Pathomechanisms in hepatocellular carcinoma: characterisation of leukocyte recruitment, the role of the mRNA-binding protein tristetraprolin, and nuclear paraspeckles

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„IACTA ALEA EST.“

(CAIVS IVLIVS CAESAR)

Contents

Abbreviations

°C	degree Celsius
µ	micro (10^{-6})
3′-UTR	3′-untranslated region
7-AAD	7-Aminoactinomycin D
A	area
a	atto (10^{-18})
ACTB	beta-actin gene (human)
Alb	albumin gene (murine)
ALT	alanine transaminase
APC	allophycocyanin
Apoa4	apolipoprotein A-IV gene (murine)
ARE	AU-rich elements
ARE-BP	AU-rich elements binding protein
AST	aspartate transaminase
b	bases
BCL2	B-cell lymphoma 2 gene (human)
BCLC	Barcelona Clinic Liver Cancer
bp	base pair
BSA	bovine serum albumin
BV	brilliant violet
C	fatty acid chain length (e.g. C16 = stearic acid)
C57BL/6	C57 black 6
cDNA	complementary DNA
Clec4f	C-type lectin domain family 4, member F gene (murine)
CO_2	carbon dioxide
Cpt1a	palmitoyltransferase 1 gene (murine)

CPT1A	palmitoyltransferase 1 protein (human)
Csnk2a2	casein kinase II subunit alpha gene (murine)
Cx:y	ratio of total length to number of double bonds in fatty acids (e.g. C10:2 = a fatty acid with the length of ten carbon atoms and two double bonds)
Cxcl1	chemokine (C-X-C Motif) ligand 1 gene (murine)
CXCL1	chemokine (C-X-C Motif) ligand 1 gene (human)
CXCL1	chemokine (C-X-C Motif) ligand 1 protein (human)
CYP	cytochrome P450
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DEN	diethylnitrosamine
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
E	exponent (scientific notation)
E2F1	transcription factor E2F1 gene (human)
EDTA	ethylenediamine-N,N,N,N'-tetra acid
ELAVL1	ELAV-like protein 1 gene (human) = human antigen R gene
Elovl6	elongation of very long chain fatty acids protein 6 gene (murine)
EpCAM	epithelial cell adhesion molecule
EPD	The Eukaryotic Promoter Database
Fabp1	fatty acid binding protein 1 gene (murine)
FACS	fluorescence-activated cell sorting
FAME	fatty acid methyl ester
Fasn	fatty acid synthase gene (murine)
Fc	fragment crystallisable region
FCB	flow cytometry buffer
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	forward-scattered light
g	gram
g	gravitational force
GC-MS	gas chromatography-mass spectrometry
GSE	Gene Expression Omnibus series
H	height
h	hour
H&E	haematoxylin and eosin
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDAC	histone deacetylase protein (human)
HDL	high density lipoprotein
HuR	human antigen R gene (human)
HuR	human antigen R protein (human)
IC50	half maximal inhibitory concentration
IGF	insulin-like growth factor
IGF2BP1	insulin-like growth factor 2 mRNA-binding protein 1 gene (human)
IGF2BP3	insulin-like growth factor 2 mRNA-binding protein 3 gene (human)
k	kilo
l	litre
LDL	low density protein
Ly6	leukocyte antigen-6
m	metre or milli (10^{-3})
M	molar
MACS	magnetic cell separation

min	minute
MKI67	Ki-67 protein (human)
Mlxipl	MLX-interacting protein-like gene (murine)
mRNA	messenger ribonucleic acid
MS	magnetic separation
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MYC	c-Myc gene (human)
n	nano (10^{-9})
N	nitrogen
n	size of a statistical sample or number of experiments
NaCl	sodium chloride
NAFLD	non-alcoholic fatty liver disease
NaN_3	sodium azide
NASH	non-alcoholic steatohepatitis
Neat1	nuclear paraspeckle assembly transcript 1 (murine)
NEAT1	nuclear paraspeckle assembly transcript 1 (human)
NONO	non-POU domain containing octamer binding protein gene (human)
Nr1h3	nuclear receptor subfamily 1 group H member 3 gene (murine)
p	probability (value)
PBS	phosphate buffered saline
PE	phycoerythrin
Ppara	peroxisome proliferator-activated receptor alpha gene (murine)
PSPC1	paraspeckle component 1 gene (human)
PSPC1	paraspeckle component 1 protein (human)
qPCR	real-time quantitative polymerase chain reaction
RBM14	RNA-binding motif protein 14 gene (human)
RBP	mRNA-binding protein
RNA	ribonucleic acid

ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
Scd1	stearoyl-CoA desaturase 1 gene (murine)
SEM	standard error of mean
seq	sequencing
Srebf1	sterol regulatory element-binding transcription factor 1 gene (murine)
Srebf2	sterol regulatory element-binding transcription factor 2 gene (murine)
SSC	side-scattered light
TBARS	thiobarbituric acid reactive substances
TCGA	The Cancer Genome Atlas
TG	triglycerides
TLC	thin-layer chromatography
Tnf	tumor necrosis factor alpha gene (murine)
TNF-a	tumour necrosis factor (alpha) protein (human)
TPM	transcripts per million
Tris	α,α,α-tris-(hydroxymethyl)-methylamine
TTP	tristetraprolin gene (human)
Ttp	tristetraprolin gene (murine)
TTP	tristetraprolin protein (human)
Ttp	tristetraprolin protein (murine)
v	variant
v/v	volume per volume
VEGFA	vascular endothelial growth factor A gene (human)
w/v	weight per volume
x	fold
XIAP	X-linked inhibitor of apoptosis protein gene (human)
ZFP36	zinc finger protein 36 homolog gene (human) = tristetraprolin gene

Abstract

Hepatocellular carcinoma (HCC) represents the second most common cause of cancer-related death worldwide, not least due to late diagnosis and insufficient systemic treatment options. Since HCC is linked to inflammation, one aim of this study was to characterise inflammatory events in early hepatocarcinogenesis. The data revealed a dramatic monocyte recruitment and altered lipid composition upon treatment with the carcinogen diethylnitrosamine (DEN), which is one of the most frequently used agents in mouse models inducing HCC. Further aims were to decipher actions of the mRNA-binding protein tristetraprolin (TTP, *ZFP36*) and of the long non-coding RNA nuclear paraspeckle assembly transcript 1 (*NEAT1*) in liver cancer. In human gene expression data sets comparing HCC to normal liver tissue, *TTP* was downregulated, whereas *NEAT1* was upregulated. The experimental data suggest that hepatocytic *Ttp* promotes tumour formation, but that TTP inhibits tumour progression, i.e. migration, proliferation, and oncogene expression in human hepatoma cells. Chemoresistance induced *NEAT1* and the formation of paraspeckles and transcripts encoding paraspeckle proteins are strongly predictive for a poor prognosis in human HCC. Taken together, this work provides evidence for TTP, *NEAT1*, and paraspeckles being new interesting targets for therapeutic options in HCC.

Zusammenfassung

Das Hepatozelluläre Karzinom (HCC) ist weltweit die zweithäufigste Ursache für krebsbedingten Tod, auch aufgrund einer späten Diagnose und unzureichender systemischer Behandlungsmöglichkeiten. Da Entzündung im HCC eine wichtige Rolle spielt, war ein Ziel dieser Studie, entzündliche Vorgänge in der frühen Hepatokarzinogenese zu charakterisieren. Die Daten zeigten eine starke Monozytenrekrutierung und veränderte Lipidzusammensetzung bei Behandlung mit dem Karzinogen Diethylnitrosamin (DEN), einem der am häufigsten verwendeten Karzionogenen in Mausmodellen, die HCC induzieren. Weitere Ziele waren die Aufklärung der Effekte des mRNA-bindenden Proteins Tristetraprolin (TTP, *ZFP36*) und der langen nicht-kodierenden RNA nuclear paraspeckle assembly transcript 1 (*NEAT1*) bei Leberkrebs. In menschlichen Genexpressionsdatensätzen, die HCC mit normalem Lebergewebe vergleichen, wurde *TTP* herunterreguliert und *NEAT1* hochreguliert. Die experimentellen Daten legen nahe, dass hepatozytisches Ttp die Tumorbildung fördert, wohingegen TTP die Tumorprogression, d. h. Migration, Proliferation und Onkogenexpression, in humanen Hepatomzellen hemmt. Die Chemoresistenz-induzierte Expression von *NEAT1*, Paraspeckles und Transkripten, die für Paraspeckle-Proteine kodieren, sind für eine schlechte Prognose beim menschlichen HCC stark prädiktiv. Zusammengefasst liefert diese Arbeit Hinweise darauf, dass TTP, *NEAT1* und Paraspeckles neue interessante Angriffspunkte für Therapieoptionen bei HCC darstellen.

1 Introduction

1.1 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the predominant form of liver cancer and the second most common cause of cancer-related death worldwide (Figure 1; Bruix et al., 2015; Tang et al., 2017). Interestingly, more men than women are affected by HCC (Tang et al., 2017), which may be caused by an elevation of hepatic inflammatory events in men (Naugler et al., 2007). As a very heterogeneous and complex tumour, HCC is characterised by multiple genetic aberrations (Umeda et al., 2017). The majority of patients are at initial diagnosis in later stages according to the Barcelona Clinic Liver Cancer (BCLC) classification (Wörns et al., 2009). This late diagnosis is one of the reasons why chemoresistance, which is linked to poor prognosis, is abundant in HCC and results in poor response rates to systemic drug treatment (Cheng et al., 2016; Jiao and Nan, 2012; Wörns et al., 2009; Yau et al., 2008).

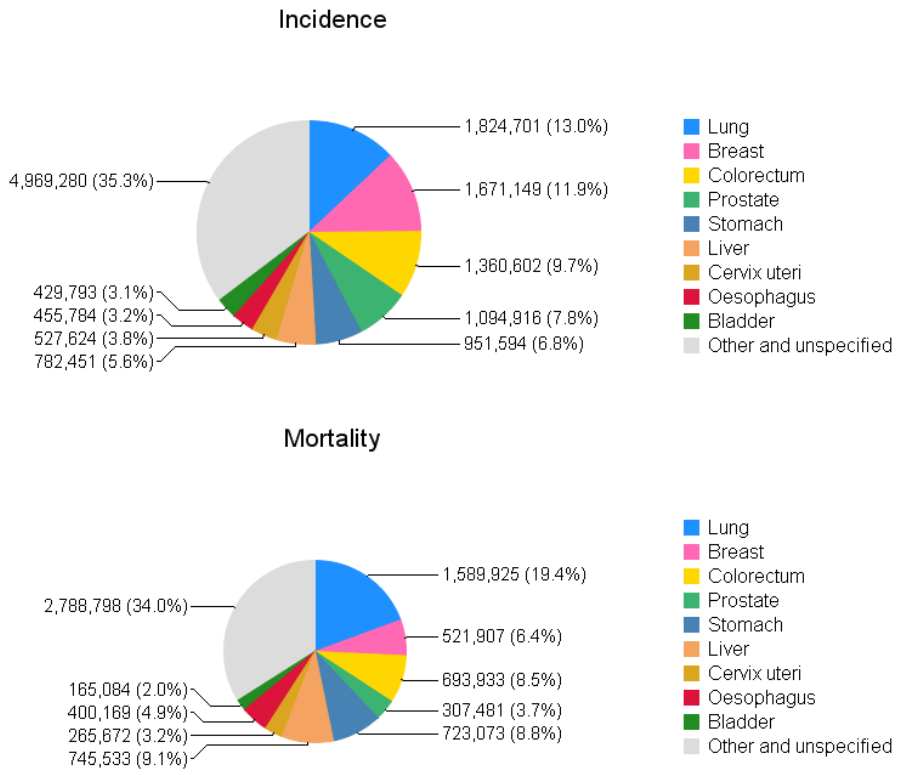


Figure 1: Incidence and mortality of liver cancer (Ferlay et al., 2013)

1.1.1 Inflammation in hepatocarcinogenesis

Many factors, such as infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), carcinogens, diabetes, obesity, non-alcoholic fatty liver disease (NAFLD), alcohol abuse, and cirrhosis induce liver injury. If untreated, these injuries further transform to chronic liver damage, which contributes to hepatic chronic inflammation. In order to restore liver function, compensatory proliferation occurs (Sun and Karin, 2013). However, excessive compensatory proliferation can induce severe pathological changes including fibrosis, cirrhosis, and hepatocarcinogenesis (Sun and Karin, 2013). In addition, it is assumed that chronic inflammation promotes tumour progression (Balkwill and Mantovani, 2001; Nakamoto et al., 1998).

1.1.2 Obesity in hepatocarcinogenesis

Obesity seems to be one important risk factor for the development of HCC (Marengo 2016). In obesity, adipocytes begin to swell due to an overload with triglycerides (TG). Eventually, they are stressed and can dedifferentiate, which results in the release of free fatty acids from TG by lipolysis and the reduced expression of adiponectin (Zimmermann et al., 2009; Larter et al., 2010). This subsequent lipid accumulation in the adipose tissue supports the development of insulin resistance. The mix of hyperinsulinemia and hyperglycemia further promotes hepatic synthesis of fatty acids and contribute to even higher hepatic TG levels (Reeves et al., 2016). In addition, the accumulation of free fatty acids in the liver may induce inflammation by increasing the levels of several cytokines such as tumour necrosis factor alpha (TNF-a) (Arkan et al., 2005). Therefore, obesity causes insulin resistance mediated prolonged cell survival on the one hand and fatty acid mediated continuous generation of cytokines and reactive oxygen species (ROS) inside the liver on the other hand. These changes are likely to contribute to or even to promote hepatocarcinogenesis (Reeves et al., 2016).

1.1.3 Chemical hepatocarcinogenesis

Carcinogenesis may be induced by chemical compounds found in everday products. The N-nitroso compound diethylnitrosamine (DEN) for instance can be found in beer and whiskey, balloons and condoms, tobacco smoke, meat, salted fish, and latex gloves (Altkofer et al., 2005; Feng et al., 2009; Rywotycki, 2007; Sen et al., 1980; Tricker et al., 1991; Zou et al., 1994). Although there is a lack of studies about the effect of DEN in human carcinogenesis, data suggest that DEN is at least correlated

with cancers of the gastrointestinal tract after oral absorption, e.g. gastric cancer (Jakszyn and Gonzalez, 2006). DEN induces tumour initiation *via* DNA adduct formation after long-term treatment in animals (Verna et al., 1996). In rats, DEN treatment causes liver injury after a medium-term (2-8 weeks) treatment, which may progress to fibrosis, cirrhosis, and finally metastasising liver tumours (Liu et al., 2009). In mice, DEN-induced HCC is one of the most frequently used animal models to study liver cancer (Fausto and Campbell, 2010), and DEN has also been used to induce hepatic inflammation in mice (Naugler et al., 2007).

1.2 The mRNA binding protein tristetraprolin

The fate of many proteins, some of which play an important role in inflammatory processes, is strongly regulated by post-transcriptional modifications (Feigerlová and Battaglia-Hsu, 2017). In addition, the post-transcriptional control of gene expression is a crucial factor for proper cell homeostasis (Cooper et al., 2009). The regulation of messenger RNAs (mRNAs) prevents incorrect synthesis of proteins that could eventually lead to the development of a disease (Cooper et al., 2009; Castello et al., 2013). Different factors, such as mRNA-binding proteins (RBPs) are able to regulate the fate of mRNAs. RBPs may interact with distinct elements in the mRNA and are able to either stabilise or destabilise the mRNA (Iadevaia and Gerber, 2015). The deregulation of RBPs has been linked to different cancers including HCC (Gutschner et al., 2014; Jeng et al., 2008; Wang et al., 2016).

RBPs usually contain one or several characteristic RNA-binding motifs (e.g. a zinc finger motif), which enables them to specifically interact with their target mRNA. In order to implement specific control by targeting at a subset of mRNAs, RBPs mainly interact with sequences located in the 3'-untranslated region (3'-UTR) of mRNAs (Iadevaia and Gerber, 2015; Imig et al., 2012).

Tristetraprolin (TTP, gene name *ZFP36*), a 34 kDa member of the CCCH class of tandem zinc finger proteins, is expressed in a variety of tissues (Blackshear, 2002) and accelerates the decay of its target transcripts (Baou et al., 2011). This mRNA-destabilising role could be specially observed in a Ttp-deficient mouse model, in which *Tnf* mRNA was significantly stabilised in macrophages, which led to a significant elevation in circulating Tnf and contributed to symptoms of a systemic inflammatory syndrome (Carballo et al., 1998). This syndrome includes erosive arthritis, conjunctivitis, dermatitis, medullary and extramedullary myeloid hyperplasia associated with cachexia, glomerular mesangial thickening, and high titers of anti-DNA and antinuclear antibodies (Taylor et al., 1996). In addition, TTP expression is frequently repressed in human cancers, such as gastric, lung, breast, ovarian, and colon cancer (Hitti et al., 2016; Sanduja et al., 2012), and a loss of functional TTP was shown to modulate different tumourigenic phenotypes (Brennan et al., 2009).

1.3 The lncRNA nuclear paraspeckle assembly transcript 1 and nuclear paraspeckles

The range of ncRNAs is enormous and exceeds by far the number of protein-coding mRNAs (<2%) (Fatica and Bozzoni, 2014). A large proportion of these ncRNAs are longer than 200 nucleotides, which are often polyadenylated and defined as long ncRNAs (lncRNAs) (Rinn and Chang, 2012; Batista and Chang, 2013). These lncRNAs can be found in both the nucleus and the cytoplasm and are able to perform a broad range of tasks including post-transcriptional modification (Fatica and Bozzoni, 2014; Faghihi et al., 2008).

The nuclear paraspeckle assembly transcript 1 (*NEAT1*) is located in nuclear paraspeckles, which can be found in the nucleus (Clemson et al., 2009; Mao et al., 2011; Souquere et al., 2010). The two transcript variants of *NEAT1*, which have been described in the literature, are the short *NEAT1_v1* (*NEAT1ε*, 3.7 kb in length) and the long *NEAT1_v2* (*NEAT1-202*, *NEAT1β*, 23 kb in length) (Naganuma et al., 2012). Furthermore, Ensembl lists 201 and 203-205 as additional transcript variants (Figure 2). The transcripts *NEAT1_v1* and *NEAT1_v2* play an important role in paraspeckle formation: While the biosynthesis of paraspeckles is mainly *NEAT1_v2*-dependent, both transcripts are vital for the integrity of paraspeckles (Naganuma et al., 2012). Despite *NEAT1_v1/v2*, proteins, such as paraspeckle component 1 (PSPC1), non-POU domain containing octamer binding protein (NONO), and RNA-binding motif protein 14 (RBM14) are also paraspeckle components. Due to the lack of an altered phenotype in *Neat1* knockout mice, the biological role of paraspeckles is, as yet, widely unknown (Nakagawa and Hirose, 2012). Still, paraspeckles were reported to inhibit DNA damage-induced cell death and therefore contribute to tumourigenesis (Gao et al., 2014). Different human malignancies including lung cancer, colorectal cancer, prostate cancer, breast cancer, and HCC showed an increased expression of *NEAT1* (Yu et al., 2017). Additionally, a *NEAT1*-dependent enhancement of chemoresistance was previously reported in different cancer cell lines (Adriaens et al., 2016).

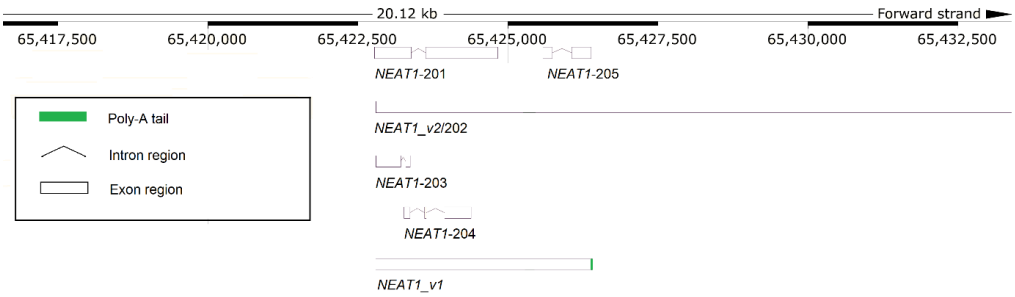


Figure 2: Size and location of *NEAT1* transcript variants on the *NEAT1* gene locus on chromosome 11 (schematic). Please note that the scheme is not drawn in scale.

1.4 Aim of the present work

HCC is a heterogeneous and complex tumour, and many mechanisms regarding its formation and progression remain elusive. Although it has been shown that inflammatory processes as well as lipid accumulation contribute to hepatocarcinogenesis, very little is known about the composition of inflammatory cells and lipids in an HCC promoting environment. Genetic aberrations are important factors in hepatic tumour progression, but only little is known about the role of TTP and *NEAT1*-dependent paraspeckles in hepatic carcinogenesis and tumour progression.

The following hypotheses were tested:

1) DEN induces leukocyte recruitment to the liver and alters lipid metabolism
2) TTP plays a role in hepatocarcinogenesis and tumour progression
3) *NEAT1* and paraspeckles are induced in HCC chemoresistance and serve as prognostic marker

2 Characterisation of diethylnitrosamine-induced leukocyte recruitment and lipid alterations in mouse livers

2.1 Abstract

Diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) in mice is one of the most frequently used animal models in liver cancer studies. Although it is commonly accepted that the toxic agent DEN induces inflammation and tumour development, the effects of DEN treatment on the composition of immune cells and hepatic lipids are mostly unknown. We therefore performed a comprehensive flow cytometric and analytical study to characterise immune cell and lipid composition in the livers of a DEN short-term mouse model to enhance the understanding of the mechanisms involved in a developing liver neoplasia.

Liver and body weight, serum transaminases, ROS, and caspase-3-like activity were analysed in nine-week-old male C57BL/6 mice, which were intraperitoneally injected with either 100 mg / kg body DEN in 0.9% NaCl or a sham-control and sacrificed 48 h after injection. Lipids and lipogenic genes were characterised *via* GC-MS, TLC, and qPCR. Leukocyte composition and chemokine expression was determined *via* flow cytometry and qPCR.

The total amount of leukocytes as well as the number of myeloid dendritic cells, neutrophils, and monocytes was significantly elevated in the livers of DEN-treated animals. In contrast, the proportion of macrophages was significantly reduced. The hepatic mRNA expression of the chemokine (C-X-C Motif) Ligand 1 (*Cxcl1*) showed increased levels in DEN-treated animals. Both hepatic lipid composition and serum levels were significantly altered in DEN-treated animals compared to sham treated mice. The amount of hepatic triglycerides as well as of specific single fatty acids were increased in the DEN-treated animals. Serum triglyceride levels were lowered in these animals, while serum cholesterol levels were elevated. The expression of carnitine palmitoyltransferase 1 (*Cpt1a*) was increased, whereas stearoyl-CoA desaturase 1 (*Scd1*), Fatty acid binding protein 1 (*Fabp1*), and fatty acid synthase (*Fasn*) were downregulated in livers of DEN-treated mice.

Taken together, this report is the first to show the impact of DEN treatment on hepatic leukocyte and lipid composition.

2.2 Introduction

The carcinogenic potential of N-nitroso compounds was first discovered in 1937 when it was reported that dimethylnitrosamine might induce liver damage in men (Freund, 1937). The carcinogen diethylnitrosamine (DEN) can be found in different products, including balloons and condoms (Altkofer et al., 2005), beer and whiskey (Sen et al., 1980), tobacco smoke (Tricker et al., 1991), meat (Rywotycki, 2007), salted fish (Zou et al., 1994), and latex gloves (Feng et al., 2009). Although not toxic itself, DEN unfolds its carcinogenic potential after hydroxylation via the CYP system (Verna et al., 1996). Especially CYP2E1 seems to play an important role in this bioactivation (Kang et al., 2007). In 1964, the first study including rats (Magee and Lee, 1964) introduced DEN into animal experiments: Since then, it has been widely used in animal models. DEN induces – like other N-nitroso compounds – DNA adduct formation and tumour initiation after long-term treatment (Verna et al., 1996). In rats, DEN treatment induces non-specific liver injury after a short-term (2-8 weeks) treatment progressing to fibrosis, cirrhosis, and finally metastasising liver tumours (Liu et al., 2009). In mice, DEN-induced hepatocellular carcinoma (HCC) is among the most frequently used animal models in liver cancer studies (Fausto and Campbell, 2010).

HCC, the predominant form of liver cancer, is the second most common cause of cancer-related death worldwide (Bruix et al., 2015; Tang et al., 2017). Since the 1980s, the incidence of HCC has increased in North America and Northern Europe, where it is mainly caused by type 2 diabetes, obesity, and metabolic disorders (El-Serag, 2012; Reeves et al., 2016). An accumulation of lipids caused by obesity has been linked to a poor prognosis with increased mortality in HCC (Reeves et al., 2016). In addition, it has been shown that chronic inflammation is a major factor in the development of HCC (Sun and Karin, 2013) and may promote tumour progression (Balkwill and Mantovani, 2001; Nakamoto et al., 1998).

It is well established that DEN induces inflammation as well as tumour development. However, the effects of DEN treatment on the composition of immune cells and hepatic lipids are rather ill characterised. We therefore undertook a comprehensive flow cytometric and analytical study to characterise immune cell composition, lipid classes, and fatty acids in the livers of a DEN short-term mouse model. Our findings

contribute to the understanding of the mechanisms involved in the development of liver neoplasia.

