DEPARTAMENTO DE BIOLOGÍA MOLECULAR
FACULTAD DE CIENCIAS
UNIVERSIDAD AUTÓNOMA DE MADRID

PhD Thesis

"Dissecting the role of Gadd45b in hepatocellular carcinoma development"

Umberto Rosato

Madrid 2017

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Thesis Director

Dr. Jesús María Salvador Sánchez

Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC)

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To my parents for giving me the best gift of all: Life. To my grandparents, for giving me some of the greatest memories of my life, you will live in me till the end of my days. To the three greatest friends life has gifted me with: Matteo, Nicola and Rafal…thank you guys for everything. To Domenico, aka "Mimmo", you helped me to get up when I hit rock bottom…you have no idea how grateful I am to you. To Greg Plitt: thanks to you I have learned to navigate the sea of life, face and love the storms and keep going in the direction I want to go to. You inspired me to be the best version of myself. Your legacy will always live on.

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"We only live once, but if we work it right, once is enough" – Greg Plitt

SUMMARY

The Gadd45 family proteins play a central role as stress sensors that modulate the response of mammalian cells to stress inflicted by physiological and environmental factors. Gadd45a and Gadd45b behave either as oncogenes or as tumor suppressor genes in a cell-type dependent manner. Despite their role has been extensively studied in various cancers, the functions played by these proteins in hepatocellular carcinoma development remains unknown. We addressed this issue by studying HCC development in a cohort of wild-types *Gadd45a* and *Gadd45b* null mice injected with diethylnitrosamine (DEN) a hepatocarcinogenic agent. We show that loss of either Gadd45a or Gadd45b strongly inhibits DEN-induced hepatocarcinogenesis. The lack of Gadd45b or Gadd45a in mice decreased hepatocyte death and compensatory proliferation, after DEN treatment. IL-6 is required for proliferation upon DEN-injection and the decrease of IL-6 production in both *Gadd45a* and *Gadd45b* null mice was linked to a decrease in JNK activation. Furthermore, we show that ablation of Gadd45b in Kupffer cells, but not in hepatocytes, is sufficient to recapitulate the decrease in proliferation and IL-6 production observed in whole body *Gadd45b* null mice. Altogether, these results provide a novel role for both Gadd45b and Gadd45a as oncogenes during DEN-induced HCC, and establish Gadd45b as critical player in regulating IL-6 production during liver injury.

RESUMEN

La familia de proteinas GADD45 tiene un papel central como sensor de estrés, modulando la respuesta de las células de mamíferos frente a los estímulos fisiológicos y ambientales. Tanto Gadd45a como Gaad45b, pueden comportarse como oncogenes o genes supresores tumorales dependiendo del tipo celular en el que actúen. Su papel se ha estudiado ampliamente en varios tipos de cáncer, sin embargo la función que llevan a cabo dichas proteínas en el desarrollo del carcinoma hepatocelular continúa siendo desconocida. Nosotros abordamos este tema empleando una cohorte de ratones WT y deficientes en *Gadd45a* y *Gadd45b*, inyectados con DEN, un agente hepatocarcinógeno. En este estudio se muestra cómo la pérdida tanto de Gadd45a como de Gadd45b inhibe fuertemente el desarrollo del hepatocarcinoma inducido por la inyección de DEN. La ausencia de Gadd45a o Gadd45b disminuye la muerte de hepatocitos y la consiguiente proliferación compensatoria, inducida por la DEN. La IL-6 es esencial para el proceso de proliferación en el modelo de la DEN. La disminución de la producción de IL-6 en ratones deficientes en *Gadd45a* o *Gadd45b* se ha asociado a una menor activación de JNK. Además, mostramos cómo la falta de Gadd45b en las células de Kupffer, pero no en hepatocitos, es suficiente para provocar esa disminución en la producción de IL-6 y en la proliferación compensatoria, que podíamos observar en los ratones *Gadd45b* knockout. Con todo ello, estos resultados proveen un nuevo papel para *Gadd45a y Gadd45b* como oncogenes durante el desarrollo del hepatocarcinoma inducido por DEN y establecen a *Gadd45b* como un regulador clave en la producción de IL-6 durante el daño hepático

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ABBREVIATIONS

AP1	Activator protein 1
AKT	Akt-8 virus oncogene cellular homolog
BCL-XL	B-cell lymphoma-extra large
BRCA1	Breast cancer 1
CAR	Constitutive androstane receptor
CDK4	Cyclin-dependent kinase 4
DEN	Diethylnitrosamine
DHE	Dihydroethidium
DMBA	7,12-Dimethylbenzantrancene
EGF	Epidermal growth factor
F4/80	Macrophage-restricted F4/80 protein
FOXO2A	Forkhead box O2A
Gadd45	Growth arrest and DNA damage inducible-45 proteins
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
IKK	IκB kinase
IKKα	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-6	Interleukin 6
IκB	Inhibitor of NF-κB
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MCL-1	Myeloid leukemia cell differentiation protein
MDR2	Multidrug resistance protein-2
MEFs	Mouse embryonic fibroblasts
MKK3	Mitogen-activated protein kinase kinase 3
MKK4	Mitogen-activated protein kinase kinase 4
MKK6	Mitogen-activated protein kinase kinase 6
MKK7	Mitogen-activated protein kinase kinase 7
MMTV	Mouse mammary tumor virus
NF-κB	Nuclear factor kappa B

NSAIDs	Nonsteroidal anti-inflammatory drugs
PB	Phenobarbital
PDGF	Platelet-derived growth factor
PH	Partial hepatectomy
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
RB	Retinoblastoma protein
ROS	Reactive oxygen species
SMAD	Contraction of the C. elegans protein SMA and the Drosophila protein MAD (mothers against decapentaplegic)
STAT3	Signal transducer and activator of transcription 3
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TR4/NR2C	N-methyl-D-aspartate receptor subunit 2C
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
XPC	Xeroderma pigmentosum protein group C

LIST OF FIGURES

INTRODUCTION

1. Liver: anatomy and functions

Extending across the entire abdominal cavity, the liver is a roughly triangular organ weighting approximately 1.5 kilograms. It consists of four lobes, the left and the right, located on the frontal side, and the quadrate and the caudate, located on the posterior side. The gallbladder, a small organ whose main purpose is to store bile, is located just beneath the right lobe (Fig I-1).

Blood is delivered to the liver by the hepatic portal vein, where it divides into the liver's complex system of arteries and arterioles that provides cells with all the required nutrients (Fig. I-2). The hepatic arteries then converge into the hepatic vena cava, which brings the blood to the heart.

The liver is anatomically divided into hexagonal functional units called lobules. At the center of the hexagonal structures lies the central vein, whereas at each of the 6 corners lie 6 hepatic portal veins coupled to 6 hepatic arteries, these blood vessels are connected by capillary-like tubes called sinusoids. At the center of the hexagon, traversed by the sinusoids, lie the liver cells.

The liver is a paramount and vital organ performing essential functions for the body homeostasis. Among the numerous functions (around 500 to date) *(*Zakim*, 2002*) described are: storage of sugar and glucose to provide vital energy for the body, storage of essential vitamins and iron, blood detoxification from harmful substances, red blood cells elimination, metabolization of thousands of xenobiotics and the production of bile, a compound aiding digestive processes by emulsifying lipids. Furthermore, the liver is responsible for the breakdown of hormones like insulin, and the breakdown of bilirubin, facilitating its excretion into bile. The liver possess the remarkable ability of regeneration: even if 3/4 of the liver are removed, the remaining liver cells exit quiescence and initiate proliferative processes aimed at restoring the original liver mass. It is important to notice that in mammals, the lobes that are removed are unable to regrow, and the restoration of the mass is the result of proliferation of the lobes not being removed.

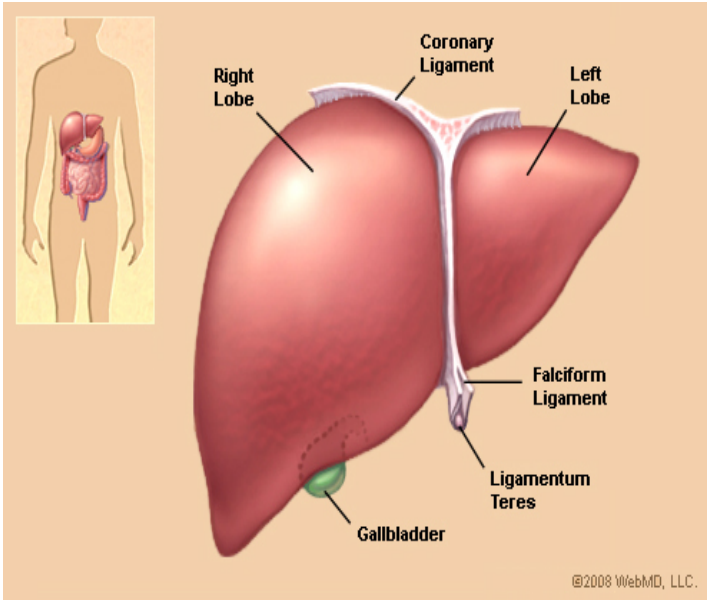

Figure I-1. Illustration of a human liver and its position in the body. (Copyright © 2008 WebMd LLC)

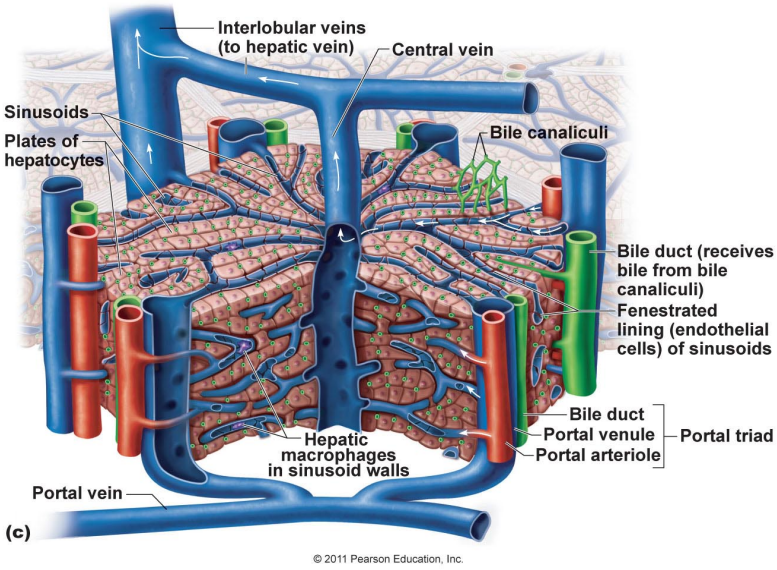

Figure I-2. Representation of the structure of a hepatic lobule (Copyright © 2011 Pearson Education)

1. The three types of liver cells

The liver consists of three cell populations: hepatocytes, Kupffer cells and stellate cells, illustrated in Figure I-3

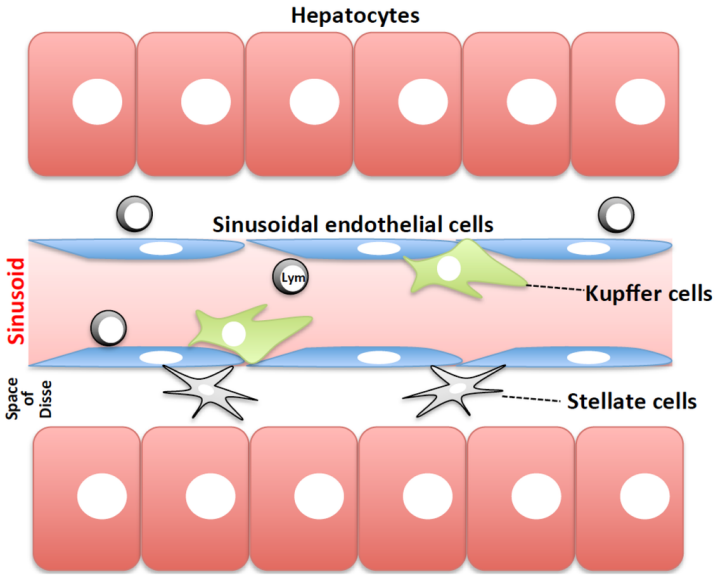

Figure I-3. Representation of the various cellular types present in the liver: Hepatocytes, Kupffer cells and Stellate cells (Tsutsui & Nishiguchi, 2014)

1.1 Liver hepatocytes

By constituting around 70% of the liver, the hepatocytes are the main cell type associated with liver-specific functions. They are round, cubical cells, typically 20-30 µm of diameter. Tetraploidy or polyploidy represent common features in hepatocytes nuclei. Hepatocytes metabolize exogenous compounds and are the main sites of synthesis for transferrin complement and glycoprotein (Zakim, 2002). They can perform gluconeogenetic processes and the formation of triglycerides and fatty acids from carbohydrate.

In normal conditions, they are quiescent cells, but can be primed to reenter cell cycle following liver injury. Their central role in xenobiotic metabolism and the numerous functions performed renders this cell type prone to injury. Hepatocyte injury can result in

hepatocyte death via apoptosis or necrosis. Apoptosis contributes to liver homeostasis by removing damaged cells, however, deregulation of such process are common to many liver pathologies (Malhi et al, 2010; Yagmur et al, 2007).. Enhanced hepatocyte apoptosis is tightly coupled with inflammation and fibrosis (Hikita et al, 2009; Takehara et al, 2004) and increase in hepatocyte apoptosis stimulates proliferation of nearby hepatocytes, a process essential for cancer development. Apoptosis is commonly initiated and executed by activation of intracellular protease enzymes called caspases. Apoptosis can be initiated either in an extrinsic or intrinsic manner. In the former, caspases are activated by binding of cell membrane death receptor activation to their extracellular ligands, such tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), and (TRAIL). In the latter, cellular stress enhanced mitochondrial membrane permeability, allowing Cytochrome C release into the cytosol, which triggers caspase activation. In the liver, crosstalk among hepatocytes and Kupffer cells is crucial in mediating responses to stress.

Hepatocyte proliferation for instance, constitutes the fundamental driving process of liver regrowth in response to chronic or acute liver injury causing hepatocyte death (Fausto, 2005). The initiation of this response involves the production of priming factor such as interleukin 6 (IL-6), secreted by Kupffer cells. These priming factors are thought to prime hepatocyte to respond to growth factors such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF), which stimulate hepatocyte to reenter cell cycle (Zimmermann et al, 2005).

1.2 Kupffer cells

Kupffer cells are resident liver macrophages making around 30%? of the total liver cells (Bilzer et al, 2006). They reside in the sinusoids and derive from bone marrow monocytes that upon migration establish into the liver becoming its resident macrophages. The most important activators of this cell group are the complement factors C3a and C5a, β-glucans and lipopolysaccharide from bacteria, (Bilzer et al, 2006). Once activated, they can secrete a vast array of cytokines mediating responses to stress. Kupffer cells participate in processes such as host defense, by clearing bacteria from the blood stream;

indeed, they constitute the first macrophage population that comes in contact with bacteria and endotoxins deriving from the gastrointestinal tract. They are essential for the optimal liver regenerative ability by producing TNF-α (Yamada et al, 1997) and IL-6 (Cressman et al, 1996), can promote alcoholic liver injury and contribute to the pathogenesis of non-alcoholic fatty liver disease (Adachi, 1994). In response to liver stress such as p, such as DEN injection or partial hepatectomy (PH), Kupffer cells secrete factors, such as IL-6 and TNF-α, which are required for hepatocyte to reenter cell cycle, enabling correct liver regeneration (Prins et al, 2005). Once released, these cytokine trigger liver regeneration mainly by stimulating the nuclear Factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathway. Liver regeneration is indeed compromised in mice depleted of Kupffer cells due to lower expression of TNF-α (Murata et al, 2008). Diethylnitrosamine (DEN) and acute liver failure are two characterized stress responses involving a Kupffer cell dependent regeneration. During DEN acute liver injury for instance, necrotic hepatocytes release IL-1α, which in turn stimulates Kupffer cells to produce IL-6, a paramount cytokine promoting hepatocyte division and liver regeneration. Taking advantage of their origin from bone marrow, Kupffer cells genes function can be studied thanks to bone marrow transplant, by lethally irradiating mice and transplant the bone marrow deficient in the gene of interest. Kupffer cells have indeed a lifespan of around 40 days and, following bone marrow transplantation, can repopulate the liver in a period comprised between 14 to 21 days (Naito et al, 1991).

1.3 Stellate cells:

They are perycites located in the area between the sinusoid and the hepatocytes, called the Disse space. Representing roughly 5% of the liver's total number of cells (Hellerbrand et al, 2013), and in normal conditions are thought to be quiescent. Activation occurs in response to liver stress and to collagen production that can eventually contribute to liver fibrosis.

2 Hepatocellular Carcinoma

2.1 The biology of HCC

Hepatocellular Carcinoma (HCC) is the primary malignancy of the liver, accounts for 75% of primary liver cancers and is the third leading cause of cancer death. The survival rate is around 50% and chronic liver injuries such as the ones caused by hepatitis B, hepatitis C, cirrhosis and excessive alcohol consumption can enhance the probability of onset of this disease. HCC has a higher incidence in males than in females, probably because of hormonal reasons, and is most common between the ages of 30 and 50 (Forner et al, 2012). This cancer is most common in Asia, where 5 to 10% of the population is infected by hepatitis B (Alavian & Haghbin, 2016). Nowadays, vaccination against hepatitis B, limiting alcohol consumption and having a healthy diet represent the best form of prevention, especially for patients having a familiar history of HCC. HCC can be either poorly differentiated, with giant and anaplastic cells, or differentiated, where cells resemble hepatocytes. Different techniques are used for the diagnosis of this cancer: ultrasound is used mainly for screening, whereas CT scan or an MRI is more appropriate in people with a high suspicion of liver cancer. The liver image reporting and data system (LI-RADS) is the system of HCC classification based on CT scan: LR1 and LR2 are the less severe diagnosis, indicating that the tumor is benign or probably benign respectively. LR3 LR4 LR5 are the more severe diagnosis, indicating a non-benign tumor and LR5 being the more severe grade. Criteria such as size, tumor vascularity, involvement of liver vessels and presence of extrahepatic metastases are crucial factor in determining treatment approach. Various therapies are available for HCC treatment, both surgical and non-surgical. The surgical approaches consist of liver transplant or tumor resection, the former being extremely dependent on patient selection whereas the latter presents a 50 to 60% recurrence rate and only 10 to 20% of liver tumors can be removed surgically. The non-surgical approaches involve radiotherapy and chemotherapy. The sole agent approved by the FDA to treat HCC is sorafenib, a receptor tyrosine kinase inhibitor, which inhibits tumor proliferation and angiogenesis and enhances apoptosis in tumor cells (Abdel-Rahman et al, 2014).

