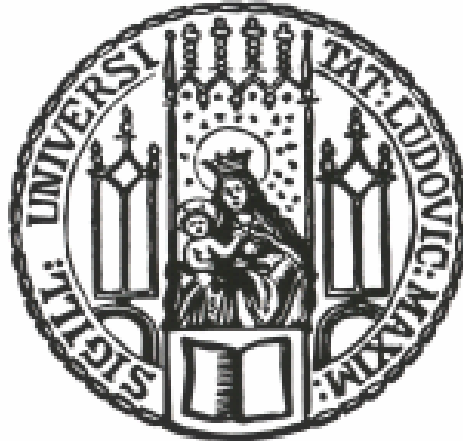


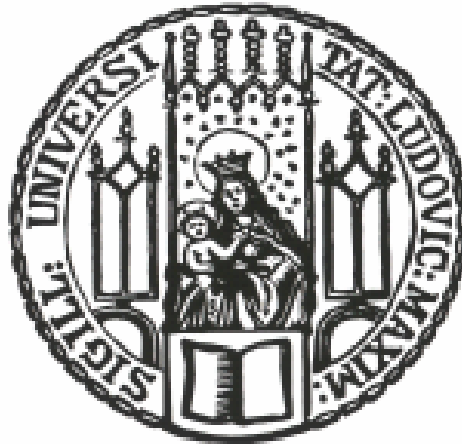
Thesis for the attainment of the doctoral degree
from the Faculty of Biology, Ludwig-Maximilian-University, Munich



**C-JUN DOWNREGULATION SENSITIZES HEPATOMA
CELLS TO RECEPTOR INDUCED APOPTOSIS THROUGH
PREVENTING FADD PHOSPHORYLATION**

By
Nilüfer Sonuc
Munich, 2008

Dissertation zur Erlangung des Doktorgrades
der Fakultät für Biologie der Ludwig-Maximilian-Universität München



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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von PD. Dr. Frank Kolligs betreut.

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die Dissertation von mir selbstständig ohne unerlaubte Hilfe angefertigt ist.

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Dedicated to my husband

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Abbreviation

AB	antibody
ATP	Adenosintriphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CRE	cyclization recombination
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
E. coli	Escherichia coli
ES	cells embryonic stem cells
FCS	fetal calf serum
g	gram
GAPDH	Glycerinaldehyd-3-phosphat-dehydrogenase
GFP	green fluorescent protein
GITC	guanidinium isothiocyanate
h	hour
IL-1	interleukine- 1
HRP	horseradish peroxidase
kD	kilo dalton
kb	kilo base
l	liter
LB	Luria Broth

M	molar
mA	milliampere
mg	milligram
min	minute
ml	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
nm	nanometer
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
psi	pound per square inch
PVDF	Polyvinylidenfluorid
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
rpm	revolution per minute
RPMI 1640	Roswell Park Memorial Institute medium
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s	second
SDS	sodium dodecyl sulphate
siRNA	small interfering ribonucleic acid
TBS	Tris-buffered saline

TBE	Tris- Boric acid- EDTA
UV	ultraviolet
μg	microgram
μl	microliter
μm	micrometer
WT	wild-type

I. Introduction

Apoptosis (= programmed cell death) is a key regulator of tissue homeostasis. An imbalance between cell death and proliferation may result in tumour formation. Also, killing of cancer cells by cytotoxic therapies such as chemotherapy, γ -irradiation or ligation of death receptors is predominantly mediated by triggering apoptosis in target cells. In addition to the intrinsic apoptotic pathway, elements of death receptor signaling pathways have been identified to contribute to the efficacy of cancer therapy. Failure to undergo apoptosis in response to anticancer therapy may lead to resistance. Also, deregulated expression of death receptor pathway molecules may contribute to tumourgenesis and tumour escape from endogenous growth control. Understanding the molecular events that regulate apoptosis provides new opportunities for pathway- based rational therapy.