2.3 Materials and Methods

2.3.1 Animals

All animal procedures were performed in accordance with the local animal welfare committee (37/2014). Mice were kept under controlled conditions regarding temperature, humidity, 12 h day/night rhythm, and food access ad libitum. 32 nine-week-old male C57BL/6 mice were intraperitoneally injected with either 100 mg / kg body weight DEN in 0.9% NaCl (Sigma Aldrich, Taufkirchen, Germany) or a 0.9% NaCl solution as a sham-control. 48 hours after the injection, the mice were sacrificed. The livers of 20 of the 32 animals were checked for immune cell composition via flow cytometry. The livers of 12 of the 32 animals were checked for lipid classes and fatty acids. All of the animals were analysed regarding serum parameters, body and liver weight.

2.3.2 Flow cytometry

In general, flow cytometry analysis was performed according to Wang et al. (2011). For determination of leukocyte composition, the liver was cut into small pieces and incubated with 0.04% collagenase type IV in RPMI 1640 medium (both obtained from Sigma Aldrich) for 45 min at 37°C while shaking. Afterwards, the liver was pressed through a 100 µm cell strainer (Fisher Scientific, Schwerte, Germany) and 40 ml pre-cooled 1xPBS was added. The suspension was centrifuged (5 min, 4°C, 500 *g*), supernatant was discarded and another 40 ml of 1xPBS were added. About 1 ml of the suspension was extracted for determination of $CD45^+$ cells, and the rest was centrifuged again. After discarding the supernatant, 20 ml isotonic Percoll solution (33.75 ml Percoll, 3.75 ml 10xPBS, 62.5 ml PBS) were added and the suspension was centrifuged (12 min, room temperature, 700 *g*). The supernatant was discarded and 8 ml TAC buffer (17 mM Tris, 140 mM ammonium chloride) was added. After 20 min inversion at room temperature, the suspension was filtered again through a 100 µm cell strainer. After adding 1 ml FCS-EDTA solution (10 mM EDTA in 100 ml FCS) and centrifugation (10 min, 4°C, 500 *g*), the supernatant was discarded and the cells were resuspended in 200 µl MACS buffer (0.5% BSA and 2 mM EDTA in PBS). After adding CD45 microbeads (10 µl per 10^7 cells, Miltenyi, Bergisch Gladbach, Germany), the suspension was incubated for 15 min on ice. The cells were washed

with 5 ml 1xPBS and centrifuged (10 min, 4°C, 500 *g*), and the supernatant was discarded. The cells were resuspended in 500 µl MACS buffer and transferred to a pre-eluted MS column (attached to the magnet). After washing three times with 500 µl MACS buffer, the column was removed from the magnet and 1 ml MACS buffer was added to collect the $CD45^+$ cells. The cells were counted, diluted (500,000 / vial), and centrifuged (3 min, 4°C, 500 *g*). Cells were resuspended in flow cytometry buffer (FCB; PBS containing 2.5% (v/v) bovine calf serum and 0.05% (w/v) NaN_3) and incubated with mouse BD Fc Block (BD Biosciences, Heidelberg, Germany), as recommended by the supplier. 0.5 µg of each antibody were added: APC rat anti-mouse Ly6G Clone 1A8 (#560599), APC-R700 rat anti-mouse CD11b Clone M1/70 (#564985), BV421 rat anti-mouse Ly6C Clone AL-21 (#562727), BV510 mouse anti-mouse NK 1.1 Clone PK136 (#563096), PE hamster anti-mouse CD11c Clone HL3 (#55740, all from BD Biosciences), and FITC human anti-mouse F4/80 clone REA126 (#130-102-327 from Miltenyi). After incubation for 15 min on ice in the dark, the suspension was centrifuged (3 min, 4°C, 500 *g*), the supernatant was discarded, and the pellet was washed with 1 ml FCB followed by centrifugation. After discarding the supernatant, cells were resuspended in 300 µl FCB and 10 µl cell viability solution (BD Biosciences) were added to each sample 10 min before flow cytometric analysis.

The same protocol was used for the determination of $CD45^+$ cells, but minor changes were applied: 1 million cells of the total liver homogenate were analysed using 1 µg of the FITC mouse anti-mouse CD45.2 Clone 104 antibody (#561874, BD Biosciences) and 20 µl of the cell viability solution.

The stained cells were examined on a BD LSRFortessa™ cell analyser (BD Biosciences), and results were analysed using the FACS Diva and the FACSuite software (BD Biosciences). For each antibody, the number of positive cells was normalised to the total number of cells.

To determine the composition of the leukocytes, the following gating strategy was applied: FSC^{low} debris and erythrocytes, and multiplets with a non-linear SSC-A/SSC-H ratio were excluded. Viability was determined by 7-AAD staining. Viable cells ($7\text{-}AAD^-$) were analysed for CD11b and Cd11c expression. Myeloid dendritic cells (Mosayebi and Moazzeni, 2011) were defined as $CD11b^+$ $CD11c^{hi}$ cells, and neutrophils were identified as $Ly6G^+$ cells within the $CD11b^+$ $CD11c^-$ population.

$CD11b^+$ $CD11c^-$ $Ly6G^-$ $NK1.1^-$ cells were further divided into subpopulations according to their Ly6C and F4/80 expression, i.e. macrophages ($Ly6C^{lo}$ $F4/80^{hi}$) and monocytes ($Ly6C^{hi}$ $F4/80^{lo}$), following Blériot et al. (2015) and Ramachandran et al. (2012). All gates were defined by using fluorescence minus one (FMO) controls (Supplementary Figure 1).

The isolation and gating strategy was established in collaboration with Dr. Jessica Hoppstädter.

2.3.3 qPCR

Isolation of total RNA and reverse transcription was performed as described previously (Kiemer et al., 2009). Real-time quantitative polymerase chain reaction (qPCR) was performed in a CFX96 cycler (Bio-Rad, Munich, Germany) with 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). All samples were analysed in triplicate. Primers and conditions are listed in Supplementary Table 1. Efficiency for each experiment was determined using a standard dilution series as described previously (Kiemer et al., 2009). The absolute gene expression was normalised to *Csnk2a2* mRNA values. *Csnk2a2* was identified as the most stable housekeeping gene out of 3 housekeeping genes according to geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004).

2.3.4 TLC lipid class analysis

Thin-layer chromatography (TLC) lipid class analysis was performed as described previously (Dembek et al., 2017; Kessler et al., 2016; Laggai et al., 2013).

2.3.5 Fatty Acid Measurement by Gas Chromatography-Mass Spectrometry (GC-MS)

Snap-frozen liver tissue samples were lyophilised (approximately 2 mg dry weight) and hydrolysed by the fatty acid methyl ester (FAME) method. Fatty acid measurement using gas chromatography–mass spectrometry was performed as described previously (Laggai et al., 2014) by Dr. Katja Gemperlein (Department of

Microbial Natural Products, Helmholtz Institute for Pharmaceutical Research Saarland, Saarbrücken, Germany).

2.3.6 Serum parameters

Serum levels of cholesterol, high-density lipoproteins (HDL), triglycerides, aspartate transaminase (AST), and alanine transaminase (ALT) were determined at the 'Zentrallabor des Universitätsklinikums des Saarlandes' (Saarland University, Homburg, Germany).

2.3.7 Caspase-3-like activity assay

Caspase-3-like activity assay was performed as described previously (Kessler et al., 2013). Fluorescence was measured 3 h after incubating at 37°C by a GloMax® Discover System (Promega, Mannheim, Germany).

2.3.8 TBARS assay

Quantification of thiobarbituric acid reactive substances (TBARS) was performed as described previously (Simon et al., 2014).

2.3.9 Statistical analysis

Data analysis and statistics of experimental data were performed using the Origin software (OriginPro 8.1G; OriginLabs, Northampton, MA, USA). All data are displayed as boxplots with 25th/75th percentile boxes, geometric medians (line), means (square), and 10th/90th percentiles as whiskers. Statistical differences were estimated by independent two-sample t-test or Mann-Whitney test depending on normal distribution, which was tested by the Shapiro–Wilk method. All tests are two-sided, and differences were considered statistically significant when p-values were less than 0.05.

2.4 Results

2.4.1 Effects of DEN on leukocytes

The total amount of leukocytes and their composition in the livers was analysed by flow cytometry. The obtained data revealed that the total number of leukocytes ($CD45^+$) was significantly higher in the DEN-treated animals (Figure 1A, Table 1). Furthermore, the number of myeloid dendritic cells ($CD11b^+$ $CD11c^{hi}$), neutrophils ($CD11b^+$ $CD11c^-$ $NK1.1^-$ $Ly6G^+$), and monocytes ($CD11b^+$ $CD11c^-$ $NK1.1^-$ $Ly6G^-$ $Ly6C^{hi}$ $F4/80^{lo}$) was significantly increased in the DEN-treated animals (Figure 1B, C; Table 1). In contrast, the proportion of macrophages ($CD11b^+$ $CD11c^-$ $NK1.1^-$ $Ly6G^-$ $Ly6C^{lo}$ $F4/80^{hi}$) was significantly decreased in the livers of DEN-treated animals (Figure 1B, C; Table 1). Therefore, the monocyte / macrophage ratio was highly elevated in the DEN-treated mice (Table 1). Since chemokines are important factors of leukocyte recruitment, the hepatic mRNA expression of the chemokine (C-X-C Motif) ligand 1 (*Cxcl1*) was analysed and showed increased levels in DEN-treated animals. The analysis of the C-type lectin domain family 4, member F (*Clec4f*), which is a specific marker for Kupffer cells (Scott et al., 2016), revealed no differences ($p = 0.62$) between the differently treated animals (Figure 1D).

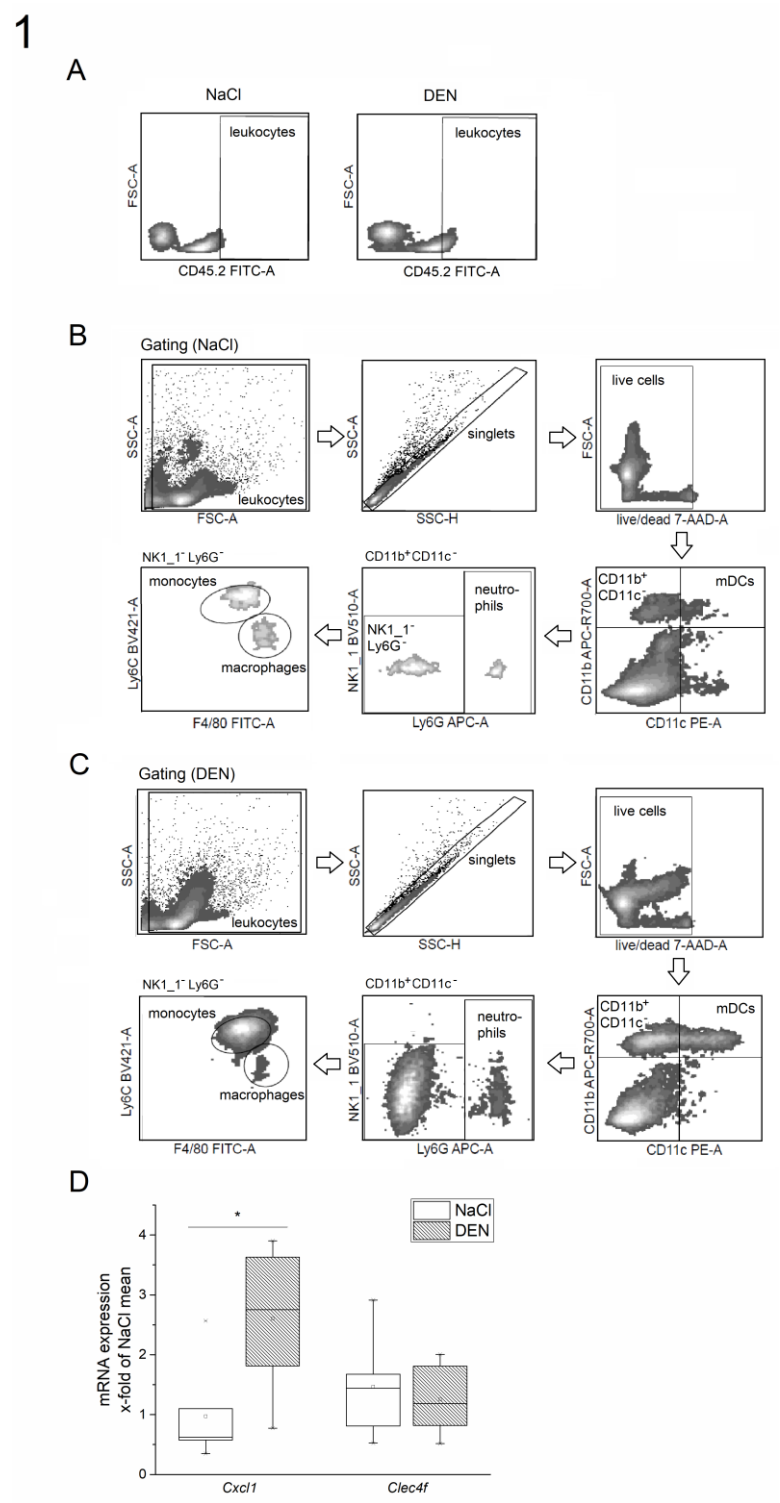


Figure 1. Effects of DEN on hepatic leukocyte counts and the composition of the myeloid compartment. (A): The total amount of CD45 positive cells in liver tissues was determined by flow cytometry. (B, C): CD45 positive hepatic cells isolated from NaCl- (B) and DEN- (C) treated mice were analysed by flow cytometry. (D): *Cxcl1* and *Clec4f* mRNA expression by qPCR normalised to *Csnk2a2* mRNA. n = 10 for A-C; n = 6 for D. Statistical difference: *: $p \leq 0.05$.

	mean (NaCl)	SEM (NaCl)	mean (DEN)	SEM (NaCl)	p-value
leukocytes [% of total hepatic cells]	0.52	± 0.049	1.31	± 0.207	0.002
myeloid DCs [% of leukocytes]	2.624	± 0.337	14.605	± 1.23	1.42E-7
Neutrophils [% of leukocytes]	2.519	± 0.351	4.222	± 0.534	0.016
macrophages [% of leukocytes]	1.912	± 0.361	0.969	± 0.09	0.021
monocytes [% of leukocytes]	2.969	± 0.34	17.551	± 1.736	2.81E-7
monocyte / macrophage ratio	2.218	± 0.439	19.135	± 1.961	2.39E-7

Table 1. Total leukocyte amount [% of total hepatic cells], proportion of myeloid cells [% of leukocytes / $CD45^+$ cells], and monocyte /macrophage ratio. P-values below 0.05 were considered significant.

2.4.2 Effects of DEN on lipids

Since inflammatory events in the liver are often linked to lipid accumulation, we assessed the hepatic lipid composition as well as the serum levels of lipids. Both were significantly altered in DEN-treated animals compared to sham-treated mice (Figure 2). The amount of hepatic triglycerides was significantly increased, whereas none of the other lipid classes showed significant differences between sham-treated and DEN-treated mice (Figure 2A). Serum triglyceride levels were significantly lowered in these animals (Figure 2B), while serum cholesterol levels were elevated (Figure 2B). Serum HDL levels did not differ significantly between both groups (Figure 2B).

2

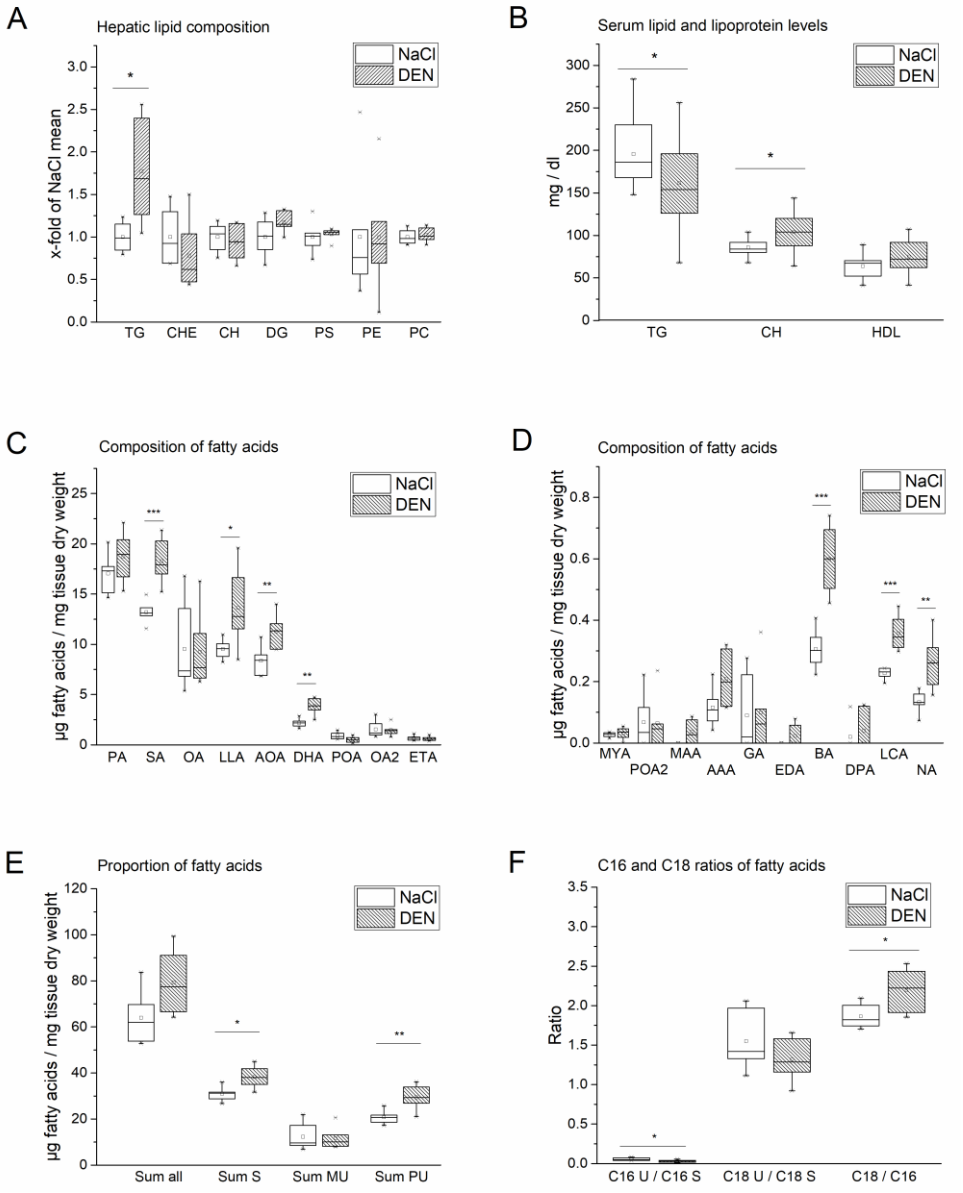


Figure 2. Effects of DEN on lipid composition. (A): Hepatic lipid composition: triglyceride (TG), cholesterolester (CHE), cholesterol (CH), diglyceride (DG), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC). (B): Triglyceride (TG), cholesterol (CH), and high-density lipoprotein (HDL) serum levels. (C): Composition of fatty acids in liver tissue (1): palmitic acid (PA,16:0), stearic acid (SA ,18:0), oleic acid (OA, 18:1), linoleic acid (LLA, 18:2), arachidonic acid (AOA , 22:4), docosahexaeinic acid (DHA, 22:6), palmitoleic acid (POA, 16:1), oleic acid isomer 2 (OA2, 18:1), eicosatrienoic acid (ETA, 20:3). (D): Composition of fatty acids in liver tissue (2): myristic acid (MYA, 14:0), palmitoleic acid isomer 2 (POA2, 16:1), margaric acid (MAA, 17:0), arachidic acid (AAA, 20:0), gondoic acid (GA, 20:1), eicosadienoic acid (EDA, 20:2), behenic acid (BA, 22:0), dicosapentaenoic acid (DPA, 22:5), lignoceric acid (LCA, 24:0), nervonic acid (NA, 24:1). (E): Proportion of fatty acid classes in liver tissue (S = saturated; MU = monounsaturated; PU = polyunsaturated). (F): C16 and C18 fatty acid ratios in liver tissues. n = 6 for A, C-F; n = 16 for B. Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

GC-MS analyses of hydrolysed lipids revealed significantly higher levels of several fatty acids in DEN-treated mice (Figure 2C, D). A more pronounced accumulation of monounsaturated as well as polyunsaturated fatty acids was observed (Figure 2E). In particular, arachidonic acid (C20:4), a precursor of eicosanoids, docosahexaenoic acid (C22:6), linoleic acid (C18:2), stearic acid (C18:0), behenic acid (C22:0), lignoceric acid (C24:0), and nervonic acid (C24:1) were increased (Figure 2C, D). The C16 unsaturated / C16 saturated fatty acid ratio was lowered, and the C18 / C16 fatty acid ratio was elevated in the livers of DEN-treated animals (Figure 2F).

To get further insights into the lipogenic effect of DEN, the hepatic mRNA expression of several genes involved in lipid metabolism was analysed. The expression of carnitine palmitoyltransferase 1 (*Cpt1a*), which catalyses fatty acid transport into the mitochondrion and regulates fatty acid oxidation in the liver (McGarry and Brown, 1997), was increased in DEN-treated mice. The rate limiting enzyme in the synthesis of monounsaturated fatty acids (Miyazaki and Ntambi, 2003) stearoyl-CoA desaturase 1 (*Scd1*), fatty acid binding protein 1 (*Fabp1*), which enhances cellular long chain fatty acid uptake (Guzman et al., 2013), and the key enzyme of *de novo* lipogenesis (Chakravarthy et al., 2007) fatty acid synthase (*Fasn*) were downregulated in livers of DEN-treated mice (Figure 3A). In addition, apolipoprotein A-IV (*Apoa4*), which transports long chain fatty acids as a part of chylomicrons (Wang et al., 2015), tended to be upregulated ($p = 0.08$) in the DEN-treated animals. The expression levels of nuclear receptor subfamily 1 group H member 3 (*Nr1h3*), which catalyses cholesterol conversion into bile acids (Kalaany and Mangelsdorf, 2006), sterol regulatory element-binding transcription factor 1 (*Srebf1*), which controls the expression of fatty acid, phospholipid and triglyceride biosynthetic genes (Shimano et al., 1997), sterol regulatory element-binding transcription factor 2 (*Srebf2*), which mainly regulates cholesterol biosynthesis (Horton et al., 1998), peroxisome proliferator-activated receptor alpha (Ppara), which is a key regulator in fatty acid uptake, utilisation, and catabolism (Kersten, 2014), MLX-interacting protein-like (*Mlxipl*), which regulates glucose metabolism and the synthesis of fatty acids and triglycerides (Iizuka et al., 2004), and elongation of very long chain fatty acids protein 6 (*Elovl6*), which is involved in the elongation of saturated and monounsaturated fatty acids (Matsuzaka et al., 2002), were not altered between both groups (Figure 3B).

3

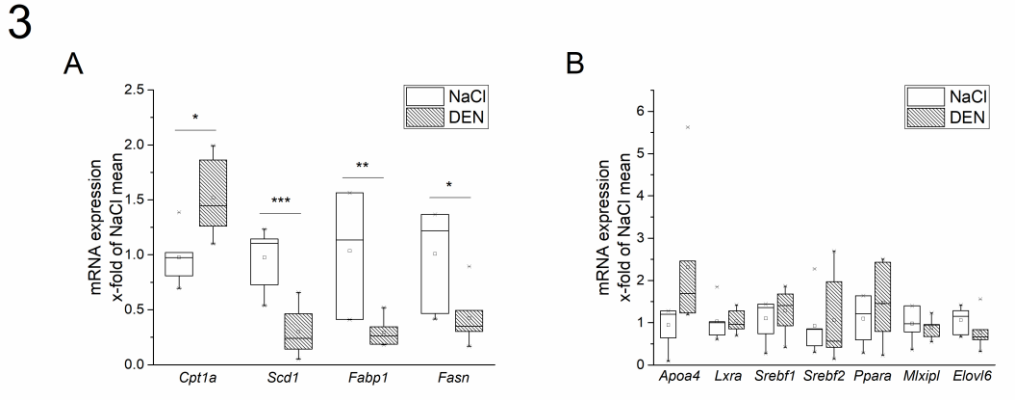


Figure 3. Effects of DEN on hepatic expression of genes involved in lipid metabolism. (A): Expression of *Cpt1a*, *Scd1*, *Fabp1*, and *Fasn* by qPCR (significant differences). (B): Expression of *Apoa4*, *Nr1h3*, *Srebf1*, *Srebf2*, *Ppara*, *Mlxipl*, and *Elovl6* by qPCR (no significant differences). n = 6. Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

2.4.3 Effects of DEN on weight, serum transaminases, ROS, and caspase-3-like activity

To determine the amount of liver damage induced by DEN, the serum was analysed for the transaminases AST and ALT – two routinely used markers for acute liver injury. Both were significantly elevated in the DEN-treated animals (Figure 4A).