Given the low survival rate of HCC after the diagnosis, is essential to understand the molecular pathways and genetic alterations leading to HCC development. An understanding of these phenomenon can contribute to identify new therapeutic targets and novel strategies for HCC treatment.

In regard to genetic alterations, amplification of the chromosomes 1q, 8q. 6p, and 17q and the loss of chromosomes 8p, 16q, 4q, 17p, and 13q were found to be among the most prominent (Moinzadeh et al, 2005). As for molecular pathways, many were found to have crucial roles in HCC development. More than 200 codons mutations affect the p53 gene in HCC. 62% of HCC were shown to display β-catenin deregulation (Inagawa et al, 2002). These mutations prevent β-catenin from being phosphorylated, causing an overactivation of Wnt β-catenin pathway (Willert et al, 2002). Overexpression of TGF-β is commonly found in HCC (Abou-Shady et al, 1999).TGF-β displays a growth limiting function in many cellular contexts and the reason of its expression in HCC is not fully understood. A resistance of tumors to TGF-β signal or a switch of TGF-β to a tumor promotive role are reasonable hypothesis explaining this phenomenon. Various studies have shown an upregulation of Ras and a downregulation of Ras inhibitors in HCC (Nomura et al,1987; Schuierer et al, 2006). N-Ras, H-Ras and K-Ras, the three genes belonging to the Ras family group, display common mutation in the codon 61, 12 and 12 respectively (Takada et al, 1989; Challen et al, 1992; Cerutti et al, 1994). The Raf-1 essential target mediated many of the Ras associated function, is inhibited by sorafenib (Abou-Alfa et al, 2006). The Retinoblastomas suppressor protein (Rb) has also been implicated in HCC development. In human HCC cell lines and in 28% of HCCs this protein is inactivated (Suh et al, 2000; Azechi et al, 2001). Moreover, other members of the Rb network display altered expression in HCC. Cyclin D1/CDK4 complex is overexpressed in 58% of HCCs and p16, a Cyclin-dependent kinase 4 (CDK4) inhibitor is absent in 34% of HCCs (Joo et al, 2001; Liew et al, 1999).

2.2 HCC: The DEN model

Numerous mouse models, either genetic or chemically induced, have been developed in the past decades in order to study HCC patterns. The important question is to assess the extent to which these models mimic human HCC. Genetic profiles of mouse tumors induced by DEN, an alkylating agent, share many similarities to expression profiles of HCC with poor prognosis (Lee et al, 2004). Furthermore, mutations in the H-Ras protooncogene, associated with poor prognosis in humans, are commonly found in DEN induced tumors (Stahl et al, 2005). Upon intraperitoneal injection (IP) DEN is hydroxylated in the liver by cytochrome p450 to alpha-hydroxylnitrosamine. This compound causes DNA damage by reacting with DNA bases (Verna et al, 1996). Reactive oxygen species (ROS) generated by cytochrome p450 might also contribute to DEN tumorigenic potential (Valko et al, 2006). The liver possess the remarkable capacity to restore its original mass upon stress, starting 24 hours after DEN injection, a fraction of the liver cells undergoes DNA damage induced apoptosis. Neighboring cells exit the quiescent state and divide in order to restore the original cell number. This process is essential to produce the DEN-driven genetic mutations eventually leading to carcinogenesis, as DNA adducts formed by DEN only result in mutations upon DNA duplication (Fig. I-3). Therefore, defects in the apoptotic pathways or in pathways enabling cellular proliferation can significantly affect DEN-induced tumorigenesis. Puma-null mice for instance, a protein involved in the apoptotic pathway, are resistant to DEN-induced tumorigenesis due to their intrinsic inability to undergo apoptosis (Qiu et al, 2011). Cytokine-mediated crosstalk between hepatocytes and Kupffer cells is crucial for DEN-induced tumor development. Following DEN injection necrotic hepatocytes release IL-1α in the extracellular milieu (Sakurai et al, 2006), which activates Kupffer cells to secrete IL-6, which in turn primes hepatocytes to enter cell cycle (Fig. I-4) (Zimmers et al, 2003). Various factors such as genetic strain, age and sex of the mice contribute to the efficiency of the DEN tumorigenic processes. Males are more susceptible than females to HCC development. These differences in susceptibility are due to the inhibitory effects of estrogens and the positive effects of androgens on HCC development (Nakatani et al, 2001). A single IP injection of DEN is not sufficient to produce HCC in two weeks old

mice but not in adult mice (Shiota et al, 1999).

DEN is also used as the initiator agent in a two-stage model. After DEN injection mice are treated with phenobarbital (PB), which by increasing cytochrome p450 expression, oxidative stress, methylating promoter regions of tumor suppressor genes, exponentially enhances DEN carcinogenic potential. (Waxman & Azaroff, 1992; Imaoka et al, 2004; Watson & Goodman, 2002). Tumors induced by DEN in conjunction with PB display mutations in the β-catenin gene (Aydinlik et al, 2001). When 6 to 10 week old mice are injected with DEN followed by PB, the compound enhances DEN tumorigenesis. Biochemical differences between adult and infant hepatocytes might account for this phenomenon. Promotion of tumors with PB is also dependent on gender and strain differences (Weghorst & Klaunig, 1999; Goldsworthy et al, 2002). In infant mice however, DEN induces tumors in absence of PB. Indeed, when 15 days old mice are injected with a DEN dose of 25 mg/kg tumors will develop in PB-independent manner with an average latency of 40-weeks (Bakiri et al, 2013). This is the approach we used to induce tumors in our mice.

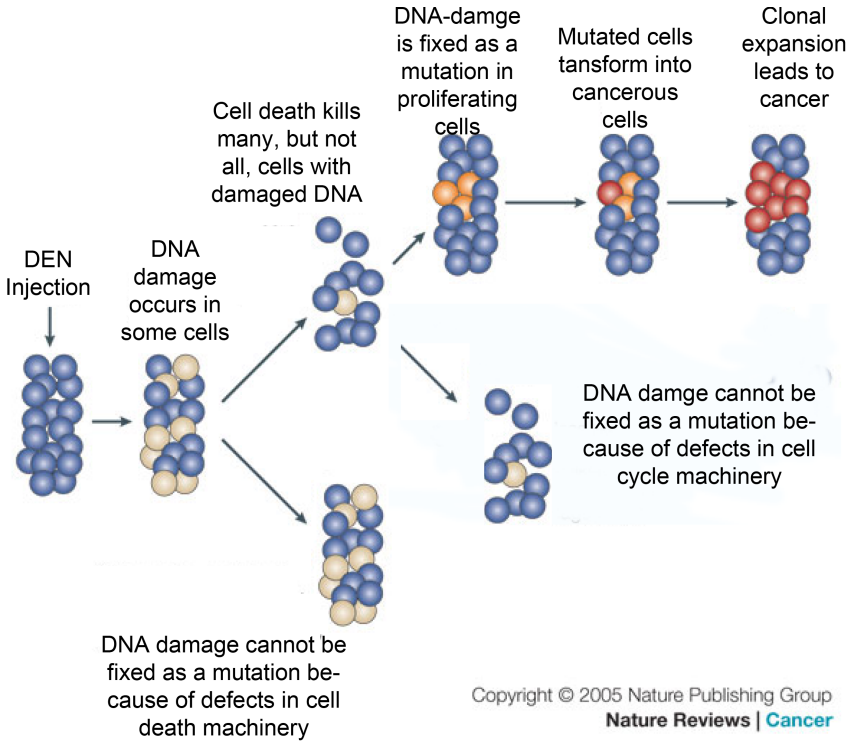

Figure I-4. Representation of the initial steps involved in DEN-induced carcinogenesis. (Adapted from Allan and Travis, 2005).

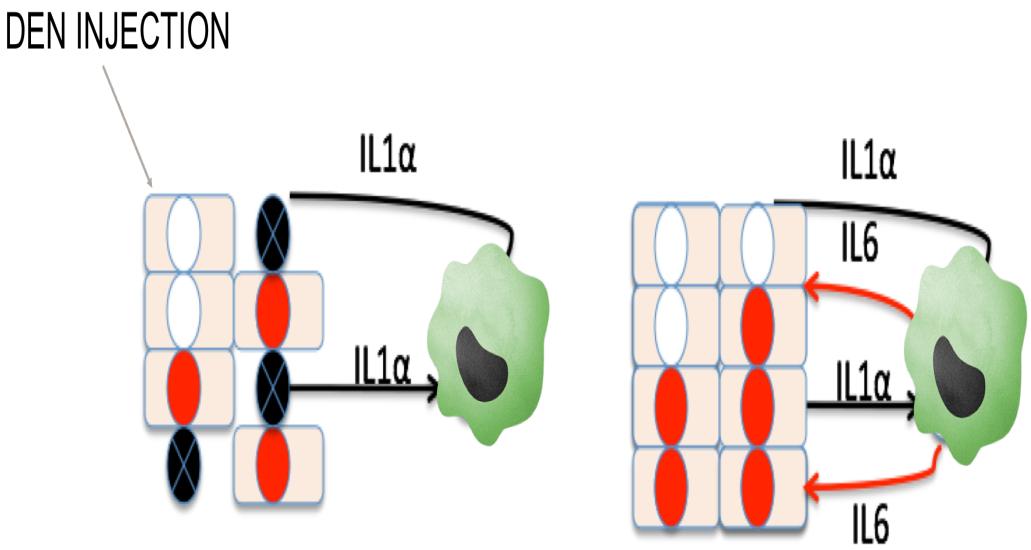

Figure I-5: Crosstalk among Kupffer cells and hepatocytes after DEN injection.: Necrotic hepatocytes release IL-1α in the extracellular milieu, which activates Kupffer cells to produce IL-6. IL-6 stimulates hepatocytes with damaged DNA to enter cell cycle, thus favoring DNA mutations.

3 The Gadd45 family proteins.

3.1 The Gadd45 family members

Growth arrest and DNA damage inducible (Gadd45) gene family consists of three members, *Gadd45a, Gadd45b and Gadd45g*, having–key roles in the integration of various intra and extracellular stimuli with their appropriate cellular responses (Liebermann and Hoffman, 2003). Gadd45 proteins are very acidic, weigh around 18 kD and share 55 to 57% of homology with each other (Abdollahi et al, 1991). They are expressed in various tissues, such as liver, brain, kidneys, muscles and testis (Zhang et al, 1999). The coding genes are located on chromosome 1, 19 and 9 for *Gadd45a, Gadd45b* and *Gadd45g* respectively. Despite the high degree of sequence homology they can differ substantially in their regulation and function (Lieberman and Hoffman, 2003). These differences can be attributed to both the different spectrum of binding factors of each family member and different regulation of expression. For instance, only Gadd45a contains binding motifs for p53 and activator protein 1 (AP1) in its genetic region (Jin et al, 2001; Daino et al, 2006). To date, no enzymatic activity in each one of the 3 family members has been identified, as a result, these proteins are believed to exert most of their cellular functions via protein-protein interaction. Besides interaction with a wide plethora of proteins, Gadd45 family members can form homodimers and heterodimers with other Gadd45 family proteins as well as oligomers (Kobayashi et al, 2005; Schrag et al, 2008). In basal conditions their expression is very low, but it can raise substantially when cells are subject to both intra and extracellular stresses (Moskalev et al, 2012).

3.2 Gadd45 in apoptosis and cellular proliferation

Gadd45 proteins are generally known to promote apoptosis in a variety of cell type and contexts. Ectopic expression of Gadd45 proteins triggers apoptosis in mouse hepatocytes in a p38 and c-Jun N-terminal kinases (JNK) dependent manner and *Gadd45b*-null hepatocytes are resistant to TGF-β-mediated apoptosis (Selvakumaran et al, 1994; Yoo et al, 2003).

To note, *Gadd45a* also inhibits apoptosis in some contexts, for instance it protects melanoma cell from cell death in response to cisplatin and UV radiation (Smith et al, 1996). *Gadd45a* expression in pancreatic cancer is associated with reduced prognosis and apoptosis resistance (Hildesheim et al, 2002). Studies on *Gadd45b* gene also highlight a proapoptotic role in many contexts. Indeed *Gadd45b* overexpression induces apoptosis in HeLa cells and mediates the apoptotic response to neural ischemia (Cretu et al, 2009). *In vitro,* ectopic expression of *Gadd45* members blocks cell growth by arresting cells in the G2/M phase or by induction of apoptosis in several human cell lines such as H1299 lung carcinoma, HeLa cervical cancer cells and NIH3T3 embryonic fibroblasts (Moskalev et al, 2012). *Gadd45b* overexpression however, protects B-cells from activation-induced cell death, indicating that this protein, like *Gadd45a*, exerts an antiapoptotic role in some context (Schmitz et al, 2013).

Induction of *Gadd45a* is generally suppressed by growth regulatory stimuli, indicating that in many instances this protein antagonizes cellular proliferation. Loss of *Gadd45a* is sufficient to transform cells with H-ras gene (Bulavin et al, 2003). Expression of *Gadd45b* is negatively regulated by protooncogenes such as c-Myc and AKT virus oncogene cellular homolog (AKT), further confirming its role in growth suppression. *In vivo* lack of *Gadd45b* can have context-specific effect on cellular proliferation. While lack of *Gadd45b* impairs regeneration after partial hepatecomty (Papa et al, 2008), it can cause a modest increase in proliferation in liver hyperplasya induced by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) treatment (Tian et al, 2011).

3.3 Gadd45 proteins in cancer

Gadd45 proteins have been mainly associated with tumor suppressive properties in various tumor types. *Gadd45a*$^{-/-}$ mice show genomic instability, increased sensitivity to carcinogenesis and are more prone to 7,12-Dimethylbenzantrancene (DMBA) induced ovarian tumors (Hollander et al, 1999; Hollander et al, 2001). Furthermore, *Gadd45a* inhibits the onset of mammary tumors by inducing JNK and p38 mediated apoptosis and senescence respectively (Tront et al, 2010). Also, disruption of *Gadd45a* expression was found in many hematopoietic malignancies (Qiu et al, 2003; Sun et al, 2003; Ying et al, 2005). *Gadd45b*$^{-/-}$ mice are more susceptible to ionizing radiation, chemical carcinogens, and display an impaired immune response to implanted melanoma cells (Ju et al, 2009). *In vitro,* ectopic expression of Gadd45 proteins block cell growth by arresting cells in the G2/M phase or by induction of apoptosis in several human cell lines such as H1299 lung carcinoma, HeLa cervical cancer cells and NIH3T3 embryonic fibroblasts. (Zhan et al, 1994; Zhang et al, 2001; Sun et al, 2003). The ability of Gadd45 family members to induce apoptosis in various cancer cell lines is crucial for various pharmacological agents, for instance *Gadd45a* expression is required in MCF7 human breast carcinoma cells for Troglitazone-induced apoptosis (Yin et al, 2004). Besides suppressing cancer development by inducing apoptosis, Gadd45 proteins can also suppress cancer by inhibiting cellular migration, for instance silencing of *Gadd45a* expression by shRNA inhibits the migration of BL185 murine cell line and HCC cell lines (Trabucco et al, 2010).

Methylation appears to be a way through which Gadd45 proteins expression is silenced. In liver cancers, for instance the promoter of *Gadd45b* is methylated in HCC (Qiu et al, 2004). As for *Gadd45a*, at least in the case of breast cancer, point mutations seem to be the likely mechanism of its inactivation (Campomenosi et al, 2000).

In many, but not all, instances therefore, Gadd45 proteins display tumor suppressive functions. Various are indeed the drugs whose chemotherapeutic effect is dependent on Gadd45 proteins. Gadd45a is induced by CD437, Genistein, Trichostatin A and Nonsteroidal anti-inflammatory drugs (NSAID), all drugs used for chemotherapeutic purposes (Oki et al, 2004; Zerbini et al, 2005; Campanero et al, 2008), and Gadd45b is

required for sorafenib-induced apoptosis in hepatocellular carcinoma cells (Ou et al, 2010)

Despite a great number of evidences indicating Gadd45 proteins are relevant in tumor suppressive function, in some cases these proteins might exert a paradoxical opposite action. For instance, while *Gadd45a* suppresses Ras driven breast tumorigenesis, it enhances tumorigenesis when breast tumors are induced through Myc overexpression. Thus, *Gadd45a* plays either a tumor promotive or suppressive role in an oncogene dependent fashion (Tront et al, 2010). Ablation of *Gadd45a* in Myc driven mammary tumorigenesis results in either apoptosis or cellular senescence.

3-4 Gadd45 proteins in liver

Gadd45b expression is rapidly increased in response to many liver challenges, such as PH, hyperplasia, and response to xenobiotic. This increase is essential to maintain liver homeostasis. In particular, several evidences suggest a key role for Gadd45b in mediating hepatocyte proliferation.

Gadd45b$^{-/-}$ mice are unable to regenerate the liver after PH (Papa et al, 2008), a surgical procedure where 3/4 of the liver are surgically removed. After PH indeed, *Gadd45b* transcription is stimulated by the TGF-β mother against decapentaplegic (SMAD) pathway and the TNF-α–NF-κB pathway (Ohmura et al, 1996). After PH, TNF-α stimulates NF-κB activation, NF-κB in turn, activates *Gadd45b* transcription. TGF-β instead, can induce *Gadd45b* transcription by activating SMAD3 and SMAD4 (Major and Jones, 2004). Upon induction during PH, *Gadd45b* inhibits MKK7, thus inhibiting JNK phosphorylation. JNK phosphorylation starts to decrease at around 8 hours in WT mice, whereas *Gadd45b*$^{-/-}$ mice display enhanced phosphorylation even after 72 hours, highlighting the important difference between transient and persistent JNK activation in liver proliferation after PH (Papa et al, 2008). TNF-α is a critical player in this process since mice lacking TNF-α receptor also fail to regenerate liver upon PH and fail to upregulate *Gadd45b* (Papa et al, 2008).

However, the effects of *Gadd45b* on hepatocyte proliferation are highly dependent on the stimulus used to induce liver proliferation. Indeed, when mice are challenged with

TCPOBOP, an activator of the constitutive androstan receptor (CAR) pathway, TCPOBOP stimulates rapid liver growth by priming hepatocytes for cellular proliferation. Contrary to PH, this effect is direct and does not require any prior physical liver injury (Tian and Locker, 2013). This process is called hyperplasia and usually is triggered by toxic exposure. This enhanced cell division makes the liver presumably more efficient in the detoxification process. *Gadd45b* induction after TCPOBOP stimulation is abrogated in $CAR^{-/-}$ mice, but remains the same in tumor necrosis factor receptor (TNFR) $TNFR1^{-/-}$ $TNFR2^{-/-}$ *and* $TNFR3^{-/-}$ indicating that PH and CAR induce *Gadd45b* expression through different pathways (Columbano et al, 2005). $Gadd45b^{-/-}$ mice display enhanced proliferation after TCPOBOP challenge as demonstrated by the doubling of Cyclin D1 expression (Tian et al, 2011).

