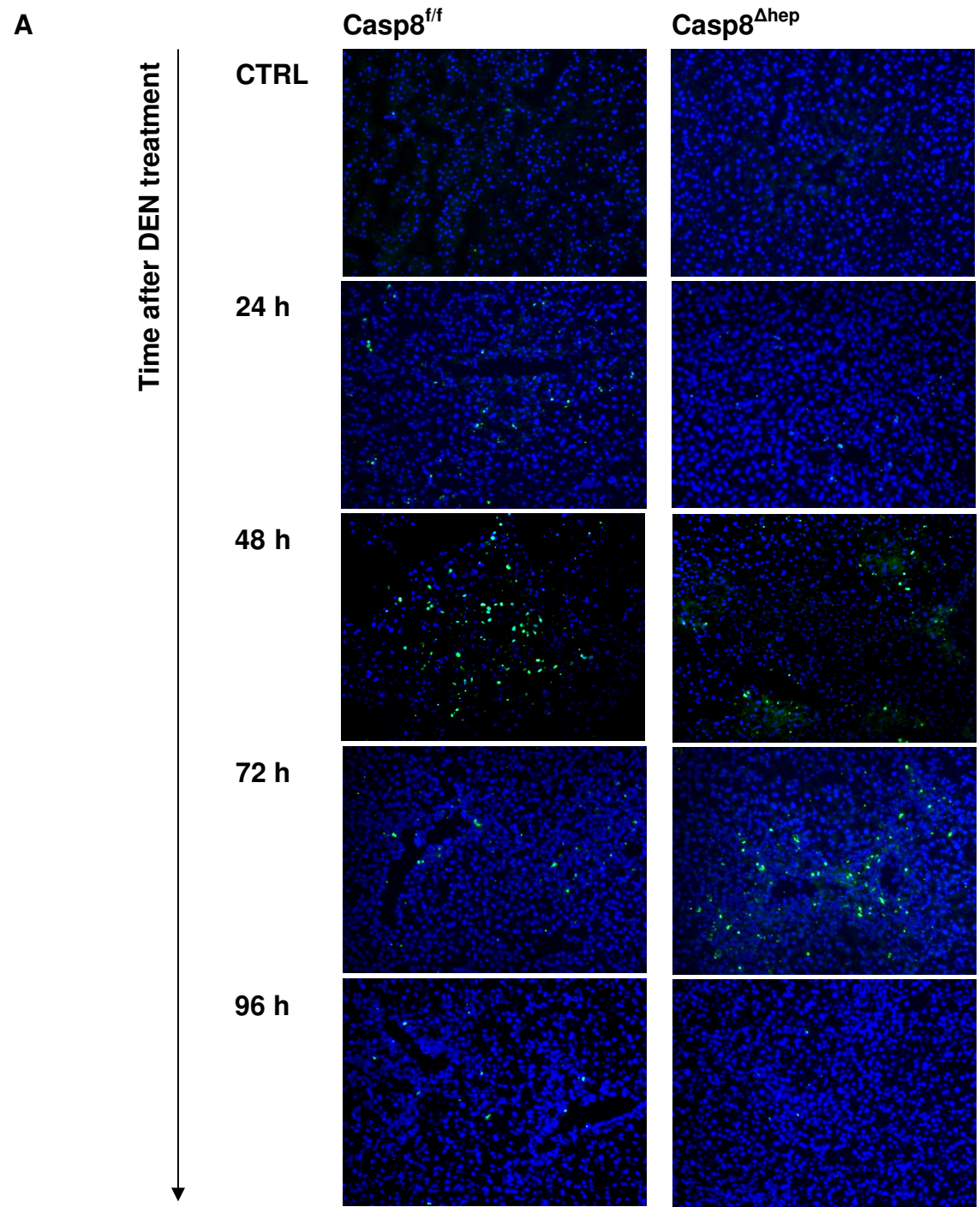


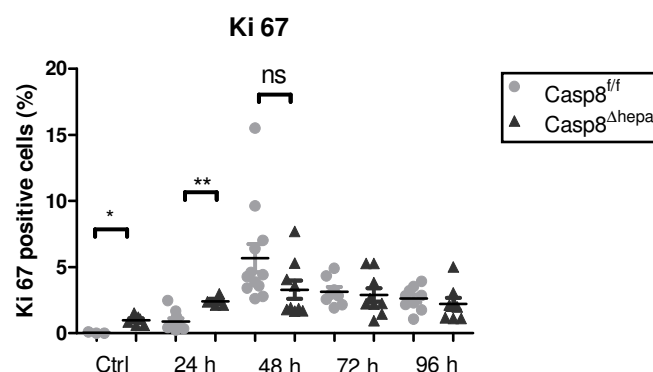
Figure 4.3: TUNEL Immunofluorescence (A) and Caspase 3 assay (C) after DEN-induced liver injury. A. Casp8^{f/f}- and Casp8^{Δhepa}-mice were injected with DEN (200mg/ kg) at the age of 6 - 8 weeks and sacrificed after 24, 48, 72 and 96 hours, respectively. Liver cryosections were stained with the TUNEL method; apoptotic cells are stained in green; total cells were counter stained with DAPI (blue). B. Quantification of TUNEL-positive cells shown in A. For each mouse, three independent magnification fields were analyzed. *: p<0.05; n.s.: not significant. C. Caspase activity was measured in 48 and 72 hours DEN treated mice as substrate turnover of a fluorescence substrate and is indicated as Arbitrary Fluorescence Unit (AFU). n.s.: not significant.

4.2.1 Lack of Casp8 accelerates onset of hepatic cell proliferation after DEN treatment

One requirement of tumorigenesis is an increased proliferation capacity (Malumbres & Barbacid 2009). Of notice, one important trigger of proliferative response could be cell loss due to apoptosis or necrosis. Although the previous experiments did not show significant differences of apoptosis in Casp8^{Δhepa} and Casp8^{f/f} mice, recent data suggested an influence of Casp8 on cell cycle regulation and NF-κB-activation (Maelfait & Beyaert 2008). Therefore, liver sections of DEN-treated Casp8^{Δhepa} and Casp8^{f/f} mice were stained for the general proliferation marker Ki-67 to visualize and quantify cells in the active phase of the cell cycle.

Interestingly, untreated Casp8^{Δhepa} mice already revealed a slight but significant increase in proliferating liver cells (Figure 4.5) suggesting that loss of Casp8 triggers moderate basal proliferation in the liver. Moreover, the data showed a significant (p = 0.002) higher amount of proliferating cells in Casp8^{Δhepa} in comparison to Casp8^{f/f} mice 24 hours after DEN treatment, whereas the proliferative response in these mice was similar to controls at later time points. Thus, Casp8^{Δhepa}-mice seem to be pre-activated for hepatic cell proliferation and show an accelerated proliferative response towards toxic liver injury.

A



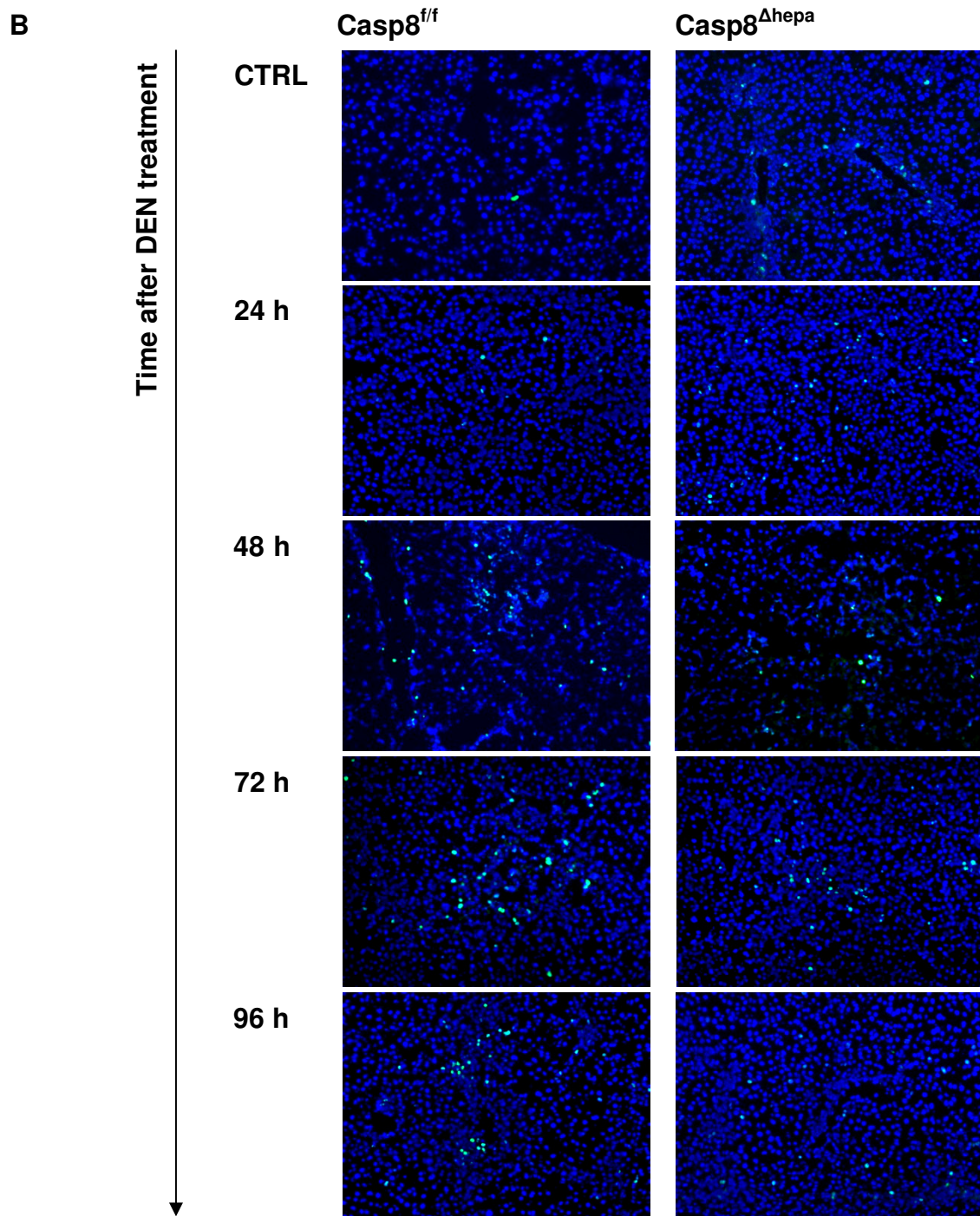


Figure 4.4: Ki-67-Immunofluorescence after DEN-induced liver injury. Casp8^{f/f} and Casp8^{Δhepa} mice were injected with DEN (200mg/ kg) at the age of 6 - 8 weeks and sacrificed after 24, 48, 72 and 96 hours, respectively. A. Quantification of Ki-67-positive cells shown in B. The percentage of TUNEL-positive cells is indicated on the y-axis. For each mouse, three independent magnification fields were analyzed. **: p<0.01; *: p<0.05; n.s: not significant. B. Liver cryosections were stained with a fluorescence-labeled Ki67-antibody; cells in active cell cycle are stained in green; total cells were counter stained with DAPI (blue).

It was recently shown that DEN-induced HCC development depends on activation of Jun N-terminal kinase 1 (JNK1) (Hui et al. 2008) which belongs to an important family of stress-activated mitogen activated kinases (MAPK). The most prominent substrate of JNK is the transcription factor cJun. Together with

members of the Fos family, cJun forms the activating protein 1 (AP-1) which is important for activation of cell cycle controlling genes and thus for induction of cell proliferation (Eferl & Wagner 2003). Therefore, aberrant JNK/cJun response could have an impact on tumor incidence. In this context, recent data showed a premature up-regulation of cJun in $\text{Casp8}^{\Delta\text{hepa}}$ mice after 2/3-hepatectomy resulting in accelerated onset of hepatocyte proliferation (Freimuth et al., unpublished data).

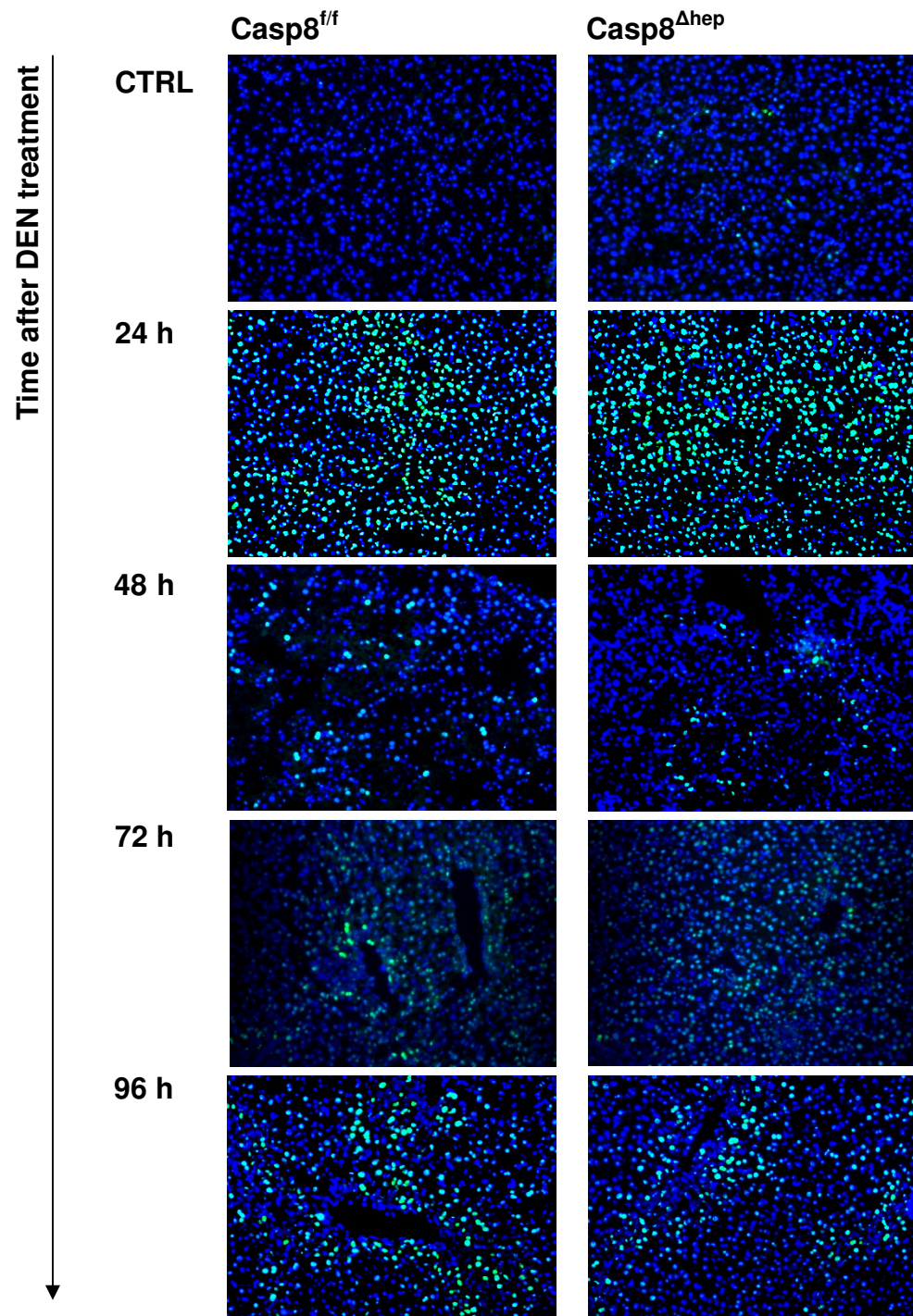


Figure 4.5: Phosphorylation and activation of cJun after DEN-induced liver injury. Casp8^{f/f} and Casp8^{Δhepa} mice were injected with DEN (200mg/ kg) at the age of 6 - 8 weeks and sacrificed after 24, 48, 72 and 96 hours, respectively. Liver cryosections were stained for phosphorylated cJun using and fluorescence-labeled antibody; p-Jun positive cells are stained in green; total cells were counter stained with DAPI (blue).