4

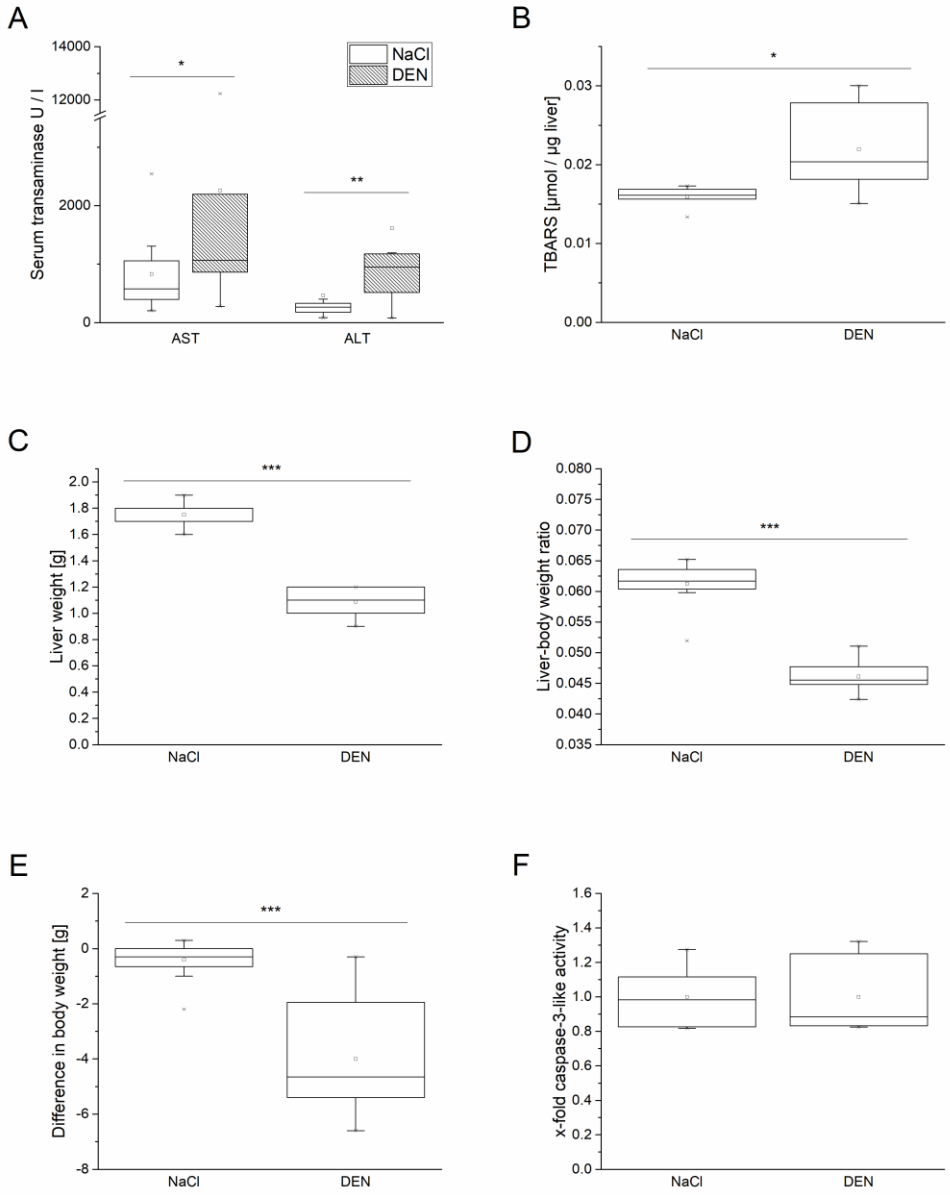


Figure 4. Effects of DEN on liver injury and hepatic cell death. (A): AST and ALT serum levels. (B): Lipid peroxidation in liver tissue by TBARS assay. (C): Liver weight. (D): Liver / body weight ratio. (E): Difference in body weight (day of treatment vs day of sacrifice). (F): Apoptosis in liver tissue, caspase-3-like activity assay. n = 16 for A; n = 6 for B-F. Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Since lipid peroxidation is increased in NAFLD (Konishi et al., 2006), we assessed lipid peroxidation as a marker for oxidative stress caused by reactive oxygen species (ROS). Accordingly, DEN treatment significantly increased lipid peroxidation as analysed by TBARS assay (Figure 4B).

After the treatment, we observed that liver weight and liver-to-body weight ratio were distinctly lower in DEN-treated animals. Furthermore, not only the liver but also the body weight were significantly decreased in DEN-treated animals (Figure 4C-E).

In order to determine whether apoptotic or necrotic events play the main role in this loss of liver tissue, a caspase-3-like activity assay was performed showing no significant difference between both groups (Figure 4F).

2.5 Discussion

Although the carcinogenic effect of DEN is widely known, the details of the DEN-induced changes in the liver are not completely understood. It has also been suggested that the toxic effects of DEN are strongly dependent on cytokine expression of immune cells (Naugler et al., 2007). In the latter study, the authors hypothesised that Kupffer cells are the main source of DEN-induced inflammatory cytokine expression leading to DEN-induced liver injury. However, they did not investigate cytokine expression with and without Kupffer cells *in vivo*. In our study, monocytes were the most abundant fraction in the analysed immune cells, whereas the number of macrophages was decreased. Similar results were found in murine livers where hepatic inflammation was induced by paracetamol, CCl_4, or bacterial infection (Blériot et al., 2015; Ramachandran et al., 2012; Zigmond et al., 2014). All of these studies report a temporary decline of Kupffer cell numbers. As resident macrophages, Kupffer cells show a regulatory and anti-inflammatory phenotype at steady state (Blériot et al., 2015). Inflammatory events result in an influx of Ly6C^{hi} monocytes, which differentiate into proinflammatory macrophages during the early response, thereby driving inflammation.

Although Maeda et al. (2005) showed that the induction of inflammatory cytokines 24 h after DEN treatment depends on Kupffer cells, they reported a diminished tumour development in mice, which were deficient for the pro-inflammatory IKK in both Kupffer cells and monocytes. Thus, no distinction can be made between the contribution of Kupffer cells and monocytes to carcinogenic event. In addition, expression of the major chemokine *Cxcl1* was increased in DEN-treated animals. In alcoholic steatohepatitis CXCL1 is upregulated, thereby promoting neutrophil infiltrations (Gao and Tsukamoto, 2016). In a combined alcohol and Western diet, *Cxcl1* characterises a neutrophilic inflammation (Lazaro et al., 2015). In line with these findings, it has been shown that in non-alcoholic steatohepatitis (NASH), the number of neutrophils and monocytes is elevated (Liang et al., 2014; Peverill et al., 2014). In HCC, *CXCL1* as well as neutrophils seem to promote tumour progression (Cui et al., 2016; Kuang et al., 2011), which underlines the tumour-promoting environment created in our model. Concordantly, treatment with an anti-Ly6G antibody leads to a reduction in tumour burden of DEN-treated mice (Wilson et al., 2015).

In inflammatory conditions of the liver, inflammation is usually accompanied and triggered by an increased lipid accumulation in hepatocytes independent of the cause of inflammation (e.g. viral hepatitis, alcoholic or non-alcoholic steatohepatitis). However, differences in the hepatic lipid composition in DEN-induced liver injury have not been well characterised so far. Hepatic lipid deposition by DEN has been described in fish (Braunbeck et al., 1992). Other studies focused on the qualitative lipid composition in DEN-induced liver tumours and did not compare quantities to healthy or adjacent normal tissue (Abel et al., 2001; Canuto et al., 1989; Yoshimura et al., 2013). In general, in an accumulation of fat in the liver, lipids are mostly incorporated in the form of triglycerides (Cohen et al., 2011). Accordingly, serum triglyceride levels were lowered in DEN-treated animals, whereas hepatic triglyceride levels were increased, suggesting a decreased triglyceride export from the liver. In addition, the sum of saturated as well as the sum of polyunsaturated fatty acids were increased in the DEN-treated mice indicating a pro-inflammatory environment (Nishiyama et al., 2017; von Schacky and Harris, 2007). The DEN-induced elevation of the C18/C16 fatty acid ratio has been shown in DEN-treated mice and NASH livers (Fengler et al., 2016; Kessler et al., 2014; Laggai et al., 2014, Puri et al., 2007). The increased ratio is at least partly due to a dramatic increase of stearic acid (C18:0), which has also been shown to be elevated in HCC tissue (Tolstik et al., 2015). Corresponding to the decreased ratios of unsaturated to saturated C16 and C18 fatty acids, *Scd1*, being responsible for the desaturation of stearic acid (Miyazaki et al., 2006), was decreased in the DEN-treated mice.

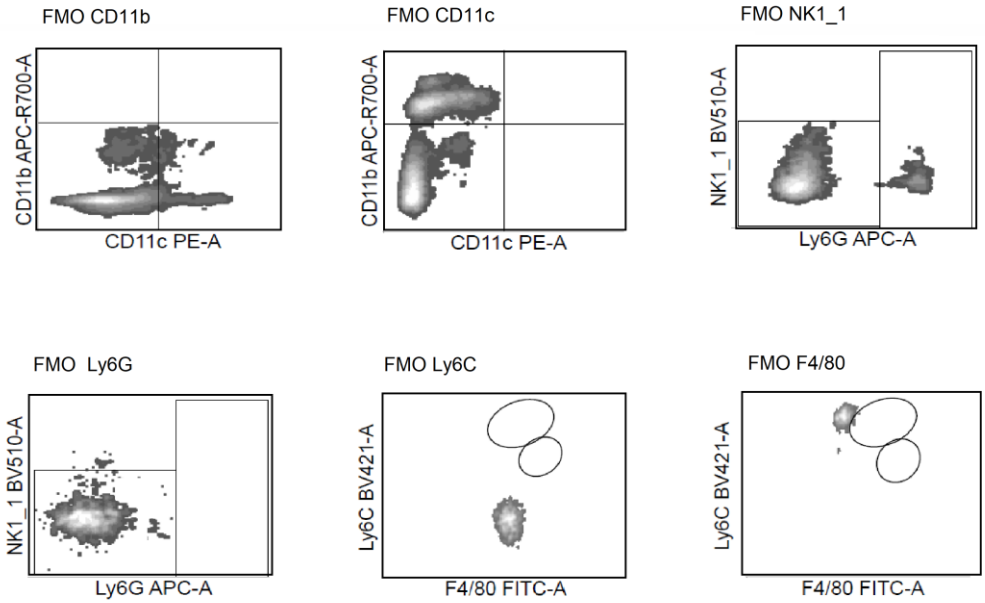
Last, *Cpt1a,* which plays an important role in the beta-oxidation of long chain fatty acids, was upregulated in DEN-treated mice. Metabolic disorders like diabetes have been shown to be caused by an increased expression but reduction of functional activity of CPT1A (Rasmussen et al., 2002). Interestingly, *Fabp1*, which inversely correlates with poor prognosis in HCC (Wang et al., 2014), was downregulated in DEN-treated mice. Quite in contrast, *Fabp1* knock out mice showed decreased hepatic triglyceride levels (Martin et al., 2005). The downregulation of *Fasn* in DEN-treated animals may be explained by a possible negative feedback mechanism caused by the increased amount of above mentioned fatty acids. Lower *Fasn* levels in hepatic lipid accumulation have been described in different etiologies of steatosis, such as NASH and drug-induced steatosis (Laggai et al., 2014; Lelliott et al., 2005; Sommer et al., 2017).

As commonly known, long-term and short-term models of DEN application induce liver injury in rodents (Liu et al., 2009; Qiu et al., 2017; Shirakami et al., 2012). A substantial liver injury was supported by the observation that liver weight and liver-to-body weight ratio were distinctly lower in DEN-treated animals. Accordingly, short-term treatment with similar doses of DEN in rats resulted in a loss of relative liver weight (Barbisan et al., 2002). This might be due to necrosis and hyperaemia taking place after short-term treatment with DEN (Liu et al., 2009). A predominant role of necrotic events is further supported by the lack of a significant difference in caspase-3-like activity and strongly increased transaminases after application of DEN suggesting a minor involvement of apoptotic events.

2.6 Conclusion

Although it has been shown before that short-term DEN treatment drives severe liver injury and acute hepatic inflammation (Liu et al., 2009; Naugler et al., 2007; Park et al., 2010), to our knowledge, our report is the first to show its impact on immune cell recruitment and hepatic lipid changes. Thus, the DEN short-term approach is a useful model to analyse the pathomechanisms and possible interventions in a tumour-promoting environment in the liver.

2.7 Supplementary data



Supplementary Figure 1. FMO controls used for flow cytometric gating.

gene	forward primer sequence 5' → 3'	reverse primer sequence 5' → 3'	gene bank accession no.	AT [°C]	product size [bp]	primer concen-tration [µM]
Apoa4	TACGTATGCTGATGGGGTGC	ATCATGCGGTCACGTAGGTC	NM_007468.2	60	132	0.2
Clec4f	CTTCGGGGAAGCAACAACTC	CAAGCAACTGCACCAGAGAAC	NM_016751.3	57	177	0.2
Cpt1a	CTCAGTGGGAGCGACTCTTCA	GGCCTCTGTGGTACACGACAA	NM_013495.2	60	105	0.25
Csnk2a2	GTAAAGGACCCTGTGTCAAAGA	GTCAGGATCTGGTAGAGTTGCT	NM_009974.3	60	85	0.4
Cxcl1	AACCGAAGTCATAGCCACACT	CCGTTACTTGGGGACACCTT	NM_008176.3	60	112	0.25
Elovl6	ACAATGGACCTGTCAGCAAA	GTACCAGTGCAGGAAGATCAGT	NM_130450.2	60	119	0.1
Fabp1	ATGAACTTCTCCGGCAAGTAC	ACTTTTTCCCCAGTCATGGTC	NM_017399.4	63	178	0.15
Fasn	ATCCTGGAACGAGAACACGATCT	AGAGACGTGTCACTCCTGGACTT	NM_007988.3	60	140	0.15
Mlxipl	CTGGGGACCTAAACAGGAGC	GAAGCCACCCTATAGCTCCC	NM_021455.4	60	166	0.25
Nr1h3	CCGACAGAGCTTCGTCC	CCCACAGACACTGCACAG	NM_013839.4 TV1; NM_001177730.1 TV2	60	81	0.2
Ppara	CCTTCCCTGTGAACTGACG	CCACAGAGCGCTAAGCTGT	NM_001113418.1	60	77	0.25
Scd1	AGATCTCCAGTTCTTACACGACCAC	CTTTCATTTCAGGACGGATGTCT	NM_009127.4	60	140	0.2
Srebf1	GGCTCTGGAACAGACACTGG	GGCCCGGGAAGTCACTGT	NM_011480.3	60	110	0.1
Srebf2	ACCTAGACCTCGCCAAAGGT	CGGATCACATTCCAGGAG	NM_033218.1	61	130	0.25

Supplementary Table 1. Sequences of primers used for murine mRNA expression analysis.

3 The mRNA-binding protein TTP promotes hepatocarcinogenesis but inhibits tumour progression in liver cancer

3.1 Abstract

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related death worldwide. The dysregulated expression of mRNA-binding proteins (RBPs) can be a key factor in tumour initiation and progression. One of the RBPs, which has been shown to be downregulated in several types of cancer, is tristetraprolin (TTP, gene name *ZFP36*). Little is known about the role of TTP in hepatic tumour initiation and progression. Using a hepatocyte-specific *Ttp* knockout mouse model and in vitro TTP overexpression, tumour initiation and progression were analysed in order to elucidate the mechanistic role of TTP in HCC.

TTP expression was analysed in three large human data sets (TCGA and GEO) comprising up to 369 tumour and non-tumour liver samples. Effects of hepatocyte *Ttp* knockout in male C57BL/6 mice on tumour initiation and inflammation was determined *via* haematoxylin and eosin staining, and flow cytometry. Effects of TTP overexpression was analysed *via* MTT-assay (chemoresistance), scratch assay (migration), flow cytometry (proliferation), and qPCR (TTP target identification) in human HepG2, PLC/PRF/5, and Huh7 hepatoma cells. In the same cell lines, the effects of hypomethylation on *TTP* expression was determined.

TTP expression was downregulated in tumour samples of all analysed data sets compared to non-tumour or cirrhotic tissue. *Ttp* knockout mice had a significantly decreased tumour burden upon treatment with the carcinogen diethylnitrosamine (DEN) and their DEN-induced leukocyte recruitment was altered. Only a minor effect of TTP overexpression on chemoresistance was observed in Huh7 cells. However, TTP overexpression distinctly decreased migration ability in PLC/PRF/5 and Huh7 cells, and proliferation in HepG2, PLC/PRF/5, and Huh7. Several oncogenes were downregulated in TTP-overexpressing hepatoma cells, e.g. B-cell lymphoma 2 (*BCL2*), c-Myc (*MYC*), and vascular endothelial growth factor A (*VEGFA*). Hypomethylation increased *TTP* expression in HepG2 and Huh7 cells, but *TTP* promoter methylation was unaltered in human HCC samples.

Taken together, this study suggests that hepatocyte TTP promotes hepatocarcinogenesis, while it shows tumour-suppressive actions in hepatic tumour progression.

3.2 Introduction

Hepatocellular carcinoma (HCC), the predominant form of liver cancer, is the second most common cause of cancer-related death worldwide (Bruix et al., 2015; Tang et al., 2017). Most cases of HCC occur in North Africa and East Asia, where HCC is mainly induced by chronic hepatitis B and C infection (Farazi and DePinho, 2006). Other risk factors are alcohol abuse, whereas in Northern Europe, the USA, and Canada, HCC is mainly caused by obesity, type 2 diabetes, and metabolic disorders (El-Serag, 2012, Reeves et al., 2016).

The initiation and progression of cancer are provoked by dysregulated expression of proteins controlling diverse cellular phenotypes: cell cycle, differentiation, apoptosis, angiogenesis, and cell invasiveness (Hanahan and Weinberg, 2011). Biosynthesis of these proteins is strongly regulated by the concentrations of their respective mRNAs in the cytoplasm, which depend on both mRNA synthesis and degradation. The cytoplasmic stability of many mRNAs is controlled by mRNA-binding proteins (RBPs), some of which have been shown to be deregulated in HCC. However, most of the studies focus on upregulated RBPs (Dang et al., 2017; Gutschner et al., 2014; Kessler et al., 2015; Li et al., 2017). A subgroup of the RBPs are the so-called AU-rich elements binding proteins (ARE-BPs), which control the stability of mRNAs by binding to AU-rich elements (ARE) located within their 3′-untranslated region (3′-UTR) (Guhaniyogi and Brewer, 2001). On the one hand, some of the ARE-BPs – like tristetraprolin (TTP or *ZFP36*) – accelerate decay of transcripts (Blackshear 2002). The strongest evidence for its mRNA-destabilising role could be observed in a Ttp-deficient mouse model, in which tumour necrosis factor α (*Tnf*) mRNA was stabilised leading to a systemic inflammatory syndrome (Carballo et al., 1998). On the other hand, some ARE-BPs – such as human antigen R (HuR or *ELAVL1*) – protect mRNAs from degradation (Baou et al., 2011). Interestingly, TTP expression is repressed in several human cancers (Hitti et al., 2016; Sanduja et al., 2012). Furthermore, a loss of functional TTP can modulate diverse tumourigenic phenotypes (Brennan et al., 2009).

Although *TTP* has also been suggested to be downregulated in human liver cancer (Hitti et al., 2016) and to be only minimally expressed in human hepatoma cells (Sohn et al., 2010), little is known about its possible role in hepatic tumour initiation and progression. Therefore, we conducted a detailed study to address these issues.

We analysed the effect of a liver specific *Ttp* knockout in mice treated with a tumour-inducing agent and checked the impact of TTP overexpression in steps of tumour progression including migration, proliferation, and chemoresistance. Our findings reveal tumour-promoting actions of TTP in tumour initiation, but tumour-suppressive actions in HCC progression.

3.3 Materials and Methods

3.3.1 Animals

All animal procedures were performed in accordance with the local animal welfare committee (permission no. 37/2014). Male C57BL/6 mice were kept under controlled conditions regarding temperature, humidity, 12 h day/night rhythm, and food access. For the long-term experiment, which mimics hepatocarcinogenesis, 80 wild type and 80 *Ttp* knockout (unpublished) two-week-old male mice were intraperitoneally injected with either a 5 mg / kg body weight diethylnitrosamine (DEN) solution or a 0.9% NaCl solution as a sham-control to determine the effects of TTP on hepatic tumour initiation. 22 weeks after the injection, the mice were sacrificed. For the short-term experiment mimicking acute hepatic inflammation, 32 wild type and 32 *Ttp* knockout nine-week-old male mice were intraperitoneally injected with either a 100 mg / kg body weight DEN solution or with a 0.9% NaCl solution as a sham-control to identify possible effects of TTP on acute hepatic inflammation. 48 hours after the injection, the mice were sacrificed. Liver specific *Ttp* knockout was induced *via* the Cre/*lox* system. In this mice, the *Ttp* allele was flanked by two *loxP* sequences, which can be detected and cut by the Cre recombinase. To make this knockout hepatocyte specific, *Cre* was linked to the albumin gene (*Alb*), which is only expressed in the liver. The control mice are *Cre* negative and are therefore able to express the *loxP*-flanked *Ttp* unrestrictedly. Liver specific *Ttp* knockout was confirmed *via* qPCR (Supplementary Figure 1). *Ttp* expression was almost absent in hepatocyte-specific knockout mice, suggesting that the predominant *Ttp* expression in the liver is found in hepatocytes. The animals were kindly provided by Dr. Perry J. Blackshear (The Laboratory of Signal Transduction, Research Triangle Park, NC, USA).

3.3.2 Histology

For histological analysis, paraffin-embedded liver tissue specimens were cut into 5 µm sections and stained with haematoxylin and eosin (H&E). This analysis was kindly performed by Prof. Dr. Johannes Haybäck (Department of Pathology, Otto-von-Guericke University, Magdeburg, Germany).

3.3.3 Cell culture

HepG2, PLC/PRF/5, and Huh7cells were cultured in RPMI-1640 medium with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glutamine (Sigma-Aldrich, Taufkirchen, Germany) at 37°C and 5% CO_2.

3.3.4 Transient TTP overexpression

For overexpression experiments, a vector (pZeoSV2(–)) containing the human TTP coding sequence tagged with the human influenza hemagglutinin tag or the vector with the antisense sequence as a control (Ref. No.: V855-01, Invitrogen, Carlsbad, California, USA) was used. The vector was kindly provided by Prof. Dr. Hartmut Kleinert (Fechir et al., 2005) and the sequence was verified by sequencing. Transient TTP overexpression in hepatoma cells was established by transfection with the vector using jetPEI™ Hepatocyte reagent (102-05N, Polyplus transfection, Illkirch, France) as recommended by the manufacturer. Successful TTP overexpression was confirmed *via* Western Blot for every experiment.

3.3.5 Cytotoxicity assay

Hepatoma cells were seeded into 96-well plates, transfected with *TTP* or control vector, and treated with different concentrations of doxorubicin (Sigma-Aldrich) or sorafenib (Biomol GmbH, Hamburg, Germany) and the respective solvent control. 24 h after the treatment, the cytostatic substances were removed and 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) (Sigma-Aldrich) in medium were added. After 2 h incubation, the formazan crystals were solved in dimethyl sulfoxide and the absorbance was measured at 550 nm with 690 nm as reference wavelength in a microplate reader (Tecan Sunrise™, Tecan Group Ltd., Männedorf, Switzerland).

3.3.6 Migration assay

Cells were seeded into 12-well plates, transfected with *TTP* or control vector, and scratched 48 h after transfection with a pipet tip. The first image was taken immediately after the scratch; the second image was taken 24 h after the scratch. Images were obtained and analysed with an Axio Observer Z1 epifluorescence

microscope equipped with an AxioCam Mrm (Zeiss, Oberkochen, Germany) using a 5x objective. Data was analysed with the TScratch software (CSElab, Zürich, Switzerland).

3.3.7 Hypomethylation assay

HepG2, PLC/PRF/5, and Huh7 cells were treated with decitabine over 3 days. The DNA-methyltransferase inhibitor (Sigma-Aldrich) was freshly added daily.

3.3.8 Human HCC analysis

Paraffin-embedded liver samples (n = 31) from randomly selected pseudonymised HCC patients who underwent liver resection at the Saarland University Medical Center between 2005 and 2010 were obtained as described previously (Kessler et al., 2013). The study protocol was approved by the local Ethics Committee (47/07). Samples had a mixed aetiology including non-alcoholic steatohepatitis (NASH), alcoholic liver disease, viral hepatitis, hemochromatosis, porphyria, and cryptogenic cirrhosis (Kessler et al., 2013).

For differential *TTP* expression between tumour (n =247) and non-tumour (n = 239) samples, the log2 of an RMA-normalised data set (GSE14520) of an AffymetrixGeneChip HG-U133A 2.0 was analysed. Similarly, differential gene expression was analysed in data set GSE25097 between healthy (n = 6), cirrhotic (n = 40), adjacent non-tumour (n = 243), and tumour tissue (n = 268), in data set GSE5975 between positive (n = 95) and negative (n = 143) EpCAM samples, and in data set GSE20238 between vascular invasive (n = 45) and non-invasive (n = 34) HCC samples. Differential expression analysis was based on the Kolmogorov–Smirnov test. Pearson correlation was applied to detect correlations between genes of interest. These data were kindly compiled and analysed by Dr. Ahmad Barghash (School of Electrical Engineering and Information Technology, German Jordanian University, Amman, Jordan).