However, *Gadd45b* expression is not always induced during liver stress. For instance, hepatitis C virus (HCV) infection causes inflammation, hepatocyte loss, compensatory proliferation and enhanced TNF-α activation, but contrary to the two cases described above *Gadd45b* expression is not induced in this case (Higgs et al, 2010).

These findings highlight that *Gadd45b* lacks an intrinsic role during liver challenges, but rather, that the role this protein might play is extremely dependent on the stimulus used to induce liver stress and the extracellular milieu.

Gadd45a is also induced by many stimuli inducing liver stress. Increase in *Gadd45a* liver expression has been detected with hepatic carcinogens such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and DMBA (Hollander et al, 2001; Su et al, 2002; Locker, 2003; Fletcher 2005). In liver, *Gadd45a* expression is regulated by genes such as p53, breast cancer 1 (BRCA1), nuclear receptor *N*-methyl D-aspartate receptor subunit 2C (TR4/NR2C) and forkhead box O2A (FOXO2A) (Zhan et al, 1998; Jin et al, 2000; Yan et al, 2012). Expression of *Gadd45g* appears to be downregulated during liver hypertrophy and also is found to be deregulated in 65% of human HCC (Sun 2003 et al; Frau et al, 2012).

4 Signaling pathways in HCC

Numerous signaling pathways have been found to be altered during HCC development. Most importantly, in recent years the importance of the crosstalk between hepatocytes and Kupffer cells during DEN-induced HCC has been highlighted. Alteration of the same pathway can have dramatic opposite effects on HCC development depending on the cell type carrying the alteration.

4-1 NF-κB pathway

The NF-κB transcription factor family in mammals consists of five proteins, p65, c-Rel, RelB, p105/p50 and p100/p52. In normal conditions, they form dimers that are held in the cytoplasm through their interaction with inhibitor of NF-κB (IκB) proteins. IκB kinase (IKK) complex, which consists of 2 catalytic subunits, inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα) and inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) (Hoffman et al, 2006) can phosphorylate IκB inducing degradation of it. Once activated NF-κB dimers can translocate into the nucleus and activate a huge array of genes mediating processes like cytokine secretion and antiapoptotic activity (Li Q et al, 1999).

Mice lacking *Ikkβ* only in hepatocytes exhibit a notable increase in hepatocarcinogenesis induced by DEN and $Ikk\beta^{-/-}$ hepatocytes are unable to translocate NF-κB dimers into the nucleus (Maeda et al, 2009). $Ikk\beta^{-/-}$ mice display a great extent of hepatocyte death following DEN injection, this death in turn, results in enhanced compensatory proliferation to restore liver mass. Furthermore, a greater level of ROS accumulation can be seen in $Ikk\beta^{-/-}$ mice, as the defect in NF-κB activation results in impaired transcription of anti-oxidant genes. These results reveal that, at least in hepatocytes, NF-κB pathway is associated to a tumor suppressive role by promoting the upregulation of antioxidant and antiapoptotic genes (Kamata et al,2005).

Another paradoxically opposite result can, however be observed when *Ikkβ* is deleted in

both hepatocytes and hematopoietic-derived Kupffer cells. In this case, upon DEN injection carcinogenesis is strongly inhibited compared to WT mice. This phenotype is the result of impaired production of protumorigenic cytokines such as IL-6 in Kupffer cells (Maeda et al, 2009). Thus, depending in which cells NF-κB pathway is inactivated, opposite results can be observed.

Many of the studies of HCC pathways have been done using the DEN chemical model. When mice carrying multi drugs resistance gene 2 (MDR2) mutation, which show spontaneous low-grade chronic inflammation eventually leading to HCC development, are crossed with mice expressing a non-degradable form of IkB⟨, the resulting mice display reduced HCC development (Pikarsky et al, 2004). Indicating that the role of NF-κB in HCC development is highly dependent on the model used to perform the study.

4-2 IL-6

IL-6 is one of the best-characterized proinflammatory cytokines and is produced by a variety of cells such as T, B-lymphocytes, Kupffer cells, fibroblasts, monocytes and keratinocytes (Ataie-Kachoie et al, 2013). Processes regulated by this cytokine include acute phase response, inflammation and positive regulation of cellular growth (Hassan et al, 1995). Its transcription can be induced by bacterial lipopolysaccharides (LPS), TNF-α, EGF and cytokines such as IL-1α. Once bound to its receptor, it mediates a wide plethora of cellular effects by the activation of the JAK-STAT3 cascade, whose effects are described below. Deregulation of IL-6 pathway has been observed in diseases such as Chron (Neurath et al, 2011) and Alzheimer (Cacquevel et al, 2004). IL-6 is upregulated in various human tumors, such as lung, prostate, breast, pancreas and ovarian, and elevated IL-6 levels are known indicator of poor prognosis on these malignancies (Heikkila et al, 2008). IL-6 contributes to tumor development by mediating the activation of oncogenic pathways such as phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT and Ras/Mapk (Grivennikov et al, 2008). IL-6 is critical for inducing HCC development in the DEN model. Indeed, necrotic hepatocytes produce IL-1α, which in turns activates Kupffer cells to produce IL-6. Mice null for *IL-6* show a reduction in DEN induced HCC when compared to WT mice. Furthermore, dietary or genetic induced

obesity promotes DEN induced HCC, and this promotion is abrogated in *IL-6*$^{-/-}$ mice, indicating that IL-6 play a conserved tumor suppressive role in HCC, independent of its etiology (Naugler et al, 2007). IL-6 can also be responsible for the HCC related difference between males and females, as ablation of IL-6 abolishes the sex-related differences in HCC. This is due to the fact that, in female mice, estrogen hormones decrease IL-6 expression (Shi et al, 2014). In humans, high levels of IL-6 is serum of patients with hepatitis B correlate with increased probability of HCC development (Nakagawa et al, 2009).

4.3 JAK-STAT signaling

JAK-STAT pathways are important components of the cytokine signaling network. JAK proteins usually interact with the intracellular domain of the cytokine receptors, which undergo conformational changes upon binding their respective ligand. This conformation change causes the displacement of the JAK proteins, which can activate the STATs by phosphorylation. The STAT family of proteins is composed by seven members, and STAT3 is recognized as a key component in the IL-6 signaling cascade (Yoshimura et al, 2006; Yu et al, 2009). Following IL-6 binding to its receptor STAT3 translocate into the nucleus activating genes mediating IL-6 driven responses (Aggarwal et al, 2009**)**. In addition to IL-6 however, many other factors, such as EGF, HGF, oncostatin-M (OSM), platelet derived growth factor (PDGF) are known to activate STAT3 expression (Abroun et al, 2015). STAT3 is constitutively activated in head and neck squamous cell carcinoma, breast, lung cancer and myeloma cell lines. STAT3 activation regulates numerous genes involved in cellular proliferation and survival, such as (B-cell lymphoma-extra large) BCL-XL, myeloid leukemia cell differentiation protein (MCL-1), Cyclin D1 and C-Myc (Murray et al, 2007; Levy et al, 2006; Bowman et al, 2001). STAT3 mutations are rarely found in cancers, and its downregulation seems to be due to mutations in its upstream effectors. For instance, STAT3 activation in epithelial cancer is due to aberrant IL-6 expression (Maehara et al, 2000). Activated nuclear STAT3 is found in 60% of human HCCs, and SOCS3 which suppresses STAT3 activity, is found to be deregulated (He et al, 2006; Hogata et al, 2009). STAT3 ablation in hepatocytes prevents DEN induced HCC development, and mice overexpressing IL-6 and IL-6 receptor spontaneously develop

tumors displaying enhance STAT3 activation (He et al, 2006).

Furthermore, some studies have shown that STAT3 participates with NF-κB in the regulation of numerous genes (Yu et al, 2009). Indeed, many of the genes associated with NF-κB oncogenic activity can be preferentially activated when NF-κB interacts with STAT3 (Lee et al, 2011). STAT3 furthermore has been shown to maintain RelA in the nucleus in virtue of their physical interaction, further stimulating the transcription of NF-κB target genes (Lee et al, 2009).

4.4 The MAP kinase cascade

Mitogen-activated protein kinases (MAPKs) control a wide array of cellular responses, such as growth, proliferation, differentiation and apoptosis, depending on cell type and stimulus. Their deregulation is known to cause numerous diseases and to contribute to cancer development.

Given their pivotal role in eukaryotic physiology, they are activated by a wide plethora of extracellular stimuli. This activation requires ligand-induced activation of specific membrane-bound receptors, such as cytokine, tyrosine and serine-threonine kinase receptors. Exposure to stresses such as ionizing radiation, osmotic shock and oncogene expression can also trigger the MAPK pathway (Kyriakis and Avruch, 2012).

ERK, Jun N-terminal kinases (JNK) and p38 MAPK are the terminal effectors of the MAPK cascade, mediating many of the processes typically associated with MAPK activation. They are activated by phosphorylation mediated by upstream kinases, whose activity is also regulated by phosphorylation. Signal transduction occurs by sequential phosphorylation of the MAP kinase kinase kinases (MAP3Ks), which activate MAP kinase kinases (MAP2Ks), which in turn phosphorylate MAPKs (Keshet and Seger, 2010). Dual phosphorylation on a conserved threonine (T) and tyrosine (Y) in a conserved T-X-Y motif,–in an activation loop of the subdomain VIII mediates MAPK activation. This specific phosphorylation mediates conformational changes stabilizing the activation loop in an open conformation which enables substrate binding and phosphorylation (Cuenda and Rousseau, 2007).

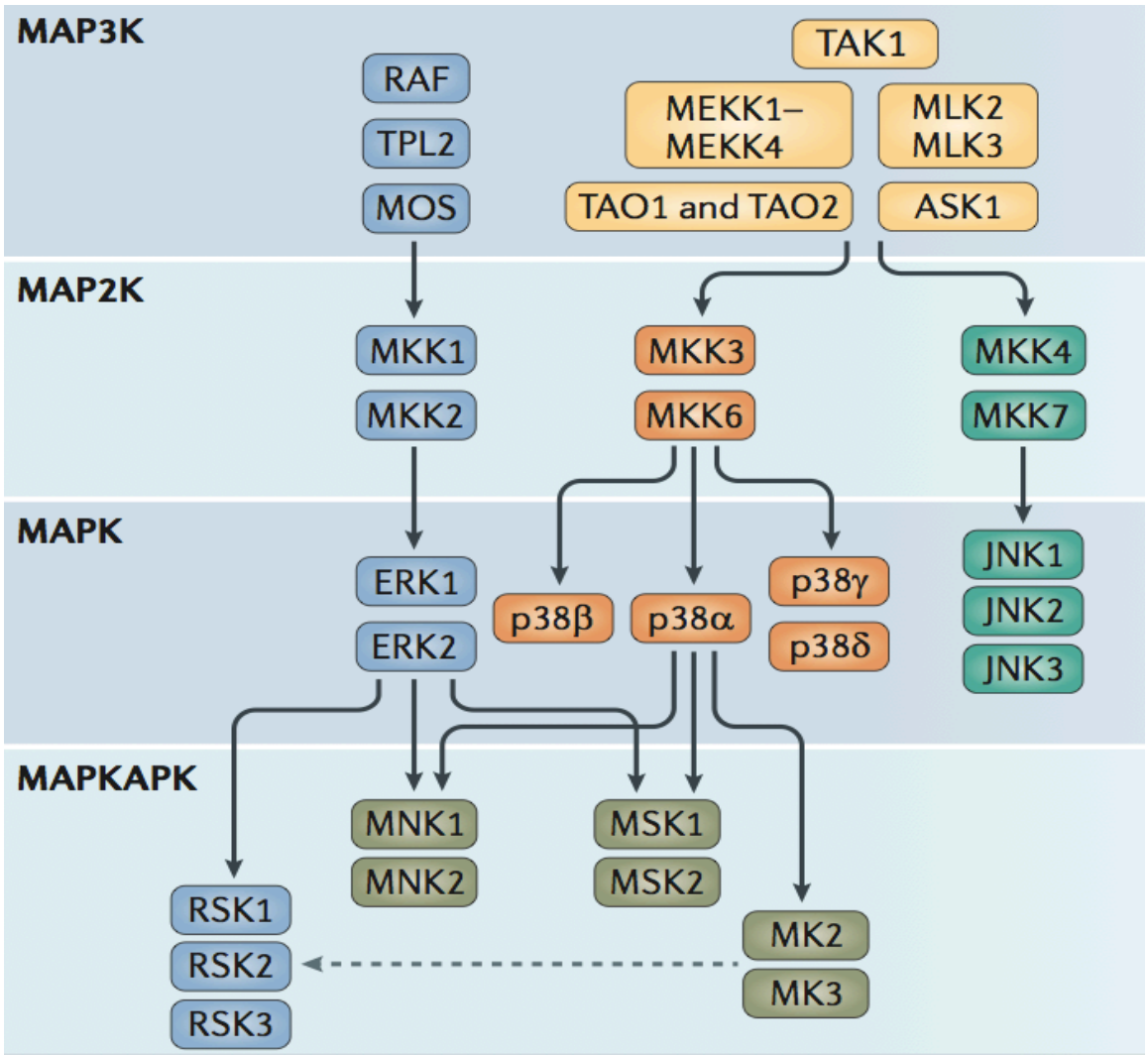

Figure I-5: Canonical mammalian MAPK signalling pathways (Arthur and Ley, 2013).

4.4.1 JNK Signaling

There are three members of the JNK family: JNK1 and JNK2, expressed ubiquitously in tissues, and JNK3, expressed primarily in the brain (Davis, 2000). JNK1 and JNK2 play overlapping, but not identical, roles in various cellular processes. Half of the human HCC display overactivated JNK (Hui et al, 2008) and pharmacological inhibition of JNK suppresses DEN-induced HCC (Mucha et al, 2009) and growth of HCC xenograft in mice. Moreover, *JNK1*$^{-/-}$ mice display reduced HCC following DEN administration (Sakurai et

al, 2006). These evidences clearly point out to an important function of JNK in the promotion of HCC. Follow-up studies demonstrate that JNK1 promotes HCC by upregulation of the C-Myc oncogene and a downregulation of p21 (Hui et al, 2008). Overactivation of the JNK pathway might also be linked to the higher tumor incidence in *IκB*$^{-/-}$ mice, as decreased NF-κB activity causes increased ROS accumulation, which in turn has been shown to extend JNK activation by inhibiting JNK phosphatase activity (Kamata et al, 2005).

However, differences between DEN-induced HCC in mouse model carrying whole body deletion of JNK and cell type specific JNK deletion can be remarkable. Hepatocyte specific ablation of both JNK1 and JNK2 has been shown to increase DEN-induced HCC, whereas combined deletion of JNK1 and JNK2 in hepatocytes and nonparenchymal cells restores the DEN-mediated HCC resistance seen in whole body JNK1$^{-/-}$ mice (Das et al, 2011). As hepatocyte-specific JNK deletion increases the development of HCC, these facts indicate that JNK deletion in nonparenchymal cells is required to reproduce the observed HCC resistance showed in JNK1$^{-/-}$ mice. A recent study done in mice carrying myeloid cell-specific JNK deletion confirms that JNK expression in this specific cell type is required for HCC development, as mice carrying JNK deletion specifically in myeloid cells are resistant to DEN induced HCC, recapitulating the phenotype observed in JNK1$^{-/-}$ mice (Han et al, 2016). These findings indicate the importance of the crosstalk between hepatocytes and myeloid cells during HCC development and that JNK performs cell type specific functions during HCC development.

4.4.2 p38 family

Four members are part of the p38 MAPK family, p38α, p38β, p38γ and p38δ (Han et al, 2007). Among them p38α is the most abundant in various cell types. p38 is mainly activated by the MAP2K mitogen-activated protein kinase kinase 3 (MKK3) and mitogen-activated protein kinase kinase 6 (MKK6). A wide body of evidence suggests that p38α acts as a tumor suppressor in many contexts. It appears to exert this function by inhibiting cell cycle progression and inducing apoptosis (Dolado et al, 2008). In many, but not all, contexts p38 seems to exert a function in antagonizing JNK activity. For instance, JNK activation is impaired in *Mkk7*$^{-/-}$ cells, but its activation can be restored by

threating mouse embryonic fibroblasts (MEFs) with p38⟨ and p38® inhibitors (Wada et al, 2008). p38α deficient myoblast are unable to differentiate due to an increased activation of the JNK/jun pathway (Perdiguero et al, 2007). Treatment of mice with LPS also induces JNK activation in p38 deficient livers correlating with increased activation of MKK3, MKK4 and MKK6 (Heinrichsdorff et al, 2008).

Fetal hematopoietic cells and embryonic fibroblasts from p38α deficient mice show increased proliferation associated with increased activation of the c-jun/JNK pathway. The same overactivation of this pathway is observed in the liver of these animals when HCC is induced by DEN injection. Human HCC samples display enhanced p38 activity compared to non-tumoral tissues (Iyoda et al, 2003), and hepatocyte-specific p38α null mice are prone to DEN-induced HCC development, highlighting the role of p38α in promoting resistance to HCC. In the same mice, p38α deletion leads to overactivation of the JNK-C-jun pathway and inactivation of either c-jun or JNK suppresses the increased proliferation of p38α deficient hepatocytes and tumor cells (Hui et al, 2007). These data clearly suggest the important role p38 plays in antagonizing JNK activity during liver stress. The same tumor inhibitory function of p38α in HCC development was confirmed in mouse models where HCC is induced by liver cirrhosis, p38α was also found to inhibit HCC by preventing ROS accumulation and liver damage (Sakurai et al, 2013).

OBJECTIVES

The principal objective of this thesis has been to study the role of Gadd45b in the development of hepatocellular carcinoma. In addition, we initiated a study about the function of Gadd45a in liver carcinogenesis.

In order to achieve this goal we had the following partial aims:

1. To examine the *in vivo* contribution of Gadd45b and Gadd45a in liver tumor development in DEN-induced HCC model.

2. To study the role of Gadd45b and Gadd45a in hepatocytes cell death following DEN injection.

3. To analyse the Gadd45b and Gadd45a involvement in the DEN-induced compensatory proliferation.

4. To investigate the implication of Gadd45b in liver inflammation by analysing immune cells infiltration, cytokines production and cell signalling in the DEN-treated livers.