In the present study the kinetics of cJun-activation after DEN induced liver injury were determined semi-quantitatively by performing immunofluorescence stainings for phosphorylated cJun on liver cryosections at distinct time points after DEN treatment (Figure 4.6). These measurements revealed a strong cJun-phosphorylation with two maxima after 24 and 72 hours, respectively. However, the staining pattern did not show substantial differences between Casp8^{Δhepa} and Casp8^{f/f} mice. These data suggests that Casp8 does not have a major impact on cJun activation at least in the DEN model.

4.2.2 Activation of the cell cycle inhibitor p21 after DEN treatment is independent of Casp8

With initiation of the present study, it was hypothesized that loss of Casp8 may affect the compensatory proliferation after acute DEN-induced liver injury. To complete these measurements, the induction of the cell cycle inhibitor p21 was also studied under these conditions. p21 is a cyclin dependent kinase inhibitor which influences cellular proliferation, differentiation and senescence as well as DNA damage repair (Abbas & Dutta 2009). Importantly, p21 down-regulation was shown to be a key event for efficient HCC development in the DEN model (Hui et al. 2008). Therefore, mRNA expression of p21 after DEN delivery was investigated in Casp8^{Δhepa} mice and compared to Casp8^{f/f} control animals.

The data demonstrate a remarkable DEN-induced up-regulation of p21-expression in both Casp8^{f/f} and in Casp8^{Δhepa} mice and did not show significant differences between the groups (Figure 4.7). In conclusion, the cyclin dependent kinase inhibitor p21 is substantially up-regulated in the acute phase of DEN-induced injury. However, this regulation is completely independent of Casp8 in this context.

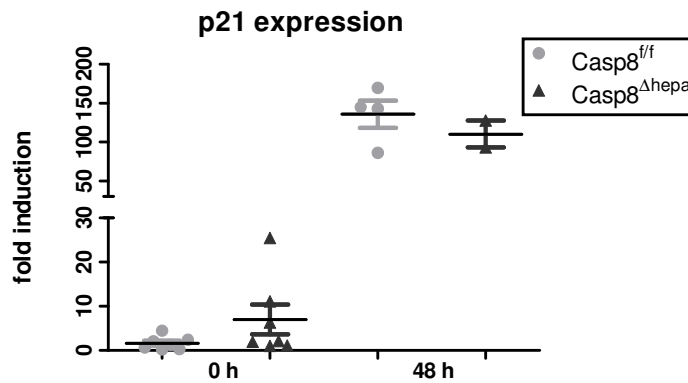


Figure 4.6: Gene-expression analysis of p21 after DEN-mediated liver injury. Casp8^{f/f} and Casp8^{Δhepa} mice were subjected to single injection with DEN (200mg/ kg) at the age of 6 – 8 weeks and sacrificed after 48 hours respectively. Gene expression of p21 was determined by quantitative real-time PCR and normalized via GAPDH expression levels. Data were calculated as fold induction in comparison to untreated Casp8^{f/f} control-mice.

In summary, the previous data hint at an unexpected moderate effect of Casp8 on the immediate response towards DEN in the liver. Casp8 deficiency results in a slightly accelerated onset of compensatory proliferation but otherwise does not have an impact on inflammation and apoptosis.

4.3.1 Loss of Casp8 does not affect tumor initiation in the liver

Application of DEN at low dose to juvenile mice at the age of 2 weeks is a well-established animal model of hepatocarcinogenesis (Diwan & Meier 1976). Under these conditions, WT mice develop liver dysplasia within 24 weeks which further progress to solid tumor nodules in a total time of approximately 40 weeks (Bralet et al. 2002). Under these conditions, dysplastic nodules can be recognized in liver tissue sections as well-defined areas of cell formations which are frequently encapsulated.

To investigate, if Casp8 acts as a tumor suppressor, cohorts of DEN-treated Casp8^{f/f} and Casp8^{Δhepa} mice were sacrificed 24 weeks after tumor induction and histologically investigated for formation of dysplastic liver lesions. For this approach, HE stained liver sections were analyzed with a software-based imaging system (Discus, Fa. Carl H. Hilgers, Königswinter, Germany) that allowed the quantification of dysplastic tissue areas within the liver section. The data is shown in Figure 4.8. Initial analysis suggested a substantial appearance of dysplastic lesions in both Casp8^{f/f} and Casp8^{Δhepa} liver (Figure

4.8 A). A more precise classification of these lesions by size revealed a similar frequency of small dysplastic areas ($<50.000 \mu\text{m}^2$) but also of medium size lesions ($50.000\text{--}200.000 \mu\text{m}^2$). Interestingly, $\text{Casp8}^{\Delta\text{hepa}}$ livers showed a higher tendency to form large liver lesions $>200.000 \mu\text{m}^2$ compared to $\text{Casp8}^{f/f}$ controls (Figure 4.8 B), although at this stage these differences were not statistically significant. However, the cumulative dysplastic liver areas in both groups were not significantly different. As this stage (24 weeks after DEN treatment) was considered the initiation phase of hepatocarcinogenesis, it was concluded from this data that Casp8 does not protect from tumor initiation.

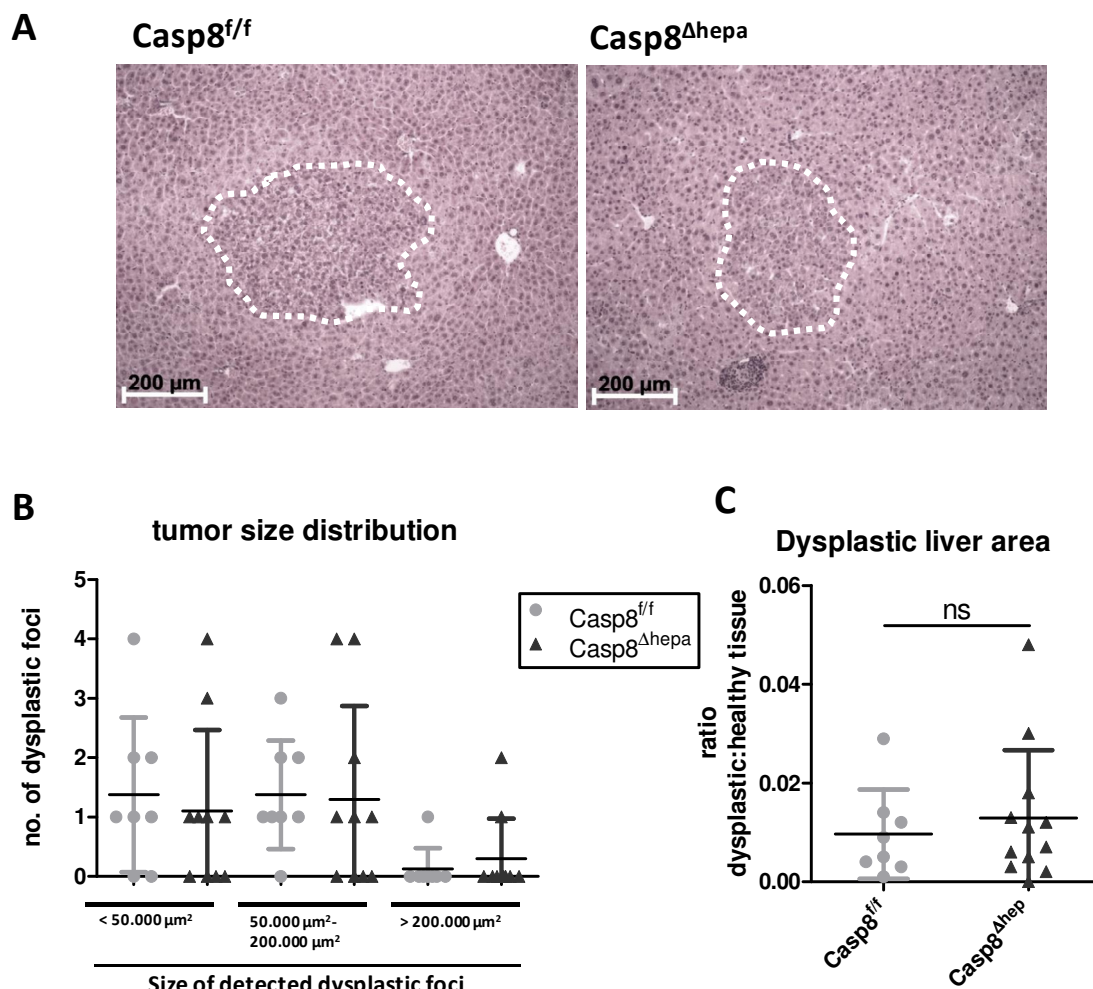


Figure 4.7: Determination of dysplastic tissue area in liver of DEN treated mice. $\text{Casp8}^{f/f}$ - and $\text{Casp8}^{\Delta\text{hepa}}$ -mice were injected with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 24 weeks. A. HE staining of representative liver sections 24 weeks after DEN-induced tumorigenesis. Pre-cancerogenous liver lesions are encircled. (B, C). HE stained liver sections from every DEN-treated animal were analyzed using Discus-Imaging software. Number and size of dysplastic tissue area was determined. B. Detected dysplastic nodules were categorized by size and compared between $\text{Casp8}^{f/f}$ - and $\text{Casp8}^{\Delta\text{hepa}}$ -mice. C. The cumulative size of dysplastic nodules in liver sections was determined and calculated as dysplasia:total-tissue area ratio for every DEN-treated mouse.

4.3.2 Loss of Casp8 triggers enhanced tumor progression

The previous findings suggested that loss of Casp8 does not affect tumor initiation in the DEN model. However, these unexpected findings did not exclude the possibility that the inactivation of Casp8 could have an impact on tumor growth. To test this hypothesis, cohorts of Casp8^{f/f} and Casp8^{Δhepa}-mice were sacrificed 40 weeks after DEN-injection. Livers were extracted and visible tumor nodules were quantified and compared.

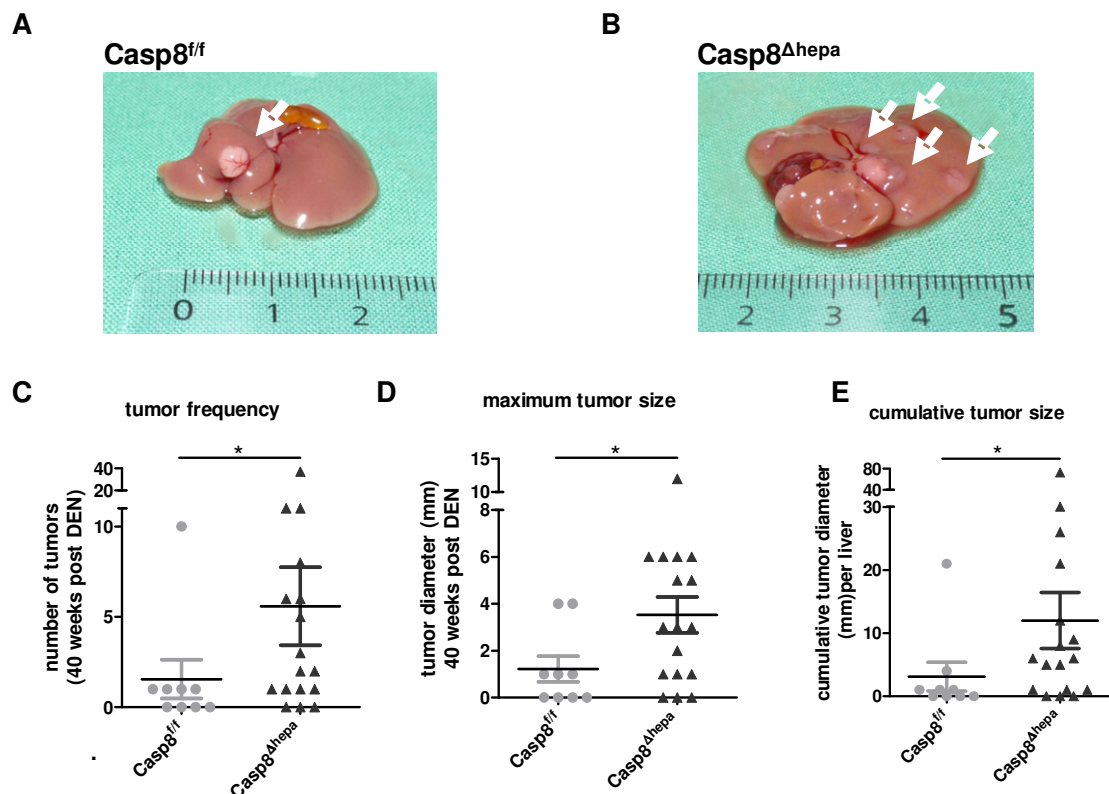


Figure 4.8: Casp8 inhibits advanced tumor progression. Casp8^{f/f} and Casp8^{Δhepa} mice were injected with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 40 weeks. Livers were extracted and macroscopic tumor nodules (white arrows) were quantified and analyzed for size. A. Representative image of a Casp8^{f/f} liver displaying typically 1 - 2 nodules. B. Representative image of a Casp8^{Δhepa} liver with frequent appearance of tumors. C. Tumor frequency in Casp8^{f/f} and Casp8^{Δhepa} mice. Each dot represents the number of tumor nodules found in one individual mouse. D. The size (mm diameter) of the largest tumor in each animal is indicated and serves as a measurement of overall tumor growth potential. E. The cumulative tumor diameter for each Casp8^{f/f} and Casp8^{Δhepa} mouse was calculated. *: p<0.05.

At this time point, macroscopically visible tumor nodules were detectable in both Casp8^{f/f} and Casp8^{Δhepa} livers (Figure 4.8 A - B). Casp8^{f/f} livers displayed an average tumor number of 1.6 per liver (SD: ± 3.2, Figure 4.9 C) and a cumulative tumor diameter of 3.1 mm/animal (SD: ± 6.8, Figure 4.9 D -

E). Interestingly, $\text{Casp8}^{\Delta\text{hepa}}$ mice revealed a significantly increased number of tumor nodules per liver (5.6; SD: ± 8.9), which were also significantly enlarged in total and cumulative size (12.0 mm; SD: ± 18.2) compared to controls (Figure 4.9 C - E). Accordingly, this data demonstrates that loss of Casp8 results in enhanced tumor progression especially between 24 - 40 weeks after tumor induction.