1. Apoptosis and Necrosis

Naturally occurring cell death is an inconspicuous, yet prevalent, event in complex multicellular organism, and was first described by anatomists and embryologists in the 19th century [Fadeel and Orrenius, 2005;Clarke and Clarke, 1996;Lockshin and Zakeri, 2001]. However, interest in the subject was modest, and the concept of physiological cell death did not receive widespread recognition until the publication of a seminal paper on apoptosis by Kerr and colleagues some 30 years ago [Kerr et al., 1972]. These authors highlighted the significance of controlled cell deletion and described the morphological features of this active and inherently programmed phenomenon, which they proposed plays a “complementary but opposite role to mitosis in the regulation of animal cell populations”. The importance of their study lies not only in the detailed morphological description of apoptosis (a term derived from the Greek word describing the falling off leaves from a tree) but also in the recognition of natural cell death as a basic phenomenon not restricted to embryogenesis [Wyllie et al., 1980].

Cell death is therefore an essential strategy for the control of the dynamic balance in living systems and two fundamentally different forms of cell death, apoptosis and necrosis, have been defined since.

Necrosis is an accidental passive process starting with disruption of the cell membrane and a progressive breakdown of ordered cell structures in response to violent environmental perturbations such as severe hypoxia/ ischaemia, extremes of temperature and mechanical trauma. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses.

Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (see Figure 1).

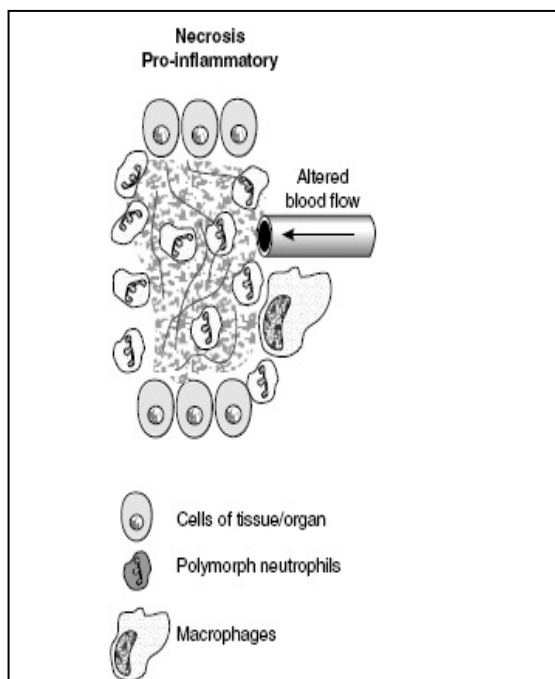


Figure 1: Diagramatic comparison of necrotic cells
Adapted from <http://www.intl.elsevierhealth.com/e-books/pdf/852.pdf>

In contrast, apoptosis or programmed cell death involves the activation of an energy-requiring intracellular machinery, which is tightly regulated and conserved throughout evolution [Yuan, 1996]. Apoptosis is defined on the basis of morphological changes including chromatin condensation, nuclear fragmentation and formation of apoptotic bodies containing intact organelles, as well as portions of the nucleus (see Figure 2).