5. To assess if specific-ablation of Gadd45b in monocytes/Kupffer cells differentially affects apoptosis, cytokine production and compensatory proliferation upon DEN injection.

MATERIALS AND METHODS

1.ANIMAL MODELS

Gadd45a$^{-/-}$ and *Gadd45b*$^{-/-}$ mice were generated on a mixed 129/C57BL/6 genetic background (Hollander et al,1999; Gupta, et al, 2005). *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ mice on a pure C57BL6/6 background were generated by backcrossing for ten generations. Mice were handled according to national and European Union animal care standards and the experiments were approved by the CNB Ethical Committee.

1.1 DEN-INDUCED CARCINOGENESIS

Chemically induced DEN tumorigenesis is a widely used protocol, which enables the development of HCC in mice. It consists of a single intraperitoneal injection of DEN in male mice. This protocol relies on an initial wave of apoptosis followed by compensatory proliferation, which enables replication of cells with damaged DNA. For this protocol, fifteen-day-old male mice on a 129/C57BL/6 genetic background were injected with 25 mg/kg DEN (Sigma). After nine months, mice were sacrificed and their livers were removed and analyzed for presence of HCCs and subjected to histological and immunological analysis.

1.2 DEN-ACUTE MODEL

This model was used to check the initial apoptotic and proliferative responses induced in liver following DEN injection. These responses happen in the first 48 hours following DEN injection. Two months old C57BL/6 wild-type (WT) mice, *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ mice were injected with 100 mg/kg of DEN and sacrificed 48 hours to monitor apoptosis and proliferation, and after 5 hours to assess RNA expression.

1.3 BONE MARROW TRANSPLANT

We wanted to create a murine model whose liver had WT hepatocytes and *Gadd45b*$^{-/-}$ Kupffer cells and viceversa, to determine whether the phenotype observed in *Gadd45b*$^{-/-}$ mice after injection was due to the lack of *Gadd45b* in Kupffer cells, hepatocytes or in both. All the experiments were carried out in the C57BL/6 background, in order to

eliminate issues related to bone marrow compatibility. Furthermore, in order to assess reconstitution efficiency, male mice were used as the donors and female mice as the recipients. This enables to evaluate the extent of bone marrow reconstitution in the recipient female mice by amplifying the specific male SRY gene, located on the Y chromosome. Donor cells were collected after sacrificing WT or *Gadd45b*$^{-/-}$ mice and extracting bone marrow cells from femur and tibiae by inserting a needle syringe in the bone cavity and performing several washes with a cold PBS-EDTA solution. Cells were filtered through a 70 μm filter to avoid clumps formation and four million cells were injected intravenously via tail vein into the recipient mice. Previously, two months old recipient mice were lethally irradiated with 10 grays at 24 hours before transplant. Engraftment of the transplanted bone marrow cells was allowed to take place for a minimum of six weeks before animals were sacrificed. WT mice were reconstituted with Gadd45b$^{-/-}$ bone marrow and Gadd45b-/- mice were reconstituted with WT bone marrow. Irradiated WT mice reconstituted with WT bone marrow were used as a control.

2. GENERAL METHODS

2.1 PROTEINS AND RNA SAMPLES PREPARATION

In order to extract protein and RNA from murine tissue, mice were sacrificed and liver was collected and mechanically disrupted in liquid nitrogen. Tissue powder was processed for protein and RNA extractions. Proteins were extracted using a cell signaling lysis buffer (Ref 9803). Protein concentration was assessed by using the MicroBCA protein assay kit (Thermofisher Ref 23235). For RNA preparation tissue powder was homogenized in TRI Reagent (Sigma Aldrich) and RNA-containing fraction was mixed with isopropanol for RNA precipitation. After. double precipitate washing with 70% ethanol and dissolving in RNase-free water, the RNA concentration was measured with Nanodrop spectrophotometer.

2.2. IMMUNOBLOTTING

Proteins were denatured using standard loading buffer, as described in Table M-1, for 10

minutes at 95°C. Polyacrylamide gel electrophoresis (PAGE) was performed according to a common procedure. Denatured protein samples, at volumes corresponding to equal protein amounts (40 μg), were loaded into 12.5% polyacrylamide gel wells and proteins were separated in a running buffer (Table M-2) connected to a source of electrical field (120 V, for 2 hours, at RT). Separated proteins were electro-blotted onto a PVDF membrane previously activated in methanol for 2 minutes, using a transfer buffer (Table M-3). The membrane was later incubated with a blocking solution, containing 5% skimmed milk dissolved in a TBS buffer (Table M-4) with 0.1% of Tween 20, at RT. The blocked membrane was incubated overnight with a primary antibody (Table M-5) diluted in a blocking solution at 4°C. Protein expression was addressed using HRP-Conjugated secondary antibodies. Band density was determined using image studio lite software.

LOADING BUFFER 4X	
Reactive	Concentration
Tris -HCL	20 mM PH 7.4
SDS	150 mM
Glicerol	0,05%
Ditioreitol	1M
β–mercaptoetanol	4%
Blue Bromophenol	1%
DILUTED IN WATER	

TABLE M.1 - Loading buffer 4x Components

RUNNING BUFFER 10X	
Reactive	Amount
Tris	30 gr
Glicine	144 gr
SDS	10 gr
DILUTED IN 1 LITER OF WATER	

TABLE M.2 - Running buffer 10x Components

TRANSFER BUFFER 10X	
Reactive	Amount
Tris	58 gr
Glicine	29 gr
SDS	3,7 gr
DILUTED IN 1 LITER OF WATER	

TABLE M.3 – Transfer buffer 10X Components

TBST 1X	
Reactive	Concentration
Tris -HCL	20 mM PH 7.4
NaCl	150 mM
Tween20	0,05%
DILUTED IN WATER	

TABLE M.4 – TBST 1X Components

Antibody	Secondary	Company	Dilution
P-JNK	Rabbit	Cell Signaling	1/1000
Cyclin D1	Mouse	Cell Signaling	1/2000
GAPDH	Rabbit	Cell Signaling	1/2000
P-STAT3	Rabbit	Cell Signaling	1/500
P-IκBα	Mouse	Cell Signaling	1/1000
P-MKK7	Rabbit	Cell Signaling	1/500
P-MKK4	Rabbit	Cell Signaling	1/500
Tot-JNK	Mouse	Cell Signaling	1/1000
P-C-JUN	Rabbit	Cell Signaling	1/1000

TABLE M.5 – Antibodies used in WB experiments

2.3. IMMUNOPRECIPITATION AND KINASE ACTIVITY

Liver protein lysates (500 μg) were incubated O.N. with anti-p-JNK antibody (1,5 μg) at 4°C with orbital agitation in a minimum of 200 μl lysis buffer volume. The next day 30 μl of protein G sepharose beads (GE healthcare) were added to the solution and incubated for 2,5 hour in orbital agitation. Lysates were then centrifuged at 18000 x g. After being washed twice in lysis buffer and twice in kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$), the samples were incubated at 30°C for 20 min in a 50 ul volume with 1 μg of c-jun substrate (Cell Signaling) and 200 μM ATP (Cell Signaling). Reaction was stopped by adding 14 μl of loading buffer 4x and lysates were boiled at 100°C for 5 minutes in order for the beads to dissociate from the antibody complex. Lysates were centrifuged at 9000 x g. Pellet was discarded and supernatant was used for western blot analysis.

2.4. REAL-TIME QUANTITATIVE PCR

cDNA for real-time quantitative PCR (qPCR) was generated from 0.5 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 10 μl of final reaction volume. The qPCR reactions were performed in triplicate using 5 μl of each cDNA 1/40 dilutions, 10 μM of each oligonucleotide and HOT FIREPol qPCR mix (Solis Biodyne) in a total volume of 8 μl in MicroAmp Optical 384-well plates (Applied Biosystems). PCR reactions were carried out in an ABI PRISM 7900HT (Applied Biosystems). The amount of amplified DNA was measured through the emission of light by the SYBR Green dye, intercalating in synthesized double stranded DNA. All samples were measured in triplicates. SDS v2.2 software was used to analyze results. Specific amplification was controlled by melting-curve analysis. The data were exported, processed to Microsoft Excel and analyzed by the comparative Ct method (DDCt). X-fold change in mRNA expression was quantified relative to unstimulated WT samples from the same experiment. *Gapdh* mRNA was used as a housekeeping gene. The sequences of the oligonucleotides used in this thesis can be found in Table M-6

Gene	Forward Sequence (5' → 3')	Reverse Sequence (5' → 3')
Gapdh	CCCATCACCATCTTCCAGGA	CGACATACTCAGCACCGGC
Il-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
IL-1α	CACCTTACACCTACCAGAGTGATTTG	TGTTGCAGCTCATTTAACCAAGTG

TABLE M.6 – Oligonucleotide Sequences

2.5. IMMUNOHISTOCHEMISTRY

For histological analyses, livers were fixed in 4% buffer formalin, dehydrated, embedded in paraffin, section (5 um) and processed for H&E staining or immunohistochemistry. Liver and tumor sections were deparaffinised using xylene for 20 min and rehydrated with 3-min incubations in ethanol at decreasing concentrations (2x 100%, 1x 90% and 1x 70%). The antigen retrieval was performed by heating up the sections in 0.01 M citrate buffer (pH 6.0) for 15 min. The samples in hot citrate buffer were placed on ice for 20 min to cool down gradually. The activity of endogenous peroxidase was blocked by incubating for 20 min in 3.3% of hydrogen peroxide (H_2O_2) in methanol at RT. To block unspecific proteins the sections were incubated, for 1 hour at 37°C, in 20% of goat serum in 3% of bovine serum albumin (BSA) in distilled water. After the blocking step, incubations in different primary antibodies were performed in accordance with manufacturer's recommendations. The antibodies were diluted in blocking solution and then added onto the tissue sections that were placed in a humid chamber for an overnight incubation at 4°C. Next day, the sections were washed with PBS-Tween 0.1% (Table M-7) three times for 5 min. Biotinylated secondary antibodies were used to bind to the primary antibodies in accordance with their specificity during 1-hour incubation at RT. Image analysis and performed using the cell profiler software.

PBS 1X	
Reactive	Concentration
Sodium phosphate	10mM PH 7.4
NaCl	150mM
DILUTED IN WATER	

TABLE M.7– PBS COMPONENTS

Apoptotic cells in liver tissue were stained using terminal deoxynucleotidyltransferase (TdT)- mediated dUTP nick-end label (TUNEL) staining. Briefly, paraffin-embedded liver sections were deparaffinised and rehydrated as described above. The slides were washed with PBS and permeabilized in PBS containing 0.5% Triton X-100. Next, the sections were pre-incubated for 15 min at RT in freshly prepared TdT 1X buffer (Table M-8) containing 1mM cobalt (II) chloride. The TdT-based reaction was carried out in a humid chamber at 37°C for 1 hour in the same buffer, containing besides 3% TdT (v/v) (Roche, ref. 03333574001) and 2% biotin-16-dUTP (v/v) (Roche, 11093070910). After this the sections were washed twice with PBS containing 0.01% Tween 20. Sections were then incubated with streptavidin conjugated with a cyanine fluorochrome (Cy5). Nuclei were stained with Hoechst33342.. The slides were observed by fluorescent microscopy using Leica DMI 6000B microscope. Image analysis was performed using the cell profiler software

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TdT Buffer 5X	
Reactive	Concentration
BSA	0,125%
Tris-Buffer PH 8.8	125mM
Sodium cacodylate trihidrate	1M
DILUTED IN WATER	
ADJUST PH to 6.6	

TABLE M.8– TdT buffer 5X components

2.7. IMMUNOFLUORESCENCE

2.7.1 F4/80

Liver tissues were frozen in liquid nitrogen. Slides were fixed in 100% cold acetone for 10 minutes, blocking was carried out for 30 minutes using PBS containing 10% goat serum and 1% BSA. Slides were incubated overnight at 4°C with anti-F4/80 antibody (Serotec) in the blocking solution. The next day slides were incubated for 45 minutes RT with mouse anti-rat Cy5 (Jackson Lab), briefly washed with PBS and incubated for 10 minutes at RT with DAPI. Slides were then observed by fluorescent microscopy using Leica DMI 6000B microscope. Image analysis was performed using the cell profiler software

2.7.2 ROS STAINING

Liver tissues from mice were frozen in liquid nitrogen, slides were allowed to thaw for 2 minutes at RT, and incubated with dihydroethidium (DHE) (10 μM) in PBS for 30 minutes at 37°C. DHE is a superoxide indicator, exhibiting blue-fluorescence in the cytosol until oxidized, where it intercalates within the cell's DNA, staining its nucleus in red. Slides were gently washed with PBS and incubated 10 minutes at RT with DAPI. Slides were then observed by fluorescent microscopy using Leica TCS SP5 confocal microscope. Image analysis was performed using the cell profiler software

3. PRIMARY CELLS ISOLATION METHODS

3.1 PRIMARY HEPATOCYTE ISOLATION

For hepatocyte isolation, all the solutions described below were kept in a bath at 37°C to equate a mouse body temperature. Mice were anesthetized with avertin and peritoneum was opened. A sterile thread was passed under the inferior vena cava just below the liver and ligated. A 24-G catheter was inserted into the inferior vena cava and the thread was tightly ligated. Vena porta was cut to allow the blood out of the vascular system. Perfusion solution (HBSS w/o Ca/Mg/phenol, Invitrogen) was sent to the liver through

the catheter for 4 minutes at a flow of 2,5 ml/min. Perfusion solution was then switched with collagenase solution (Williams E media containing 0,6 mg/ml collagenase type IV, Sigma). Collagenase solution was sent to the liver with a flow of 4 ml/min for 12 minutes. Liver was excised, put in a 60 mm dish and cells were disgregated by gentle counterstriking with two 1 ml pipettes. Suspension was filtered through a cell strainer and passed into a falcon tube. Cells were centrifuged at 35x g for 10 minutes. Supernatant was discarded and pellet was resuspended in HBSS. Live and dead hepactocytes were separated by a 10 minutes, 35 x g centrifugation in iso-osmotic Percoll (45 ml Percoll, 4,5 ml 10x HBSS, 0,5 ml HEPES). Supernatant, consisting of dead hepatocytes was discarded and the pellet was resuspended in HBSS and used for cell cultures.

3.2 KUPFFER CELL ISOLATION

For Kupffer cells, the isolation procedure was identical to the hepatocyte protocol until the first centrifugation. At this point, hepatocyte pellet was discarded and supernatant was centrifuged at 650 x g for 10 minutes. The resulting pellet consisted mainly of Kupffer cells. Pellet was resuspended in 10 ml HBSS and carefully layered on top of a 50%/25% percoll gradient and centrifuged at 1800 x g for 15 minutes. Upper layer, consisting mostly of dead cells, was removed and the middle layer, containing a ring of alive Kupffer cells was carefully transferred in a new 50 ml falcon tube and HBSS was added to reach 50 ml volume. Solution was centrifuged 650 x g for 10 minutes and the resulting pellet was used for cell cultures.

4. FLOW CYTOMETRY METHODS

4.1 NEUTROPHILES DETECTION

The protocol was identical to the Kupffer cell isolation protocol described above until the first centrifugation at 650 x g for 10 minutes. The resulting pellet consists mainly of Kupffer cells but a small population of infiltrating neutrophils can be identified by flow cytometry. Cells in the pellet were counted and placed at a density of 50000 cells/well in

a 96 well plate. Cells were centrifuged at 550 x g for 5 minutes and incubated 30 minutes at 4°C with anti Ly6C and Ly6G antibodies conjugated with FITC and PE respectively. Cells were centrifuged at 550 x g for 5 minutes, and cells were passed through the flow cytometer (Gallios, Beckman) and double positive cells were identified using the Kaluza software

5. CELL CULTURE METHODS

5.1 HEPATOCYTE CULTURE

Hepatocytes were plated in 96 wells at a density of 12500 cells/well. Cells were cultured in Hepatocyte Feeding Media (DMEM/F12 GlutaMAX supplemented with BSA, HEPES, Na-pyruvate, sodium bicarbonate, proline, galactose, ITS and EGF). Cells were kept in an incubator at 37°C and 5% CO_2.

5.2 KUPFFER CELL CULTURE

Kupffer cells were plated in 96 well plates at a density of 120000 cells/well. Cells were cultured in RPMI, supplemented with 10% FBS, non-essential amino acids, sodium-piruvate, L-glutamine.Cells were kept in an incubator at 37°C and 5% CO_2.

5.*3 IN VITRO* CYTOKINE PRODUCTION ASSAY

Kupffer cells at a density of 120000 cells/well were cultured in a 96 well plate. Cells were serum starved O.N. in 0,5% serum RPMI media. The next day 10% serum RPMI media containing either 100 ng/ml of IL-1α or IL-1β was added to the cells, supernatant was collected after 24 hours and IL-6 secretion was measured with IL-6 ELISA kit (Affimetrix) according to the manufacturer protocol.

5.4 LDH RELEASE ASSAY

Hepatocytes were isolated and kept in culture for 2 days, on day 3 feeding media containing either 100 ng/ml TNF-α or TGF-β (R&D) was added. 24 hours later, supernatant was collected and LDH was measured with LDH release assay (Promega) according to the manufacturer protocol.

6. STATISTICAL ANALYSIS

For statistical analyses GraphPad Prism 7 was used. Differences between WT and *Gadd45b*$^{-/-}$ or *Gadd45a*$^{-/-}$ mice were examined for statistical significance using the Student´s test. Differences were considered statistically significant for (*) $P<0.05$ (**) $P<0.01$ (***) $P<0.001$. In figure R1-B and R7-B, Fisher test was applied to compare differences between tumor-positive mice of different genotype. If not stated otherwise, non-significant differences between the corresponding groups from different genotypes have not been shown on the graphs.