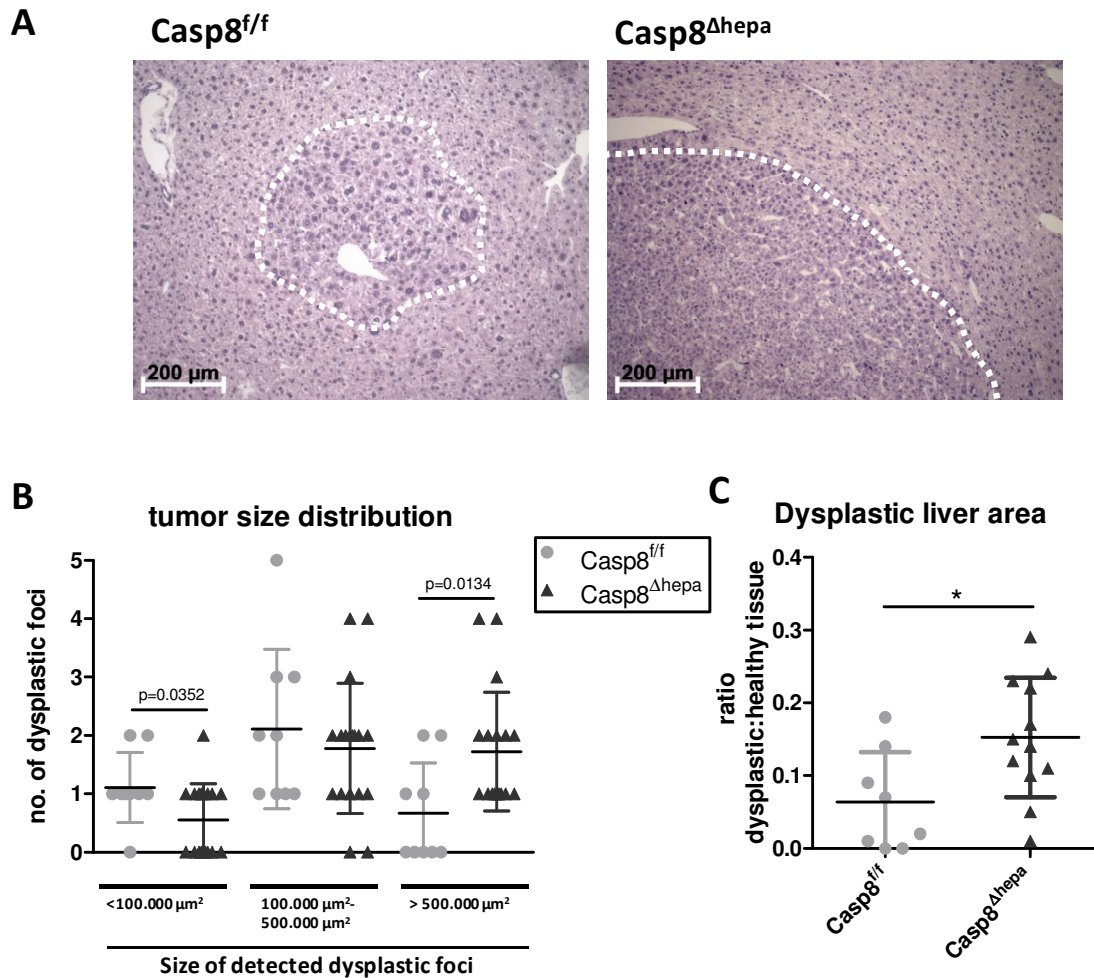


Figure 4.9: Loss of Casp8 results in enhanced formation of dysplastic liver lesions 40 weeks after DEN-mediated tumor induction: $\text{Casp8}^{\text{f/f}}$ and $\text{Casp8}^{\Delta\text{hepa}}$ mice were injected with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 40 weeks. Liver sections were stained with HE and analyzed microscopically. A. Representative HE staining showing moderate dysplasia in $\text{Casp8}^{\text{f/f}}$ mice (encircled, left) and large dysplastic lesions in $\text{Casp8}^{\Delta\text{hepa}}$ mice (right, partially encircled). (B, C). HE stained liver sections from every DEN-treated animal were analyzed using Discus-Imaging software. Number and size of dysplastic tissue area was determined. B. Detected dysplastic nodules were categorized by size and compared between $\text{Casp8}^{\text{f/f}}$ and $\text{Casp8}^{\Delta\text{hepa}}$ mice. C. The cumulative size of dysplastic nodules in liver sections was determined and calculated as dysplasia:total-tissue area ratio for every DEN-treated mouse. *: $p < 0.05$.

To further confirm these results, liver sections were stained with HE and analyzed for number and size of dysplastic nodules as already described in 4.3.1. In sharp contrast to the findings at early tumor initiation (compare Figure 4.8), the liver histology in Casp8^{Δhepa} mice revealed significantly more dysplasia compared to Casp8^{ff} controls (Figure 4.10 A - B). A more detailed analysis of dysplastic foci by size demonstrated that Casp8^{Δhepa} liver comprised less small-sized foci but significantly more large-sized lesions compared to control mice (Figure 4.10 C). In summary, this data confirms that ablation of Casp8 triggers tumor growth selectively during advanced hepatocarcinogenesis.

4.3.3 DEN-induced hepatocarcinogenesis is independent of Casp8-mediated extrinsic apoptosis

Casp8 is the apical caspase involved in extrinsic, death-receptor mediated apoptosis (Salvesen 1999). Accordingly, it was hypothesized that ablation of Casp8 would affect DEN-driven hepatocarcinogenesis if extrinsic apoptosis is involved in this process. However, the previous experiments suggested that Casp8 is not relevant for the immediate response towards to DEN-induced liver injury as no differences in apoptosis levels were found between Casp8^{Δhepa} mice and controls. To investigate, if extrinsic apoptosis plays a role for tumor initiation or tumor progression, TUNEL-analysis was performed with liver sections derived from DEN-treated Casp8^{ff} and Casp8^{Δhepa} mice 24 and 40 weeks after treatment, respectively.

The results are shown in Figure 4.10. Although the number of TUNEL-positive cells slightly increased in both in Casp8^{ff} and Casp8^{Δhepa} mice throughout tumor progression, significant differences between the groups could not be detected (Figure 4.10 B). Thus, Casp8 mediated apoptosis does not play a major role for DEN-induced liver cancer development.

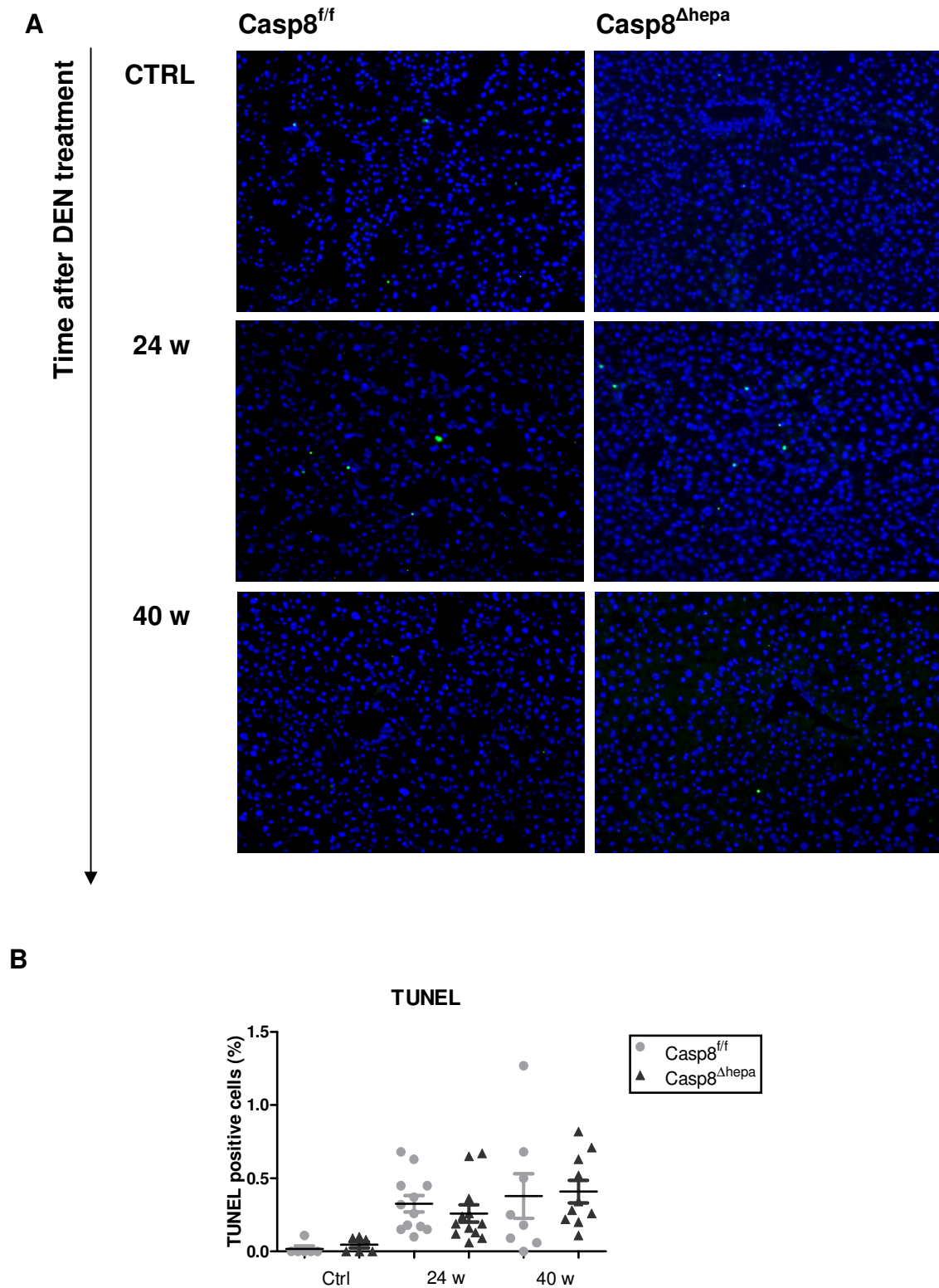


Figure 4.10: TUNEL immunofluorescence after DEN-induced liver injury. A. Casp8^{f/f} and Casp8^{Δhepa} mice were injected with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 24 and 40 weeks, respectively. Liver cryosections were subjected to TUNEL analysis; apoptotic cells are stained in green; total cells were counter stained with DAPI (blue). B. Quantification of TUNEL-positive cells shown in A. For each mouse, three independent magnification fields were analyzed.

To further support the previous finding that apoptosis does not play a major role in DEN-induced hepatocarcinogenesis in Casp8^{ff} mice or Casp8^{Δhepa} animals, the gene expression of pro-apoptotic mediators in early and advanced liver cancer was determined by quantitative real-time PCR. For this approach, six well characterized pro-apoptotic genes were selected: Fas, TRAIL, their cognate receptors Fas-R and TRAIL-R2 and the intrinsic apoptosis genes Bax and Noxa, respectively.

Fas-ligand (Fas) is a type II transmembrane protein and a prototypical member of the tumor necrosis factor (TNF) family (Nagata & Golstein 1995). Binding of Fas to its receptor usually induces extrinsic apoptosis e.g. in hepatocytes in a Casp8-dependent manner (Timmer et al. 2002). Similarly TRAIL is also capable of inducing death-receptor mediated apoptosis by binding its cognate receptors TRAIL-R1 (Death Receptor 4 (DR-4)) or TRAIL-R2 (DR-5), respectively (Malhi et al. 2006). Of notice, in contrast to human, mice do not express TRAIL-R1 but a receptor similar to human's TRAIL-R2 (Wu et al. 1999). Moreover, it has been demonstrated in several studies that TRAIL receptor expression is regulated by the tumor suppressor p53 e.g. following genotoxic stress (Wu et al. 1997) and thus is induced in tumor cells or in hepatocytes during chronic inflammation. Bax and Noxa belong to the pro-apoptotic members of the Bcl-2 family members and are therefore involved in controlling intrinsic apoptosis and the mitochondrial permeability transition (Hassan et al. 2008).

The results of the measurements are shown in Figure 4.11. Interestingly, none of the investigated genes was up-regulated in Casp8^{ff} mice at tumor initiation (24 weeks after DEN) or during advanced tumor progression further supporting the idea that neither extrinsic nor intrinsic apoptosis plays a major role in DEN-induced hepatocarcinogenesis. However, a sub-population of Casp8^{Δhepa} mice revealed an increased expression of Fas 24 weeks after DEN treatment (Figure 4.11 A). Although the overall Fas expression at this time point was not significantly different from the control group, these data suggest that loss of Casp8 in hepatocytes could prime the liver for enhanced infiltration of Fas-expressing immune cells. In line with these findings, Casp8^{Δhepa} livers also showed significantly higher expression of Fas-receptor 24 weeks after DEN treatment (Figure 4.11 B).

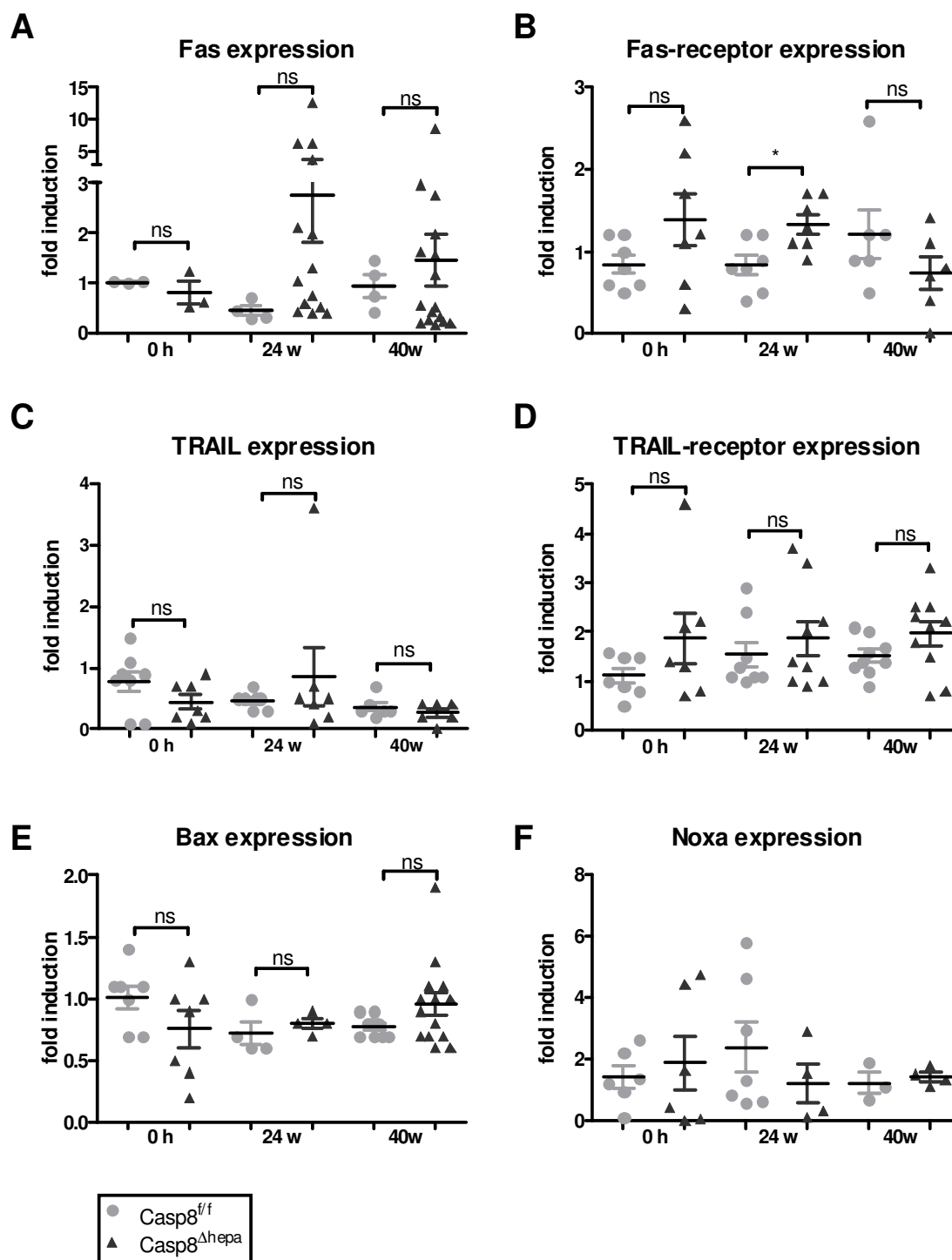


Figure 4.11: Gene expression analysis of pro-apoptotic genes after DEN-mediated tumor induction. Casp8^{f/f} and Casp8^{Δhepa} mice were subjected to single injection with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 24 and 40 weeks, respectively. Gene expression was determined by quantitative real-time PCR and normalized via GAPDH expression levels. Data were calculated as fold induction in comparison to untreated Casp8^{f/f} control-mice. Each dot represents the gene expression of one individual mouse. A. Expression of Fas ligand (Fas). B. Expression of Fas-receptor. C. Expression of TRAIL ligand. D. Expression of TRAIL-receptor. E. Expression of Bax. F. Expression of Noxa. *: p<0.05; ns: not significant.