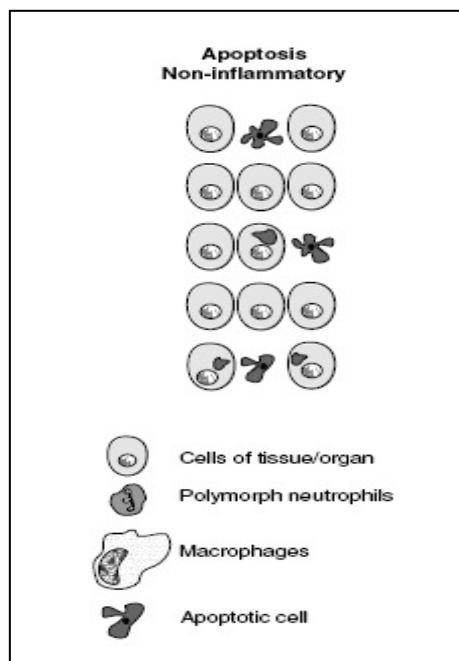


Figure 2: Diagrammatic comparison of apoptotic cells

Adapted from <http://www.intl.elsevierhealth.com/e-books/pdf/852.pdf>

The apoptotic bodies are enclosed by an intact plasma membrane and rapidly recognized, ingested and degraded by phagocytes or neighbouring cells. As apoptosis typically does not induce inflammation or tissue scarring, it is well suited for a role in normal cell turnover during embryogenesis and in adult tissues [Jacobson et al., 1997].

Apoptosis and necrosis are usually considered separate entities, but an alternate view is emerging that apoptosis and necrosis are frequently the consequence of the same initiating factors and signalling pathways. Rather than being separate entities, apoptosis and necrosis in their pure form may represent extremes on a continuum of cell death [Lemasters, 1999; Lemasters, 2005].

2. Molecular mechanism of apoptosis

Most of the morphological changes that were observed by Kerr *et al.* [Kerr et al., 1972] are caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These death proteases are homologous to each other, and are part of a large protein family known as the caspases [Alnemri et al., 1996]. Caspases are highly conserved through evolution, and can be found from humans all the way down to insects, nematodes and hydra [Budihardjo et al., 1999;Cikala et al., 1999;Earnshaw et al., 1999]. Over the past decade, many key events in caspase regulation have been documented at the molecular and cellular level [Danial and Korsmeyer, 2004].

Two main pathways that activate caspases have been identified. The first (“extrinsic pathway”) begins at the cell surface and involves ligand-induced activation of death receptors, such as TNFRI and CD95, which then recruit and activate caspases. The second (“intrinsic pathway”) involves mitochondrial integration of cellular stress signals and subsequent release into the cytosol of cytochrome c, which activates caspases via the adapter molecule “apoptotic protease activating factor-1” (APAF-1).

2.1. Receptor mediated apoptosis (Extrinsic pathway)

The *extrinsic* apoptosis pathway follows the activation of specific membrane receptors, the death receptors. Although death receptors can promote cell growth under at least some situations [Algeciras-Schimmich et al., 2002], the ability of these receptors to induce apoptosis is critical in several disease processes and has been the focus of most work today.

Death receptors are members of the TNF receptor gene superfamily, which consists of more than 20 proteins with a broad range of biological functions including the regulation of cell

death and survival, differentiation or immune regulation [Ashkenazi and Dixit, 1998;Krammer, 2000;Walczak and Krammer, 2000] (see Figure 3).