RESULTS

1.1 Gadd45b is required for DEN-induced tumorigenesis

To address the role of Gadd45b in HCC development, a cohort of wild type and *Gadd45b*$^{-/-}$ mice was injected with the carcinogen DEN. Mice were sacrificed after nine months to assess tumor development (Fig. R-1A). Notably, we found significant differences in the percentage of tumor free mice between WT and *Gadd45b*$^{-/-}$ mice. In fact, 30% of all *Gadd45b*$^{-/-}$ mice versus only 3% of wild type mice did not display any sign of tumorigenesis after exposure to DEN (Fig. R-1A,B). Furthermore, when the groups of tumor positive animals were compared, 41% of wild type mice developed between 10 and 20 tumors, and this number dropped to less than 10% for *Gadd45b*$^{-/-}$ mice. Most importantly, no mice in *Gadd45b*$^{-/-}$ genotype developed more than 20 tumors, while this number was as high as 20% in the wild type background (Fig. R-1C). The liver/bodyweight ratio can be taken as an indication of the liver tumoral mass, indeed, tumors present in the liver increase liver mass, augmenting the liver/bodyweight ratio. This number was significantly lower in *Gadd45b*$^{-/-}$ mice compared to WT mice (Fig. R-1D), indicating that *Gadd45b*$^{-/-}$ tumors had lower cellular mass than WT tumors. Adenomas are benign tumors of the liver, and only a small percentage can evolve into HCC over time. Benign tumors, by definition, lack the ability to invade neighboring tissues or metastasize and, therefore, cannot be defined as cancerous. Remarkably, HCC could be identified in 40% of the WT mice whereas no mouse belonging to the *Gadd45b*$^{-/-}$ genotype displayed any sign of HCC (Fig. R-1E). All the tumors identified in *Gadd45b*$^{-/-}$ mice were adenomas. We therefore can say that *Gadd45b*$^{-/-}$ mice were HCC free after nine months of exposure to DEN. These evidences point out to a new and unexpected tumor promotive role of Gadd45b in DEN-induced tumorigenesis.

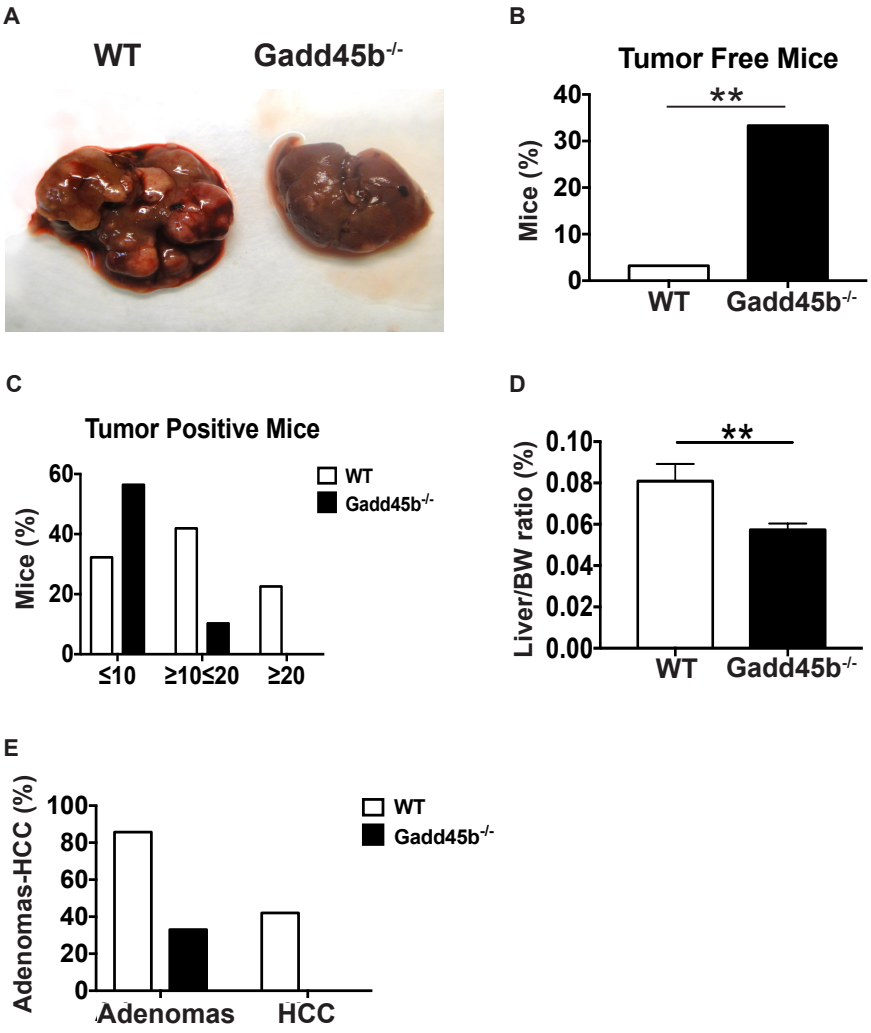

Figure R-1: Liver tumor formation in WT and *Gadd45b*$^{-/-}$ mice after DEN treatment. Two weeks old wild-type and *Gadd45b*$^{-/-}$ mice were treated with a single intraperitoneal injection of DEN (25 mg/kg) and livers were harvested after nine months. (**A**) Representative picture of DEN-induced liver tumors in wild-type (n=32) and *Gadd45b*$^{-/-}$ (n=39) mice. (**B**) The percentage of tumor-free mice after 9 months of exposure to DEN. Statistically significant differences between WT and *Gadd45b*$^{-/-}$ mice are indicated (**) P<0,01. P value is from a Fisher´s exact test. (**C**) The percentage of tumor-positive mice after nine months. (**D**) The liver/body weight ratio was calculated and expressed as the mean percentage ± S.E.M. Differences between WT and *Gadd45b*$^{-/-}$ mice were examined for statistical significance using the Student´s test, (**) P<0,01. (**E**) The percentage of HCC and adenomas nine months after DEN injection in WT (n=8) and *Gadd45b*$^{-/-}$ mice (n=9).

1.2 Gadd45b deficiency prevents hepatocyte death and compensatory proliferation following DEN administration

It is well known that carcinogen induced hepatocyte cell death and subsequent cellular proliferation are crucial processes leading to tumor development. In the case of DEN-treatment, both events are initiated in the first 48 hours after DEN administration. As *Gadd45b*$^{-/-}$ mice develop no tumors upon DEN injection, we reasoned that early induction of *Gadd45b* could be critical for the onset of these processes. Indeed, *Gadd45b* mRNA expression was significantly increased upon DEN injection (Fig. R-2A). We examined hepatocyte death in WT and *Gadd45b*$^{-/-}$ mice after exposure to DEN. Sections of liver were stained for cell death using the TUNEL assay (Fig. R-2B). In addition, we studied caspase activation by immunoblot analysis. We found that lack of *Gadd45b* in mice impaired cell death. TUNEL staining and cleaved caspase-3 expression revealed reduced hepatocyte death in DEN-treated *Gadd45b*$^{-/-}$ mice compared to WT mice (Fig. R-2B).The observed decrease in cell death could be the result of defects in the apoptotic mechanisms leading to hepatocyte death in *Gadd45b*$^{-/-}$ mice. To test this hypothesis, we purified primary hepatocytes from WT and *Gadd45b*$^{-/-}$ mice and treated them with TNF-α and TGF-β, two bona fide proapoptotic cytokines. Apoptosis was analyzed by measuring lactate dehydrogenase (LDH) released in the media. LDH is a soluble enzyme found inside living cells that is released in the extracellular space when cell membrane integrity is compromised. *In vitro,* purified primary *Gadd45b*$^{-/-}$ hepatocytes showed reduced sensitivity to TNF-α and TGF-β induced cell death (Fig. R-2C) pointing out to an intrinsic resistance mechanism of apoptosis independent from extracellular milieu. The liver is a body organ capable of regeneration, the reduced hepatocyte cell death of *Gadd45b*$^{-/-}$ mice upon DEN injection might affect compensatory proliferation, a mechanism required to induce the initial mutations eventually leading to cancer development. In the initial phase of DEN-induced carcinogenesis, the apoptotic and proliferative waves are almost simultaneous and expression of proliferative markers such as Cyclin D1 and Ki67 can be observed 48 hours upon injection. In many cases, a lower apoptotic response results in a decrease in the compensatory proliferation. We therefore

examined the proliferative response after DEN-treatment in WT and *Gadd45b*$^{-/-}$ mice. The lack of *Gadd45b* in mice caused both reduced positivity to Ki67 immunostaining and reduced amount of Cyclin D1 by western blot analysis (Fig. R-2D). In summary, our data indicate that *Gadd45b* expression is required for hepatocyte cell death and subsequent compensatory proliferation triggered by DEN-induced stress.

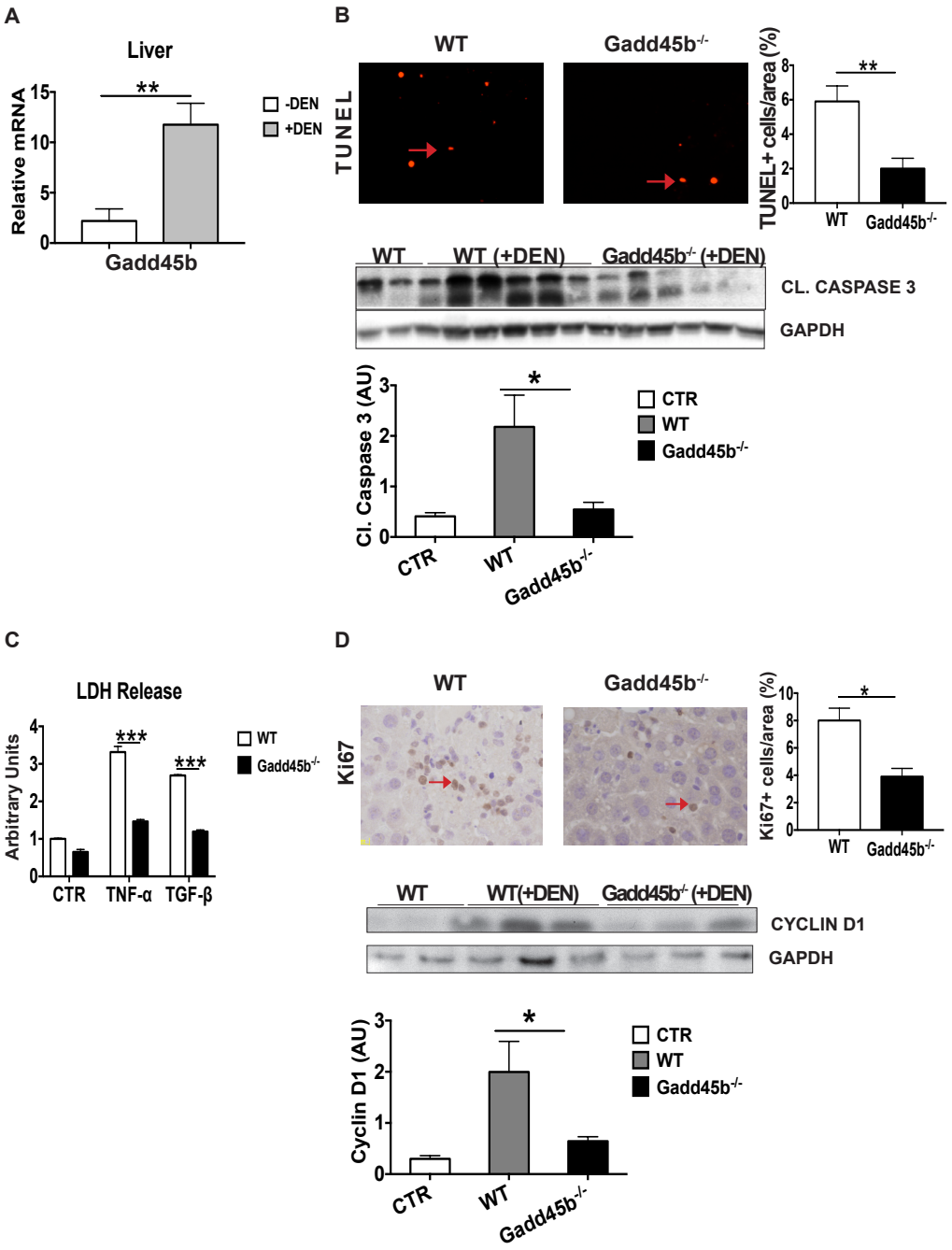

Figure R-2 Lack of *Gadd45b* decreases hepatocyte death and proliferation after DEN injection. (**A**) Quantitative RT-PCR showing *Gadd45b* mRNA expression in liver of WT mice 5 h after 100 mg/kg DEN treatment *in vivo*. The statistical data for *Gadd45b* mRNA was normalized to *Gapdh* mRNA. Data represent mean ± S.E.M. of three independent experiments, n=3 mice in each group, (P<0,01). (**B**) Top, TUNEL stain of livers from WT and *Gadd45b*$^{-/-}$ mice 48 h following 100 mg/kg DEN injection, n=3 mice per genotype. Statistical analysis of double positive cells with 5 fields of view for each group, data represent mean ± S.E.M. (P<0,01). Bottom, Cleaved Caspase 3 expression level (CL-caspase3), in the same conditions as the top figure, was analyzed by Western blot (n=6 for DEN-treated groups, n=2 for untreated controls) and GAPDH expression was used as a loading control. This result is representative of three independent experiments. Densitometry analysis of cleaved caspase 3 signal is normalized to GAPDH. Data represent mean ± S.E.M. (P<0,05). (C) LDH release in the supernatant of WT and *Gadd45b*$^{-/-}$ primary hepatocytes either untreated or treated for 24 h with 100 ng/ml TNF-α and TGF-β (n=3 in all groups), data represent mean ± S.E.M. (P<0,001). (**D**) Top, analysis of hepatocyte proliferation by immunostaining for Ki67 of livers from WT and *Gadd45b*$^{-/-}$ mice 48 h following 100 mg/kg DEN injection, n=3 mice per genotype. Statistical analysis of Ki67 positive cells with 5 fields of view for each group, data represent mean ± S.E.M. (P<0,05). Bottom, expression of Cyclin D1 was monitored by Western blot analysis and GAPDH expression was used as a loading control. Densitometry analysis of Cyclin D1 signal is normalized to GAPDH. Data represent mean ± S.E.M. (P<0,05).

1.3 Reduced IL6 production in *Gadd45b*$^{-/-}$ mice upon DEN administration

Necrotic hepatocytes release a vast array of inflammatory mediators, among those, IL-1⟨ is known to be necessary, but not sufficient, to activate Kupffer cells to release IL-6. This cytokine stimulates hepatocyte division, connecting cell death to cellular proliferation. We therefore examined the production and expression of inflammatory

cytokines in WT and Gadd45b-null mice. Whereas IL-6 serum level in *Gadd45b*$^{-/-}$ mice is significantly lower compared to WT mice, upon DEN injection, only a non-significant trend of decrease can be found for the IL-1α (Fig. R-3A). Since IL-1α is mainly produced by hepatocytes and IL-6 by Kupffer cells, we analyzed whether lack of Gadd45b might affect their expression in both cell types. When hepatocytes and Kupffer cells from WT and *Gadd45b*$^{-/-}$ mice were purified upon DEN injection, quantitative RT-PCR analysis showed reduced *IL-6* gene transcription in *Gadd45b*$^{-/-}$ mice compared to WT mice. However, transcription of hepatic *IL-1α* remained unaltered between the two groups (Fig. R-3B). Therefore, reduced *IL-6* expression in *Gadd45b*$^{-/-}$ mice can account for the reduced compensatory proliferation upon DEN genotoxic stress.

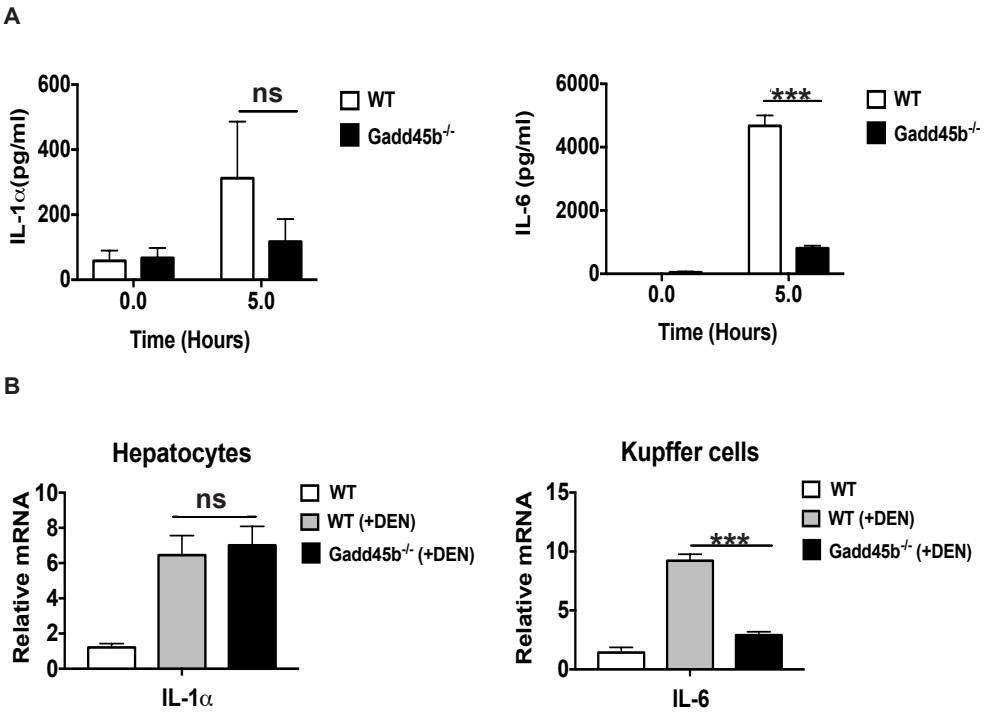

Figure R-3. ***Gadd45b*** **is required for IL-6 production induced by DEN treatment**. **A**) Luminex assay showing cytokines serum levels in WT and *Gadd45b*$^{-/-}$ mice at the indicated time points, after 100 mg/kg DEN injection . Left concentration of IL-1α (n=3 for each genotype and time), right, concentration of IL-6, (n=3 for each genotype and time; P<0,001). Data represent mean ± S.E.M. (**B**) WT and *Gadd45b*$^{-/-}$ mice were injected with DEN 100 mg/kg (n=3 each group), liver was harvested after 5 hours and

mRNA expression was analyzed in hepatocytes and Kupffer cells, using uninjected WT mice as control. Left, IL-1α expression in hepatocytes from WT and *Gadd45b*$^{-/-}$ mice compared with uninjected WT mice was analyzed by quantitative RT-PCR. The statistical data for mRNA was normalized to β-actin mRNA. Data represent mean ± S.E.M. Right, IL-6 expression in Kupffer cells from WT and *Gadd45b*$^{-/-}$ mice compared with uninjected WT mice was analyzed by quantitative RT-PCR. mRNA was normalized to β-actin (P<0,001), data represent mean ± S.E.M.