The RNAseq expression data from The Cancer Genome Atlas (TCGA) pan cancer dataset comparing *TTP* expression in tumour and non-tumour liver tissue was produced *via* Toil (Vivian et al., 2017). RSEM (Li and Dewey, 2011) reported transcripts per million values were downloaded *via* the UCSC Xena Browser

(https://xenabrowser.net) and comprised 369 primary solid tumour as well as 50 matched non-tumour tissue samples. These data were kindly compiled and analysed by Dr. Markus List (Department of Computational Biology and Applied Algorithmics, Max Planck Institute for Informatics, Saarbrücken, Germany).

TCGA analysis of DNA methylation in HCC was performed as described before (Kessler et al., 2015). The data set contained 41 non-tumour and 95 tumour samples. We considered methylation only in the promoter regions (defined within 2,000 bp upstream of the transcription start site provided in The Eukaryotic Promoter Database (EPD)). Averages were considered for regions covered by multiple probes. These data were kindly compiled and analysed by Dr. Ahmad Barghash (School of Electrical Engineering and Information Technology, German Jordanian University, Amman, Jordan).

3.3.9 qPCR

Isolation of human total RNA and reverse transcription was performed using the High Pure RNA Isolation Kit or the High Pure RNA Tissue Kit (Roche Diagnostics, Mannheim, Germany) and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) according to the manufacturers' instructions. Real-time quantitative polymerase chain reaction (qPCR) was performed in a CFX96 cycler (Bio-Rad, München, Germany) with 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). All samples were estimated in triplicate. Primers and conditions are listed in Supplementary Table 1. Efficiency for each experiment was determined using a standard dilution series as described previously (Kiemer et al., 2009). The absolute gene expression was normalised to *ACTB* (for human samples) or *Csnk2a2* (for murine samples) mRNA values.

3.3.10 Flow cytometry

Flow cytometric analysis of murine liver leukocyte composition was performed as described previously (Wang et al., 2011; Chapter 2.3.2). To determine the amount of leukocytes, 1 µg of the FITC mouse anti-mouse CD45.2 Clone 104 antibody (#561874, BD Biosciences, Heidelberg, Germany) was used. To detect the

composition of leukocytes, 0.5 µg of each antibody were added: APC rat anti-mouse Ly6G Clone 1A8 (#560599), APC-R700 rat anti-mouse CD11b Clone M1/70 (#564985), BV421 rat anti-mouse Ly6C Clone AL-21 (#562727), BV510 mouse anti-mouse NK 1.1 Clone PK136 (#563096), PE hamster anti-mouse CD11c Clone HL3 (#55740, all from BD Biosciences), and FITC human anti-mouse F4/80 clone REA126 (#130-102-327 from Miltenyi, Bergisch Gladbach, Germany). To determine the composition of the leukocytes, the following gating strategy was applied: FSClow debris and erythrocytes, and multiplets with a non-linear SSC-A/SSC-H ratio were excluded. Viability was determined by 7-AAD staining. Viable cells (7-AAD$^-$) were analysed for CD11b and Cd11c expression. Myeloid dendritic cells (Mosayebi and Moazzeni, 2011) were defined as CD11b$^+$ CD11c^{hi} cells, and neutrophils were identified as Ly6G$^+$ cells within the CD11b$^+$ CD11c$^-$ population. CD11b$^+$ CD11c$^-$ Ly6G$^-$ NK1.1$^-$ cells were further divided into subpopulations according to their Ly6C and F4/80 expression, i.e. macrophages (Ly6C^{lo} F4/80^{hi}) and monocytes (Ly6C^{hi} F4/80^{lo}), following Blériot et al. (2015) and Ramachandran et al. (2012). All gates were defined by using fluorescence minus one (FMO) controls. Flow cytometric analysis of human hepatoma cell proliferation was performed as described previously (Schultheiß et al., 2017) using the PE Mouse Anti-Human Ki-67 Set (#5113743, BD Biosciences). The isolation and gating strategy was established in collaboration with Dr. Jessica Hoppstädter.

3.3.11 Statistical analysis

Data analysis and statistics of experimental data were performed using the Origin software (OriginPro 8.1G; OriginLabs, Northampton, MA, USA). All data are displayed either as columns with mean values±SEM or as individual values and boxplots±interquartile range with mean (square) and median (line). Statistical differences were estimated by independent two-sample t-test or Mann-Whitney test depending on normal distribution, which was tested by the Shapiro–Wilk method, or Fisher-exact test for categorical data. All tests are two-sided, and differences were considered statistically significant when p-values were less than 0.05.

3.4 Results

3.4.1 TTP expression in human HCC tissue

Since *TTP* has been shown to be downregulated in different human cancer types (Sanduja et al., 2012) an extensive expression analysis of *TTP* in HCC was performed. We analysed *TTP* expression in a microarray data set comprising almost 250 human HBV-derived HCC samples. The data revealed that the vast majority of HCC tissues exhibited much lower *TTP* mRNA levels than the non-tumour tissue (Figure 1A). A TCGA data comprising 369 HCC (primary solid tumour) to 50 non-tumour liver tissue also showed a substantially decreased expression of *TTP* in HCC tissue (Figure 1B). Moreover, a data set comparing HCC tissue to healthy, cirrhotic, and non-tumour tissue of HCC patients revealed decreased levels of *TTP* mRNA in HCC but not in cirrhosis (Figure 1C). Besides the analyses of these publicly available –omics datasets, we analysed *TTP* mRNA expression by qPCR in a set of human liver tumour samples from mixed aetiologies and revealed the same results (Figure 1D).

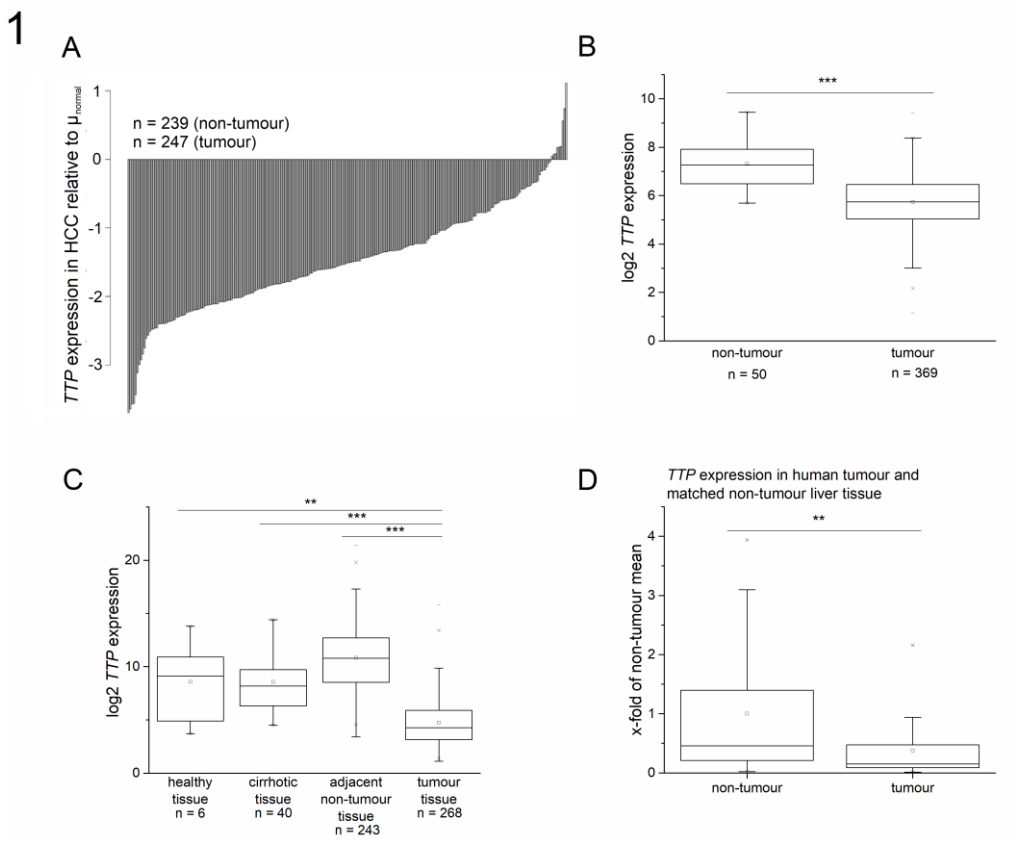


Figure 1. *TTP* expression in human tumour and non-tumour liver tissue. (A): *TTP* expression in 247 human HCC samples relative to the mean of 239 non-tumour liver tissue samples (μ normal) (GSE14520). (B): *TTP* expression in tumour (n = 369) and non-tumour (n = 50) tissue (TCGA). (C): *TTP* expression in healthy, cirrhotic, adjacent non-tumour, and tumour liver tissues (500 samples; GSE25097). (D): *TTP* mRNA levels isolated of tumour and adjacent non-tumour tissues (n = 31). Statistical difference: **: $p \leq 0.01$; ***: $p \leq 0.001$.

3.4.2 Effects of TTP on tumour initiation

Since TTP was strongly downregulated in human HCC tissue, liver specific *Ttp* knockout mice were generated to test the hypothesis that this knockout will promote tumourigenesis. *Ttp* knockout and wild type mice were treated with DEN at the age of two weeks to induce tumours and were sacrificed at the age of six months. In contrast to our hypothesis, however, the tumour incidence was significantly lower in the DEN-treated *Ttp* knockout animals compared to the DEN-treated wild type animals (Fig 2A) while there was no statistical difference regarding tumour incidence between the genotypes in the sham-treated groups. In addition, the number of tumours per animal was significantly decreased in the DEN-treated *Ttp* knockout mice compared to the DEN-treated wild type mice (Fig 2B, C). Almost half of the

tumours of the sham-treated wild type animals grew trabecular or mixed, whereas all other tumours were solid (Fig 2D). The histological evaluation of inflammatory infiltrates revealed no difference between the different groups (Fig 2E-G).

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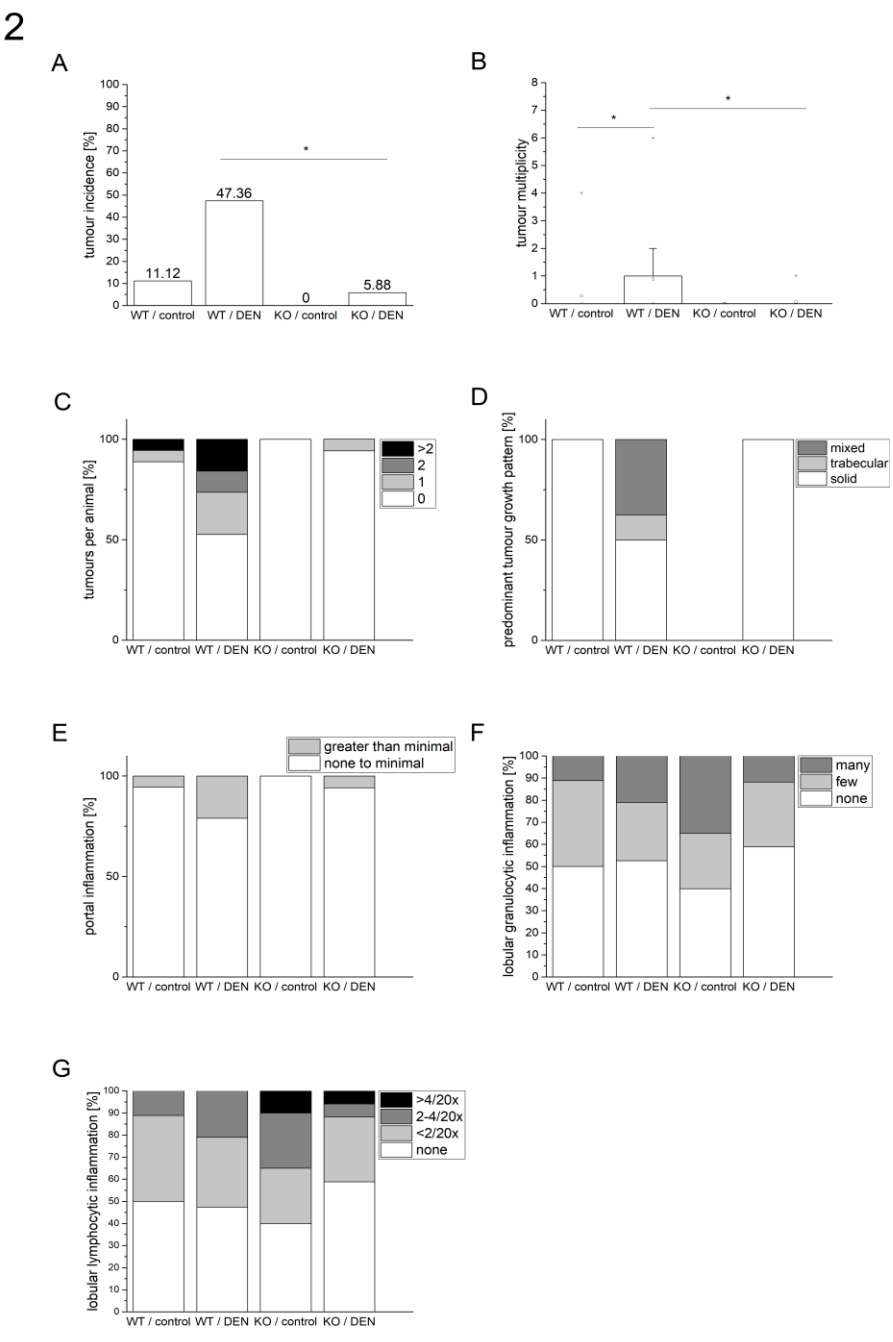

Figure 2. Amount and pattern of tumours and inflammation in DEN- or sham-treated wild type (WT) and *Ttp* knockout (KO) mice. (A): tumour incidence. (B, C): tumour multiplicity. (D): predominant tumour growth pattern. (E): portal inflammation. (F): lobular granulocytic inflammation. (G): lobular lymphocytic inflammation (Scoring: none, <2, 2-4, or >4 visible at least in one area with a 20x objective). n = 18 (WT/control), 19 (WT/DEN), 20 (KO/control), 17 (KO/DEN). Statistical difference: *: $p \leq 0.05$.

3.4.3 Effects of TTP on DEN-induced leukocyte recruitment

DEN-induced hepatocarcinogenesis is typically characterised by inflammatory events (Naugler et al., 2007) and, TTP has been suggested to affect leukocyte recruitment (Ghosh et al., 2010). We therefore hypothesised that protection from DEN-induced liver cancer in *Ttp* knockout mice is facilitated via attenuated leukocyte recruitment. Therefore, the amount of leukocytes inside the liver as well as their composition were analysed in the above described long-term DEN experiment *via* flow cytometry. The DEN-treated wild type mice showed an increased number of leukocytes compared to their sham-treated controls as assessed by staining for the pan-leukocyte marker CD45 (Wang et al., 2011). No difference due to *Ttp* knockout was observed (Figure 3A). The amount of macrophage and monocyte was decreased in DEN-treated *Ttp* knockout mice compared to DEN-treated wild type mice, and neutrophils also tended ($p = 0.06$) to be decreased (Figure 3B). No statistical effect on myeloid dendritic cells and the monocytes / macrophages ratio could be observed in all groups (Figure 3B, C).

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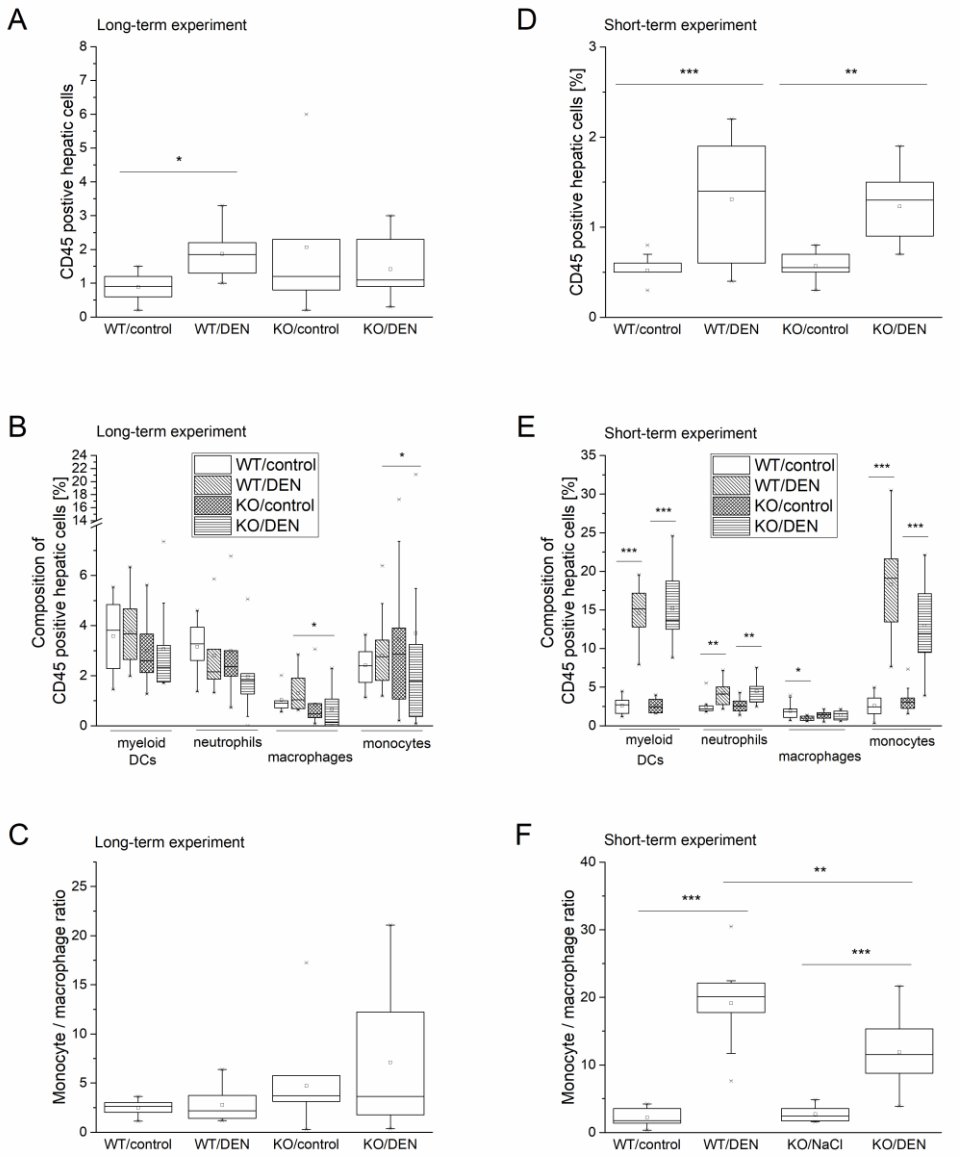

Figure 3. Effects of DEN on the hepatic amount and composition of leukocytes in wild type (WT) and *Ttp* knockout (KO) mice. Leukocyte number and composition was analysed by multi-color flow cytometry. (A): Total amount of CD45 positive cells (long-term experiment). (B): The proportion of myeloid dendritic cells (myeloid DCs), neutrophils, macrophages, and monocytes within CD45 positive cells was evaluated (long-term experiment). (C): Monocyte / macrophage ratio of CD45 positive cells (long-term experiment). (D): Total amount of CD45 positive cells (short-term treatment). (E): Composition of CD45 positive cells, i.e. myeloid dendritic cells (myeloid DCs), neutrophils, macrophages, and monocytes (long-term experiment). (F): Monocyte / macrophage ratio of CD45 positive cells (long-term experiment). n = 10. Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

To elucidate the impact of TTP on acute hepatic inflammation as initiating event in hepatocarcinogenesis (Kessler et al., 2015; Kessler et al., 2014; Naugler et al., 2007), *Ttp* knockout and wild type mice were treated with a high dose of DEN at the age of nine weeks to induce inflammation and were sacrificed 48 h after the treatment. First, the amount of leukocytes inside the liver as well as their composition were analysed. DEN increased the total amount of leukocytes and increased the composition of myeloid dendritic cells, neutrophils, and monocytes in *Ttp* knockout and wild type animals in a similar way (Figure 3D, Figure 3E). However, the proportion of macrophages was decreased in DEN-treated wild type mice compared to their control, whereas no difference could be observed between sham- and DEN-treated *Ttp* knockout mice (Figure 3E). DEN treatment also increased the monocyte / macrophage ratio, but the effect was reduced in *Ttp* knockout mice compared to wild type mice (Figure 3F).

3.4.4 Effects of TTP on chemoresistance

In order to clarify the role of TTP during tumour progression, TTP expression was investigated with respect to chemoresistance. TTP-overexpressing as well as control HepG2, PLC/PRF/5 and Huh7 cells were treated with different concentrations of sorafenib and doxorubicin. The results suggested a strong impact of TTP overexpression on chemosensitivity in all three cell lines (Figure 4A-F). However, the viability of untreated TTP-overexpressing cells was significantly lower than the number of untreated control cells in all three cell lines (Figure 4A-F). Therefore, the evaluation was adjusted in a way that TTP-overexpressing and control cells were normalised to the control cells. This revealed a less dramatically decreased but still significantly different chemoresistance (Supplementary Figure 2). Due to this effect of TTP on chemoresistance in hepatoma cells, we wondered whether TTP expression was related to EpCAM expression in HCC, since EpCAM has been suggested as a marker for HCC chemoresistance (Noda et al., 2009). Therefore, we analysed the expression of *TTP* in EpCAM positive vs EpCAM negative HCC tissues but found no differences ($p = 0.29$; Supplementary Figure 3).

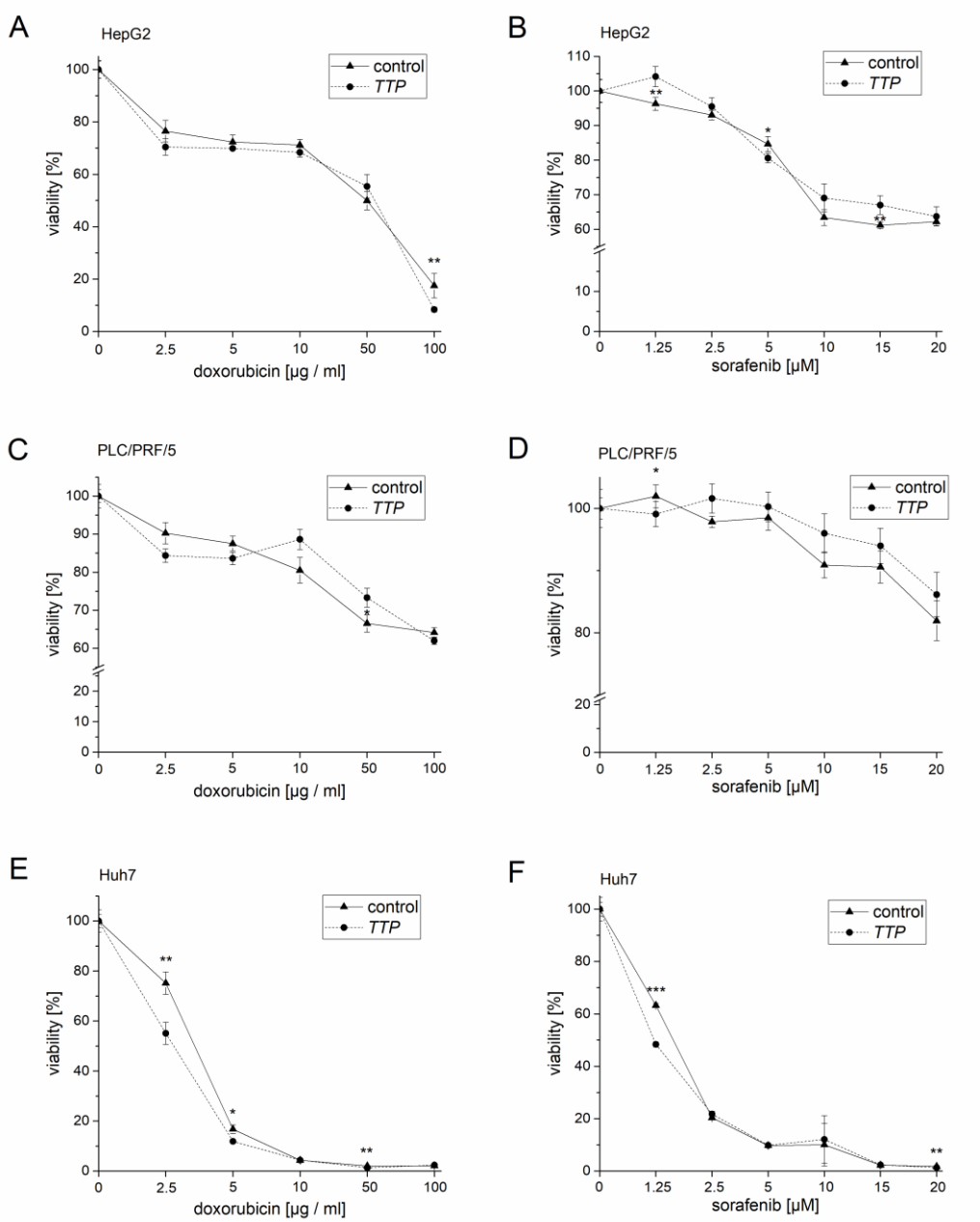

Figure 4. Effects of TTP overexpression on chemoresistance in hepatoma cells. Cells were transfected with either a *TTP* or a control vector. 24 h after transfection, cells were treated with different concentrations of doxorubicin (0 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml) or sorafenib (0 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 15 µM, 20 µM). Cell viability was determined *via* MTT assay. (A): HepG2 cells treated with doxorubicin. (B): HepG2 cells treated with sorafenib. (C): Huh7 cells treated with doxorubicin. (D): Huh7 cells treated with sorafenib. (E): PLC/PRF/5 cells treated with doxorubicin. (F): PLC/PRF/5 cells treated with sorafenib. n = 3; quadruplicates. Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

3.4.5 Effects of TTP on proliferation

The MTT assay in TTP-overexpressing cells suggested anti-proliferative actions of TTP. We therefore aimed to test the hypothesis of anti-proliferative actions of TTP by MKI67 staining and flow cytometry in stably overexpressing cell lines. However, cells transfected with the overexpressing plasmid did not grow at all. Thus, proliferation ability of transient TTP-overexpressing cells was investigated. The proliferation in HepG2, PLC/PRF/5, and Huh7 cells was dramatically decreased after TTP overexpression (Figure 5A, B).