Accordingly, the Fas signaling cascade is activated at least in a sub-population of Casp8^{Δhepa} mice after DEN challenge, although the signals cannot induce apoptosis due to the lack of Casp8. It is therefore possible that these signals may activate alternative pathways (e.g. NF-κB) in the absence of Casp8. This aspect will be further addressed in the discussion.

However, in good agreement with the data derived from the TUNEL experiments, significant de-regulation of TRAIL/TRAIL-R5, Bax or Noxa was not observed in Casp8^{Δhepa} livers at any time point investigated (Figure 4.11 C - F) which was very similar to the findings obtained from WT controls. In summary, the initial hypothesis that enhanced tumor progression in Casp8^{Δhepa} mice occurs due to reduced apoptosis of premalignant cells could not be confirmed since apoptosis seems to play only a minimal role in the DEN model.

4.3.4 Determination of liver cell proliferation in Casp8^{Δhepa} mice after DEN-treatment

Uncontrolled excessive cell proliferation is a hallmark of cancer (Hanahan & Weinberg 2000). Due to the increased tumor progression in Casp8^{Δhepa} mice, a higher amount of proliferating cells in the liver was expected in these mice 40 weeks after DEN-treatment. In agreement with this idea, Ki67-staining indicated more hepatocyte proliferation in Casp8^{Δhepa} mice at 40 weeks after DEN-injection compared to Casp8^{f/f}-controls (Figure 4.12), although the differences were not statistically significant ($p = 0.08$). Moreover, the results of the Ki67-staining confirmed the previous histological analysis in 24 week old mice (compare Figure 4.8) in which no differences in tumor initiation were observed between Casp8^{f/f} and Casp8^{Δhepa} mice 24 weeks after DEN-treatment. Therefore, the data suggests that a slight increase in overall hepatocyte proliferation in Casp8^{Δhepa} mice occurring between 24 and 40 weeks after DEN-treatment might be sufficient to explain enhanced tumor progression.

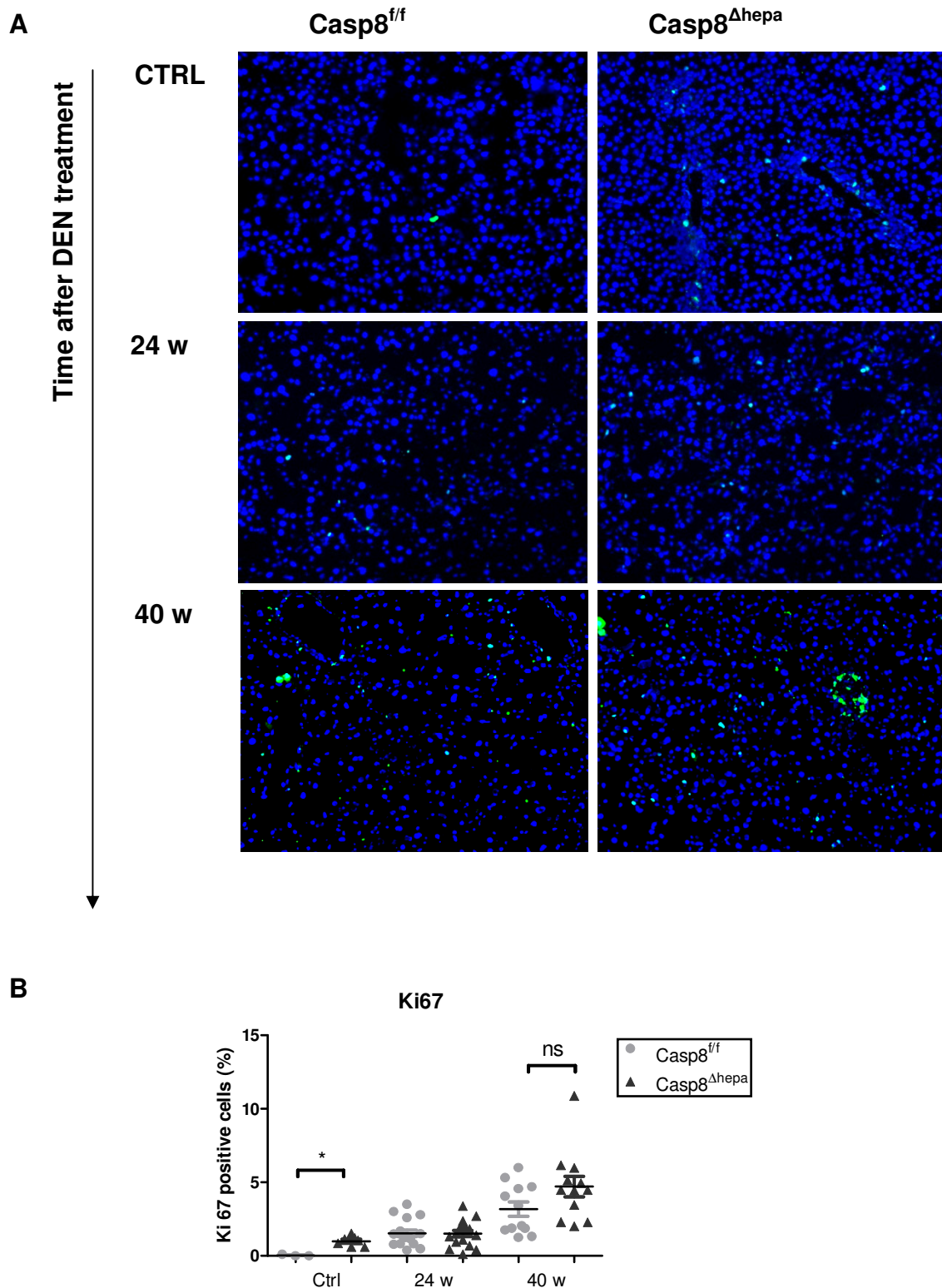


Figure 4.12: Ki67-immunofluorescence after DEN-treatment. $Casp8^{f/f}$ - and $Casp8^{\Delta hepa}$ -mice were injected with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 24 and 40 weeks, respectively. A. Liver cryosections were stained with a Ki67-antibody; cells in active cell cycle are stained in green; total cells were counter stained with DAPI (blue). B. Quantification of Ki67-positive cells shown in A. For each mouse, three independent magnification fields were analyzed. *: $p < 0.05$; ns: not significant.

Recent data showed that loss of Casp8 triggered accelerated and prolonged onset of the JNK/c-Jun-pathway after 2/3-hepatectomy, which resulted in premature induction of the cell cycle and liver regeneration (Julia Freimuth, unpublished data). In order to explain the enhanced tumor progression in Casp8^{Δhepa}-mice observed in the present study, it was speculated that constitutive activation of the JNK/c-Jun-pathway could contribute to these findings. Therefore, c-Jun phosphorylation was investigated in Casp8^{f/f} and Casp8^{Δhepa} mice 24 and 40 week after DEN-treatment by immunofluorescence staining using a phosphorylation-specific antibody.

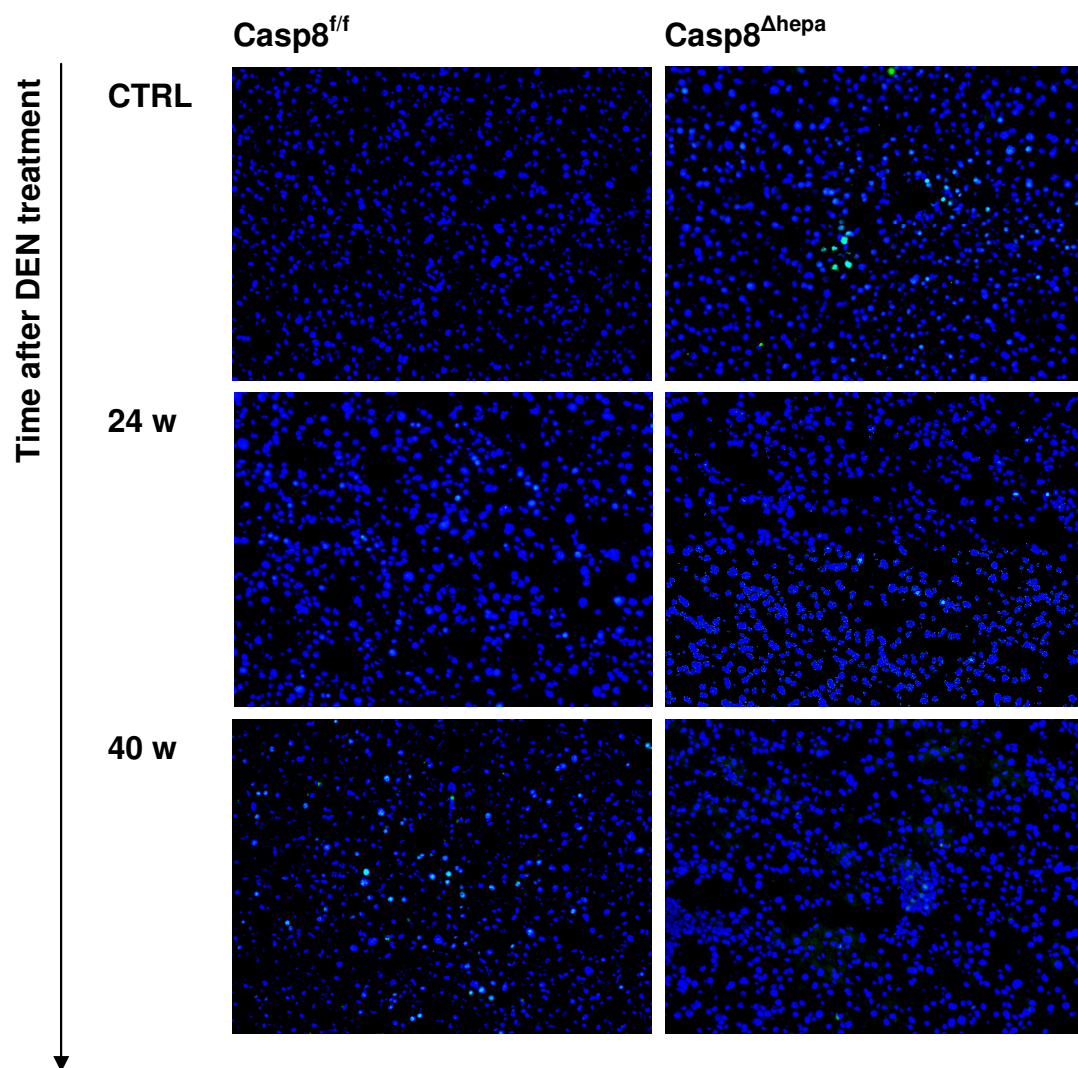


Figure 4.13: Determination of cJun phosphorylation after DEN-mediated tumor induction: Casp8^{f/f} and Casp8^{Δhepa} mice were injected with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 24 and 40 weeks, respectively. Liver cryosections were stained with an antibody selectively detecting phosphorylated cJun; p-Jun-positive cells are stained in green; total cells were counter stained with DAPI (blue).

Of note, untreated Casp8^{Δhepa} control mice revealed some basal cJun activation which was not detected in the Casp8^{f/f} counterpart (Figure 4.13). However, this is in good agreement with the recent observation that Casp8^{Δhepa} mice develop moderate spontaneous liver inflammation (Liedtke et al. 2011). Regarding tumor initiation (24 weeks post DEN) and advanced tumor progression (40 weeks post DEN) substantial differences in cJun activation were not observed. Nonetheless, focal concentrations of p-cJun positive cells were observed in assumed dysplastic liver lesions or inflammatory infiltrates 40 weeks after DEN which were moderate or large in Casp8^{f/f} mice and small in Casp8^{Δhepa} animals (Figure 4.13). Due to the high variation of these staining patterns, this data did not allow any conclusion regarding potential interactions between Casp8 and the JNK/cJun-pathway in the DEN model.

4.3.5 Determination of the inflammatory response in Casp8^{Δhepa} mice in DEN-induced hepatocarcinogenesis

Several studies suggested that hepatocellular carcinoma mostly develops in a pro-inflammatory environment (Karin et al. 2006 – 2007). Although the DEN model is typically not associated with chronic inflammation, recent reports demonstrated that pro-inflammatory mediators such as IL-6 and TNF play a crucial role in DEN-induced hepatocarcinogenesis (Naugler & Karin 2008).

To test, if Casp8 is involved in this process, mRNA-expression levels of IL-6 and TNF were determined in Casp8^{f/f} and Casp8^{Δhepa} mice 24 and 40 weeks after DEN treatment, respectively. In agreement with previous studies, IL-6 expression was slightly up-regulated during the course of tumor development, while no significant differences between the control group and Casp8^{Δhepa} mice could be observed (Figure 4.14 A., left panel). Similarly, TNF expression levels in both groups were identical 24 - 40 weeks after DEN treatment, whereas basal TNF expression was significantly increased in Casp8^{Δhepa} mice as reported recently (Liedtke et al. 2011). However, a more detailed analysis of IL-6 expression in the Casp8^{Δhepa} cohort 40 weeks post DEN identified a sub-population of animals (n = 5) with strongly elevated IL-6 levels. It was therefore assumed that the individual IL-6 expression could correlate with the number of tumor foci in the same animal. To test this

hypothesis, a linear regression analysis was performed. The data is shown in Figure 4.14 B and demonstrates that the expression level of IL-6 does not significantly correlate with number of tumor foci.