1.4 Gadd45b deficiency reduces inflammation but not oxidative stress upon DEN treatment

ROS generated by both initiated cells and inflammatory cells are thought to accelerate HCC development by inducing DNA damage and hepatocyte injury (He et al, 2011). Indeed, excessive ROS production promotes hepatocyte death by various mechanisms, including prolonged JNK activation by inactivation of JNK phosphatases (Kamata et al, 2005). Furthermore, Kupffer cells-mediated IL-6 secretion is known to facilitate macrophages and neutrophils recruitment to the injury site (Kong et al, 2016). Given the decrease in IL-6 observed in *Gadd45b*$^{-/-}$ mice upon DEN injection, we analyzed if *Gadd45b* affects these processes. After 48 hours of DEN treatment, *Gadd45b*$^{-/-}$ mice displayed reduced infiltrate in terms of macrophages and neutrophils (Fig. R-4A). When ROS production was analyzed instead, no differences (red signaling) were found between the WT and *Gadd45b*$^{-/-}$ genotypes, indicating that *Gadd45b* tumor promotive effect was not caused by increased oxidative stress (Fig. R-4B).

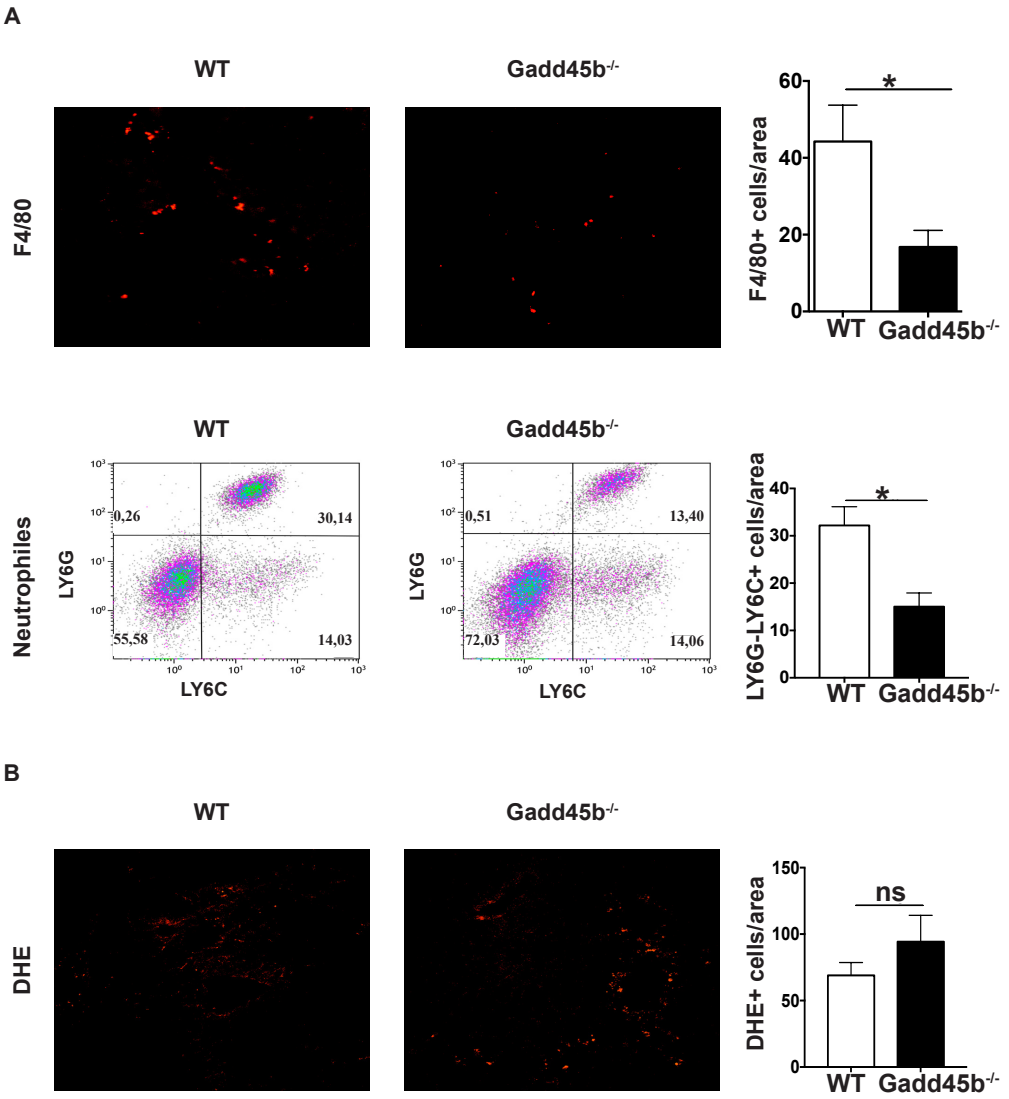

Figure R-4 Lack of *Gadd45b* reduces cell infiltration but does not affect oxidative stress. **A**) WT and *Gadd45b*$^{-/-}$ mice were injected with DEN 100 mg/kg (n=3 each group), livers were harvested after 5 hours and macrophages and neutrophils infiltration was analyzed. Top, F4/80 immunostaining: statistical analysis of F4/80 positive cells with 5 fields of view for each group, data represent mean ± S.E.M. (P<0,05). Bottom, single-cell suspensions from livers are stained and analyzed by FACS for the indicated cell surface markers. Statistical data indicated the percentage of neutrophils identified as LY6C^{high} LY6G^{high} cells, data represent mean ± S.E.M. (P<0,05) of at least three independent experiments. (**B**) DHE stain of livers from WT and *Gadd45b*$^{-/-}$ mice 48 hours following 100 mg/kg DEN injection (n=3 each group). Statistical analysis of DHE-positive cells with 5 fields of view for each group, data represent mean ± S.E.M.

1.5 *Gadd45b* stimulates JNK activity during DEN induced tumorigenesis.

JNK is known to promote tumorigenesis during DEN-induced liver injury by enhancing Cyclin D1 expression (Sakuray et al, 2006). Interestingly in liver, cell-type specific deletion of JNK can have opposite effects on DEN-induced HCC development. Hepatocyte-specific deletion of JNK has been found to enhance the development of tumors following DEN-injection. However, whole liver *JNK1*$^{-/-}$ mice, carrying JNK deletion in both hepatocyte and nonparenchimal cells, are resistant to DEN-induced tumorigenesis (Sakurai et al, 2006). Forty eight hours post DEN injection, phosphorylation of JNK is markedly decreased in *Gadd45b*$^{-/-}$ mice compared to WT (Fig. R-5A). In the same context, kinase activity assay shows reduced activity of JNK in *Gadd45b*$^{-/-}$ livers (Fig. R-5B). Phosphorylation of JNK is mainly, but not only, mediated by its upstream MAP2K mitogen-activated protein kinase kinase 7 (MKK7) and mitogen-activated protein kinase kinase 4 (MKK4). However, no differences could be found between WT and *Gadd45b*$^{-/-}$ mice in MKK7 and/or MKK4 phosphorylation,. In addition, the lack of Gadd45b resulted in a dysregulation of the IL-6 signalling pathway, confirmed by reduced phosphorylation of STAT3, and decreased NF-κB activity, consistent with the reduced phosphorylation of IκBα.

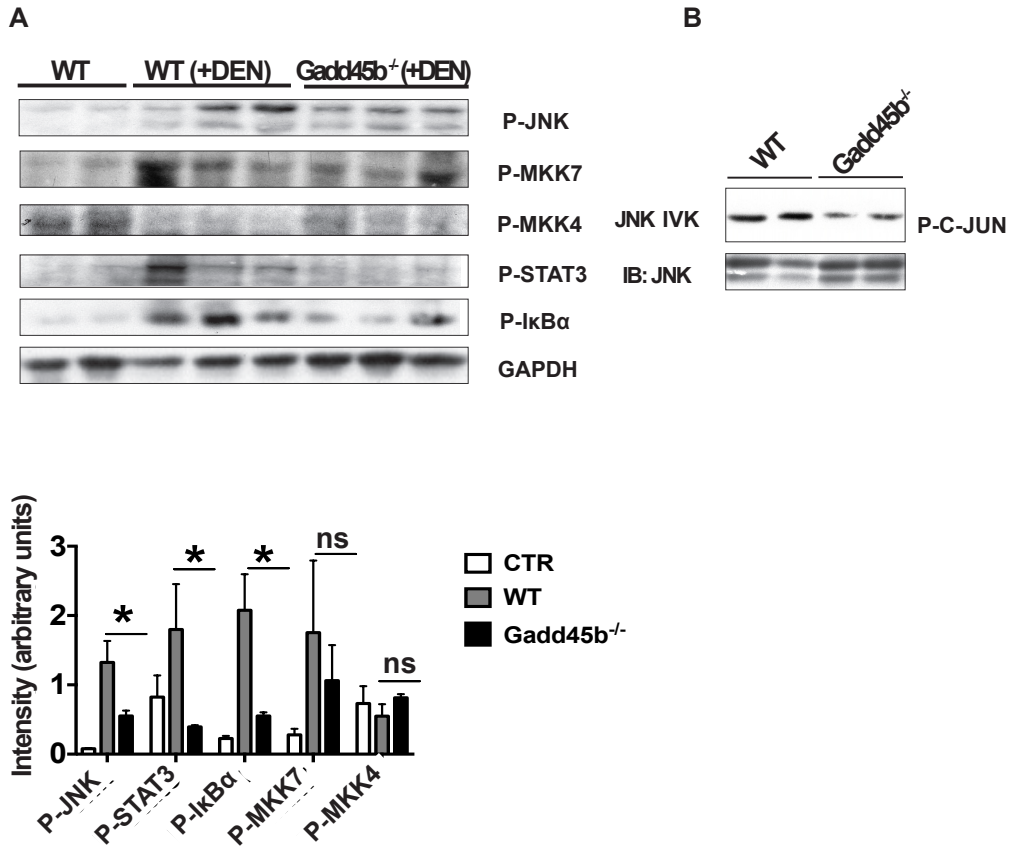

Figure R-5 *Gadd45b* is required for JNK and STAT3 activation after DEN treatment. **A**) Top, WT and *Gadd45b*$^{-/-}$ mice were injected with DEN (100 mg/kg). Phosphorylation of STAT3, MKK4, MKK7, JNK and IκBα were assessed by Western blot analysis. Uninjected WT mice were used as a control. Bottom, Densitometry analysis of proteins in the top figure. Signal is normalized to GAPDH. Values represent mean ± S.E.M., n=3 mice for treated groups, n=2 for the control group (P<0,05). (**B**) Cell extracts from WT and *Gadd45b*$^{-/-}$mice were immunoprecipitated (IP) with anti-JNK antibody and *in vitro* immune complex kinase assay was performed using C-JUN as a bone fide JNK substrate.

1.6 *Gadd45b* expression in Kupffer cells is required for IL-6 production and subsequent cellular proliferation

Specific ablation of the same gene either in hepatocytes or in Kupffer cells can have opposite effects on DEN-induced carcinogenesis. Lack of JNK only in hepatocytes or in hepatocytes and myeloid cells altogether either increases or decreases DEN-induced tumorigenesis, respectively (Das et al, 2011). This particular phenotype has been linked to IL-6 production. IL-6 is a paramount cytokine and its expression by Kupffer cells is required for DEN-mediated HCC development. Blockage of JNK selectively in hepatocyte has been found to enhance Kupffer cells mediated IL-6 secretion whereas combined deletion of JNK in both hepatocyte and Kupffer cells represses it. As we observed a blockade of JNK signaling in liver from *Gadd45b*$^{-/-}$ animals associated with decreased IL-6, we investigated if this phenotype is maintained when *Gadd45b* is selectively deleted in hepatocytes or in myeloid cells. As Kupffer cells originate from bone marrow, we took advantage of the bone marrow transplant, where mice are lethally irradiated and transplanted with a donor bone marrow. Liver repopulation by Kupffer cells from donor origins happens within 6 weeks. We lethally irradiated *Gadd45b*$^{-/-}$ and WT mice and reconstitued them with WT or *Gadd45b*$^{-/-}$ bone marrow respectively, this enables the generation of mice carrying selective deletion of *Gadd45b* either in hepatocytes (*Gadd45b*$^{\Delta hep}$) or in monocytes/Kupffer cells (*Gadd45b*$^{\Delta Kup}$). Upon DEN injection, we found that compensatory proliferation was substantially compromised in *Gadd45b*$^{\Delta Kup}$ mice, as noted by decreased levels of proliferative markers Ki 67 and Cyclin D1 (Fig. R-6A,B). Cyclin D1 levels in these mice were equal to uninjected controls, whereas Cyclin D1 levels in *Gadd45b*$^{\Delta hep}$ were significantly increased ~~up to 4 folds~~ compared to *Gadd45b*$^{\Delta Kup}$ mice. As compensatory proliferation is mediated by IL-6, we analyzed IL-6 expression in these animals following DEN injection. (Fig. R-6C). IL-6 serum level was decreased in *Gadd45b*$^{\Delta Kup}$ compared to *Gadd45b*$^{\Delta hep}$ mice. Thus, specific deletion of *Gadd45b* in Kupffer cells impairs DEN induced compensatory proliferation by decreasing IL-6 production. As for DEN-induced apoptosis we have observed no differences between *Gadd45b*$^{\Delta Kup}$ compared to *Gadd45b*$^{\Delta hep}$ mice following DEN injection (Fig. R-6D).

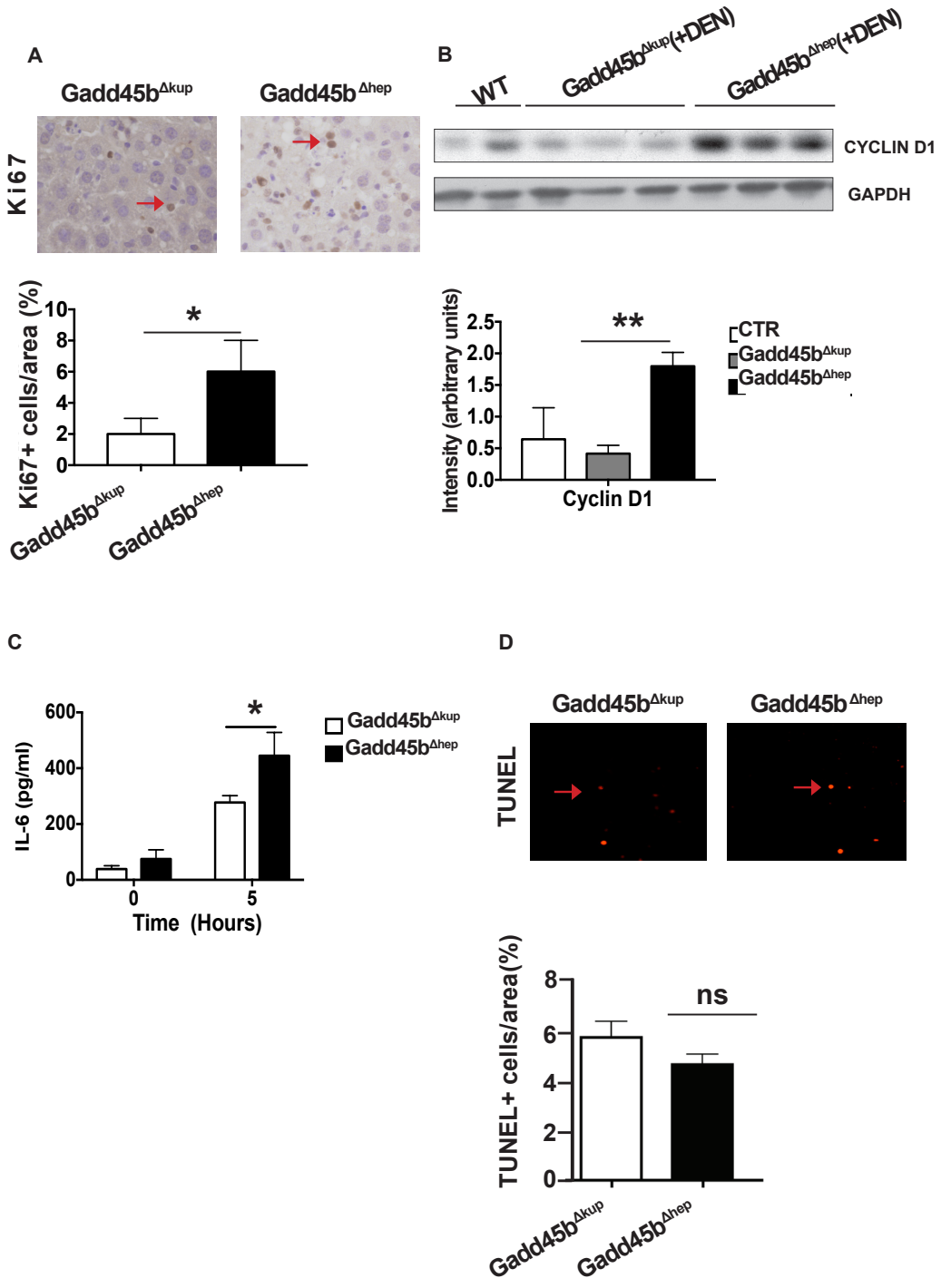
A
Gadd45b^Δkup
Gadd45b^Δhep
Ki67
Ki67+ cells/area (%)
10
8
6
4
2
0
*
Gadd45b^Δkup
Gadd45b^Δhep
B
WT
Gadd45b^Δkup(+DEN)
Gadd45b^Δhep(+DEN)
CYCLIN D1
GAPDH
Intensity (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
**
CTR
Gadd45b^Δkup
Gadd45b^Δhep
Cyclin D1
C
IL-6 (pg/ml)
600
400
200
0
*
Gadd45b^Δkup
Gadd45b^Δhep
0
5
Time (Hours)
D
Gadd45b^Δkup
Gadd45b^Δhep
TUNEL
TUNEL+ cells/area(%)
8
6
4
2
0
ns
Gadd45b^Δkup
Gadd45b^Δhep

Figure R-6 *Gadd45b* expression in Kupffer cells is required for efficient IL-6 production. Reconstitution of the bone marrow in recipients *Gadd45b*$^{+/+}$ mice with *Gadd45b*$^{-/-}$ donors (*Gadd45b*$^{\Delta Kup}$) and in recipients *Gadd45b*$^{-/-}$ mice with *Gadd45b*$^{+/+}$ donors (*Gadd45b*$^{\Delta hep}$). Mice were injected with DEN (100 mg/kg) for 48 hours: proliferative, apoptotic process, alongside with IL-6 secretion were assessed. (**A**) Top, Ki67 immunostaining of livers from *Gadd45b*$^{\Delta Kup}$ and *Gadd45b*$^{\Delta hep}$ mice, n=3 each group. Statistical analysis of Ki67 positive cells with 5 fields of view for each group, data represent mean ± S.E.M. (P<0,05). (**B**) Top, Analysis of Cyclin D1 expression in *Gadd45b*$^{\Delta Kup}$ and *Gadd45b*$^{\Delta hep}$ mice by Western blot after DEN injection. Uninjected WT mice were used as a control. GAPDH was used as a loading control. Bottom, densitometry analysis of Cyclin D1. Signal is normalized to GAPDH. Values are mean ± S.E.M., n=3 mice the treated groups, n=2 in the control group (P<0,01). (**C**) Blood was collected from *Gadd45b*$^{\Delta Kup}$ and *Gadd45b*$^{\Delta hep}$ mice at the indicated times after DEN injection and IL-6 cytokine secretion was analyzed by ELISA, n=3 each group, data are mean ± S.E.M. (P<0,05). (**D**) TUNEL stain of livers from *Gadd45b*$^{\Delta Kup}$ and *Gadd45b*$^{\Delta hep}$ mice, n=3 for each group. Statistical analysis of double positive cells with 5 fields of view for each group, data are mean ± S.E.M..