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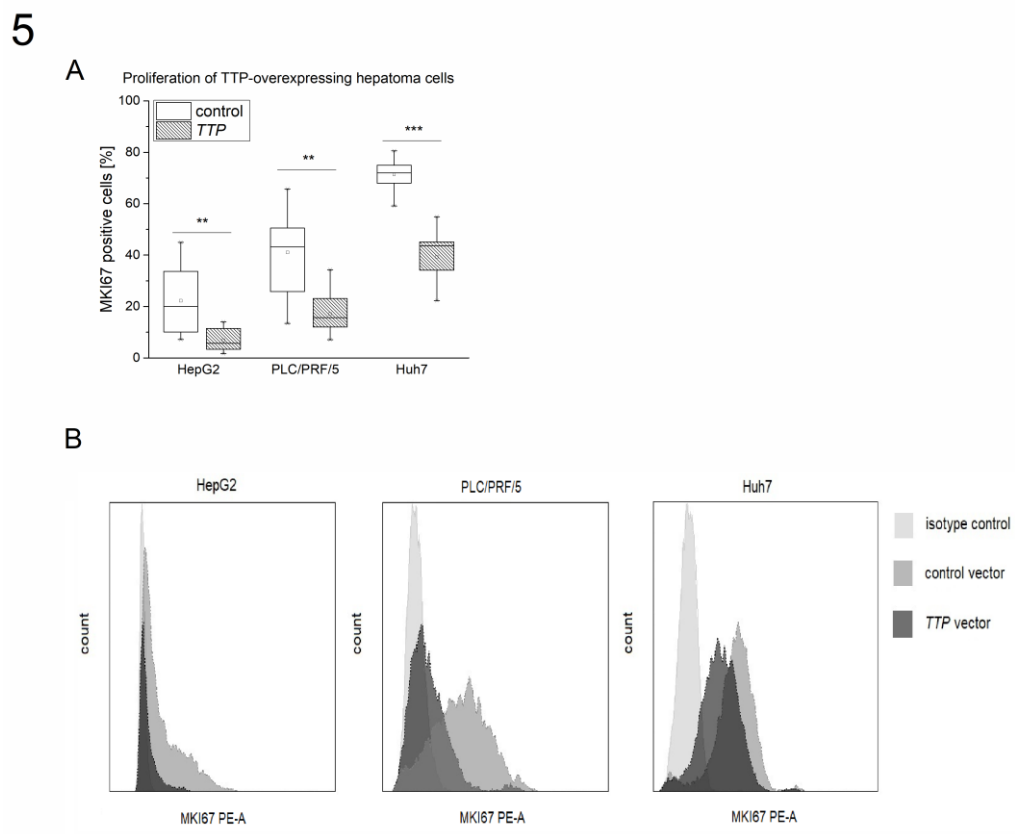

Figure 5. Proliferation of TTP-overexpressing hepatoma cells. (A): Proliferation of cells transfected with either a *TTP* or a control vector. (B): Flow cytometric analysis of the proliferation marker MKI67 in TTP-overexpressing (*TTP*) and control cells (control vector). The isotype controls represent the control cells. Representative histograms of MKI67 flow cytometric analyses are shown. n = 3; triplicates. Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

3.4.5 Effects of TTP on migration

Due to the dramatic impact of TTP on proliferation, we hypothesised that TTP might also influence the migration ability in hepatoma cells. Therefore, a scratch assay was

performed. The migration ability of PLC/PRF/5 and Huh7 cells, but not of HepG2 cells was inhibited by TTP (Figure 6A-D).

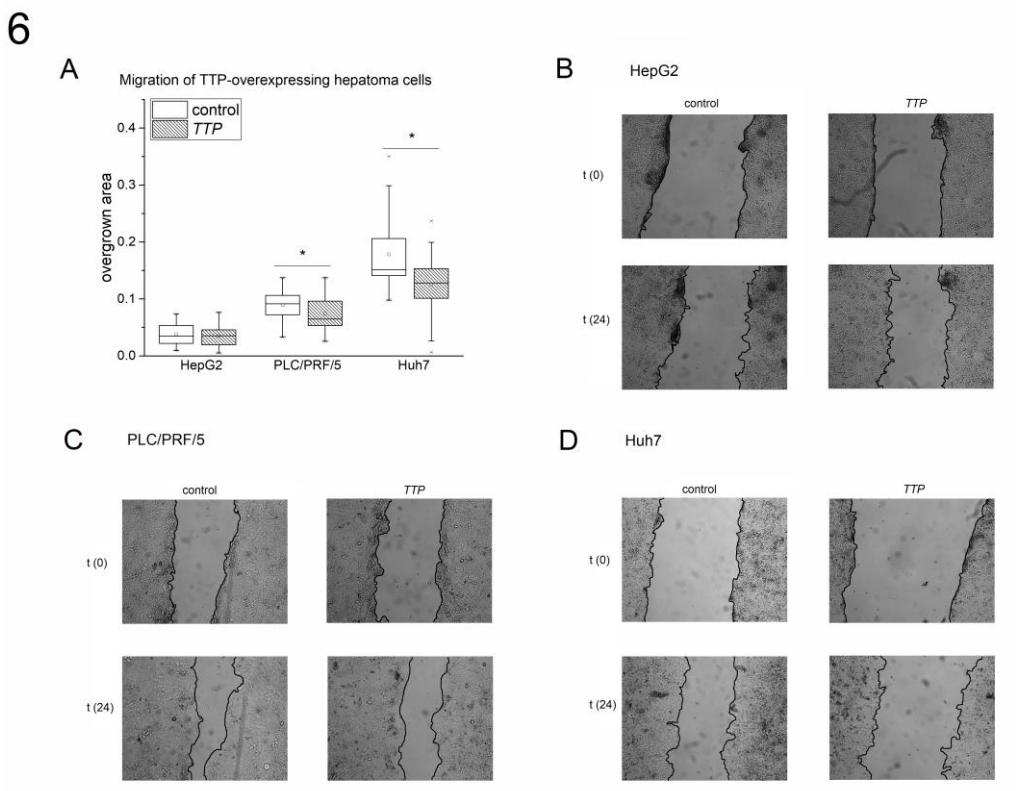

Figure 6. Migration of TTP-overexpressing hepatoma cells. (A): Migration of cells transfected with either a *TTP* or a control vector. (B-D): Representative images of transfected HepG2 (B), PLC/PRF/5 (C), and Huh7 cells (D). Images were taken with a 5x objective immediately after the scratch (t (0)) or 24 h after the scratch (t (24)). For better visualisation, lines indicating overgrown areas were inserted. n = 5-6; quadruplicates. Statistical difference: *: $p \leq 0.05$.

3.4.6 Genes affected by TTP

Since TTP represents an mRNA destabilising factor, we hypothesised that TTP's tumour-supressing actions were caused by an altered expression of its target genes, i.e. that TTP overexpression resulted in a downregulation of oncogenes. Therefore, the expression of the oncogenes B-cell lymphoma 2 (*BCL2*), c-Myc (*MYC*), transcription factor E2F1 (*E2F1*), vascular endothelial growth factor A (*VEGFA*), and X-linked inhibitor of apoptosis protein (*XIAP*), which have been shown to be TTP targets in non-liver tissues (Wang et al., 2016; Selmi et al., 2015), was checked. To confirm the validity of these targets, we analysed the sequences of their 3'-UTRs and found TTP binding-motifs (Mukherjee et al., 2014) in abundance (Supplementary

Material 1). Interestingly, AREs could also be found in several yet unknown TTP targets, which had been suggested as tumour promoters and were therefore also analysed in TTP-overexpressing cells. One of them is the long transcript variant of nuclear enriched abundant transcript 1 (*NEAT1_v1/v2*), *NEAT1_v2* (Supplementary Material 1). *NEAT1* is one of the least stable long non-coding RNAs (Clark et al., 2012), a presumed tumour promoter, and associated with chemoresistance (Adriaens et al., 2016; Guo et al., 2015). Two other ARE containing genes represent RBPs themselves: the insulin-like growth factor 2 mRNA-binding protein 1 (*IGF2BP1*) and the insulin-like growth factor 2 mRNA-binding protein 3 (*IGF2BP3*) (Supplementary Material 1), which both promote hepatic tumour progression (Gutschner et al., 2014; Jeng et al., 2008). Last, the expression of the TTP antagonist HuR (*ELAVL1*) was determined (Wang et al., 2016). In HepG2 and Huh7 cells, all analysed genes tended to be lower expressed after TTP overexpression (Figure 7A, B). *MYC*, *IGF2BP3*, and *VEGFA* were significantly lowered in TTP-overexpressing HepG2 cells (Figure 7A). In TTP-overexpressing Huh7 cells, *IGF2BP1* was the only gene that was significantly decreased (Figure 7B). *BCL2*, *IGF2BP1*, *NEAT1_v2*, and *VEGFA* were significantly lowered in PLC/PRF/5 cells (Figure 7C). The expression of *HuR* showed no statistical difference in all three cell lines (Figure 7A-C). Since *VEGFA*, a promoter of invasion in HCC (Li et al., 1998), was lower expressed in HepG2 and PLC/PRF/5 cells, we hypothesised that TTP might play a role in HCC vascular invasion. Therefore, *TTP* expression was determined in human HCC samples showing vascular invasion compared to samples without vascular invasion. In fact, the results show a significantly decreased *TTP* expression in tissues showing vascular invasion (Figure 7D).

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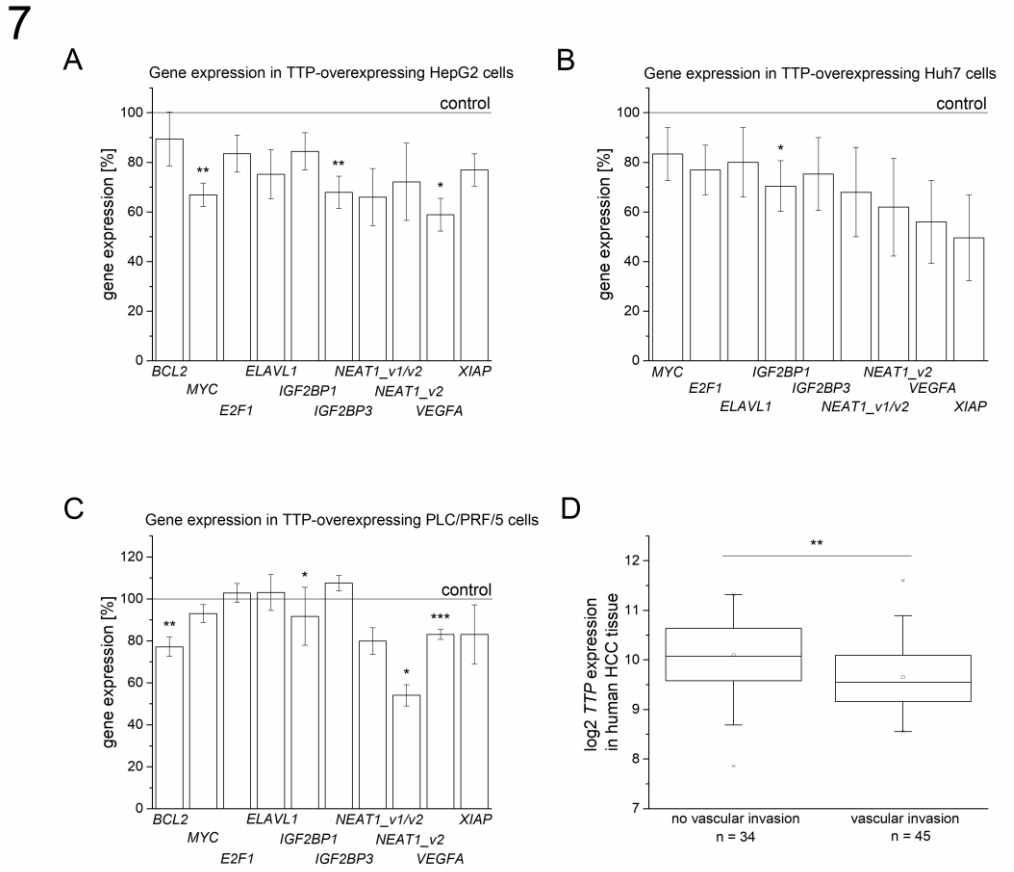


Figure 7. Oncogene expression in TTP-overexpressing hepatoma cells and *TTP* expression in invasive vs non-invasive HCC. Expression levels normalised to cells treated with a control vector were determined in HepG2 (A), Huh7 (B), and PLC/PRF/5 cells (C) by qPCR. *BCL2* was not determined in Huh7 cells since mRNA expression was below the detection limit. n = 3; triplicates. (D): *TTP* expression in human HCCs grouped into tumours positive (n = 45) or negative (n = 34) regarding vascular invasion (GSE20238). Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

3.4.7 TTP expression in hypomethylated hepatoma cells and promoter methylation

It has been supposed that *TTP* is regulated *via* methylation of its promoter and that treatment with the DNA methyltransferase inhibitor azacytidine increases *TTP* expression in Huh7 cells but does not affect *TTP* expression in HepG2 and PLC/PRF/5 cells (Sohn et al., 2010). To confirm these results, we treated these three cell lines with the more potent inhibitor decitabine (Hollenbach et al., 2010). As shown before, no significant effect after treatment could be seen in PLC/PRF/5 cells (Figure 8A). In Huh7 cells, *TTP* expression could be induced upon decitabine treatment (Figure 8A). An elevated expression of *TTP* at multiple concentrations

could also be observed in HepG2 cells (Figure 8A). Elevated levels of the epigenetically regulated lncRNA *H19* in all three cell lines served as a positive control (Supplementary Figure 4; Schultheiß et al., 2017).

Since differences in *TTP* expression regarding methylation could be observed in two of the three analysed hepatoma cell lines, a TCGA data set comprising 50 normal and 109 tumour samples was analysed comparing methylation levels of the *TTP* promoter in hepatic tumour and non-tumour tissue. The analysis of the TCGA data set, however, revealed no difference regarding the *TTP* promoter methylation between non-tumour and tumour tissue (p = 0.51) (Figure 8B).

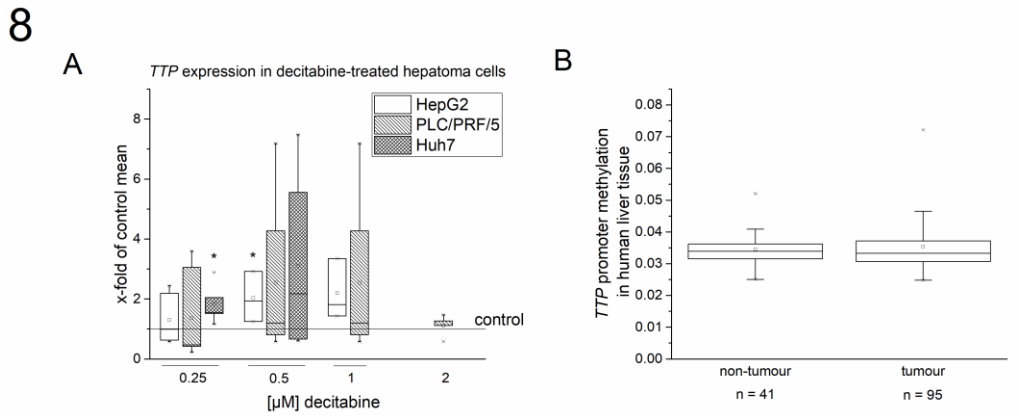


Figure 8: Effects of hypomethylation on *TTP* expression in hepatoma cells and TTP promoter methylation in liver tissue. (A): *TTP* mRNA expression levels in HepG2, PLC/PRF/5, and Huh7 cells treated with decitabine. The effect of decitabine was not determined in HepG2 cells at concentrations above 1 µM and in Huh7 cells at concentrations above 0.5 µM due to its toxicity. n ≥ 2, duplicates. (B) TTP promoter methylation in human liver tumour (n=95) and non-tumour (n=41) samples (TCGA). Statistical difference: * p < 0.05.

3.5 Discussion

It is known that TTP expression is frequently repressed in different human cancers (Hitti et al., 2016; Sanduja et al., 2012) and that a loss of functional TTP can modulate diverse tumourigenic phenotypes (Brennan et al., 2009). In this study, we were able to confirm a strong downregulation of *TTP* in HCC tissues in three large cohorts and in a smaller set of human liver tumour compared to adjacent non-tumour samples as previously suggested by others (Hitti et al., 2016). Therefore, we hypothesised that a hepatic knockout of *Ttp* might increase the amount of tumours in murine livers. However, *Ttp* knockout animals showed a lower number of tumours compared to the wild type animals.

Although total *Ttp* knockout mice show a severe inflammatory phenotype (Carbello et al., 1998), a hepatocyte-specific knockout of *Ttp* seems to have a rather inhibitory effect on inflammation as observed in the short-term mouse experiment, where the amount of macrophages was unaltered, whereas the monocytes / macrophages ratio of DEN-treated knockout mice was decreased compared to the DEN-treated wild type mice. A temporary increase of monocytes accompanied by a decrease of resident liver macrophages is well described in other models inducing hepatic inflammation (Blériot et al., 2015; Ramachandran et al., 2012; Zigmond et al., 2014). In the long-term experiment, the amount of macrophages and monocytes was decreased in the DEN-treated *Ttp* knockout mice compared to wild type mice. This may be explained by the presence of repopulating Kupffer cells of monocyte origin in the DEN-treated wild type mice following monocyte infiltration during acute inflammation (Blériot et al., 2015; Scott et al., 2016; Zigmond et al., 2014). DEN-treated *Ttp* knockout mice did not show such a compensatory effect because the amount of macrophages was probably not decreased in acute inflammation as indicated by the unaltered proportion of macrophages in DEN-treated *Ttp* knockout mice compared to sham-treated knockout mice in the short-term experiment. Although TTP knockdown has been shown to induce monocyte infiltration into 3D tumour spheroids and macrophage infiltration into xenograft-induced murine breast cancer (Milke et al., 2013), our data rather suggest that hepatocytic TTP promotes tumourigenesis by driving proinflammatory monocyte infiltration and thus inflammation.

Interestingly, the distinct decrease of *TTP* expression in tumour but not in cirrhotic tissue suggests *TTP* as a useful marker detecting the progression from cirrhotic state to malignancy.

An important role of TTP in hepatic tumour progression is supported by its decreased expression in vascularised HCC tissue. This and the fact that *VEGFA* – an important factor for vascularisation and reported target of TTP in colon cancer (Claesson-Welsh and Welsh, 2013; Wang et al., 2016) – was downregulated in TTP-overexpressing hepatoma cells, indicate that TTP might play a role in the vascularisation ability of liver cancer, which is a hallmark in the beginning of tumour progression (Hanahan and Weinberg, 2011).

It is well known that cell migration is a critical factor for cancer metastasis (Vicente-Manzanares and Horwitz, 2011), which may occur in the early stages of tumour progression (Balic et al., 2006; Li et al., 2007). TTP has been shown to inhibit the migration ability in prostate cancer, ovarian cancer, gastric cancer, and head and neck squamous cell carcinoma cells (Lee et al., 2014; van Tubergen et al., 2011; Wang et al., 2016; Yoon et al., 2016). Additionally, TTP was suggested to decrease the metastatic potential in breast cancer (Al-Souhibani et al., 2010). In this study, we were able to show that overexpression of TTP also inhibited the migration ability and proliferation in hepatoma cells. A negative effect of TTP on proliferation has been shown before in non-hepatic cells, such as gastric cancer, pancreatic cancer, melanoma and glioma cells (Wang et al., 2016), but – to our knowledge – we are the first to show these effects in hepatic cells. This inhibition of proliferation may be a major reason why a stable overexpression of the *TTP*-containing plasmid was not possible to establish in HepG2, Huh7, and PLC/PRF/5 cells.

TTP has been shown to downregulate several well-established markers for tumour progression like *BCL2*, *VEGFA*, and *MYC* in non-liver tissue (Wang et al., 2016). In line with these findings, we observed a decreased expression of *MYC* and *VEGFA* in HepG2 cells, and a decreased expression of *BCL2* and *VEGFA* in PLC/PRF/5, cells which were overexpressing TTP. Since HuR stabilises all of the above mentioned genes (Baou et al., 2011; Chai et al., 2016), an inhibition of HuR by TTP may result in the same effects. However, the unaltered expression of *HuR* in these cells suggests an *HuR*-independent mechanism. The different expression levels of the analysed genes comparing the three cell lines might be explained by the distinct heterogeneity of liver cancer itself (Li and Wang, 2016). According to this, the three analysed cell

lines also have rather different phenotypes (Ghasemi et al., 2013; Hsu et al., 1993; Kanno et al., 2015).

Chemoresistance is widespread in HCC and another important factor for tumour progression (Wörns et al., 2009). We hypothesised that chemoresistance may also be affected by TTP, since many of its downstream targets (e.g., *BCL2*, *VEGFA*, and *MYC*) are associated with a poor chemosensitivity (Wang et al., 2016). In addition, PLC/PRF/5 cells showed a decreased expression of the long transcript variant of the long-non coding RNA *NEAT1*, *NEAT1_v2*. Interestingly, *NEAT1* has been reported to enhance chemoresistance in different cancer cell lines, including hepatoma cells (Adriaens et al., 2016; unpublished data). Since there are only few studies addressing the role of *TTP* in chemoresistance (Wang et al., 2016), we analysed the effect of an approved drug for systemic liver cancer therapy, sorafenib, as well as the effect of doxorubicin, which is widely used in chemoembolization (Dhanasekaran et al., 2010; Wörns et al., 2009) on TTP-overexpressing hepatoma cells. Our data show that overexpression of TTP distinctly decreases the viability of hepatic Huh7 cells after doxorubicin or sorafenib treatment, but alters chemosensitivity in HepG2 and PLC/PRF/5 cells to a lower extent. The unaltered expression of TTP in EpCAM positive – a marker for HCC chemoresistance (Noda et al., 2009) – compared to EpCAM negative HCC tissues supports a less important role of TTP in HCC chemoresistance.

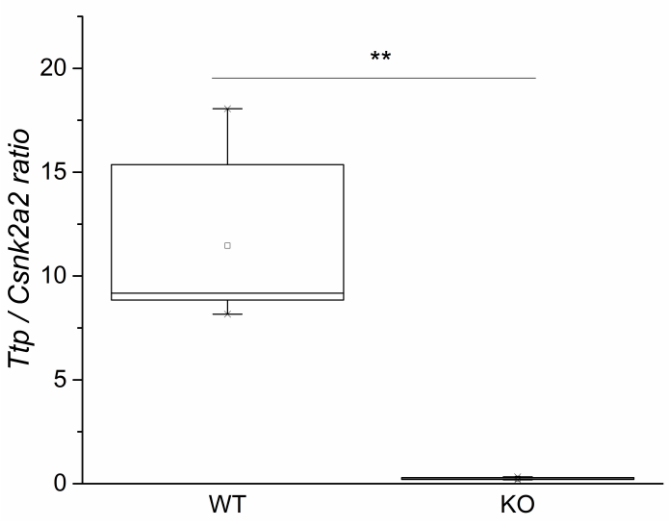
Regarding the regulation of TTP in HCC, previous findings suggested that methylation of the *TTP* promoter is a key factor in its downregulation in hepatoma cells (Sohn et al., 2010; Tran et al., 2016). In line with previous findings, we could detect an increased *TTP* expression in Huh7 and an unaltered *TTP* expression in PLC/PRF/5 cells treated with decitabine (Sohn et al., 2010; Tran et al., 2016). In contrast to Sohn's results, we were also able to show an increased TTP expression in the hypomethylated HepG2 cells, which may be explained by the higher potency of decitabine compared to azacytidine (Hollenbach et al., 2010). This assumption is supported by the increased expression of H19 in decitabine-treated PLC/PRF/5 cells, which was not observed in the azacytidine-treated PLC/PRF/5 cells (Schultheiß et al., 2017). Sohn et al. (2010) also described an increased methylation at a single CpG site in HCC samples compared to non-tumour samples (n =24). Still, our analysis of *TTP* methylation in the whole promoter region of human liver tissue showed no difference between non-tumour (n = 41) and tumour tissue (n = 95). Interestingly,

another study shows that TTP expression is regulated by histone deacetylases (HDACs) and not by DNA methylation in colon cancer cells (Sobolewski et al., 2015). HDACs promote transcriptional repression and have been associated with silencing of many tumour suppressor genes, since many HDACs are overexpressed in cancer (Sobolewski et al., 2015). Therefore, also in the liver *TTP* expression might not only be influenced by its promoter methylation but by HDACs.