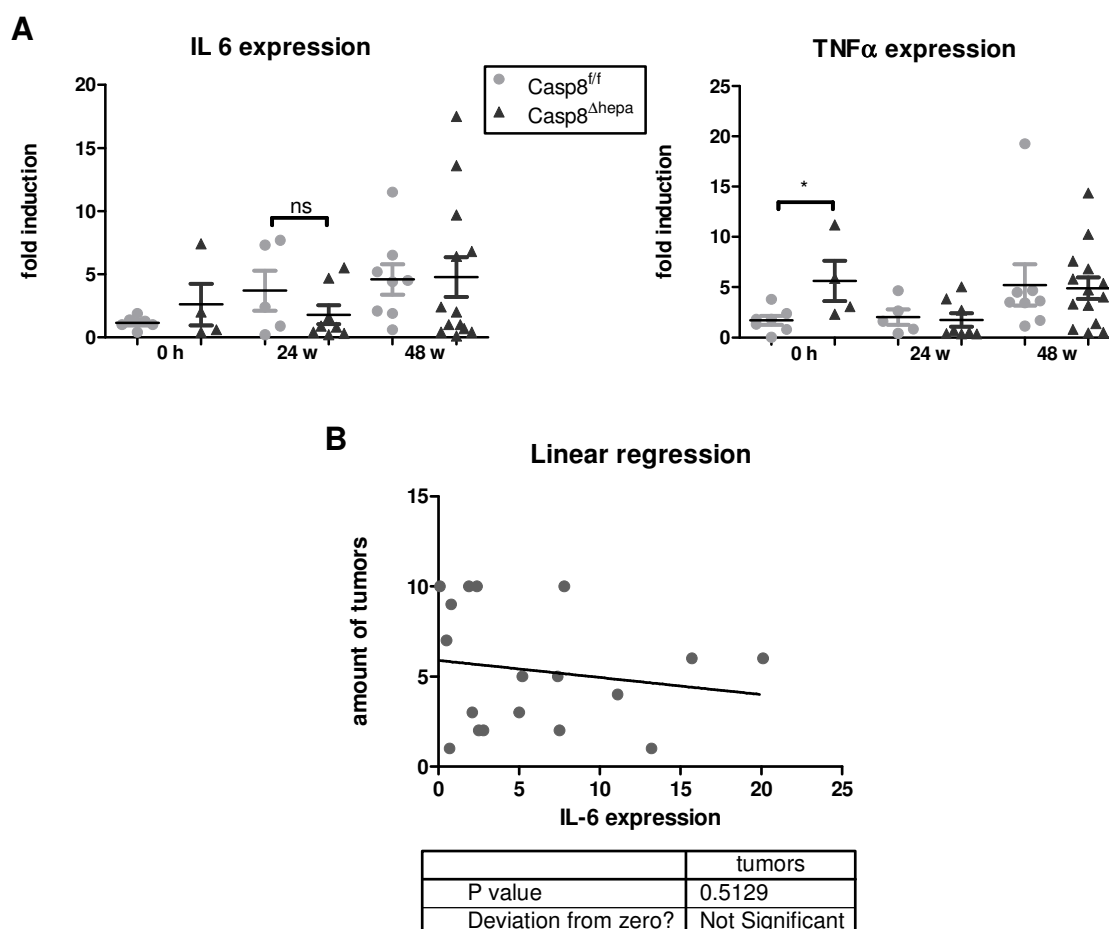


Figure 4.14: Analysis of TNFα and IL-6 gene expression after DEN mediated tumor induction: A. Casp8^{f/f} and Casp8^{Δhepa} mice were subjected to single injection with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 24 and 40 weeks, respectively. Gene expression was normalized via GAPDH expression levels. Data were calculated as fold induction in comparison to untreated Casp8^{f/f} control-mice. B. Linear regression analysis. For each DEN-treated Casp8^{f/f} or Casp8^{Δhepa} liver the relative IL-6 expression level (fold induction) was plotted against the number of tumor foci. Regression analysis was performed using PRISM software. P values < 0.05 were considered statistical significant.

To further evaluate inflammation in DEN-treated Casp8^{f/f} and Casp8^{Δhepa} mice, liver sections were stained with a fluorescence-labeled CD45 antibody. CD45, also known as protein tyrosine phosphatase receptor type C (PTPRC) is a leukocyte antigen and thus serves as a marker for overall leukocyte infiltration. Stained sections were analyzed by fluorescence microscopy and

subjected to quantification by counting positive cells in randomly selected magnification fields.

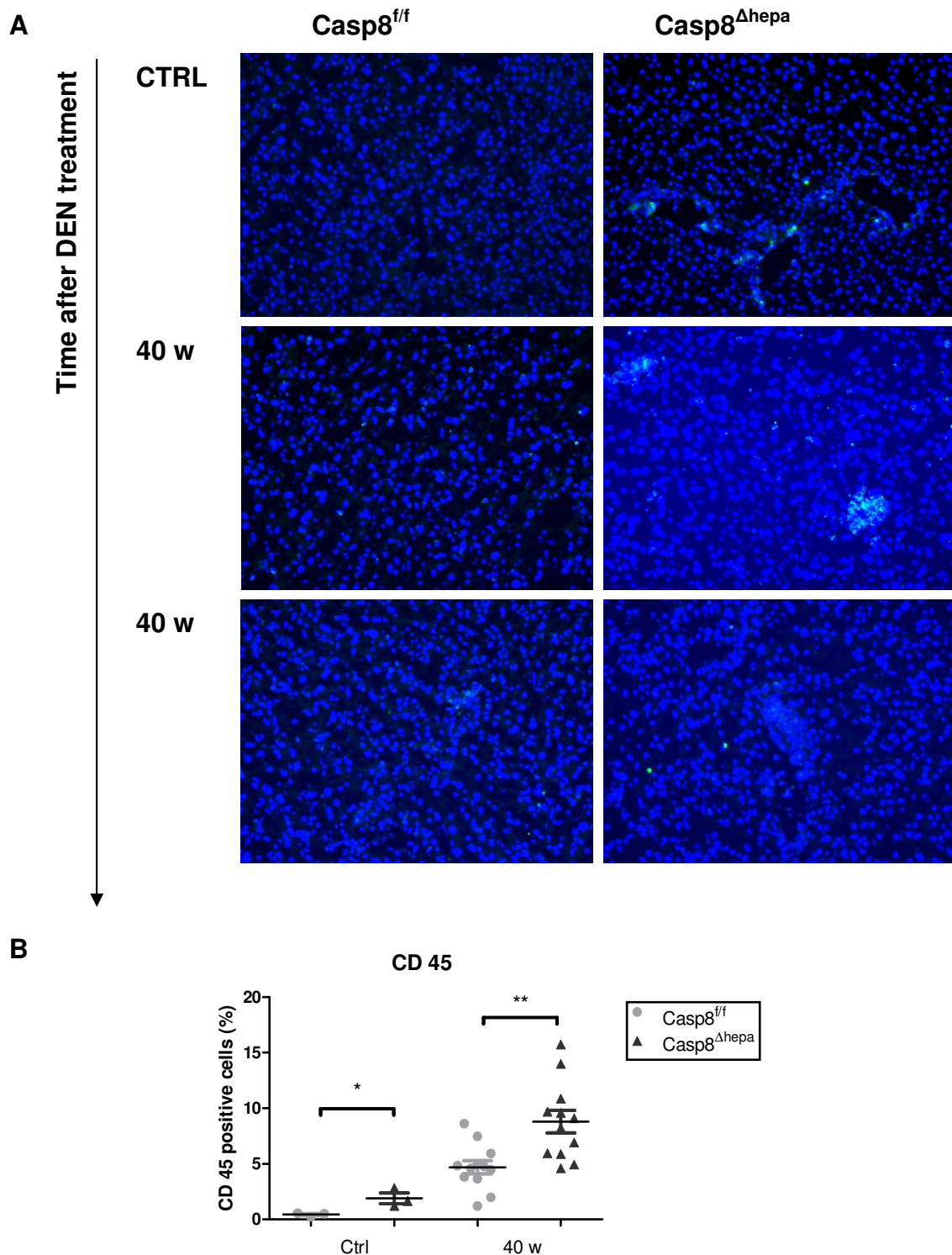


Figure 4.15: CD 45 Staining after DEN mediated tumor induction. A. Casp8^{f/f} and Casp8^{Δhepa} mice were injected with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 40 weeks. Liver cryosections were stained with immunofluorescence-labeled CD45-antibody; CD45-positive leukocytes are stained in green; total cells were counter stained with DAPI (blue). B. Quantification of CD45-positive cells shown in A. For each mouse, three independent magnification fields were analyzed. **: p<0.01; *: p<0.05.

In good agreement with elevated basal TNF expression levels, Casp8^{Δhepa} livers revealed also increased numbers of CD45-positive cells in untreated samples (Figure 3.16, top). Interestingly, these mice also showed significantly increased CD45-positive infiltrates in livers with advanced HCC (40 weeks post DEN), which was barely evident in the control group. Accordingly, enhanced tumor formation in Casp8^{Δhepa} mice is associated with inflammation suggesting that Casp8 mediates anti-inflammatory properties in the DEN model.

4.3.6 DNA array analysis links the loss of Casp8 to over-expression of DMBT1

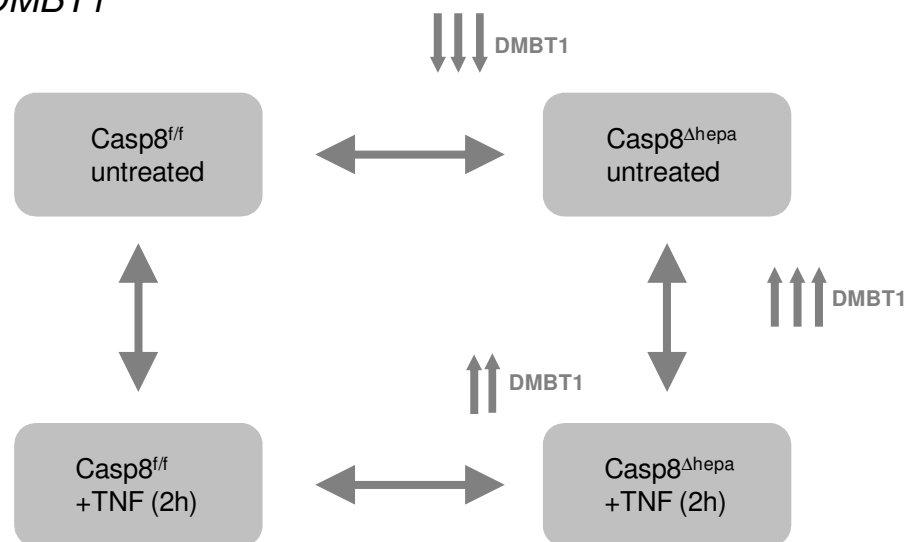


Figure 4.16: Casp8^{f/f} and Casp8^{Δhepa} mice (n = 3 each) were treated with recombinant murine TNF for 2 hours or left untreated. Liver samples were subjected to RNA isolation and subsequent DNA array analysis in cooperation with Prof. M. Müller (Wageningen) of DMBT1 of untreated and TNF treated Casp8^{f/f} and Casp8^{Δhepa} mice.

The present data hinted at tumor suppressive properties of Casp8 due to higher tumor progression and proliferating rate in Casp8^{Δhepa} mice. However, the molecular mechanisms, how Casp8 antagonized advanced tumor growth, remained obscure and the previous experiments suggested that non-apoptotic functions of Casp8 might be responsible for these effects. To detect yet unknown Casp8-dependent pathways, DNA array analyses from untreated and TNF-treated Casp8^{f/f} and Casp8^{Δhepa} mice were performed independent of the present work (C. Liedtke, M. Müller and C. Trautwein, unpublished data). This approach revealed that a yet poorly investigated gene designated Deleted in Malignant Brain Tumors 1 (DMBT1) was de-regulated by ablation of Casp8 in a TNF-dependent manner (Figure 4.16). The data revealed that DMBT1 was strongly up-regulated by TNF in Casp8^{Δhepa} mice, but not in WT controls.

Therefore it was hypothesized that enhanced HCC progression in Casp8^{Δhepa} mice could be related to aberrant DMBT1 expression. Consequently, DMBT1 expression in DEN-treated mice with early or advanced HCC was investigated in more detail.

4.3.7 Casp8 prevents DMBT1-dependent CD133+ cancer stem cell activation

The role of DMBT1 in the liver is poorly understood so far. Initially, it was described in the context with medullablastoma formation (Mollenhauer et al. 1997). In further studies it was shown to be deleted or down-regulated in lung, gut, and breast cancers (Mori et al. 1999, Takeshita et al. 1999, Wu et al. 1999b, Mollenhauer et al. 2004). Interestingly, DMBT1 was over-expressed in regenerating liver cells (Bisgaard et al. 2002). Beside its assumed function in innate immunity and mucosal protection, it was postulated that DMBT1 is involved in epithelial and stem cell differentiation (Tynan et al. 2005).

To test the possibility that DMBT1 is involved in enhanced HCC progression in Casp8^{Δhepa} mice, DMBT1 mRNA levels were determined in Casp8^{f/f} and Casp8^{Δhepa} mice 24 and 40 weeks after DEN-treatment (Figure 4.17 A). Interestingly, DMBT1 was substantially up-regulated (up to 5755 fold) in subpopulations of Casp8^{Δhepa} mice after 24 and 40 weeks following DEN-treatment. Although the differences were not statistically significant due to high variation between the mice, Casp8^{Δhepa} animals could be categorized into 2 subpopulations with either silencing of DMBT1 (expression 0.1 fold or lower compared to un-treated controls) or dramatic over-expression (10 - 5755 fold), which perfectly reflects the variable distribution of tumor foci and size in these animals 40 weeks after DEN treatment (compare Figure 4.9).

Over-expression of DMBT1 in Casp8^{Δhepa} mice was confirmed on the protein level using *in situ* immunofluorescence stainings on extracted liver sections 24 and 40 weeks after DEN treatment, respectively. As shown in Figure 4.17 B, DMBT1 expression in Casp8^{f/f} livers was mostly restricted to the large liver vessels. In contrast, livers of DEN-treated Casp8^{Δhepa} mice revealed overall larger stained tissue areas which were not only restricted to vascular structures.

Very recent data indicated an involvement of DMBT1 in differentiation of oval cells – a postulated liver stem cell population - into hepatocytes (Deng et al. 2012). Based on the findings of the present study demonstrating increased DMBT1 expression in Casp8^{Δhepa} livers with strong HCC progression, it was hypothesized that Casp8 could antagonize DMBT1-mediated activation of liver stem cells and subsequent stem cell mediated tumor progression.

One accepted marker for liver stem cells, but also for tumor stem cells in general, is CD133+ (also known as Prominin 1). CD133+ expression identifies a population of oval cells with the gene expression profile and function of primitive, bipotent liver stem cells (Ma et al. 2007, Yin et al. 2007, Fujii et al. 2010, Tsuchiya et al. 2009). Interestingly, CD133+ has been recently identified as part of the cancer stem cell population responsible for tumorigenesis in brain, prostate, and colon cancer (Singh et al. 2003, Singh et al. 2004, Hemmati et al. 2003, Richardson et al. 2004, O'brien et al. 2007, Ricci-Vitiani et al. 2007). Several studies have linked CD133+ expression to liver cancer stem cells, demonstrating that CD133+ cells from established HCC cell lines displayed significant tumorigenic capacity (Ma et al. 2007, Yin et al. 2007).

To test if high DMBT1 expression could be associated with stem cell activation, gene expressing analysis of CD133+ in Casp8^{f/f} and Casp8^{Δhepa} mice 24 and 40 weeks after DEN-treatment was performed. The data demonstrated an up-regulation of CD133+ in both Casp8^{f/f} and Casp8^{Δhepa} mice 40 weeks after DEN-treatment. However, Casp8^{Δhepa} mice revealed again to subpopulations of livers/HCC showing either normal CD133+ expression comparable to controls or marked up-regulation (5 - 25 fold in comparison to Casp8^{f/f} mice, Figure 4.17 C). To track CD133+ in these livers, CD133+ immunofluorescence stainings in liver sections were performed demonstrating a focal strong expression of CD133+ on the surface of Casp8^{Δhepa} liver cells, which was not evident in controls (Figure 4.17 D). The simultaneous over-expression of DMBT1- and CD133+ expression in Casp8^{Δhepa} mice indicated a possible connection between these two molecules. Therefore, a linear regression analysis of these signals was performed showing a significant correlation of both factors (Figure 4.17 E). Accordingly, the data suggests that loss of Casp8 triggers increased expression of DMBT1 in the liver leading to CD133+ positive (cancer) stem cell activation.