2.1 *Gadd45a* is required for DEN-induced hepatocyte death and compensatory proliferation

Gadd45a shares 55% of homology with Gadd45b but its role in liver homeostasis has not been as extensively characterized. *Gadd45a* is induced by a wide variety of cellular stresses, including UV-radiation, X-rays, hypoxia and oncogenic stress and various alkylating agents and disruption of its expression was observed in various malignancies (Tamura et al, 2012, Alexay et al, 2012). *Gadd45a*$^{-/-}$ mice show genomic instability, do not spontaneously develop tumors, but display increased sensitivity to induced carcinogenesis, such as DMBA-induced ovarian tumors confirming its pivotal role in maintaining genome integrity (Hollander et al, 1999, 2001). To date however, the role of *Gadd45a* in DEN-induced HCC development remains still unaddressed. We therefore examined whether this alkylating agent might affect Gadd45a expression in liver. We

found that *Gadd45a* expression was increased around 20 times after DEN injection (Fig. R-7A). To check the biological relevance of this phenomenon, and whether this increase plays a role during DEN-induced tumorigenesis, we analyzed the hepatocyte cell death and proliferative response in WT and *Gadd45a*$^{-/-}$ mice 48 hours after DEN injection. The lack of Gadd45a in mice caused a decreased hepatocyte cell death after DEN treatment, as confirmed by reduced positivity to TUNEL stain and lower Cleaved Caspase 3 signal in the Western blot analysis (Fig. R-7B). This decrease in hepatocyte death resulted in a decreased compensatory proliferation, as *Gadd45a*$^{-/-}$ mice showed reduced Ki67 signal and reduced expression of Cyclin D1 (Fig. R-7C). Thus, Gadd45a expression is required for DEN-induced hepatocyte death and compensatory proliferation. Both processes are connected by the release of different cytokines by necrotic cells. It is established that the release of IL-1α by necrotic hepatocytes upon DEN-stress activates Kupffer cells to produce IL-6 which in turn stimulates hepatocytes to exit the quiescent state and start the proliferative phase. We therefore examined the serum levels of inflammatory cytokines in WT and *Gadd45a*$^{-/-}$ mice. Our studies demonstrated that Gadd45a deficiency caused decreased levels of IL-1α and IL-6 after DEN injection. This result might indicate that the decreased hepatocyte cell death in *Gadd45a*$^{-/-}$ mice, upon DEN injury, resulted in a reduced production of IL-1α that in turn stimulates Kupffer cells to release IL-6. However, the reduced IL-6 serum level could also be the result of impaired IL-6 production pathways due to the lack of *Gadd45a* in Kupffer cells. Together, our data indicate that Gadd45a deficiency caused decreased cell death (Fig. R-7B), reduced hepatocyte cell proliferation (Fig. R-7C) and decreased levels of inflammatory cytokines such as IL-1α and IL-6 (Fig. R-7D).

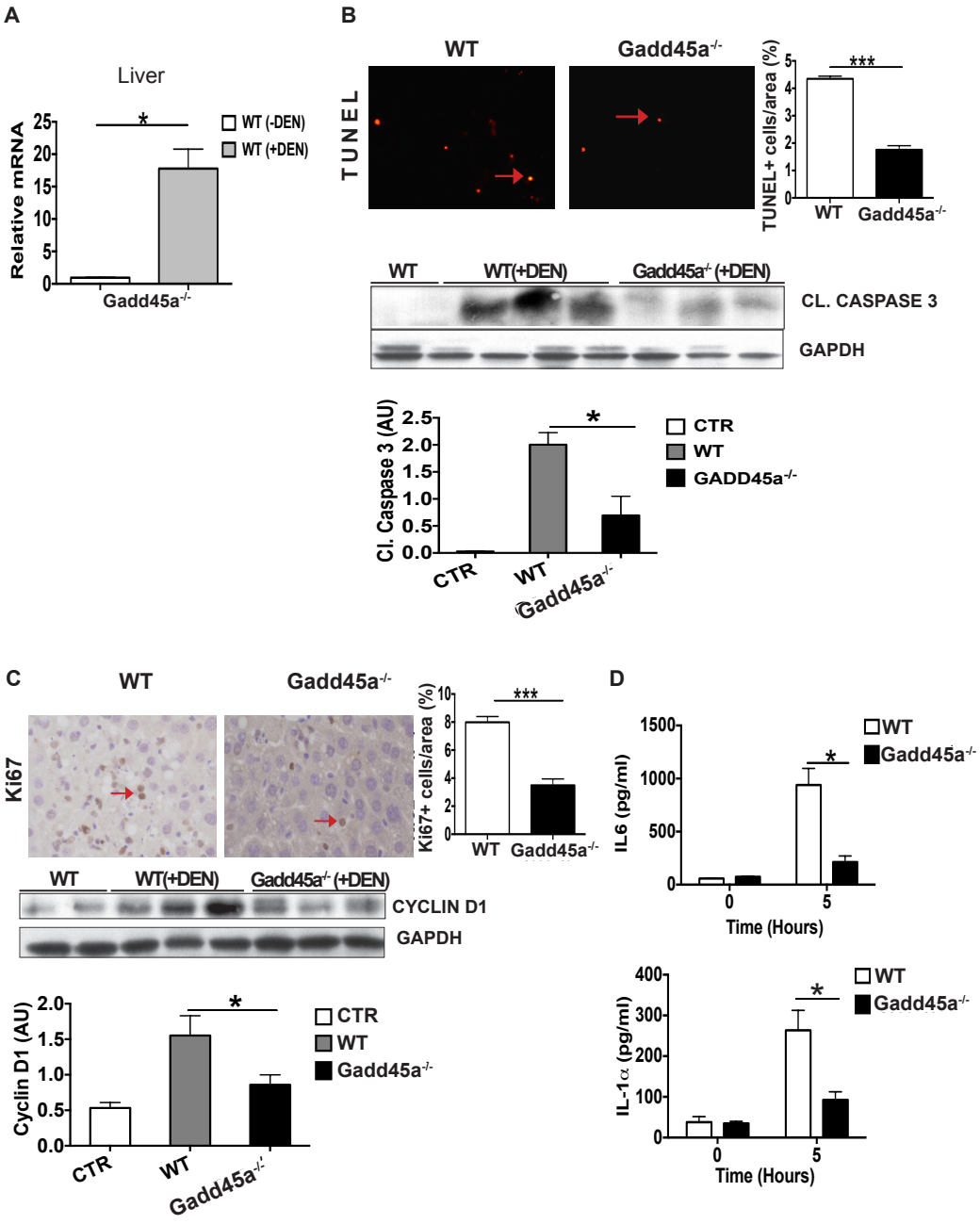
A
Liver
Relative mRNA
WT (-DEN)
WT (+DEN)
Gadd45a-/-
B
WT
Gadd45a-/-
TUNEL
TUNEL+ cells/area (%)
WT
Gadd45a-/-
WT
WT(+DEN)
Gadd45a-/- (+DEN)
CL. CASPASE 3
GAPDH
Cl. Caspase 3 (AU)
CTR
WT
GADD45a-/-
C
WT
Gadd45a-/-
Ki67
Ki67+ cells/area (%)
WT
Gadd45a-/-
WT
WT(+DEN)
Gadd45a-/- (+DEN)
CYCLIN D1
GAPDH
Cyclin D1 (AU)
CTR
WT
Gadd45a-/-
D
WT
Gadd45a-/-
IL6 (pg/ml)
Time (Hours)
WT
Gadd45a-/-
IL-1α (pg/ml)
Time (Hours)

Figure R-7. Effects of *Gadd45a* deficiency in hepatocyte cell death and compensatory proliferation. **A**) *Gadd45a* mRNA expression in the liver of untreated WT mice and 5 hours following DEN injection (100 mg/kg) was analyzed by quantitative RT-PCR. mRNA was normalized to *Gapdh*. Values are mean ± S.E.M., n=3 mice in each group (P<0,05). (**B**) Top, TUNEL stain of livers from WT and *Gadd45a*$^{-/-}$ mice 48 hours following 100 mg/kg DEN injection, n=3 each group. Statistical analysis of double positive cells with 5 fields of view for each group, data represent mean ± S.E.M. (P<0,001). Bottom, Caspase 3 cleavage in the same conditions as the top figure was analyzed by Western blot and GAPDH expression was used as a loading control. Densitometry analysis of Western blot signal is normalized to GAPDH. Values are mean ± S.E.M., n=3 in the treated groups, n=2 in the control group, (P<0,05). (**C**) Top, Ki67 immunostaining of livers from WT and *Gadd45a*$^{-/-}$ mice 48 hours following DEN injection, n=3 each group. Statistical analysis of Ki67 positive cells with 5 fields of view for each group, data are mean ± S.E.M. (P<0,001). Bottom, Expression of Cyclin D1 in the same samples was analyzed by Western blot and GAPDH expression was used as a loading control. Densitometry analysis of Western blot. Signal is normalized to GAPDH . Data represent mean ± S.E.M., n=3 in the treated groups, n=2 in the control group (P<0,05). (**D**) Blood was collected from both WT and *Gadd45a*$^{-/-}$ mice at 0 and 5 hours after DEN injection. IL-1α and IL-6 cytokine secretion was analyzed by ELISA, n=3 for the treated group, n=2 for the control group. Data represent mean ± S.E.M. (P<0,05).

2.2 Gadd45a promotes DEN-induced tumorigenesis

Gadd45a stimulates hepatocytes cell death and proliferation following DEN injection. The early induction of these processes is known to have an important impact in the development of HCC. To study whether *Gadd45a* deficient genotype is resistant to DEN-induced HCC, a cohort of 2 weeks old WT mice and *Gadd45a*$^{-/-}$ mice was injected with DEN (25 mg/kg) and mice were sacrificed after 9 months to assess tumor development. While all WT mice develop tumors, nearly 30% of *Gadd45a*$^{-/-}$ mice are tumor free 9 months after DEN injection (Fig. R-8A,B). When tumor positive animals in the two groups were compared, 45% of WT mice developed more than 10 tumors, in contrast this number was just 5% for the *Gadd45a*$^{-/-}$ mice. Moreover, 60% of *Gadd45a*$^{-/-}$ mice developed less than five tumors, whereas this number represented only 17% for the WT mice (Fig. R-8C). The liver to bodyweight ratio was smaller in *Gadd45a*$^{-/-}$ mice compared to WT, suggesting that *Gadd45a*$^{-/-}$ mice developed tumors with lower cellular mass (Fig. R-8D). Remarkably, HCC could be identified 40% of the WT mice and none of the *Gadd45a*$^{-/-}$. We therefore can say that after 9 months of DEN injection *Gadd45a*$^{-/-}$ mice were HCC free. These evidences point out to a new role for *Gadd45a* in the promotion of DEN-induced tumorigenesis.

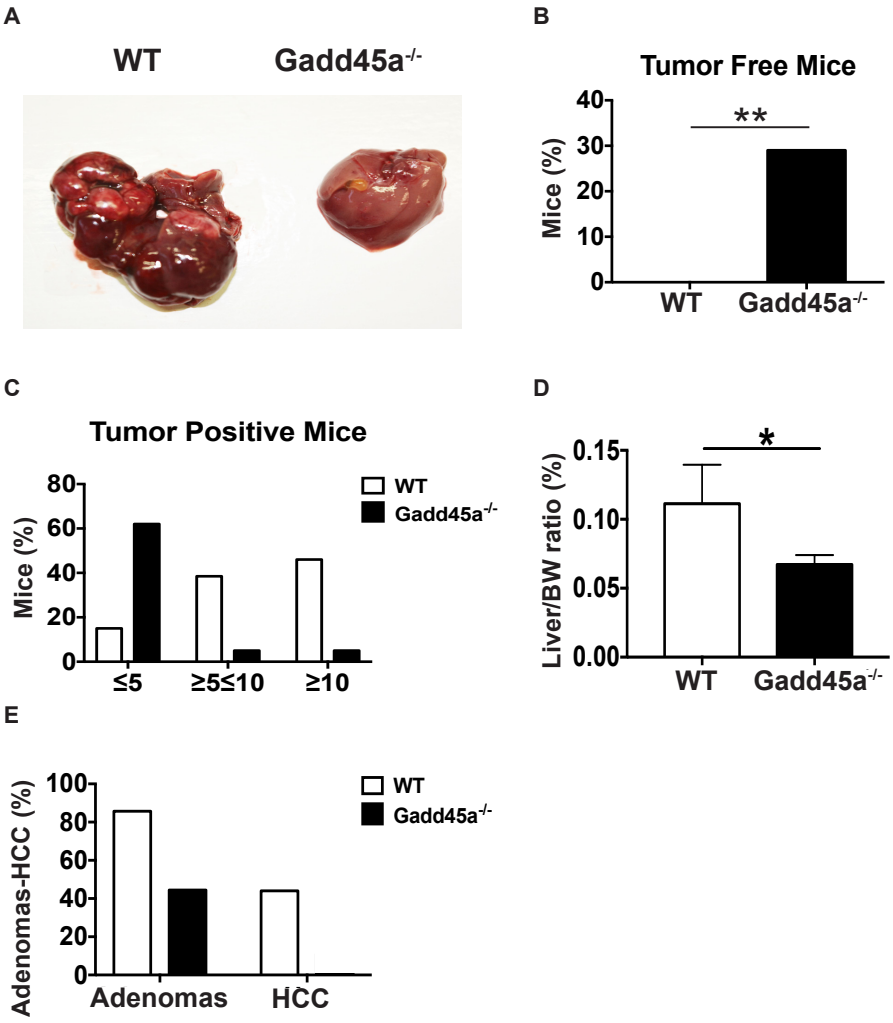

Figure R-8 *Gadd45a* promotes liver tumorigenesis. (**A**) Representative picture of DEN-induced liver tumors in WT and *Gadd45a*$^{-/-}$ mice. 2 weeks old WT (n=13) and *Gadd45a*$^{-/-}$ mice (n=22) were treated with a single injection of DEN (25 mg/kg) and livers were harvested after nine months. (**B**) Percentage of tumor-free mice at nine months. Statistically significant differences between WT and *Gadd45a*$^{-/-}$ mice are indicated (**) P<0,01. P value is from a Fisher´s exact test. (**C**) The percentage of tumor-positive mice after nine months. (**D**) The liver/body weight ratio was calculated and expressed as the mean percentage ± S.E.M. Differences between WT and *Gadd45a*$^{-/-}$ mice were examined for statistical significance using the Student´s test, (*) P<0,05. (**E**) The percentage of HCC and adenomas nine months after DEN injection in WT (n=7) and *Gadd45a*$^{-/-}$ mice (n=8).

2.3 *Gadd45a* stimulates DEN-induced JNK phosphorylation and NF-κB activity

As discussed above, ablation of JNK and NF-κB have been linked to a decrease in DEN-induced tumorigenesis. Moreover, both JNK and NF-κB activity are decreased in *Gadd45b*$^{-/-}$ mice after DEN injection. Given the homology with GADD45B and the similar phenotype observed upon DEN injection, we reasoned that *Gadd45a* might also be involved in JNK regulation. Indeed, after 48 hours DEN injection, both JNK phosphorylation and NF-κB activity (confirmed by a decrease in IκB⟨ phosphorylation) were decreased (Fig. R-9). This suggests that *Gadd45a* and *Gadd45b* might cooperate in a synergistic manner to promote DEN-induced tumorigenesis.

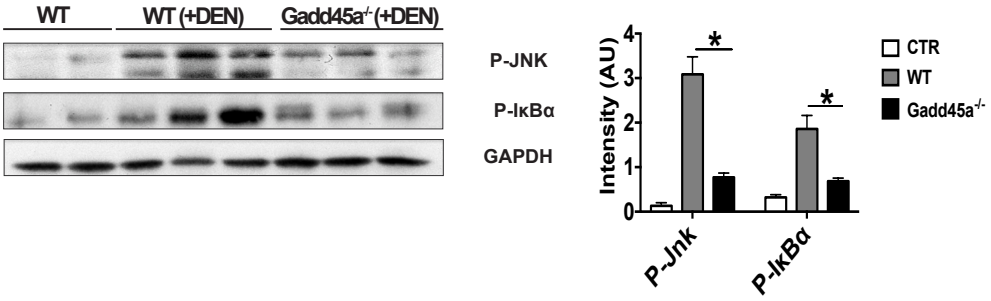

Figure R-9. ***Gadd45a* is required for DEN-induced JNK phosphorylation**. WT and *Gadd45a*$^{-/-}$ mice were injected with DEN (100 mg/kg). Phosphorylation of JNK and IκBα, along wιth total JNK expression were assessed by Western blot analysis. Uninjected WT mice were used as a control. Densitometry analysis of proteins is normalized to GAPDH . Data represent mean ± S.E.M., n=3 mice for the treated groups, n=2 for the control group, (P<0,05).

DISCUSSION

Role of *Gadd45a* and *Gadd45b* in DEN-induced HCC

Gadd45 proteins play a key role in the regulation of cell survival, apoptosis, proliferation, and maintenance of genomic stability in response to physiological and genotoxic stress, including oncogenic stress (Hoffman and Liebermann, 2009). In the current study, we evaluated the function of *Gadd45b* and *Gadd45a* in liver tumorigenesis, by using the DEN model, which represents a well-characterized method of HCC induction. Our results indicate that both *Gadd45b* and *Gadd45a* mediate DEN-induced hepatocyte death, proliferation and carcinogenesis.