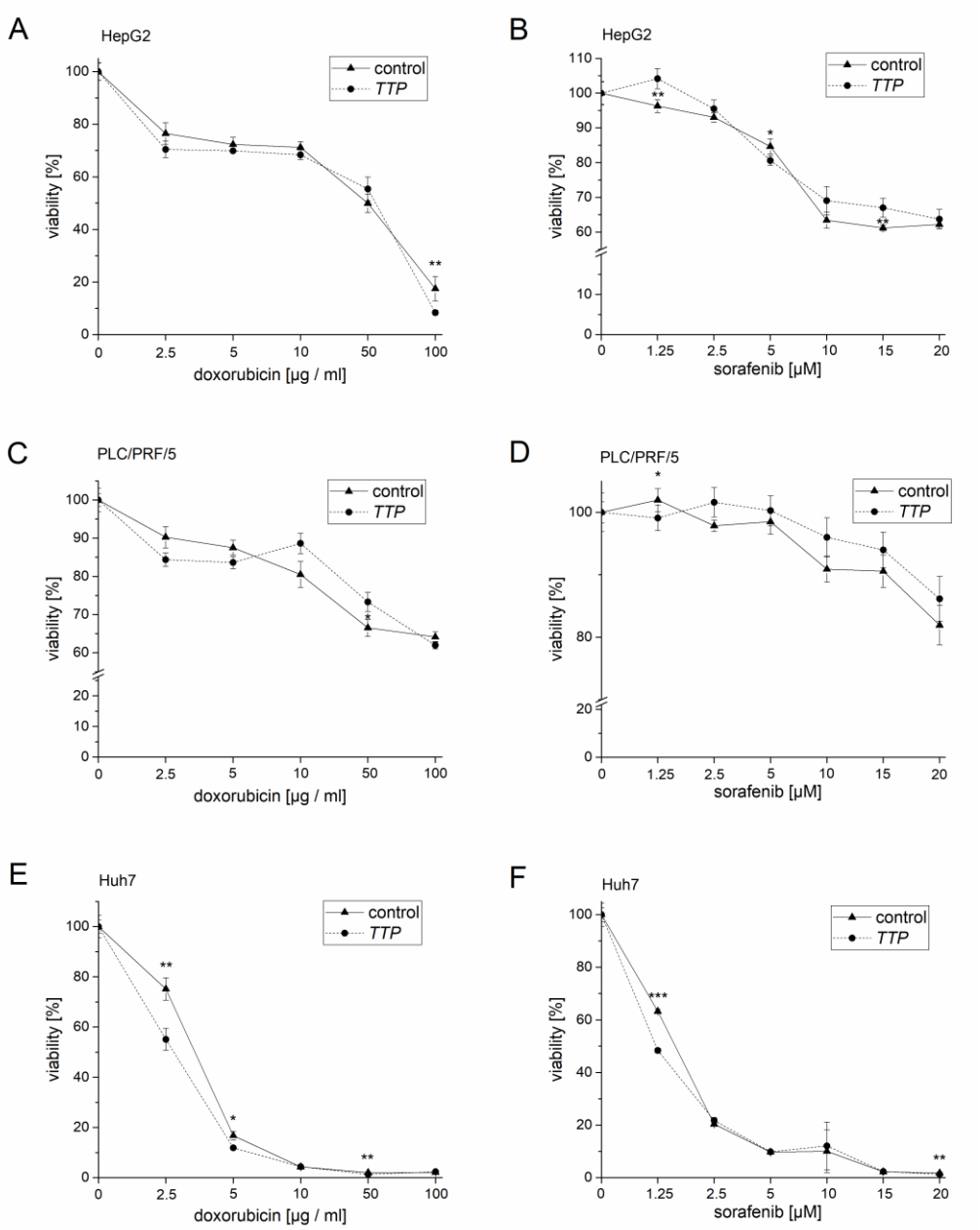
3.6 Conclusion

Our data suggest that hepatocytic TTP plays an inflammation-dependent role in promoting hepatic tumour initiation but has a major negative effect on hepatic tumour progression. In addition, *TTP* may be a useful marker detecting the progression from cirrhosis towards HCC.

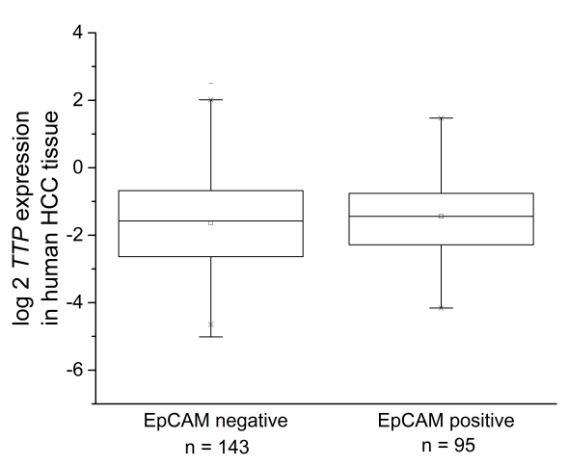
3.7 Supplementary data



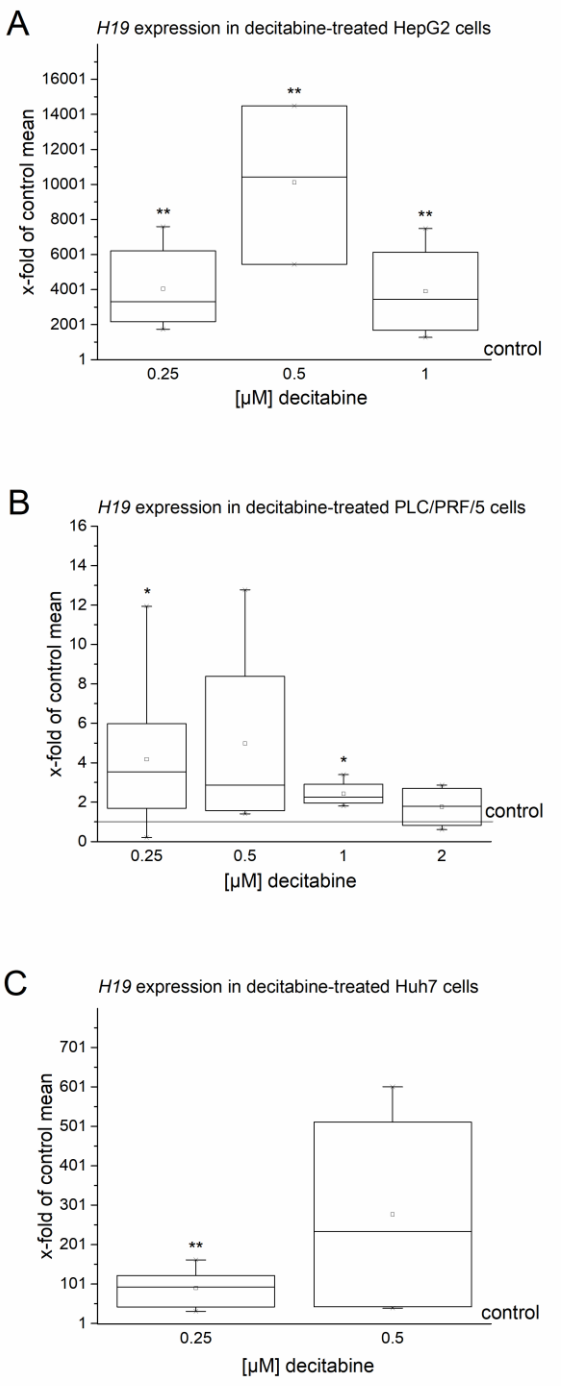
Supplementary Figure 1: *Ttp* mRNA levels in *Ttp* knockout and wild type animals. *Ttp* / *Csnk2a2* mRNA ratio in wild type (WT) and knockout (KO) animals injected with NaCl. n = 6. Statistical difference: ** $p \leq 0.01$.



Supplementary Figure 2. Effects of TTP overexpression on chemoresistance in hepatoma cells (normalised to control vector). Cells were transfected with either a *TTP* or a control vector. 24 h after transfection, cells were treated with different concentrations of doxorubicin (0 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml) or sorafenib (0 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 15 µM, 20 µM). Cell viability was determined *via* MTT assay. Both groups (*TTP* and control vector) are normalised to the viability of the control vector transfected cells without addition of doxorubicin or sorafenib (=100%). (A): HepG2 cells treated with doxorubicin. (B): HepG2 cells treated with sorafenib. (C): Huh7 cells treated with doxorubicin. (D): Huh7 cells treated with sorafenib. (E): PLC/PRF/5 cells treated with doxorubicin. (F): PLC/PRF/5 cells treated with sorafenib. n = 3; quadruplicates. Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.



Supplementary Figure 3. TTP expression in EpCAM-positive (n = 95) and EpCAM-negative (n = 143) human tumour and non-tumour liver tissue (GSE5975).



Supplementary Figure 4: Effects of hypomethylation on *H19* expression in hepatoma cells. *H19* mRNA expression levels in HepG2 (A), PLC/PRF/5 (B), and Huh7 (C) cells treated with decitabine. The effect of decitabine was not determined in HepG2 cells at concentrations above 1 µM and in Huh7 cells at concentrations above 0.5 µM due to its toxicity. n ≥ 2, duplicates. Statistical difference: * $p < 0.05$, ** $p < 0.01$.

Supplementary Material 1: ARE in TTP target genes.

The following motifs are enriched in TTP targets (Mukherjee et al., 2014): ATTTA (blue), TTTTT (yellow), **CTTTT (fat)**, TTTTC (bordeaux), CTTTC (purple), TATTTATT (green), TTATTTATT (underlined) and TATT (orange).

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MYC variant 1 (NM_002467.5)

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VEGFA variant 1 (NM_001171623.1)

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IGF2BP1 variant 1 (NM_006546.3)

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IGF2BP3 (NM_006547.2)

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NEAT1 variant 2 (NR_131012.1)

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4 Hepatocellular carcinoma and nuclear paraspeckles: induction in chemoresistance and prediction for poor survival

4.1 Abstract

Hepatocellular carcinoma (HCC) represents the second most common cause of cancer-related death worldwide, not least due to its high chemoresistance. The long non-coding RNA nuclear paraspeckle assembly transcript 1 (*NEAT1*), localised in nuclear paraspeckles, has been shown to enhance chemoresistance in several cancer cells. Since data on *NEAT1* in HCC chemosensitivity are completely lacking and chemoresistance is linked to poor prognosis, we aimed to study *NEAT1* expression in HCC chemoresistance and its link to HCC prognosis.

NEAT1 expression was determined in either sensitive, sorafenib, or doxorubicin resistant HepG2, PLC/PRF/5, and Huh7 cells by qPCR. Paraspeckles were detected by immunofluorescence staining of paraspeckle component 1 (PSPC1). The expression of transcript variants of *NEAT1* and of the paraspeckle-associated proteins *PSPC1*, non-POU domain containing octamer binding protein (*NONO*), and RNA-binding motif protein 14 (*RBM14*) was analysed in the TCGA liver tissue data set.

NEAT1 was overexpressed in all three sorafenib and doxorubicin resistant cell lines. Paraspeckles were present in all chemoresistant cells, whereas no signal was detected in the sensitive cells. *NEAT1* as well as transcripts encoding *PSPC1*, *NONO*, and *RBM14* were increased in tumour tissue. *PSPC1*, *NONO*, and *RBM14* transcripts highly correlated with poor survival, whereas *NEAT1* did not.

Our data show an induction of *NEAT1* in HCC chemoresistance and a high correlation of transcripts encoding paraspeckle-associated proteins with poor survival in HCC. Therefore, *NEAT1, PSPC1, NONO,* and *RBM14* might be promising targets for novel HCC therapies, and the paraspeckle-associated proteins might be clinical markers and predictors for poor survival in HCC.

4.2 Introduction

Liver cancer – with its predominant form hepatocellular carcinoma (HCC) – represents the second most common cause of cancer-related death worldwide (Bruix et al., 2015; Tang et al., 2017). Although improvements in the survival of patients with HCC have been achieved in the past two decades, the prognosis of HCC remains one of the worst amongst all cancers (De Angelis et al., 2014; Tang et al., 2017). One major issue regarding the therapy of HCC is its chemoresistance: no effective conventional systemic chemotherapy for patients with advanced hepatocellular carcinoma has been established until now, resulting in poor prognosis of these patients (Yau et al., 2008). The response rate for sorafenib, the only approved drug besides regorafenib for systemic treatment in the late stage of the disease, is below 3.5%, and a systemic combination of sorafenib and doxorubicin also leads to poor response rates up to a maximum of 6% (Wörns et al., 2009). Chemoresistance is closely linked to a poor prognosis, not only in HCC, but also in other cancer types (Cheng et al., 2016; Hamilton and Rath, 2014; Jiao and Nan, 2012).

It is known that only 2% of the genome encodes for proteins, whereas the considerably larger part of the transcribed human genome consists of non-coding sequences (Consortium IHGS, 2004). One of these sequences is the long non-coding RNA nuclear paraspeckle assembly transcript 1 (*NEAT1*). *NEAT1* is located in nuclear paraspeckles (Mao et al., 2011), which are found in the nucleus' interchromatin space (Clemson et al., 2009; Souquere et al., 2010). The two transcript variants of *NEAT1* described in the literature are the short *NEAT1_v1* (*NEAT1ε*, 3.7 kb in length) and the long *NEAT1_v2* (*NEAT1-202*, *NEAT1β*, 23 kb in length). Ensembl lists four additional transcript variants (*NEAT1-201*, *203 – 205*) (Figure 1). Although *NEAT1_v1* and *NEAT1_v2* transcript variants are essential for the integrity of paraspeckles, the long transcript variant is more important for *de novo* paraspeckle assembly (Naganuma et al., 2012). The biological role of paraspeckles is widely unknown, not least due to the lack of an altered phenotype in *Neat1* knock-out mice (Nakagawa and Hirose, 2012). Recently, it has been suggested that *NEAT1* enhances chemoresistance in several cancer cells (Adriaens et al., 2016). Since data on *NEAT1* in HCC chemosensitivity are completely lacking, we aimed to study *NEAT1* expression as well as *NEAT1*-dependent paraspeckle formation in HCC and in chemoresistant compared to chemosensitive HCC cell lines.

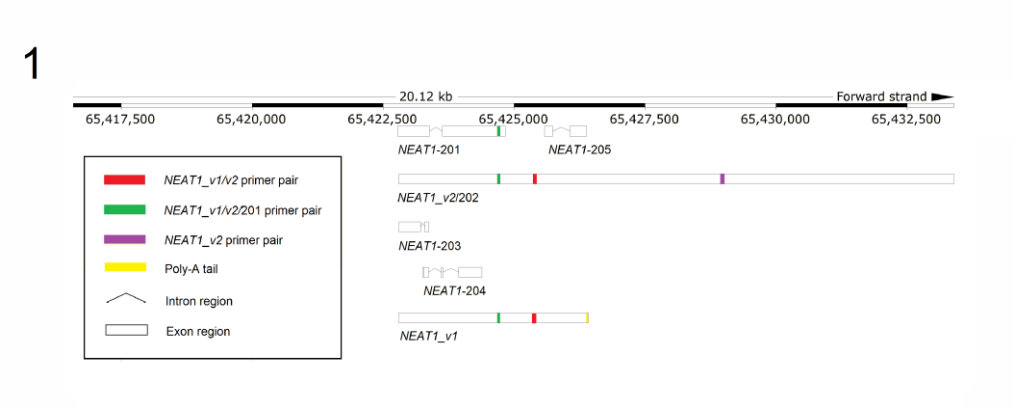

Figure 1. *NEAT1* gene locus. Size and location of *NEAT1* transcript variants on the *NEAT1* gene locus on chromosome 11 (schematic). Coloured areas indicate regions amplified in the respective qPCR reaction. Please note that the scheme is not drawn in scale.

With the recently described effect of increased HCC cell proliferation induced by *NEAT1_v1/v2* in HCC cells (Fang et al., 2017; Liu et al., 2017), we also sought to determine whether its expression might serve as a prognostic clinical marker.

4.3 Materials & Methods

4.3.1 TCGA data

RNAseq expression data were obtained from The Cancer Genome Atlas pan cancer dataset produced *via* Toil (Vivian et al., 2017). RSEM (Li and Dewey, 2011) reported transcripts per million values were downloaded *via* the UCSC Xena Browser (https://xenabrowser.net) and comprised 369 primary solid tumour as well as 50 matched non-tumour tissue samples for gene expression (see also Supplementary Table 1 for *NEAT1* transcript variants). Only transcript variants with an average expression rate of log2(TPM + 0.001) > 0 were considered for analysis in the R statistical environment (v. 3.4.2). For survival analysis we considered two groups (split at 50% quantile) and three groups (split at 25% and 75% quantiles), respectively. Significance of differences between survival curves was computed with a log rank test using the survival R package. Further, we determined pairwise Pearson correlation of transcript expression and plotted them using the corrplot R package. These data were kindly compiled and analysed by Dr. Markus List and Dr. Marcel H. Schulz (Department of Computational Biology and Applied Algorithmics, Max Planck Institute for Informatics, Saarbrücken, Germany).

4.3.2 Cell culture

HepG2, PLC/PRF/5, and Huh7 cells were cultured in RPMI-1640 medium with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glutamine (Sigma-Aldrich, Taufkirchen, Germany) at 37°C and 5% CO_2. To induce and maintain chemoresistance, cells were regularly treated with either doxorubicin (Sigma-Aldrich) or sorafenib (Biomol, Hamburg, Germany) as previously described (Schultheiß et al., 2017). Chemoresistance was regularly confirmed *via* IC50 determination by MTT assay. All cell lines were tested regularly for mycoplasma contamination and found negative. All cell lines were authenticated by the DSMZ (Braunschweig, Germany). The cells were cultured and treated in collaboration with Mrs Christina S. Hubig (Department of Pharmaceutical Biology, Saarland University, Saarbrücken, Germany).

4.3.3 RNA isolation and qPCR

Total RNA was extracted using Qiazol lysis reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Residual genomic DNA was removed by treatment with DNase I (Ambion / Invitrogen, Carlsbad, California, USA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) as recommended by the supplier. Real-time quantitative polymerase chain reaction (qPCR) was performed in a CFX96 cycler (Bio-Rad, München, Germany) with 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). All samples were estimated in triplicate. The following primers were used (see also Figure 1): *NEAT1_v1/v2* forward: TGCTACAAGGTGGGGAAGACTG; *NEAT1_v1/v2* reverse: CCCACACCCCAAACAAAACAA; *NEAT1_v1/201/v2* forward: CCCCTTCTTCCTCCCTTTAAC; *NEAT1_v1/201/v2* reverse: CCTCTCTTCCTCCACCATTAC; *NEAT1_v2* forward: TTTCAAAGGGAGCAGCAAGGG; *NEAT1_v2* reverse: ACGGCACAGGCAAATAAGACAC. Annealing temperature was 60°C for *NEAT1_v1/v2* and *NEAT1_v1/201/v2,* and 64°C for *NEAT1_v2*. Primer concentrations for all three were 0.25 µM. Efficiency for each experiment was determined using a standard dilution series. Standards from 10 to 0.0001 amol of the PCR product cloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin, USA), were run alongside the samples to generate a standard curve. The absolute gene expression was normalised to *ACTB* mRNA values.

4.3.4 Immunofluorescence

Cells were grown on coverslips overnight and fixed with 4% paraformaldehyde for 15 min on ice. After permeabilisation with 1% Triton X 100 for 15 min, unspecific binding was blocked using a combination of 2% bovine serum albumin and 10% FCS for 1.5 h. The cells were incubated with paraspeckle component 1 (PSPC1) antibody (1:20 dilution; sc-374181, Santa Cruz, CA, USASA) overnight at 4°C. After washing, the secondary antibody, goat anti-mouse AlexaFluor 488 (Invitrogen, Karlsruhe, Germany), was added for 1.5 h at room temperature. After washing and adding DAPI (Sigma-Aldrich) for nuclear staining, coverslips were mounted with FluorSave™ (Calbiochem, Sandhausen, Germany). Images were obtained and analysed with an

Axio Observer Z1 epifluorescence microscope equipped with an AxioCam Mrm (Zeiss, Oberkochen, Germany). All cell images were obtained using either a 63x objective (for Huh7 and PLC/PRF/5 cells), or a 100x objective (for HepG2 cells). Data were obtained and analysed using the AxioVision software (Zeiss).

4.3.5 Statistical analysis

Data analysis and statistics were performed with OriginPro 8.6G (OriginLab Corporation, Northampton, USA). Values were expressed as box plots with 25th/75th percentile boxes, geometric medians (line), means (square), and 10th/90th percentile as whiskers. Statistical differences were calculated using an independent two-sample t-test or Mann-Whitney test as indicated depending on whether the data were normally distributed.

4.4 Results

To determine a possible role of *NEAT1* in HCC chemoresistance, we established human hepatoma cells (HepG2, PLC/PRF/5 and Huh7) resistant to the chemotherapeutics sorafenib or doxorubicin (Schultheiß et al., 2017) and checked *NEAT1_v1/v2, NEAT1_v1/201/v2*, and *NEAT1_v2* expression in these cells. *NEAT1_v1/v2* was significantly overexpressed in all three sorafenib and doxorubicin resistant cell lines (Figure 2A, B). In addition, sorafenib resistant HepG2 and Huh7 cells as well as doxorubicin resistant PLC/PRF/5 and Huh7 cells showed an increased expression of *NEAT1_v1/201/v2* and *NEAT1_v2* (Figure 2A, B).

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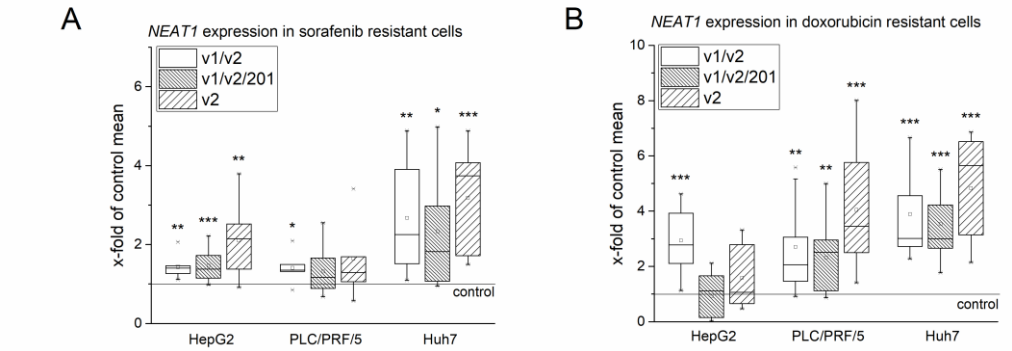


Figure 2. *NEAT1* expression in chemoresistant hepatoma cell lines. *NEAT1_v1/v2*, *NEAT1_v1/v2/201*, and *NEAT1_v2* expression determined by qPCR in sorafenib resistant (A) and doxorubicin resistant (B) cells (n = 3, triplicates). Statistical difference: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

NEAT1_v2 was found to be important for *de novo* paraspeckle assembly (Naganuma et al., 2012). Due to overexpression of *NEAT1_v2,* we speculated that the chemoresistant hepatoma cell lines should have an increased paraspeckle formation. Therefore, cells were stained for the paraspeckle specific protein paraspeckle component 1 (PSPC1). As expected, positive signals were detected in all chemoresistant cells, whereas no signal could be detected in the control cells (Figure 3). The most distinct paraspeckle staining was observed in Huh7 cells, which showed the highest *NEAT1_v2* expression (Figure 2A, B). Our data suggest that doxorubicin resistance induces paraspeckle formation slightly more intensely compared to sorafenib resistance, which is in accordance with the higher *NEAT1_v2* RNA expression in doxorubicin resistant PLC/PRF/5 and Huh7 cells (Figure 2B, 3). In

addition, doxorubicin resistant HepG2 cells tended to have fewer PSPC1-positive cells than sorafenib resistant HepG2 cells. PLC/PRF/5 and Huh7 cells showed no differences regarding this aspect (Figure 3).