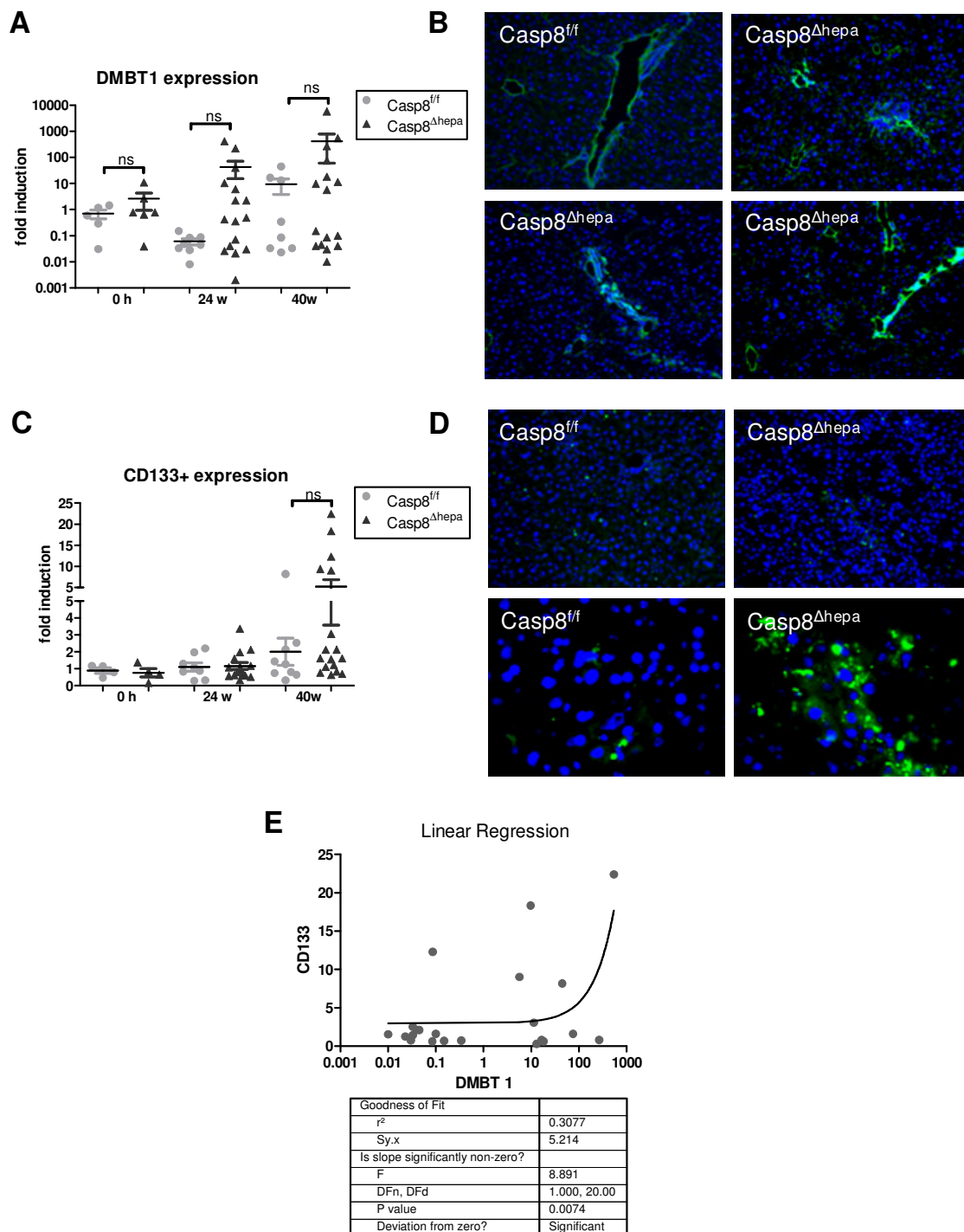


Figure 4.17: Analysis of DMBT1 and CD133+ expression after DEN-mediated tumor induction. Casp8^{f/f} and Casp8^{Δhepa} mice were subjected to single injection with DEN (25mg/ kg) at the age of 2 weeks and sacrificed after 24 – and 40 weeks respectively. Gene expression was determined by quantitative real-time PCR and normalized via GAPDH expression levels. Data were calculated as fold induction in comparison to untreated Casp8^{f/f}-control-mice. (A, B). Determination of DMBT1 expression. A. DMBT1 gene expression. Please note that the fold induction is indicated in a logarithmic scale. B. Liver cryosections were stained with immunofluorescence-labeled DMBT1-antibody. DMBT1 is stained in green; total cells were counter stained with DAPI (blue). (C, D). Determination of CD133+ expression. C. CD133+ gene expression. D. Immunofluorescence staining for CD133+ localized on the surface of liver

cells. Green: CD133+; Blue: DAPI. ns: not significant. E. Linear regression analysis between DMBT1 and CD133+.

4.3.8 *Loss of Casp8 triggers NF-κB-activation in chemically induced hepatocarcinogenesis*

Recent studies demonstrated that the DMBT1 promoter contains regulatory elements which can bind the transcription factor NF-κB. Accordingly, DMBT1 can be up-regulated in a NF-κB dependent manner, e.g. by stimulation with TNF or LPS as shown in epithelial cell lines (Rosenstiel et al. 2007). NF-κB is a transcription factor involved in immunity, inflammation, cell survival, apoptosis (Sarkar et al. 2008), cancer cell invasion and angiogenesis (Helbig et al. 2003, Huang et al. 2001, Samant et al. 2007). It consists of the two subunits p50 and p65 which are sequestered to the cytoplasm in the inactive state and are translocated to the nucleus after appropriate stimulation, where they trans-activate the expression of target genes. Of notice, NF-κB activity is increased especially in poorly differentiated human cancer (Karin 2006).

To test the hypothesis that increased DMBT1 expression and overall enhanced tumor progression in Casp8^{Δhepa} mice could be related to NF-κB activation, nuclear extracts from Casp8^{ff} and Casp8^{Δhepa} livers were isolated 40 weeks after DEN treatment and subjected to electrophoretic mobility shift assays (EMSA) using a NF-κB specific consensus sequence. Interestingly, 50 % (5/10) of Casp8^{ff} samples revealed explicit NF-κB activation while this was the case in 80 % (8/10) of Casp8^{Δhepa} livers (Fig 3.18 A). Moreover, the overall strength of activation (band intensity) was higher in Casp8^{Δhepa} livers compared to the matching Casp8^{ff} control which suggests that Casp8 may act as a NF-κB antagonist in DEN-induced hepatocarcinogenesis.

In order to detect hepatic cells with activated NF-κB, immunostainings of livers with HCC were performed using an antibody specific for the p65 subunit. Surprisingly, nuclear p65 was not detected within tumor nodules at all, but was restricted to tumor-surrounding hepatocytes especially in Casp8^{Δhepa} livers and less evident in Casp8^{ff} tissue 40 weeks after DEN treatment (Figure 4.18 B).

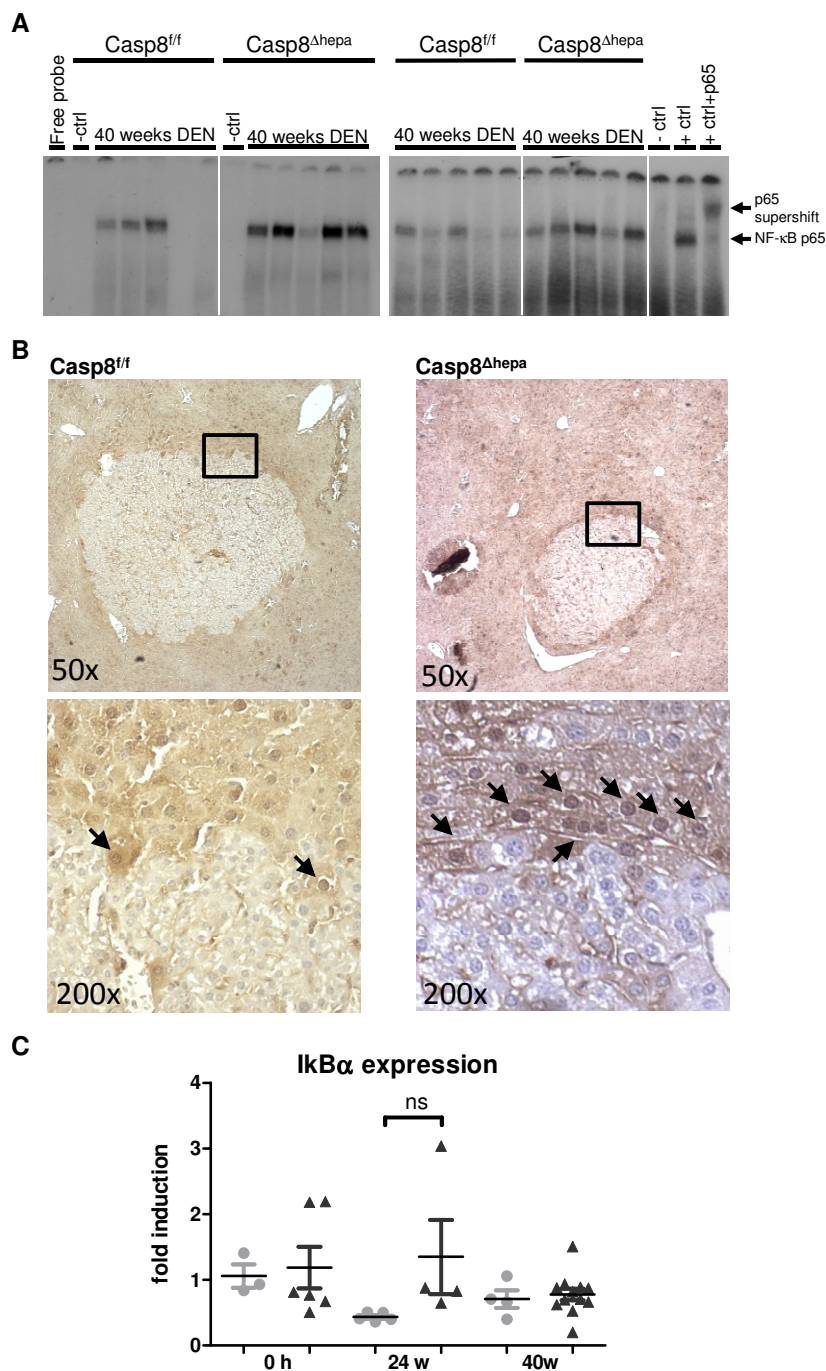


Figure 4.18: Enhanced tumor growth in Casp8^{Δhepa} mice is associated with sustained NF-κB activation and nuclear translocation of p65 in tumor-surrounding liver tissue. Casp8^{f/f} and Casp8^{Δhepa} mice were subjected to single injection with DEN (25mg/ kg) at the age of 2 weeks and sacrificed after 40 weeks. A. EMSA analysis with NF-κB consensus sequence and nuclear extracts derived from Casp8^{f/f} and Casp8^{Δhepa} livers. The results of two independent experiments is shown in total representing data from n = 10 animals per group. Free probe: labeled consensus oligo was not incubated with nuclear protein; -ctrl: negative control derived from untreated Casp8^{f/f} and Casp8^{Δhepa} livers, respectively. +ctrl: positive control derived from Casp8^{Δhepa} liver 2 hours after partial hepatectomy showing strong NF-κB activation. P65

supershift: To prove specific complex formation with NF- κ B, nuclear extracts were incubated with NF- κ B consensus oligo and an antibody directed against NF- κ B subunit p65. Formation of a new slow migrating complex (supershift) demonstrates NF- κ B binding. B. Liver sections were subjected to a p65-antibody. P65-positive cells are stained in brown. Top: Overview, original magnification 50X. A region of interest is boxed. Bottom: Enlarged view, original magnifications 200X. P65-positive cells are highlighted by arrows. C. Gene expression analysis of I κ B α in livers from Casp8^{f/f} and Casp8 ^{Δ hepa} mice. Expression was normalized to GAPDH and calculated as fold induction in comparison to untreated Casp8^{f/f} control mice.

The inhibitor of NF- κ B, I κ B α , is one of the first target genes of NF- κ B and usually ensures that NF- κ B is only transiently activated through a negative feedback loop (Legarda-Addison et al. 2009). Accordingly, I κ B α induction may indicate either NF- κ B activation or repression depending on the cellular context. However, during tumor progression (24 - 40 weeks after DEN treatment) significant differences in I κ B α gene expression between Casp8^{f/f} and Casp8 ^{Δ hepa} livers were not detected at any time point as evidenced by quantitative real-time PCR (Figure 4.18 C). This data demonstrates that in general I κ B α gene expression is not changed in DEN-induced tumor progression and thus does not contribute to the different NF- κ B activation and tumor progression observed in Casp8 ^{Δ hepa} mice.

4.3.9 Enhanced hepatic tumor formation in Casp8 ^{Δ hepa} mice is associated with down-regulation of cell cycle inhibitors p21 and p27

Tumorigenesis is usually associated with de-regulated cell cycle involving either up-regulation of cell cycle promoters (e.g. the Cyclin D1, Sugimura 1992, Tanigami et al. 1992, Zhang et al. 1993, Nishida et al. 1994), or down-regulation of cell cycle inhibitors such as p21 and p27 (Malumbres & Barbacid 2001). Several reports indicated that Casp8 also bears non-apoptotic functions and may affect cell cycle regulation (Maelfait & Beyaert 2008) although the precise mechanisms are poorly understood. To test, if ablation of Casp8 directly triggers cell cycle activation during hepatocarcinogenesis, the expression of p21 and p27 was determined in detail in untreated Casp8 ^{Δ hepa} mice and Casp8^{f/f} controls (baseline expression) but also in animals treated with DEN for 24 weeks (tumor initiation) or 40 weeks (advanced tumor progression). The cell cycle inhibitor

p21 inactivates cyclin E/A/Cdk2 kinase complexes and is directly regulated by p53. Similarly, the cyclin-dependent kinase inhibitor p27 inhibits especially cyclin E/CDK2-complexes and it has been shown that down-regulation of p27 is associated with poor prognosis of patients with tumors in breast, prostate, bladder, lung, glia, liver, larynx, ovary, stomach, and other tissues (Nickeleit et al. 2007). Surprisingly, HCC development was not associated with aberrant p21 gene expression in DEN treated Casp8^{f/f} controls or Casp8^{Δhepa} mice at any time point investigated as demonstrated by quantitative real-time PCR (Figure 4.19 A). Analysis of p27 expression in Casp8^{f/f}-mice revealed a reduced expression both 24 and 40 weeks after DEN treatment compared to basal expression levels (Figure 4.19 B). These findings are consistent with the idea that HCC development correlates with increased cell cycle activity. However, in Casp8^{Δhepa}- mice gene expression of p27 was already significantly down-regulated at basal levels, but more importantly also strongly reduced during advanced HCC progression (40 weeks post DEN) when compared to Casp8^{f/f}-controls. These findings indicate that Casp8 might be necessary to maintain sufficient p27 gene transcription in liver homeostasis but also during liver cancer formation.

Cell cycle mediators such as cyclins and cyclin-dependent kinase inhibitors are not only regulated by control of gene transcription but also on the level of protein translation and through post-translational modifications (e.g. phosphorylation, ubiquitin-mediated degradation etc.). Therefore, protein expression of p21 and p27 was determined in liver samples from Casp8^{f/f} controls and Casp8^{Δhepa} mice with advanced tumor progression (40 weeks after DEN-treatment) by western blot analysis (Figure 4.19 C). Efficient Casp8 deletion in Casp8^{Δhepa} mice was confirmed by probing these samples with a Casp8-specific antibody. Interestingly, the experiment revealed that in the majority of Casp8^{f/f}-samples (6/7) p21 protein was clearly expressed while p21 was only barely expressed (1/7 samples) in Casp8^{Δhepa} liver. This finding demonstrates that Casp8 is not relevant for transcriptional control of p21, but important for regulating its protein stability through yet unknown mechanisms. Regarding p27 protein expression, similar results were obtained. p27 was detectable in all Casp8^{f/f}-samples, but barely visible in Casp8^{Δhepa} liver samples 40 weeks after DEN treatment. Accordingly, in Casp8^{Δhepa} mice the p27 protein

expression levels were in good agreement with the gene expression profiles (compare Figure 4.26 B), again hinting at a role of Casp8 for the transcriptional regulation of p27.