Over the last decade there are increasing evidences that *Gadd45* family can play a role as either tumor suppressor or tumor promotor genes depending on the tissue, cell type or transforming events. Mice with *Gadd45a* deletion show genomic instability (Hollander et al, 1999), increased sensitivity to carcinogenesis and are more prone to DMBA induced ovarian tumors (Hollander et al, 2001). *Gadd45a* suppresses Ras-driven tumorigenesis in breast cancer model by relying on p38-dependent senescence and jnk-dependent apoptosis. Furthermore, deletion of *Gadd45a* in a xeroderma pigmentosum, complementation group C (XPC) null model increases tumor malignancy (Hollander et al 2005) and overexpression of H-ras coupled with *Gadd45a* knockdown is sufficient to transform cells (Bulavin et al, 2003). In addition, *Gadd45b*$^{-/-}$ mice are more susceptible to ionizing radiation, chemical carcinogens, and display an impaired immune response to implanted melanoma cells (Ju et al, 2009). Although it has been reported reduced expression of *Gadd45a* and *Gadd45b* in several tumors and tumor cell lines, mutations in these genes are occasional in cancer. Usually, reduced expression of *Gadd45a* and *Gadd45b* in several types of human cancer correlates with promoter methylation. For instance, *Gadd45a* promoter is hypermethylated in breast cancer when compared with normal breast epithelium (Wang et al, 2005). In prostate cancer, *Gadd45a* promoter is hypermethylated and *Gadd45a* expression is downregulated (Ramachandran et al, 2009). Disruption of *Gadd45a* expression also was found in several hematopoietic malignancies (Qiu et al, 2003; Sun et al, 2003; Ying et al, 2005). However, despite their association with tumor suppression in many contexts, Gadd45 proteins can also exert tumor-promotion functions. *Gadd45a* expression is elevated in several pancreatic ductal

adenocarcinoma (PDA) cell lines and loss of expression reduced growth and survival of cell lines in culture (Schneider et al, 2006). *Gadd45a* expression was elevated in 54% of human pancreatic ductal carcinomas (Yamasawa et al, 2002) and overexpression of *Gadd45a* and p53 loss of function also contributed to poor prognosis compared with patients with low levels of *Gadd45a* (Yamasawa et al, 2002). In addition, it has been reported high levels of *Gadd45a* in many cancer cell lines including breast (HS578T), lung (NCI-H226), lymphoid (K562), prostate (PC3) and renal (TK10) cell lines (Hildesheim and Fornace, 2002).

Regarding liver carcinogenesis, the role of *Gadd45a* and *Gadd45b* has not been studied in detail until now. It has been reported that *Gadd45b* and *Gadd45a* promoters are both methylated in HCC. Downregulation of *Gadd45b* expression is correlated with hypermethylation of the *Gadd45b* promoter and this is associated with hepatitis C virus (HCV)-associated hepatocellular carcinomas (Higgs et al, 2010), suggesting a tumor-suppressive role for both proteins in HCC.

Notably, we found that *Gadd45b*$^{-/-}$ and *Gadd45a*$^{-/-}$ mice display a substantial decrease in tumors number compared to WT mice, nine months after DEN injection. Similarly in both groups, 30% of mice are tumor free whereas virtually all WT mice develop tumors. Even when the groups of *Gadd45b*$^{-/-}$ and *Gadd45a*$^{-/-}$ mice that develop tumors are compared, the tumors number is substantially lower than the WT mice. Remarkably, when tumors where analyzed histologically, HCC was found in 40% of WT mice, but not a single HCC could be found in *Gadd45b*$^{-/-}$ or *Gadd45*a$^{-/-}$ mice. The tumors identified in both *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ mice were only adenomas. Therefore, our study provides the first direct evidences highlighting a novel role of both *Gadd45b* and *Gadd45a* as oncogenes during DEN-induced liver stress.

Given the methylation status and the reduced expression of *Gadd45a* and *Gadd45b* in human HCC, this result might seem surprising. Our data indicates that Gadd45 proteins have a specific role as oncogenes when liver cancer is induced by an alkylating agent, but we can not exclude different roles when liver tumorigenesis is induced by stress factors caused by human lifestyle. Using a mouse model of breast carcinogenesis it has been found that *Gadd45a* can act as either tumor promoter or tumor suppressor, depending on whether tumors are induced by overexpressing c-MYC or RAS respectively (Tront et al,

2006, 2010). Lack of *Gadd45a* accelerated the onset of RAS-driven mammary tumor formation. However, in the mouse mammary tumor virus (MMTV)-Myc mouse model of breast carcinogenesis, loss of *Gadd45a* decelerated the onset of breast tumor formation (Tront et al, 2010).

Role of *Gadd45b* and *Gadd45a* in DEN-induced apoptosis

In liver *Gadd45b* has been associated with an antiapoptotic function with various sources of liver stress. PB produces liver tumors in mice by activating CAR, which in turn represses cell death by interacting with GADD45B and repressing MKK7-mediated phosphorylation of JNK1 (Yamamoto et al, 2010). Also *Gadd45b* blocks liver cell death after liver stress induced by PH (Papa et al, 2008). In our study, we noticed that expression of both *Gadd45b* and *Gadd45a* was increased upon DEN treatment. Carcinogen induced cell death was significantly decreased in both groups compared to WT, as observed by the reduced signal for Cleaved Caspase 3 and reduced positivity to TUNEL staining. Also, in the case of *Gadd45b*, primary hepatocytes show reduced cell death after TNF-α and TGF-β treatment. These findings highlight a new role for *Gadd45b* and *Gadd45a* as genes exerting a proapoptotic function in DEN-induced tumorigenesis. In light of what has been discussed above, the proapoptotic function of *Gadd45b* might seem surprising. However, it is worth noticing that both PH and PB induced liver stress in a DNA-damage independent manner and, therefore, rely on the extrinsic apoptotic pathway. DEN instead, induces cell death in a DNA-damage dependent fashion. Therefore, the most likely explanation is that *Gadd45b* carries a proapoptotic or antiapoptotic function according to whether cell death is induced by the intrinsic or extrinsic pathway respectively. Interaction with proteins differentially induced in one setting or the other might be responsible for the switch.

Role of *Gadd45a* and *Gadd45b* in DEN-induced compensatory proliferation

Whereas the role of *Gadd45a* in liver proliferation has not been established, various evidences point out to *Gadd45b* as a critical factor, enhancing or suppressing liver cellular proliferation in a stimulus-dependent manner. For instance, *Gadd45b*$^{-/-}$ mice display reduced liver proliferation following PH, but a slight increase when liver proliferation is induced by treatment with TCPOBOP (Tian et al, 2011). Is important to notice that proliferation after PH happens following removal of 3/4 of the liver, whereas TCPOBOP is a mitogenic factor which makes hepatocytes enter cell cycle without any prior liver insult (Tian, 2011). Hence, the pathways stimulating cellular proliferation in one context or in another are very different. Indeed, whereas proliferation after PH is JNK dependent (Papa et al 2008), proliferation after TCPOBOP does not depend on JNK. In our model, after 48 hours DEN injection, proliferation was reduced in both *Gadd45b*$^{-/-}$ and *Gadd45a*$^{-/-}$ genotypes confirmed by reduced Ki67 immunostaining and Cyclin D1 signal. This is consistent with the observed inhibition of DEN induced cell death. To our knowledge, our results show the first evidence that *Gadd45a* is a positive regulator of hepatocyte proliferation *in vivo*. Our finding that *Gadd45b* is required for liver proliferation is in line with the same observed role after PH, and is in agreement with the phenotype observed in JNK1$^{-/-}$ mice after DEN injection (Sakurai et al, 2006). Indeed, JNK1$^{-/-}$ mice display reduced cellular proliferation upon DEN challenge confirming JNK as a positive regulator of hepatocyte proliferation.

Role of *Gadd45a* and *Gadd45b* in IL-6 production

IL-1α and IL-6 are two paramount cytokines that connect DEN-induced cell death with compensatory proliferation. Upon DEN injection, necrotic hepatocytes release IL-1α, which in turn activates Kupffer cells to secrete IL-6. IL-6 has been associated with a tumor-promoting role in many tumors and elevated IL-6 levels usually indicates poor prognosis (Heikkila et al, 2008). IL-6 stimulates hepatocytes to reenter cell cycle. IL-1α null receptor and *IL6*$^{-/-}$ mice show indeed a resistance to DEN-induced tumorigenesis (Sakurai et al, 2008; Naugler, 2007). Importantly specific deletion of IL-6 gene in

Kupffer cells suppresses the initiation of DEN-induced tumorigenesis and impairs the recruitment of cells to the damage site (Kong et al, 2016). Given the lower hepatocyte cell death and compensatory proliferation observed in *Gadd45b*$^{-/-}$ and *Gadd45a*$^{-/-}$ mice after DEN injection, we investigated if these genes play a role in the regulation of these cytokines. Whereas IL-1α secretion was decreased in the *Gadd45a*$^{-/-}$ mice upon DEN injection, only a statistical trend of decrease could be observed for *Gadd45b*$^{-/-}$ null mice. We noticed however, that IL-6 production was decreased in both *Gadd45b*$^{-/-}$ and *Gadd45a*$^{-/-.}$ Also, reduced phosphorylation of STAT3 was observed in *Gadd45b*$^{-/-}$ mice, further confirming decreased IL-6 signaling in these mice. *Gadd45b*$^{-/-}$ mice showed reduced cellular infiltration in terms of neutrophils and macrophages when compared to WT mice, a phenotype consistent with the role of IL-6 in promoting immune cell infiltration.

This observation indicates that reduced compensatory proliferation observed in both genotypes after DEN injection can be linked to decrease in IL-6 production, constituting the first evidence of *Gadd45a* and *Gadd45b* promoting IL-6 expression in liver after DEN stress.

Role of *Gadd45b* and *Gadd45a* in JNK activation

JNK is a key protein involved in DEN-induced tumor development. Initial studies showed that JNK1$^{-/-}$ mice are resistant to DEN-induced tumorigenesis and this resistance was associated with reduced expression of Cyclin D1 and the protumorigenic cytokine IL-6 (Sakurai et al, 2006). Later studies showed that hepatocyte-specific JNK$^{-/-}$ mice are instead more susceptible to DEN-induced HCC. In order to recover the phenotype observed in whole body JNK$^{-/-}$ mice, deletion of JNK in myeloid cells was necessary. Mice carrying compound deletion of JNK in hepatocytes and nonparenchymal cells were indeed resistant to DEN-induced HCC and display impaired IL-6 secretion (Das et al, 2011), and mice carrying JNK deletion specifically in myeloid cells were resistant to DEN-induced HCC (Han et al, 2016). These findings imply a crucial role of JNK in Kupffer cells-mediated IL-6 production. Given the reduced IL-6 observed in *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ mice upon DEN injection and the conserved interaction of Gadd45

proteins with MAP kinase cascade, we examined if JNK is decreased in the liver of *Gadd45a* and *Gadd45b* deficient animals following DEN injection.

In our model, we have observed decreased JNK phosphorylation, in both *Gadd45b*$^{-/-}$ and *Gadd45a*$^{-/-}$ mice 48 hours upon DEN injection when compared to WT mice. We therefore examined the phosphorylation of the upstream activators of JNK, MKK7 and MKK4. In *Gadd45b*$^{-/-}$ mice, MKK7 activation did not vary between both genotypes and the WT, whereas we could observe no MKK4 activation at 48 hours. This might imply that *Gadd45b* stimulates JNK phosphorylation through an alternative pathway. Indeed we have also found a decrease in the NF-κB signaling in both *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$, but we currently do not know if this difference can be linked to the observed decrease in JNK phosphorylation. Another possibility is that MKK7 and MKK4 may activate JNK at earlier time points. The activation of these two kinases at various points after DEN injection is currently being investigated in our lab. In addition to the canonical MAPKK activation, JNK activation is increased by stress-induced ROS release (Kamata et al, 2005). ROS indeed can inactivate JNK-specific phosphatases. Therefore, decrease in the JNK signaling can be due to a decrease in ROS formation after challenge with DEN injection. This however, at least in the *Gadd45b*$^{-/-}$ animals, appears not to be the case, as no differences in ROS accumulation can be observed in WT and *Gadd45b*$^{-/-}$ mice following DEN injection. As mentioned above, JNK deletion in Kupffer cells is necessary to downregulate IL-6 production as mice carrying hepatocyte-specific JNK deletion increase IL-6 production and develop more HCC (Das et al, 2011). We therefore addressed whether expression of *Gadd45b* specifically in Kupffer cells might restore IL-6 level and subsequent compensatory proliferation. Thanks to bone marrow transplant, we generated mice carrying *Gadd45b*-specific deletion in monocytes/Kupffer cells *Gadd45b*$^{\Delta kup}$ or in hepatocytes *Gadd45b*$^{\Delta hep}$. IL-6 production is much higher when *Gadd45b* expression is restored in Kupffer cells compared to hepatocytes, indicating that *Gadd45b* expression in Kupffer cells is necessary to increase IL-6 production. This increase in IL-6 expression in turn, is responsible for the increased proliferation observed in the livers of *Gadd45*$^{\Delta hep}$ animals following DEN injection. At 48 hours, *Gadd45b*$^{\Delta kup}$ animals showed an amount of Cyclin D1 similar to uninjected WT controls, indicating

that compensatory proliferation is minimal or absent. Thus, our model points out to *Gadd45b* as a key regulator of IL-6 expression in Kupffer cells. IL-6 is critical in carcinogen mediated liver cancer development, promoting cancer cell proliferation and inhibiting the apoptosis of cancer cells by activation of STAT3. *Gadd45b*-mediated IL-6 expression in Kupffer cells may be essential for DEN induced carcinogenesis. Our data indicates that Gadd45b is an upstream activator of JNK which is required for IL-6 dependent STAT3 phosphorylation. Our results are consistent with a protumorigenic role for *Gadd45b* in liver cancer by increasing JNK activity in Kupffer cells leading to IL-6 dependent STAT3 activation. To analyze in more detail how Gadd45b regulate JNK activation, immunohistochemical analysis of JNK phosphorylation in Kupffer cells from $Gadd45b^{\Delta kup}$ or $Gadd45b^{\Delta hep}$ is currently ongoing.

Concluding remarks

In summary our data constitute the first direct evidence of Gadd45a and Gadd45b as genes promoting DEN-induced HCC by enhancing DEN-induced cell death and compensatory proliferation. After DEN treatment, Gadd45a and Gadd45b induce JNK activation in hepatocytes, triggering cell death and promoting IL-1**α** release. Upon binding of IL-1**α** to its receptor, Gadd45b stimulates IL-6 production in Kupffer cells, and we propose that it carries out this function by activating JNK. We are currently investigating if Gadd45a realizes a similar function in Kupffer cells by generating $Gadd45a^{\Delta kup}$ and $Gadd45a^{\Delta hep}$.Once secreted by Kupffer cells, IL-6 binds to its receptor on hepatocytes and promotes hepatocytes proliferation, an essential process required for DEN-induced tumorigenesis (Figure D-1).

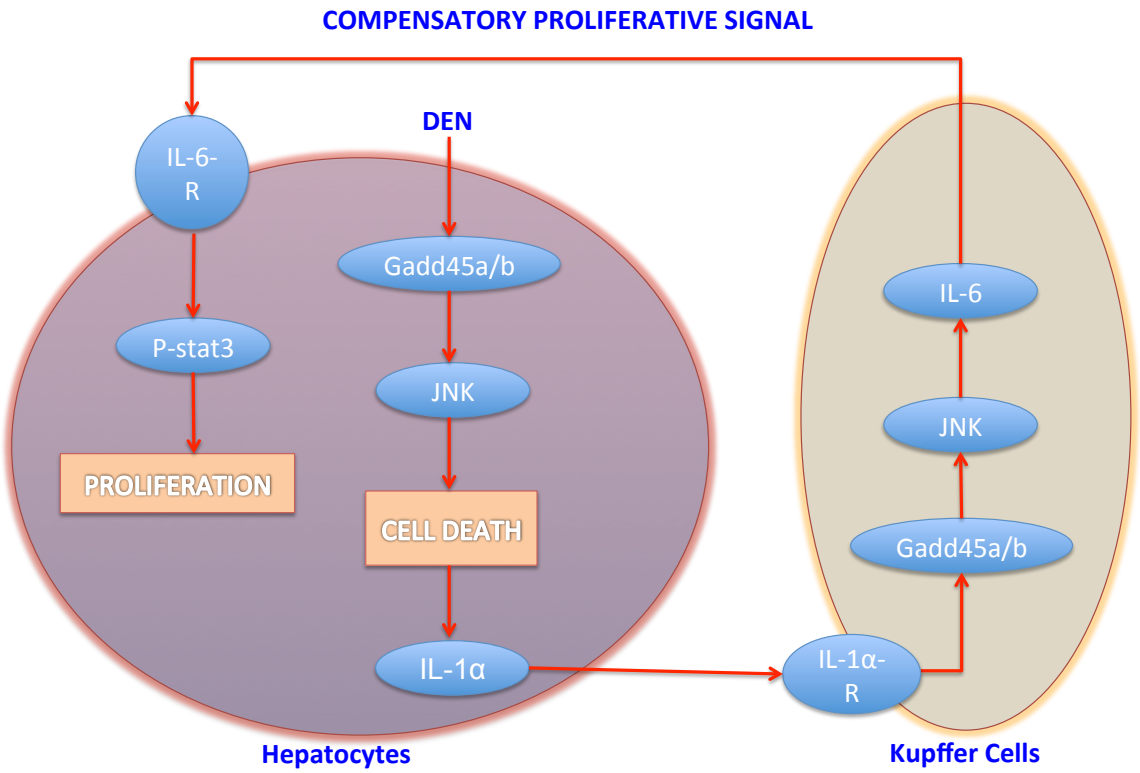

Figure D1. Model illustrating *Gadd45b* role in promoting compensatory proliferation after DEN-Injection by stimulating JNK activation.

CONCLUSIONS

1) *Gadd45b* and *Gadd45a* exert a protumorigenic role during DEN-induced HCC. Lack of *Gadd45b* and *Gadd45a* suppress the initiation of hepatocellular carcinoma in mice.

2) *Gadd45b* and *Gadd45a* promote hepatocyte cell death in response to DEN treatment. TUNEL assays and cleaved caspase 3 expression revealed a significant decrease in hepatocyte death in livers of DEN-treated Gadd45b- and Gadd45a-null mice compared to WT mice.

3) *Gadd45b* and *Gadd45a* are required for efficient compensatory proliferation following DEN injection, as the absence of each gene inhibits Cyclin D1 and Ki67 expression.

4) *Gadd45b* controls DEN-induced hepatocyte proliferation by upregulating IL-6 expression in Kupffer cells.

5) *Gadd45b* promotes recruitment of macrophages and neutrophils to the liver after DEN injection.

6) *Gadd45b* and *Gadd45a* are necessary for DEN-induced JNK activation and IL-6 signaling. Loss of Gadd45b attenuates both JNK activation in liver and IL-6 production by Kupffer cells and this may be crucial to suppress liver cancer development.

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