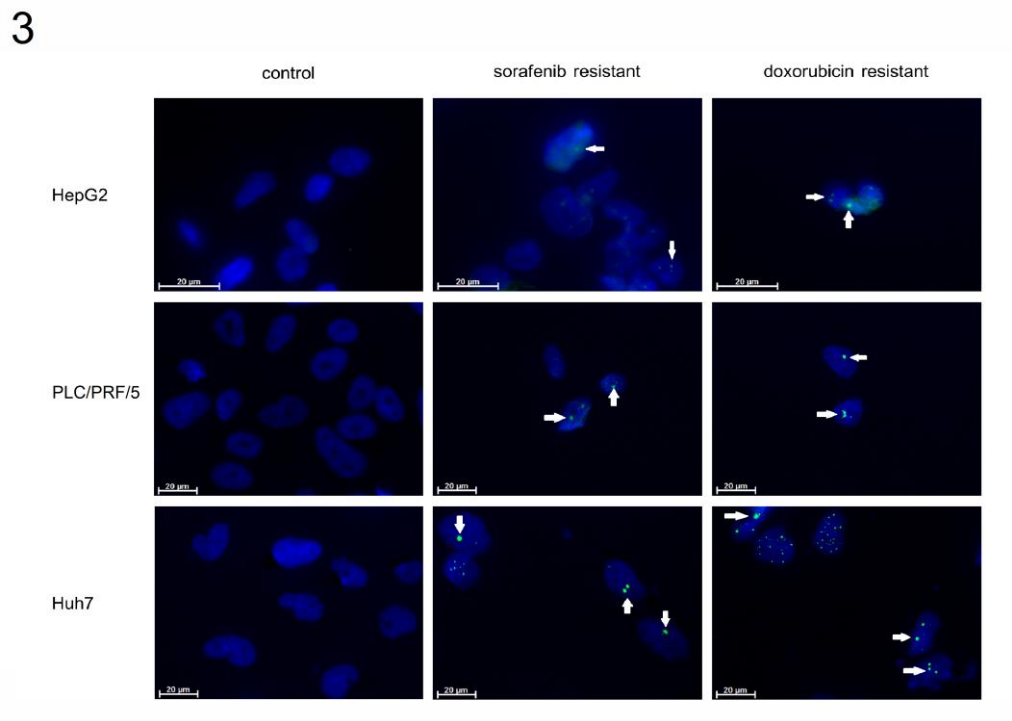


Figure 3. Paraspeckle formation in chemoresistant human hepatoma cells. Figure shows a representative image of doxorubicin and sorafenib resistant HepG2, PLC/PRF/5 and Huh7 cells. DAPI: blue, PSPC1: green.

Our data show a distinct connection of chemoresistance, *NEAT1* induction, and paraspeckle formation in three different HCC cell lines. Accordingly, two recently published papers described that *NEAT1_v1/v2* knockdown resulted in elevated apoptosis and reduced viability / proliferation in different HCC cell lines (Fang et al. 2017; Liu et al., 2017). Since our data indicate that chemosensitive cell lines do not form paraspeckles, one might speculate that *NEAT1* might also act independently of paraspeckles. Since previous studies had reported elevated *NEAT1* in HCC in samples from up to 95 patients (Guo et al., 2015; Wang et al., 2017), we hypothesised that *NEAT1* expression might serve as a prognostic marker for HCC patients.

To test this hypothesis, we analysed the expression of total *NEAT1* (including its transcript variants *201-205*) as well as its single transcript variants (Figure 1) in the TCGA dataset comprising 369 HCC tissues and 50 non-tumour tissues. We could confirm that the expression of total *NEAT1* was significantly increased in the tumour tissues as were the transcript variants *NEAT1-201, NEAT1-202,* and *NEAT1-205*, while the expression of *NEAT1-203* and *NEAT1-204* was below the threshold (Figure 4A; Supplementary Table 2).

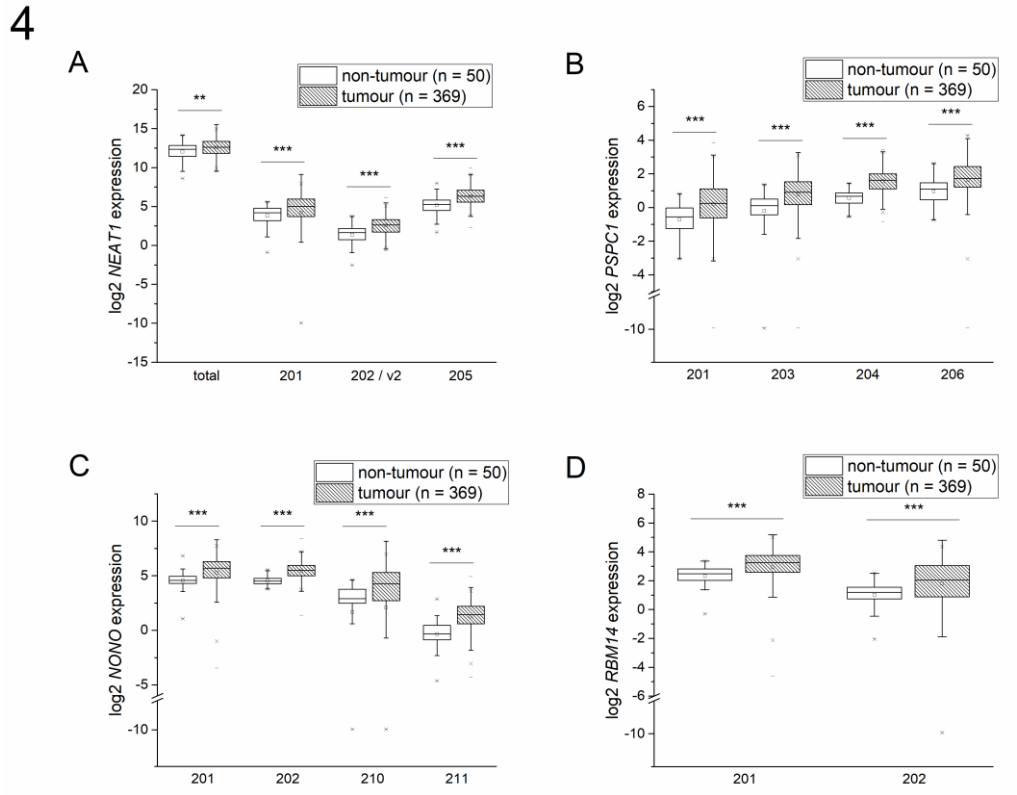


Figure 4. *NEAT1*, *PSPC1*, *NONO*, and *RBM14* expression in human liver tissue. Expression of total *NEAT1* and its transcript variants (A) and the transcript variants of *PSPC1* (B), *NONO* (C), and *RBM14* (D) in human tumour and non-tumour tissue from a TCGA data set. The figure only shows the transcript variants with an average expression rate of log2(TPM + 0.001) > 0. Statistical difference: **: $p \leq 0.01$; ***: $p \leq 0.001$.

Interestingly, though, there was no significant correlation with survival for any of the differentially expressed *NEAT1* transcripts (Table 1; Supplementary Figure 1).

gene	transcript variant	p-value ≤ 50% vs > 50% quantile	p-value > 25% & ≤ 75% vs ≤ 25% vs > 75% quantile
NEAT1	201	0.1	0.25
	202	0.53	0.19
	205	0.88	0.25
PSPC1	201	0.4	0.4
	203	**0.0022**	**0.0092**
	204	0.075	0.16
	206	**0.026**	0.071
NONO	201	0.055	0.099
	202	**0.00019**	**0.00072**
	210	0.065	0.18
	211	**0.022**	0.068
RBM14	201	**0.0025**	**0.007**
	202	0.16	0.2

Table 1. Survival analysis of *NEAT1*, *PSPC1*, *NONO*, and *RBM14* expression levels. Results of survival / time ratio depending on the expression levels (comparing two or three groups) of the transcript variants of *NEAT1*, *PSPC1*, *NONO*, and *RBM14* with an average expression rate of log2(TPM + 0.001) > 0. p-values ≤ 0.05 were considered as significant and are printed in bold letters.

With *NEAT1* representing a critical regulator and component of paraspeckles, we wondered whether the expression of genes encoding paraspeckle proteins was upregulated in HCC. For that, we analysed the expression of transcripts encoding *PSPC1*, non-POU domain containing octamer binding protein (*NONO*), and RNA-binding motif protein 14 (*RBM14*) in the TCGA samples. All transcripts described in Ensembl release 91 (Zerbino et al., 2017) were analysed, whereby only transcripts reaching the threshold mean expression of log2(TPM + 0.001) > 0 were taken into consideration (Supplementary Table 2).

We found that the expression of transcripts encoding for all three paraspeckle proteins was significantly elevated in HCC. For *PSPC1*, this was true for both protein coding transcripts *201* and *206* as well as for the non-coding transcripts *203* and *204* (Figure 4B). Additionally, the expression of the protein coding *NONO* transcripts *201* and *202* as well as the non-coding processed transcripts *210* and *211* were significantly elevated in HCC (Figure 4C). For *RBM14*, the two protein coding transcripts *201* and *202* were significantly elevated (Figure 4D). Most interestingly, there was a significant association with poor prognosis for transcripts encoding for all three paraspeckle proteins (Table 1; Supplementary Figure 2-4)

Except for *RBM14-202*, expression levels of all protein-coding and non-coding paraspeckle-associated transcripts showed distinct correlations with each other (Figure 5).

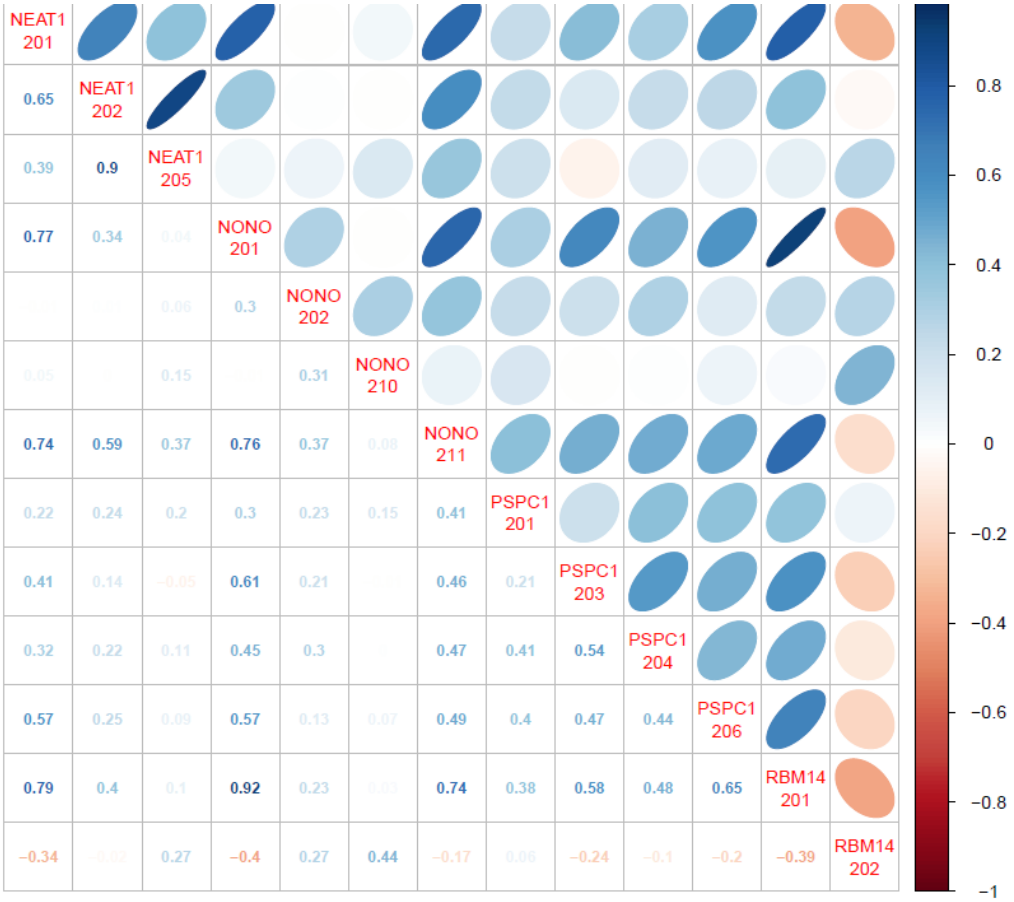


Figure 5. Pairwise correlation of transcript variants with an average expression rate of log2(TPM + 0.001) > 0 of *NEAT1*, *PSPC1*, *NONO*, and *RBM14*. The lower triangle indicates correlation coefficients numerically, whereas the upper triangle depicts them as ellipsoids. Blue indicates positive and red negative correlation as reflected by the colour legend on the right-hand side.

4.5 Discussion

An implication of *NEAT1* in the process of chemoresistance was previously described in human breast cancer MCF-7, neuroblastoma NGP, colon carcinoma HCT116, and osteosarcoma U2OS cells (Adriaens et al., 2016). Our study is the first to implicate *NEAT1* in HCC chemoresistance. We found the variants *NEAT1_v1* and *NEAT1_v2* to be significantly overexpressed in HepG2, PLC/PRF/5, and Huh7 cells that have developed resistance against either sorafenib or doxorubicin.

Although it is known that *NEAT1* plays an important role in paraspeckle formation (Naganuma et al., 2012), the complete function of paraspeckles is as yet unknown. However, it is assumed that they locate proteins inside the nucleus and are implicated in the reprogramming of a cell that takes place with differentiation. This may happen due to the inhibition of the expression of key proteins *via* nuclear RNA retention (Fox and Lamond, 2010). Paraspeckles were also reported to contribute to tumourigenesis by inhibiting DNA damage-induced cell death (Gao et al., 2014). Although paraspeckles have been described in diverse cell lines (Nakagawa and Hirose, 2012), to our knowledge, our report is the first one showing paraspeckles and their induction in liver cells. *NEAT1_v2* – which constitutes paraspeckles – was elevated in nearly all of the analysed chemoresistant hepatoma cells. For cell lines showing induction of *NEAT1_v2* but not of *NEAT1_v2* and *NEAT1_v1/201/v2* RNA, an elevated expression of *NEAT1_v1* seems probable. However, this assumption cannot be confirmed definitely due to the lack of a unique sequence of *NEAT1_v1* (Figure 1). Although *NEAT1_v1* cannot induce nuclear body formation by itself (Naganuma et al., 2012), it was suggested to increase the number of paraspeckles (Clemson et al., 2009). Another hypothesis is that *NEAT1_v1* localises in non-paraspeckle foci (so called 'microspeckles'), which may carry paraspeckle-independent functions (Gao et al., 2014). Our findings indicate an elevated expression of *NEAT1_v1* in doxorubicin resistant HepG2 cells compared to sorafenib resistant HepG2 cells. Since paraspeckles were also detected in doxorubicin resistant HepG2 cells, which showed no increased expression of *NEAT1_v2*, a positive association between *NEAT1_v1* and the number of paraspeckles seems to be likely. These results are consistent with previous findings on a link between *NEAT1_v1/v2* and *NEAT1_v2* expression and chemoresistance in several other

tumour types (Adriaens et al., 2016) and extend them towards chemoresistance in HCC.

Despite the effect of *NEAT1* on chemoresistance, it was recently reported that knockdown of *NEAT1_v1/v2* increased apoptosis and reduced both viability and proliferation in different HCC cell lines (Fang et al. 2017; Liu et al., 2017). Since we could not detect any paraspeckles in untreated, chemosensitive HCC cells, *NEAT1_v1/v2* may also have additional, paraspeckle-independent functions related to cell viability.

NEAT1 expression has been shown to be upregulated in different human malignancies, including lung cancer, colorectal cancer, prostate cancer, breast cancer, and HCC (Yu et al., 2017). However, the differentiation between the transcript variants of *NEAT1* to our knowledge was never reported. We therefore decided to analyse the expression of the transcript variants of *NEAT1* in human HCC tissue and were able to demonstrate that the transcript variants *NEAT1-201*, *NEAT1-202,* and *NEAT1-205* are elevated in HCC tissue in several hundred tumour samples compared to normal tissues. We were also able to confirm previous findings, in which an increased expression of *NEAT1_v1/v2* for up to 95 HCC *vs*. non-cancerous liver tissues – as assessed by qPCR – was reported (Guo et al., 2015; Wang et al., 2017). Although *NEAT1_v1/v2* has been reported to be associated with poor survival prognosis in breast cancer, oesophageal squamous cell carcinoma, and HCC (Chen et al., 2015; Choudhry et al., 2015; Liu et al., 2017), we could not confirm a significant association of the expression of *NEAT1* transcripts with survival in HCC in this study.

Although the elevated expression of *NEAT1* transcripts suggests *NEAT1* as a clinical marker for HCC, there is one major issue regarding this aspect: the stability of *NEAT1*. In fact, a study performing a genome-wide analysis of long non-coding RNA stability found *Neat1* to be “one of the least stable lncRNAs” (Clark et al., 2012). Hence, expression analysis of *NEAT1* in clinical practice may lead to incorrect results, while PSPC1 as a marker for *NEAT1*-dependent paraspeckle formation (Naganuma et al., 2012) may be a more valid marker for HCC prognosis.

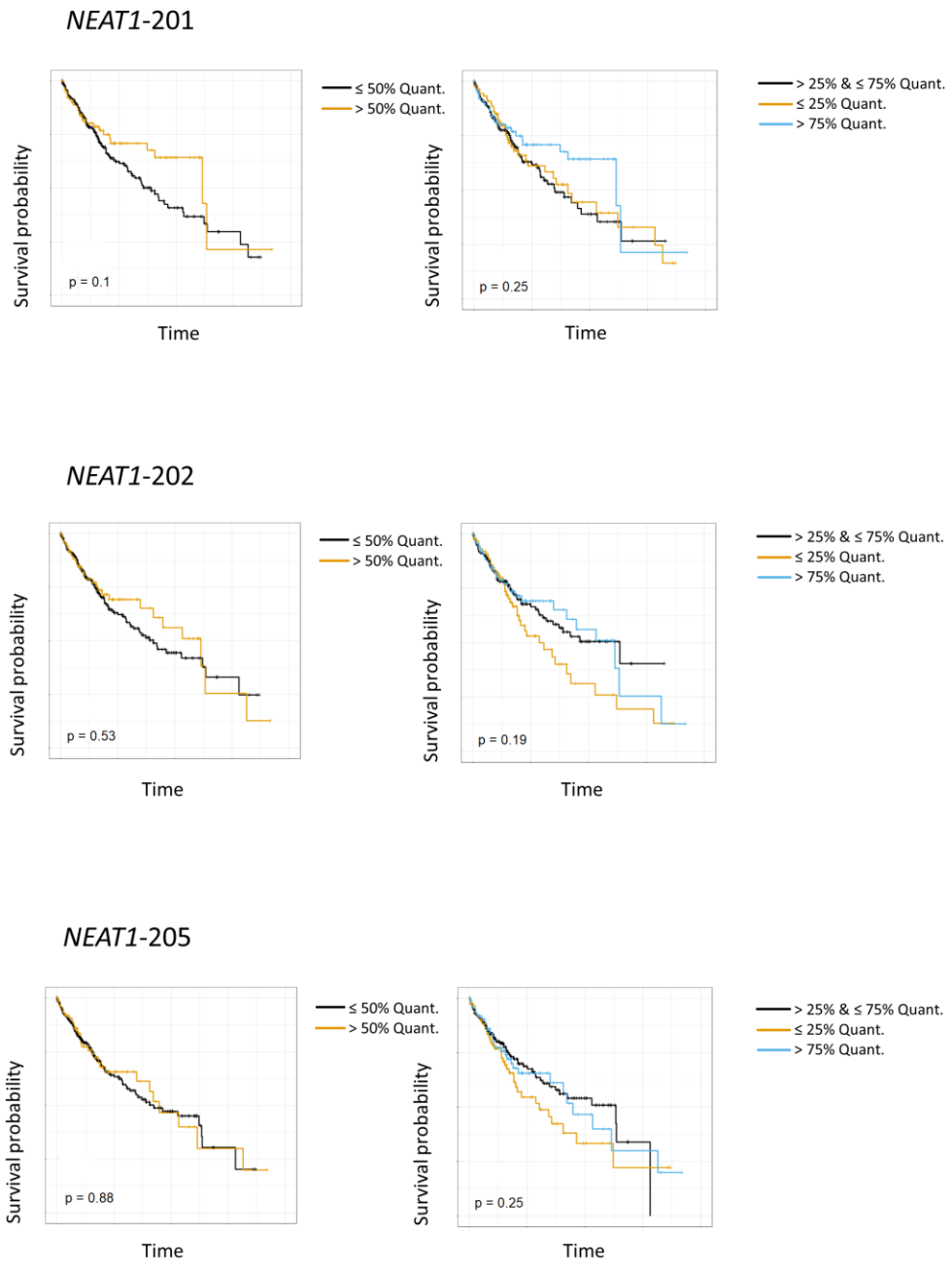
In addition to *NEAT1*, we also found transcripts of the paraspeckle-associated proteins *PSPC1*, *NONO*, and *RBM14* to be upregulated in HCC compared to non-cancerous liver tissue. PSPC1 has not only been shown to be upregulated in colorectal carcinoma (Albrethsen et al., 2010), but is also involved in the acquisition

of chemoresistance in HeLa cells (Gao et al., 2014). Another paraspeckle related protein, NONO, was reported to promote tumour growth in breast cancer (Zhu et al., 2016) and to be associated with chemoresistance in colorectal carcinoma cells (Tsofack et al., 2011). The expression of *RBM14* is upregulated in non-small cell lung carcinoma, lymphoma, pancreatic cancer and ovarian cancer (Sui et al., 2007). In addition, *RBM14* contributes to glioblastoma multiforme resistance (Kai 2016). Due to the as yet not reported upregulation of several transcripts of *PSPC1*, *NONO*, and *RBM14* in HCC compared to non-tumour tissue, we suggest them also as markers for HCC. Since at least one transcript of each of these proteins was associated with poor survival prognosis in HCC, they may also serve as prognostic markers in HCC.

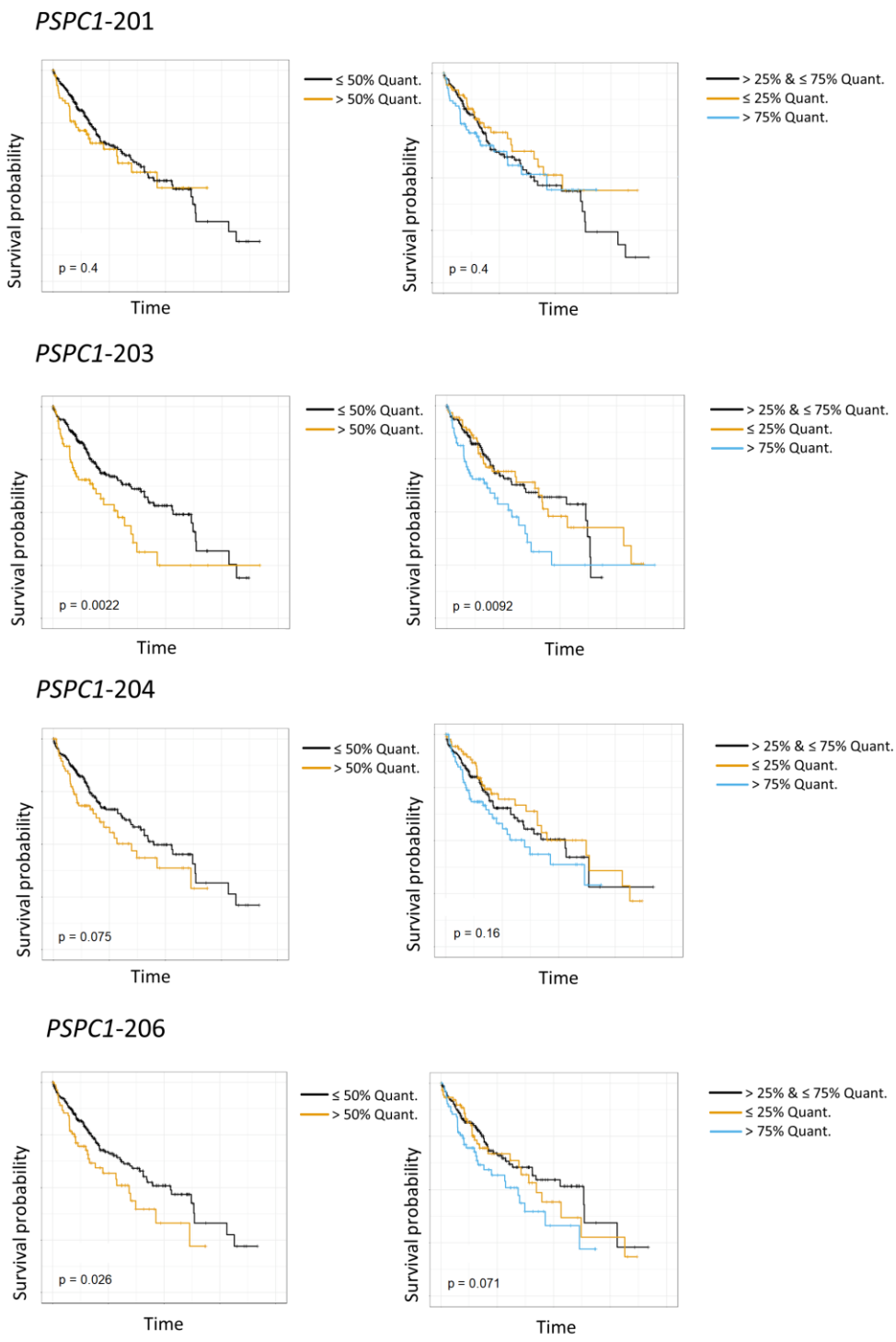
4.6 Conclusion

In conclusion, a detailed functional analysis of paraspeckle function in HCC might reveal *NEAT1* and paraspeckle-related proteins as promising targets for the development of novel HCC therapies, and *PSPC1*, *NONO*, and *RBM14* as clinical prognostic markers for HCC.