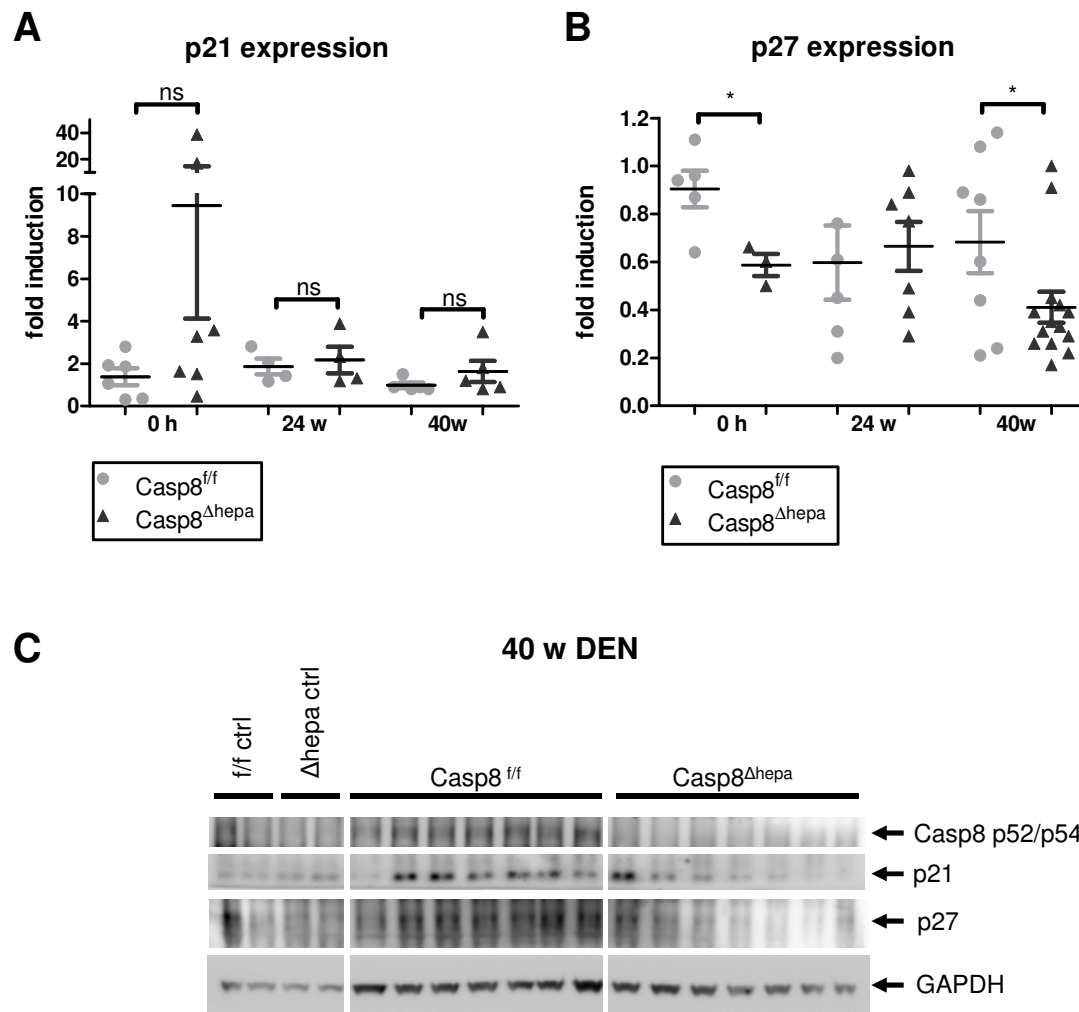


Figure 4.19: Enhanced hepatic tumor formation in Casp8^{Δhepa} mice is associated with down-regulation of cell cycle inhibitors p21 and p27. Casp8^{f/f} and Casp8^{Δhepa} mice were subjected to single injection with DEN (25 mg/ kg) at the age of 2 weeks and sacrificed after 24 and 40 weeks, respectively. (A, B). Gene expression of A. p21 and B. p27 from liver tissue at time points indicated was determined by quantitative real-time PCR and normalized via GAPDH expression levels. Data were calculated as fold induction in comparison to untreated Casp8^{f/f} control-mice. *: p<0.05. C. 40 weeks after DEN treatment, liver proteins were isolated from Casp8^{f/f} and Casp8^{Δhepa} mice and subjected to western blot analysis for Casp8, p21 and p27. Equal protein loading was confirmed by measurement of GAPDH. For each group proteins from seven independent mice were loaded.

In summary these data demonstrate that loss of Casp8 primes liver cells for enhanced proliferation through down-regulation of cyclin-dependent kinase inhibitors via several independent mechanisms.

5 Discussion

Hepatocellular carcinoma represent one of the most common primary cancers with growing incidence (Freimuth et al. 2010) and limited therapeutical options as these tumors poorly respond to chemotherapy. This indicates that hepatoma cells have acquired apoptosis resistance during malignant transformation (Liedtke & Trautwein 2012). In this line of evidence, it has been demonstrated that the pro-apoptotic mediator Caspase-8 (Casp8) is frequently inactivated in human and murine HCC tissue (Liedtke et al. 2005, Soung et al. 2005). Therefore, the aim of the present study was to test the hypothesis that Casp8 might be important for the elimination of premalignant cells and thus could be an important tumor suppressor in the liver. For this approach, genetically modified mice with a conditional hepatocyte-specific inactivation of Casp8 (Casp8^{Δhepa} mice) were subjected to the liver-specific carcinogen Diethylnitrosamine (DEN) and analyzed for the immediate acute and long term response with respect to apoptosis, inflammation and hepatic tumor formation.

The study revealed several interesting results. First, loss of Casp8 did not affect the immediate response towards acute DEN-mediated liver injury with the exception of a premature begin of proliferation in Casp8^{Δhepa} mice towards DEN-treatment. Second, the frequency and size of dysplastic liver lesions was also similar in Casp8^{Δhepa} mice and controls as analyzed 24 weeks after HCC induction. However, Casp8^{Δhepa} mice showed accelerated advanced HCC progression 40 weeks after DEN treatment with increased number and size of tumor nodules. This was associated with increased hepatic inflammation, increased NF-κB activation, down-regulation of the tumor-suppressor p21 as well as p27 and the unexpected induction of two genes encoding the putative tumor stem cell marker CD133+ and the poorly characterized factor DMBT1 (*Deleted in Malignant Brain Tumor 1*), respectively. The data hint at a novel, non-apoptotic role of Casp8 for the suppression of liver tumors and will be subsequently discussed in detail.

5.1 Loss of Casp8 does not affect the immediate response towards genotoxic acute liver injury

Administration of DEN to mice is a widely accepted model of hepatocarcinogenesis (Farazi & DePinho 2006). When applied at low dose at the age of 2 weeks, these animals typically develop tissue dysplasia and small tumor nodules within 24 weeks, which further progress to advanced HCC 40 weeks after induction. When applied at high dose to adult mice, DEN induces acute liver injury associated with predominant perlobular necrosis, but also apoptosis and a regenerative response that involves the induction of TNF, IL-6 and compensatory proliferation until the liver damage is repaired (Naugler & Karin 2008).

Very recent data demonstrated that ablation of Casp8 protects from Fas- and TNF-mediated liver injury but sensitizes the liver for enhanced necrosis in a model of hepatitis induced by the T-cell mitogen Concanavalin A (Liedtke et al. 2011). Moreover, Casp8 deficiency results in an accelerated onset of hepatocyte proliferation and liver regeneration after partial hepatectomy (PH) due to premature NF- κ B activation (J. Freimuth and C. Liedtke, unpublished results). It was therefore hypothesized that ablation of Casp8 could result in improved survival and proliferation of premalignant hepatocytes after acute DEN treatment. In partial agreement with this idea, Casp8 ^{Δ hepa} mice showed a slight but significant increase in the proliferative response to DEN, which was restricted to the first 24 hours after treatment. In agreement with recent data, Casp8 seemed to prevent premature proliferation accompanied by a longer DNA-repair time before cell division (Freimuth, unpublished data). However, the present study did not reveal any significant differences in the acute liver injury between Casp8 ^{Δ hepa} mice and control animals as demonstrated by comparable serum transaminase activities. Of special interest was the determination of the apoptotic response towards DEN as Casp8 is the apical caspase in the extrinsic apoptosis signaling cascade induced by Fas, TNF, Trail and related death ligands. Notably, Casp8 ^{Δ hepa} mice did not reveal significantly less TUNEL positive liver cells after DEN treatment compared to control mice. As TUNEL staining is not completely specific for apoptosis but may also stain necrotic cells under certain circumstances, the enzymatic caspase-3 activity in the liver was

also determined in the experimental setting and confirmed these results. Therefore the present data demonstrates that DEN induces - besides necrosis - predominantly intrinsic apoptosis which is independent of Casp8. Accordingly, Casp8 is not protective in the acute phase of genotoxic malignant transformation. Moreover, the data suggests that the regenerative response of the injured liver is controlled differentially after PH (Casp8-dependent) or DEN treatment (Casp8-independent) as loss of Casp8 results in improved liver regeneration after PH which was barely observed following DEN-induced injury.

5.2 Early hepatic tumor formation is independent of Casp8

Based on the initial hypothesis that Casp8 might be important for the elimination of premalignant hepatocytes, it was expected that Casp8^{Δhepa} mice would display enhanced tumor initiation and increased dysplasia. In contrast to these expectations, loss of Casp8 resulted in the same number and size of dysplastic liver lesions 24 weeks after DEN treatment. These unexpected findings were supported by quantification of proliferating hepatocytes *in situ* demonstrating a similar slight increase of proliferation in both Casp8^{Δhepa} mice and controls. Interestingly, TUNEL-positive, apoptotic hepatocytes were barely detected in either DEN-treated control tissue or Casp8^{Δhepa} mice 24 weeks after DEN treatment. These data suggest that the postulated role of apoptosis for the defense against chemically induced hepatocarcinogenesis might have been over-estimated in the past. This might be especially the case for extrinsic, death-receptor mediated apoptosis as lack of Casp8 had no effect on early hepatocarcinogenesis.

5.3 Casp8 protects from advanced HCC progression via non-apoptotic functions

Although Casp8^{Δhepa} mice showed a normal acute response towards DEN and the same number of dysplastic foci at early hepatocarcinogenesis, these mice showed more and larger liver tumors 40 weeks after treatment. This key finding of the present study indicates that Casp8 is rather important for inhibition of tumor cell growth than for tumor cell elimination hinting at several unexpected non-apoptotic functions of Casp8 in the progression of DEN-induced HCC. It was shown before that acute DEN-mediated liver injury is

associated with a transient pro-inflammatory response involving the activation of TNF and IL-6 related signaling pathways suggesting that increased expression of these cytokines in the acute phase of DEN injury might be related to enhanced hepatocarcinogenesis (Naugler & Karin 2008, Sander et al. 2007). Interestingly, loss of Casp8 as analyzed in the present study did not result in enhanced immediate response towards acute DEN-mediated liver injury despite stronger HCC progression. This was especially unexpected as the basal TNF expression in Casp8^{Δhepa} mice was significantly increased in comparison to control mice in agreement with recent reports (Liedtke et al. 2011). However, Casp8^{Δhepa} mice showed a remarkably increased infiltration of CD45-positive cells 40 weeks after DEN treatment although TNF- and IL-6-expression did not differ significantly between Casp8^{Δhepa} and control mice at this age. As CD45 is a well-established marker for leukocytes (Shaw 2002), these data suggest that Casp8 might have anti-inflammatory properties in advanced hepatocarcinogenesis.

A second non-apoptotic function of Casp8 involves its direct impact on the negative control of the cell cycle activity in the liver. A recent study demonstrated that Casp8 keeps the Receptor-interacting kinase RIP1 in check, e.g. by proteolytic cleavage. Accordingly, inhibition of Casp8 results in accelerated RIP1 activation and prolonged stability (Liedtke et al. 2011). In an experimental model of liver regeneration, following partial hepatectomy loss of Casp8 – and thus accelerated induction of RIP1 – was shown to be protective and resulted in improved onset of compensatory hepatocyte proliferation (J. Freimuth and C. Liedtke, personal communication). It was recently demonstrated that RIP1 down-regulates the cell cycle inhibitor p27 (Park et al. 2008). In this line of evidence, the present study demonstrated that p27 was significantly down-regulated in Casp8^{Δhepa} livers 40 weeks after DEN treatment. As this was the time point where increased HCC progression in these livers was first evident, it is tempting to speculate that loss of Casp8 might trigger enhanced cell cycle activity at least in part by inhibiting p27 in a RIP1-dependent manner. However, to further proof this hypothesis, RIP1 expression studies in advanced Casp8-deficient tumors will be necessary which was beyond the scope of this study. Moreover, abnormalities in protein regulation of a second cell cycle inhibitor were investigated. Beside cell cycle regulation, p21

modulated DNA repair processes and was important for stem cell maintenance (Abbas & Dutta 2009). Although no significant differences in mRNA expressions of p21 were detected, the protein level of p21 was decreased in Casp8^{Δhepa} mice.

As recently reported, another consequence of de-regulated RIP1 expression in Casp8-deficient mice could be enhanced NF-κB activation or programmed necrosis (Liedtke et al. 2011, Festjens et al. 2007, Vandenabeele et al. 2006). However, no signs of programmed necrosis were observed in DEN-treated Casp8^{Δhepa} mice (data not shown), whereas electrophoretic mobility shift analysis revealed increased NF-κB activity in a subset of Casp8^{Δhepa} livers with advanced HCC 40 weeks after treatment. Interestingly, a subpopulation of Casp8^{Δhepa} mice showed increased expression of the death ligand Fas 24 weeks after DEN treatment. Even though the apoptotic pathway was blocked by the lack of Casp8, other pathways could be triggered and could lead to an enhanced NF-κB activity (Ahn et al. 2001). Thus it is likely that elevated NF-κB activity contributes to enhanced tumor growth in Casp8^{Δhepa} mice. To definitely prove this hypothesis, Casp8^{Δhepa} mice with an additional block of NF-κB signaling, e.g. mice with simultaneous ablation of Casp8 and the NF-κB essential modulator (NEMO), could be treated with DEN and analyzed for reversion of enhanced HCC progression in future experiments.