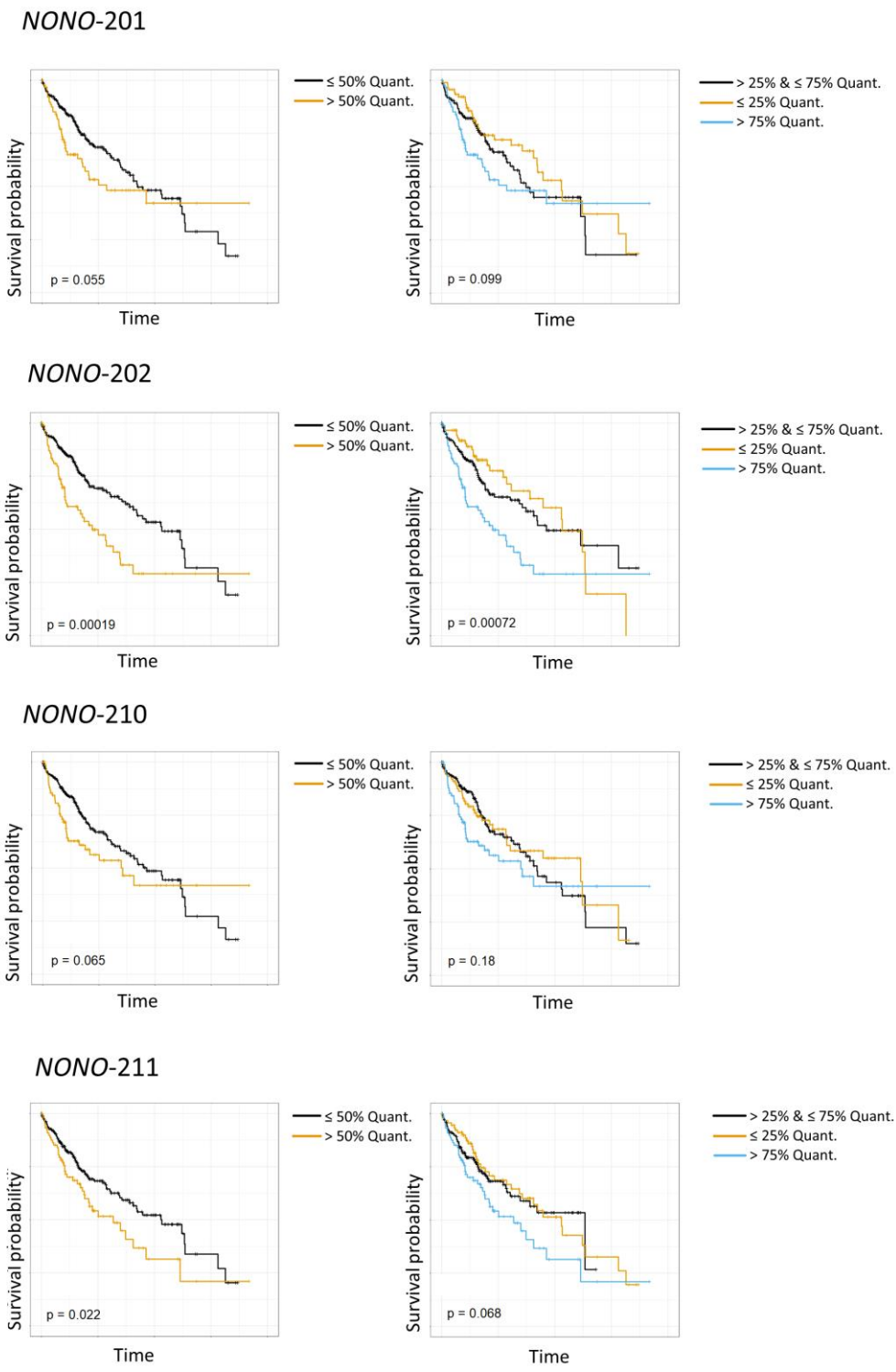
4.7 Supplementary data



Supplementary Figure 1. Kaplan-Meier curves of *NEAT1* transcript variants with an average expression rate of log2(TPM + 0.001) > 0.

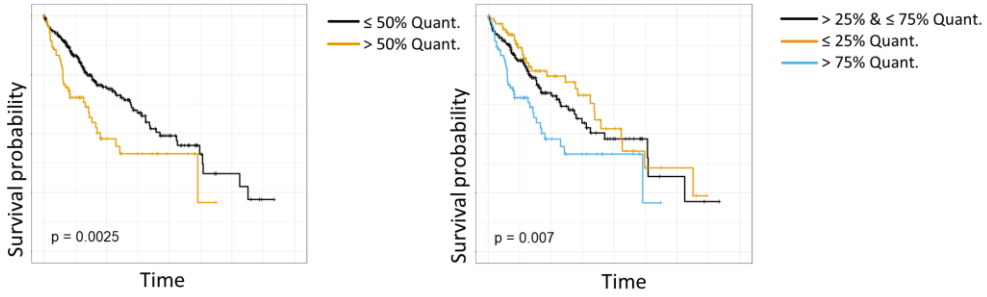


Supplementary Figure 2. Kaplan-Meier curves of *PSPC1* transcript variants with an average expression rate of log2(TPM + 0.001) > 0 .

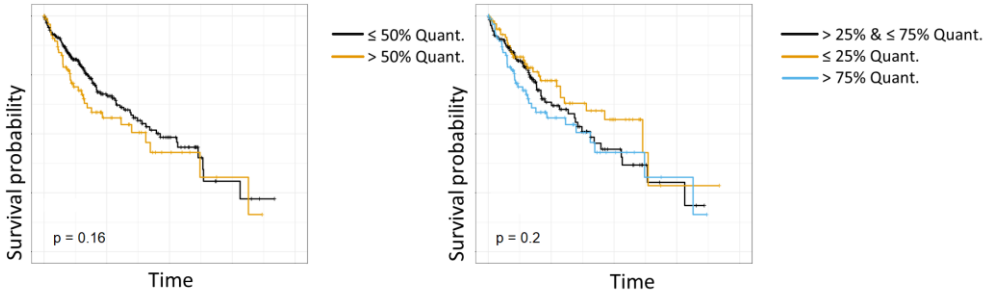


Supplementary Figure 3. Kaplan-Meier curves of *NONO* transcript variants with an average expression rate of log2(TPM + 0.001) > 0.

RBM14-201



RBM14-202



Supplementary Figure 4. Kaplan-Meier curves of *RBM14* transcript variants with an average expression rate of log2(TPM + 0.001) > 0.

transcript	NCBI Reference Sequence	Ensembl Transcript ID
NEAT1_v1	NR_028272.1	-
NEAT1_v2 / NEAT1-202	NR_131012.1	ENST00000501122.2
NEAT1-201	-	ENST00000499732.2
NEAT1-203	-	ENST00000601801.2
NEAT1-204	-	ENST00000612303.1
NEAT1-205	-	ENST00000616315.1

Supplementary table 1. NCBI Reference Sequence and Ensembl Transcript ID of *NEAT1* transcript variants.

gene	transcript variant	ensembl_transcript_id	biotype	mean expression above threshold of log2(TPM + 0.001) > 0
NEAT1	201	ENST00000499732	lincRNA	yes
	202	ENST00000501122	lincRNA	yes
	203	ENST00000601801	lincRNA	no
	204	ENST00000612303	lincRNA	no
	205	ENST00000616315	lincRNA	yes
PSPC1	201	ENST00000338910	protein coding	yes
	202	ENST00000427943	protein coding	no
	203	ENST00000471658	nonsense mediated decay	yes
	204	ENST00000492741	nonsense mediated decay	yes
	205	ENST00000497722	retained intron	no
	206	ENST00000619300	protein coding	yes
	207	ENST00000635251	retained intron	no
	208	ENST00000635562	nonsense mediated decay	no
NONO	201	ENST00000276079	protein coding	yes
	202	ENST00000373841	protein coding	yes
	203	ENST00000373856	protein coding	no
	204	ENST00000413858	protein coding	no
	205	ENST00000418921	protein coding	no
	206	ENST00000420903	protein coding	no
	207	ENST00000450092	protein coding	no
	208	ENST00000454976	protein coding	no
	209	ENST00000471419	processed transcript	no
	210	ENST00000472185	processed transcript	yes
	211	ENST00000473525	processed transcript	yes
	212	ENST00000474431	processed transcript	no
	213	ENST00000486613	processed transcript	no
	214	ENST00000490044	processed transcript	no
	215	ENST00000535149	protein coding	no
RBM14	201	ENST00000310137	protein coding	yes
	202	ENST00000393979	protein coding	yes
	203	ENST00000409372	protein coding	no
	204	ENST00000409738	protein coding	no
	205	ENST00000443702	protein coding	no
	206	ENST00000460762	processed transcript	no
	207	ENST00000461478	processed transcript	no
	208	ENST00000496694	processed transcript	no
	209	ENST00000512283	processed transcript	no

Supplementary table 2. Function and mean expression threshold of all transcript variants of *NEAT1*, *PSPC1*, *NONO*, and RBM14 listed on Ensembl.

5 Summary

Summary

Primary liver cancers including hepatocellular carcinoma (HCC) are the second most common cause of cancer related death worldwide. The incidence of HCC is specially rising in most industrialised countries, although HCC is still prevalent in Asian and African countries. An increased incidence of HCC can be observed in patients suffering from metabolic disorders.

Short-term diethylnitrosamine (DEN) treatment reduced both body and liver weight and drove severe liver injury and acute hepatic inflammation. Moreover, DEN strongly altered immune cell and hepatic lipid composition. Hence, it may be a powerful model to analyse the role of tumour supressors / promoters regarding the hepatic pathomechanisms related to immune cell and lipid composition in a tumour-promoting environment.

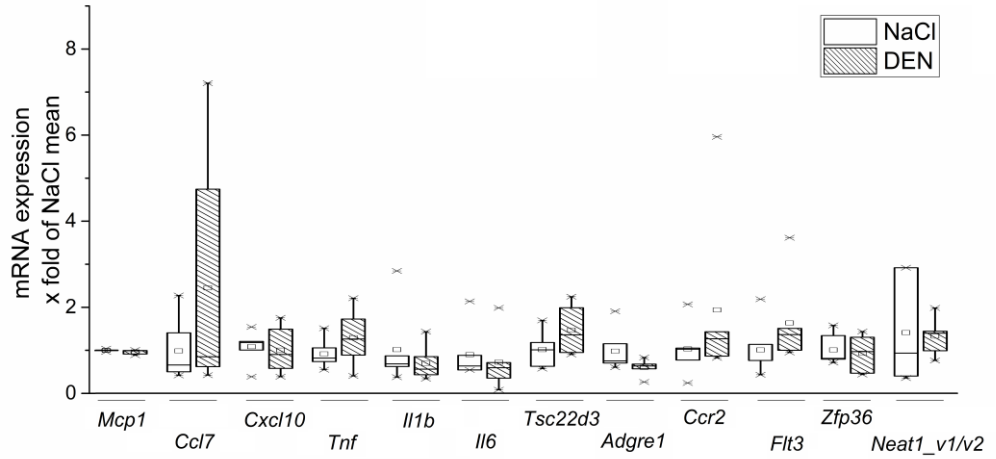
Hepatic *TTP* expression was strongly decreased in HCC tissues in three publicy available data sets compared to non-tumour samples. In addition, *TTP* expression was decreased in tumour but not in cirrhotic tissue, which may qualify TTP as a useful marker for the progression from the cirrhotic state towards HCC. Although *TTP* was decreased in HCC patients, hepatocyte *Ttp* knockout animals showed a lower number of tumours and less inflammation compared to the wild-type animals. However, TTP negatively regulated several tumour-promoting factors. The chemosensitising role of TTP in HCC could be shown by the decreased viability of TTP-overexpressing hepatoma cells, especially of Huh7 cells, after doxorubicin or sorafenib treatment. In addition, overexpression of TTP inhibited the migration ability of Huh7 and PLC/PRF/5 cells. The most distinct effect was shown in the strongly decreased proliferation in HepG2, Huh7 and PLC/PRF/5 cells, which overexpressed TTP. TTP-overexpressing hepatoma cells also showed a downregulation of several oncogenes, e.g. *BCL2*, *MYC*, and *VEGFA*. DNA hypomethylation increased *TTP* expression in HepG2 and Huh7 cells. However, the analysis of *TTP* promoter methylation in human liver tissue showed no difference between normal and tumour tissue. Therefore, TTP promotes hepatic tumour initiation but inhibits hepatic tumour progression.

An impact of *NEAT1*-dependent paraspeckle formation on the process of chemoresistance in HCC was supported by the highly abundant presence of *NEAT1*

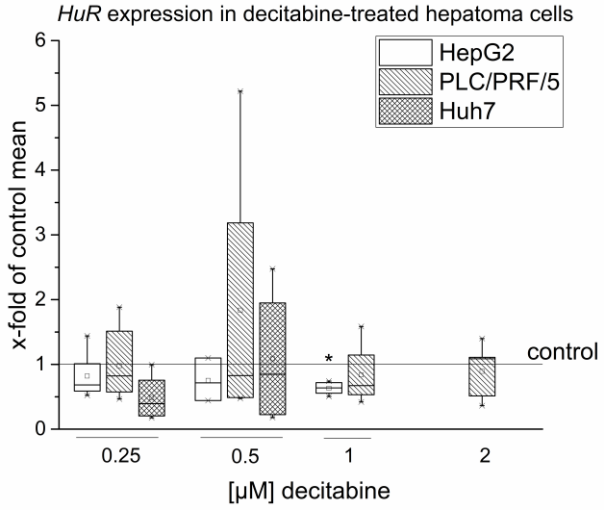
variants in either sorafenib or doxorubicin resistant accompanied by the induction of paraspeckles. In addition, the overexpression of several *NEAT1* transcripts and of transcripts of the paraspeckle-associated proteins *PSPC1*, *NONO*, and *RBM14* in HCC confirmed the clinical relevance of these results. Morover, some of these transcripts correlated with poor survival in HCC. Therefore, these genes may not only serve as targets for novel HCC therapies but might also be prognostic markers for HCC.

6 Supplementary data

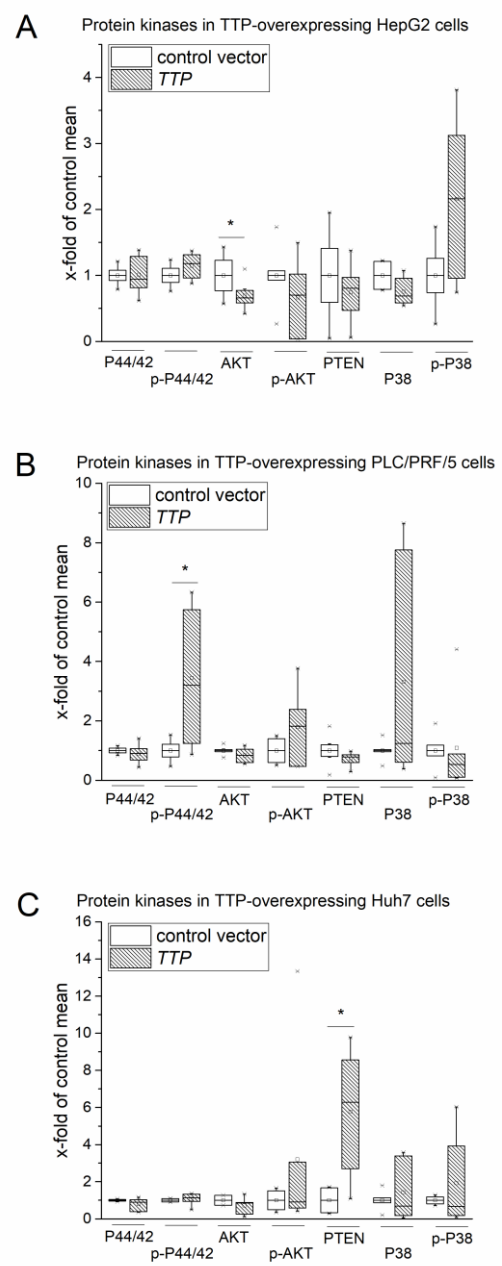
The following figures show results obtained within this thesis from gene expression analysis by qPCR and from protein analysis by flow cytometry or Western Blot. They either revealed non-statistically significant effects or are related to other projects. This is why they will not be discussed in detail.



Supplementary Fig 1. Effects of DEN on hepatic expression of genes involved in inflammation. Expression of *Mcp1* (encodes chemokine (C-C motif) ligand 2 (Ccl2)), *Ccl7* (encodes chemokine (C-C motif) ligand 7 (Ccl7)), *Cxcl10* (encodes C-X-C motif chemokine 10 (Cxcl10)), *Tnf* (encodes tumor necrosis factor alpha (Tnf-a), *Il1b* (encodes interleukin 1 beta (Il1β), *Il6* (encodes Interleukin 6 (Il-6)), *Tsc22d3* (encodes glucocorticoid-induced leucine zipper (Gilz), *Adgre1* (encodes F4/80), *Ccr2* (encodes C-C chemokine receptor type 2 (Ccr2)), *Flt3* (encodes CD135), *Zfp36* (encodes tristetraprolin (Ttp) and *Neat1_v1/v2* by qPCR. n = 6.



Supplementary Fig 2. Effects of hypomethylation on *HuR* expression in hepatoma cells. *HuR* mRNA expression levels in HepG2, PLC/PRF/5, and Huh7 cells treated with decitabine. n ≥ 2, duplicates. Statistical difference: * $p < 0.05$.



Supplementary Fig 3. Kinase signaling in TTP-overexpressing hepatoma cells. Cells were transfected with either a TTP sense construct or an antisense construct as a control. 48h after infection, protein lysates of the cells were separated via sodium dodecyl sulfate and analysed via Western Blot as described previously (Kessler et al., 2013). The following antibodies were used for detection: p44/42 (Erk1/2) (L34F12) antibody (#4696S, New England Biolabs, Ipswich, Massachusetts, USA), phospho-p44/42 MAPK (T202/Y204) 20G11 antibody (#4376S, New England Biolabs), Akt antibody (#9272S, New England Biolabs), phospho-Akt (Ser473) (D9E) XP® antibody (#4060, New England Biolabs), PTEN antibody (#9552, New England Biolabs), p38 MAPK antibody (sc-7972, Santa Cruz), and phospho-p38 MAPK antibody (#9215S, New England Biolabs). Densitometric analysis of Western blots from HepG2 (A), Plc/prf/5 (B), and Huh7 (C). Data are expressed as ratio of p44/42, Akt, PTEN, or p38 MAPK to tubulin signal intensities or as ratio of phosphorylated p44/42 (p-p44/42), Akt (p-Akt), or p38 MAPK (p-p38) to total p44/p42, Akt, or p38 MAPK signal intensities. n = 3, duplicates. Statistical difference: *: $p \leq 0.05$.

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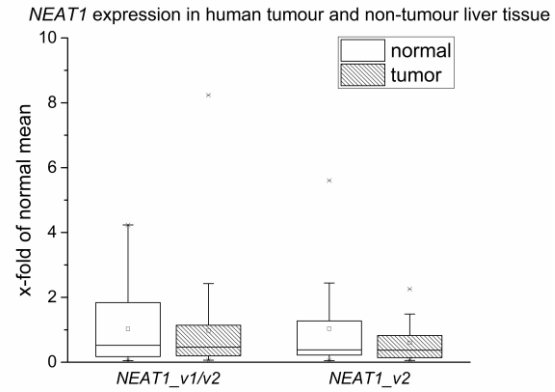
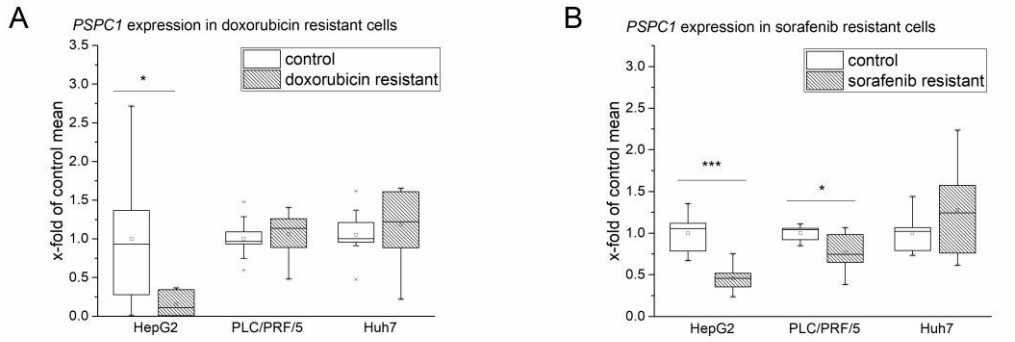
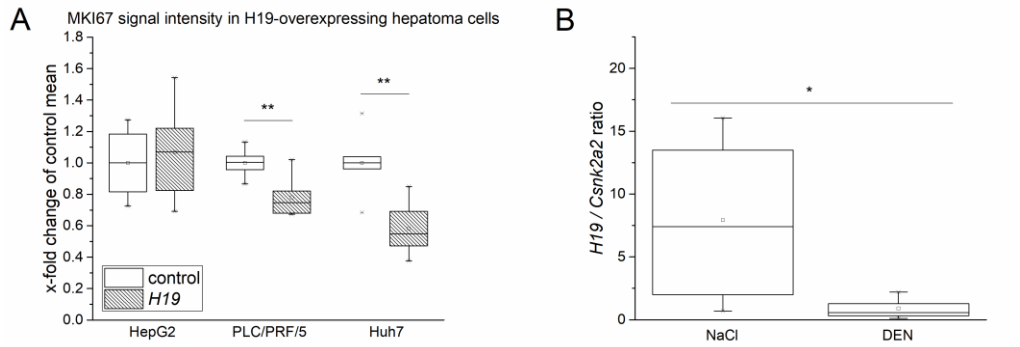


Supplementary Fig 4. *NEAT1* expression in human tumour and non-tumour liver tissue. *NEAT1_v1/v2* and *NEAT1_v2* mRNA levels isolated of tumour and adjacent non-tumour tissues (n = 31) as described in chapter 3 by qPCR normalised to *ACTB*.



Supplementary Fig 5. *PSPC1* expression in chemoresistant hepatoma cell lines. *PSPC1* expression determined by qPCR in doxorubicin resistant (A) and sorafenib resistant (B) cells (n = 3). Primers detected transcript variants *PSPC1_v1* (NM_001042414.3), *PSPC1_v4* (NM_001354909.1), and *PSPC1_v6* (NR_149052.1), which are listed on the NCBI database. The primers also detect *PSPC1-201* (ENST00000338910.8) and *PSPC1-206* (ENST00000619300.4), which are listed on Ensembl. The following primer sequences were used: *PSPC1* forward: AGACGCTTGGAAGAACTCAGA, *PSPC1* reverse: TTGGAGGAGGACCTTGGTTAC. Statistical difference: *: $p \leq 0.05$; ***: $p \leq 0.001$.



Supplementary Fig 6: Effect of H19 on proliferation and inflammation. (A) Flow cytometric analysis of the proliferation marker MKI67 in stably *H19*-overexpressing (*H19*) and vector control human hepatoma cells (control), n ≥ 2, triplicates. (B) Expression of *H19* mRNA levels in short-term DEN-treated wild-type mice, n = 6. Experiments were conducted as described previously (Schultheiß et al., 2017). Statistical difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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8 Publications

Original publications

Hosseini K, Kessler SM, Hoppstädter J, Laggai S, Gemperlein K, Müller R, and Kiemer AK. Characterisation of diethylnitrosamine-induced leukocyte recruitment and lipid alterations in mouse livers. *Submitted*.

Hosseini K, Kessler SM, Barghash A, Patial S, Hoppstädter J, Haybäck J, List M, Helms V, Schulz MH, Laggai S, Blackshear PJ, and Kiemer AK. The mRNA-binding protein TTP inhibits tumour progression in liver cancer. *Submitted.*

Hosseini K, Kessler SM, Schultheiß CS, List M, Schulz MH, Kiemer AK, and Laggai S. Paraspeckle formation and induction of lncRNA NEAT1 in hepatocellular carcinoma chemoresistance. *Submitted*.

Schultheiß CS, Laggai S, Czepukojc B, Hussein UK, List M, Barghash A, Tierling S, **Hosseini K**, Golob-Schwarzl N, Pokorny J, Hachenthal N, Schulz MH, Helms V, Walter J, Zimmer V, Lammert F, Bohle RM, Dandolo L, Haybäck J, Kiemer AK, and Kessler SM. The long non-coding RNA H19 suppresses carcinogenesis and chemoresistance in hepatocellular carcinoma. *Cell Stress*, 2017, Vol. 1, No. 1, pp. 37–54.

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Abstracts to poster presentations

Hosseini K, Kessler SM, Hoppstädter J, Laggai S, Gemperlein K, Müller R, and Kiemer AK. The carcinogen diethylnitrosamine (DEN) induces leukocyte recruitment and lipid deposition in mouse livers. Poster presentation at the 30th annual meeting of the European Macrophage and Dendritic Cell Society (EMDS), Amsterdam, Netherlands, 2016.

Hosseini K, Kessler SM, Schultheiß CS, List M, Schulz MH, Kiemer AK, and Laggai S. Paraspeckle formation and induction of lncRNA NEAT1 in hepatocellular carcinoma chemoresistance. Poster presentation at the annual meeting of the German Pharmaceutical Society (DPhG), Saarbrücken, Germany, 2017.

Hosseini K, Kessler SM, Barghash A, Patial S, Blackshear PJ, Helms V, Laggai S and Kiemer AK. The mRNA-binding protein TTP inhibits tumour progression in liver cancer. Poster presentation at the annual meeting of the German Pharmaceutical Society (DPhG), Saarbrücken, Germany, 2017.

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