Unexpectedly, the present study also revealed that in a subpopulation of DEN-treated Casp8^{Δhepa} livers, the poorly characterized factor *Deleted in Malignant Brain Tumors* (DMBT1) was substantially up-regulated on the gene expression level, which correlated with enhanced expression of the postulated tumor stem cell marker CD133+. Although the differences in expression levels between Casp8^{Δhepa} mice and controls were not statistically significant due to high variations, this observation could be relevant since increased tumor growth was also only observed in a subpopulation of Casp8-deficient livers. DMBT1 expression was investigated as preliminary data from microarray analysis hinted at a de-regulation of this factor in TNF-stimulated Casp8^{Δhepa} livers (C. Liedtke, personal communication, data not shown). The current knowledge about the function of DMBT1 in the liver is very limited. Recent studies hinted at a connection between DMBT1 and oval cells in hepatitis-B-virus related liver

diseases (Deng et al. 2012). It was suggested that an inflammation-related up-regulation of DMBT1 could stimulate the differentiation of oval cells into hepatocytes. Moreover, aberrant DMBT1-expression was associated with brain and epithelium tumors (Mollenhauer et al. 1997, Wu et al. 1997, Braidotti et al. 2004). Interestingly, the gene promoter of DMBT1 contains several NF- κ B-responsive elements (Rosenstiel et al. 2007) suggesting that DMBT1 up-regulation could be the consequence of increased NF- κ B activity. The mechanistical link between Casp8 deficiency, DMBT1 expression and enhanced HCC progression could not be clarified within the present study. However, to explain these findings in line with published data and own observations, the following hypothesis (illustrated in Figure 5.1) is proposed:

Albumin is expressed in hepatocytes and a sub-population of ductular cells such as cholangiocytes and oval cells (Bettermann et al. 2010). The latter ones are considered potential liver stem cells (Sell 1990, Michalopoulos 2007, Tanaka et al. 2011). Accordingly, *alfp-cre* mediated conditional inactivation of Casp8 could potentially result in Casp8-deficiency in hepatocytes, cholangiocytes and oval cells. Loss of Casp8 triggers enhanced RIP1 activity (Liedtke et al. 2011, J. Freimuth and C. Liedtke, unpublished data). In turn, RIP1 down-regulates the tumor-suppressor p27 (Park et al. 2008) and further up-regulates NF- κ B (Festjens et al. 2007, Biton & Ashkenazi 2011). Down-regulation of p27 in the liver is not sufficient to induce spontaneous HCC (Blain et al. 2003, Møller et al. 2000). However, together with a second oncogenic stimulus such as DEN, enhanced tumor progression in the liver seems to be plausible. One target of NF- κ B is DMBT1. Although poorly characterized yet, DMBT1 activation could trigger the activation of a more aggressive CD133+ positive stem cell population in the cholangiocyte/oval cell compartment, which is suggested by the correlation of DMBT1 and CD133+ expression levels in Casp8-deficient HCC. As a final consequence, a sub-population of Casp8 ^{Δ hepa} mice is prone to stronger HCC progression after DEN treatment.

Although several aspects of this hypothesis have to be investigated in more detail, the present study clearly suggests that a systemic inhibition of Casp8 in hepatitis patients (e.g. using low-molecular inhibitors) might bear the

risk of increased hepatocarcinogenesis, which will be discussed in the following paragraph.

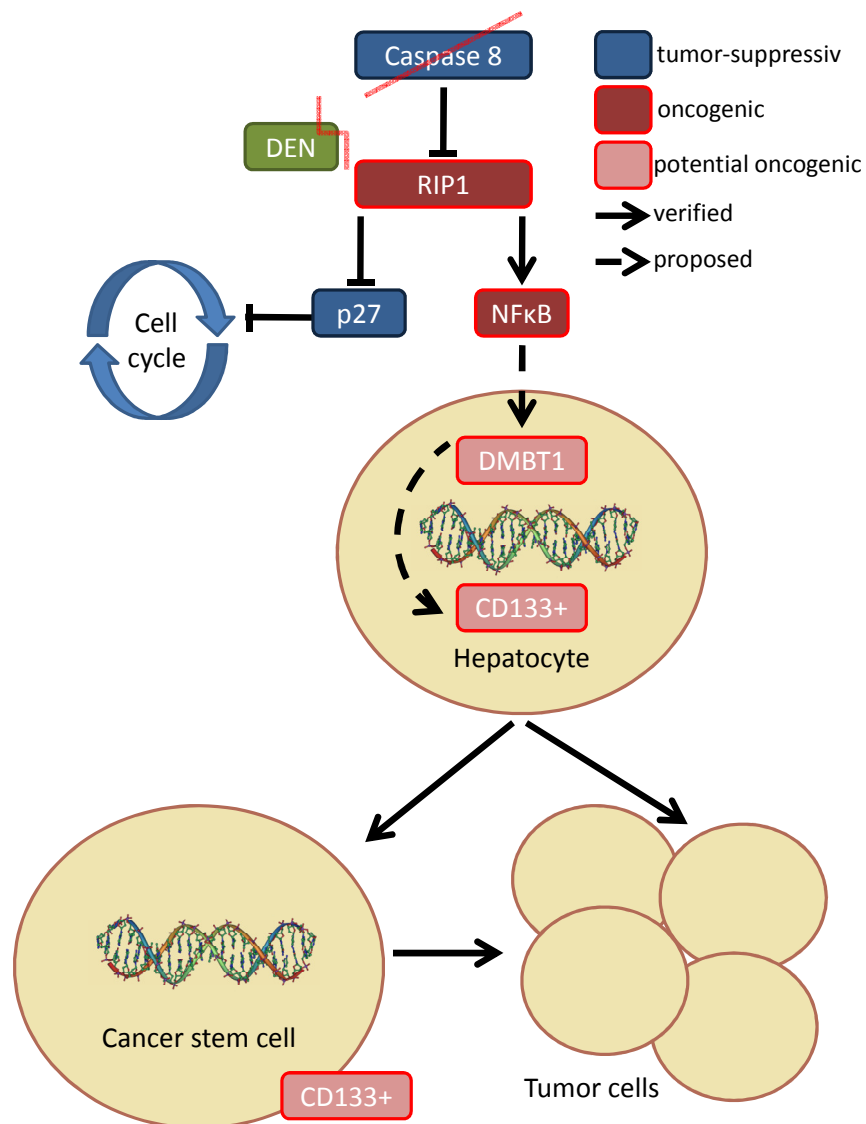


Figure 5.1: Proposed Tumor Model: Suggested model to explain enhanced HCC progression in DEN-treated Casp8^{Δhepa} mice. Further explanations are given in the text.

5.4 Protective- and harmful effects of Casp8 depend on the tissue environment

Several recent studies described different and in part even opposing effects of a Casp8 inactivation in different liver injury models. So far, at least four different settings have been investigated. In an acute liver injury induced by the Fas-activating antibody Jo2 or by a combination of lipopolysaccharide/D-galactosamine, 90% mortality in control mice, but 100 % survival of Casp8^{Δhepa} mice are detected. Therefore, ablation of Casp8 seems to be protective in a pure pro-apoptotic tissue environment (Liedtke et al. 2011).

The T-cell mitogen Concanavalin A (ConA) triggers inflammation similar to virus-induced hepatitis involving CD4-positive T-cells, natural killer cells and macrophages. Of notice, this liver injury involves predominantly liver cell necrosis with some minor apoptosis. Under these circumstances, loss of Casp8 was shown to be harmful as it triggers further tissue necrosis in the liver in a RIP1-related manner recently designated as programmed necrosis or necroptosis (Vandenabeele et al. 2010).

In a model of liver regeneration following partially hepatectomy, ablation of Casp8 was shown to be protective due to premature activation of RIP1 and NF-κB and subsequently accelerated onset of cell cycle activation (J. Freimuth and C. Liedtke, unpublished data).

Genetic inactivation of the NF-κB essential modulator (NEMO) in hepatocytes (NEMO^{Δhepa}) leads to a complete block of NF-κB-signaling and results in a complex phenotype involving spontaneous hepatitis, basal liver apoptosis and fibrosis. Interestingly, these mice spontaneously develop HCC within 9 - 12 month with 100 % incidence (Luedde et al. 2005). In this very complex *in vivo* model, additional ablation of Casp8 revealed bivalent effects (Liedtke et al. 2011): Juvenile Casp8^{Δhepa}NEMO^{Δhepa} mice developed serious liver necrosis and cholestasis in a RIP1-related manner. However, these mice were completely protected from the development of HCC suggesting that Casp8 may act as an oncogene in a chronic, pro-apoptotic environment.

In sharp contrast, the present study defined Casp8 as a moderate tumor suppressor in the DEN model. In line with these findings, the study revealed that DEN-induced hepatocarcinogenesis is barely associated with Casp8-

mediated apoptosis. The clinical relevance of these findings has to be carefully determined in future investigations. Clinical HCC usually arise on base of chronic inflammation, which is not the case for the DEN model. However, these DEN-induced tumors resemble a small group of HCCs which have a poor prognosis (Lee et al. 2006). In conclusion, Casp8 can mediate oncogenic or tumor-suppressive effects depending on stimuli and tissue environment.

5.5 Final conclusions and Outlook

At present, low molecular caspase inhibitors are considered as a potential new therapy option due to their ability to reduce hepatocytes apoptosis and thus overall liver injury, hepatic inflammation and hepatic fibrosis (Canbay et al. 2004). Several clinical studies indicated that these inhibitors could be used in patients and might be beneficial for treatment of several liver diseases (Masuoka et al. 2009). However, as discussed before, inhibition of caspases could also be a risk factor for hepatocarcinogenesis due to the assumed function of caspases to eliminate premalignant cells. The present work defined Casp8 as a tumor suppressor at least during chemically induced hepatocarcinogenesis. Interestingly, extrinsic apoptosis *per se* does not seem to play a major role for the inhibition of DEN-induced tumorigenesis. Instead, it appears that rather non-apoptotic functions of Casp8 seem to play a role in suppression of hepatocarcinogenesis. Thus, it should be carefully examined in further pre-clinical studies, if the advantage of treatment with caspase inhibitors in several liver diseases is accompanied by an increased risk of stem cell activation and hepatocarcinogenesis. Additionally, the precise classification of the underlying liver disease and microenvironment (apoptotic versus necrotic injury) becomes more important due to the several signal-dependent Casp8-functions (pro-apoptotic, anti-necrotic or anti-proliferative) and has to be carefully determined before initiation of an anti-caspase therapy.

In conclusion therapeutical inhibition of Casp8 in patients – which is already technically possible – should be handled with exceptional caution as it could even increase the risk for progression from chronic hepatitis to accelerated tumor growth.

6 Summary

Caspase-8 (Casp8) is the apical protease essential for the initiation of extrinsic apoptosis via activation of a caspase cascade. A role of Casp8 for preventing Hepatocellular Carcinoma (HCC) has been postulated. However, several studies revealed tumor suppressive, but also tumor promoting properties of Casp8 depending on the tissue environment. Therefore, the aim of this study was to investigate if Casp8 acts as tumor suppressor in the chemical model of diethylnitrosamine (DEN)-induced hepatocarcinogenesis using conditional, hepatocyte-specific Casp8 knockout (Casp8^{Δhepa}) mice.

Using a model of DEN-induced acute liver injury, it was demonstrated that loss of Casp8 did not substantially affect the immediate reaction towards genotoxic acute liver damage. However, the start of compensatory proliferation was slightly accelerated in Casp8^{Δhepa} mice hinting at a shortened phase of DNA-repair in these animals. Interestingly, loss of Casp8 was not associated with reduced liver apoptosis after acute DEN treatment demonstrating that liver damage in the DEN model is Casp8-independent. In further studies, a low-dose DEN model was applied to induce HCC in Casp8^{Δhepa} mice and wild type controls (Casp8^{f/f}). 24 weeks after HCC induction, the frequency and size of dysplastic liver lesions were similar in Casp8^{f/f} and Casp8^{Δhepa} mice demonstrating that Casp8 does not prevent tumor initiation in the DEN model. However, 40 weeks after DEN treatment HCC progression was substantially enhanced in Casp8^{Δhepa} mice as measured by increase tumor number and size. Detailed analysis revealed that Casp8^{Δhepa} mice showed increased hepatic inflammation and accelerated NF-κB-activation after DEN treatment. Moreover, loss of Casp8 in advanced HCC resulted in decreased levels of the cell cycle inhibitors p21 and p27. Finally, an induction of the putative tumor stem cell marker CD133+ was demonstrated in Casp8^{Δhepa} mice which was significantly correlated with an up-regulation of the poorly characterized factor DMBT1 (Deleted in Malignant Brain Tumor 1). In summary, the present study highlights novel, non-apoptotic properties of Casp8 for the suppression of liver tumors potentially by regulating cell cycle inhibition, hepatic stem cell- and NF-κB-activation. Accordingly, therapeutical inhibition of Casp8 in hepatitis patients – which is currently tested in clinical trials – should be handled carefully as it could increase the risk of hepatocarcinogenesis.

7 Zusammenfassung

Caspase-8 (Casp8) ist die apikale Protease im extrinsischen Apoptose-Signalweg und bewirkt die Aktivierung einer nachgeschalteten Caspasen-Kaskade. Aufgrund dieser Funktion wird eine Beteiligung von Casp8 bei der Suppression von Hepatozellulären Karzinomen (HCC) postuliert. Allerdings zeigten unabhängige Studien nicht nur tumor-suppressive, sondern auch onkogene Eigenschaften von Casp8 auf. Ziel der Arbeit war daher zu untersuchen, ob Casp8 in einem Modell der Diethylnitrosamin (DEN)-induzierten Hepatokarzinogenese Tumorsuppressor-Eigenschaften besitzt. Diese Frage sollte mit Hilfe von konditionalen, hepatozyten-spezifischen Casp8-knockout-Mäusen (Casp8^{Δhepa}) geklärt werden.

In einem Modell der akuten DEN-induzierten Leberschädigung wurde zunächst gezeigt, dass Casp8 die unmittelbare Reaktion auf diese gentoxische Leberschädigung nicht beeinflusst. Allerdings bewirkte die Deletion von Casp8 eine leicht beschleunigte Leberregeneration und dadurch indirekt eine verkürzte DNA-Reparatur. Die Deletion von Casp8 war nicht mit einer verminderten Leberapoptose assoziiert. Daher verläuft die DEN-induzierte akute Leberschädigung Casp8-unabhängig. Mit Hilfe von DEN wurden in Casp8^{Δhepa} Mäusen und Casp8^{f/f} Kontrollen HCC induziert. Es wurde gezeigt, dass Casp8 die Initiierung von Lebertumoren nicht blockiert, da beide Gruppen 24 Wochen nach Tumorinduktion eine ähnliche Anzahl und Größe von dysplastischen Leberläsionen aufwiesen. Allerdings präsentierten Casp8^{Δhepa} Mäuse 40 Wochen nach DEN-Injektion deutlich mehr und signifikant größere Tumorknoten und somit eine stärkere Tumorprogression im Vergleich zu Kontrolltieren. Detaillierte Analysen wiesen auf eine erhöhte Entzündung, verstärkte NF-κB Aktivität sowie eine Herabregulation der Zellzyklus-Inhibitoren p21 und p27 in DEN behandelten Casp8^{Δhepa} Mäusen hin. Zusätzlich führte der Verlust von Casp8 zu einer erhöhten Induktion des Stammzellenmarkers CD133+, die signifikant mit der Induktion des Proteins DMBT1 korrelierte. Zusammenfassend zeigt diese Studie neue, nicht-apoptotische Eigenschaften von Casp8 bei der Suppression von Lebertumoren auf und deutet darauf hin, dass diese Protease den Zellzyklus sowie die Aktivierung von Leberstammzellen und NF-κB steuern kann. Die Daten deuten an, dass der aktuell diskutierte Einsatz von Casp8-Inhibitoren bei Hepatitis-Patienten ein erhöhtes HCC Risiko bewirken könnte.

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10 Erklärung zur Datenaufbewahrung

Erklärung § 5 Abs. 1 zur Datenaufbewahrung

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten in der **Klinik für Gastroenterologie, Stoffwechselerkrankungen und Internistische Intensivmedizin (*Medizinischen Klinik III*)** des Universitätsklinikums Aachen hinterlegt sind.

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