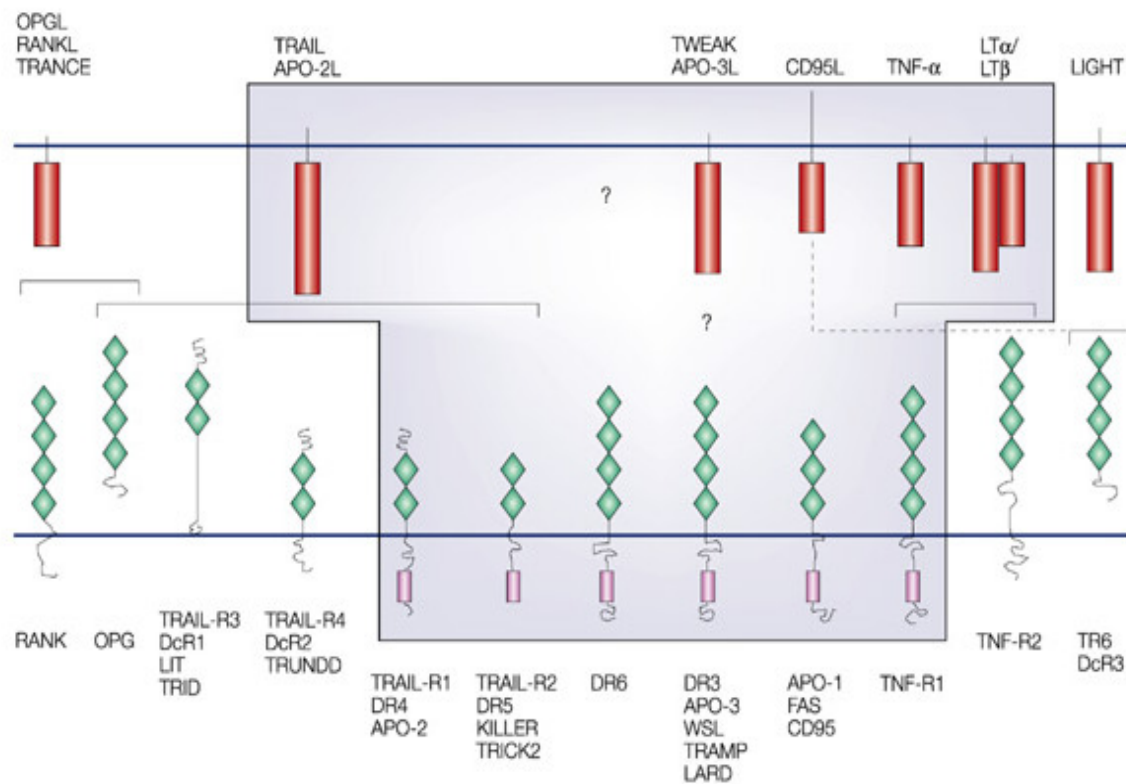


Figure 3: The TNF- and TNF- receptor superfamily. Bottom line Receptors, upper line Ligands. Pink oblong Death domain (DD). Taken from <http://www.dkfz-heidelberg.de/immunogenetik/apoptosis-cd95.htm>.

The best-characterized death receptors comprise CD95 (APO-1/Fas), the TNF- receptor 1 (TNFR1) and the related TRAIL-R1 and TRAIL-R2 (TNF- related apoptosis-inducing ligand receptors).

2.1.1 CD95 (APO-1/Fas)/CD95L (FasL)

CD95 (APO-1/Fas) first appeared in the literature in 1989, when it was described by two independent groups led by Minako Yonehara in Japan [Yonehara et al., 1989] and Peter Krammer in Germany [Trauth et al., 1989]. CD95 is a glycosylated cell-surface protein, ubiquitously expressed in various tissues, in particular in thymus, liver, heart, kidney and in

inactivated mature lymphocytes. In order to avoid unnecessary activation of the apoptotic pathway, CD95 expression and localization are tightly regulated. Only a minimal amount of CD95 is expressed on the plasma membrane in unstimulated cells, whereas the majority of the receptor is localized in the cytosol, in particular, in the Golgi complex and the trans-Golgi network [Sodeman et al., 2000; Bennett et al., 1998]. After a proapoptotic stimulus, CD95 containing vesicles translocate to the cell surface, increasing CD95 expression on the plasma membrane and initiating the apoptotic signal [Sodeman et al., 2000]. Engagement of CD95 by either agonistic antibodies or CD95L (FasL) leads to the trimerisation of the receptor, followed by recruitment of the adaptor protein FADD (Fas Associated Death Domain)/MORT-1 (mediator of receptor induced toxicity) [Wallach et al., 1999] (see section 2.2 in introduction). FADD associates with its receptor through intersection of its death domains, while its death effector domain binds to a correspondent death effector domain (DED) or a caspase recruitment domain (CARD) in the prodomain of inactive initiator caspase, such as procaspase-8 and -10. The resulting complex is called the death –inducing signaling complex (DISC) [Peter and Krammer, 2003; Kischkel et al., 1995] (Figure 4).

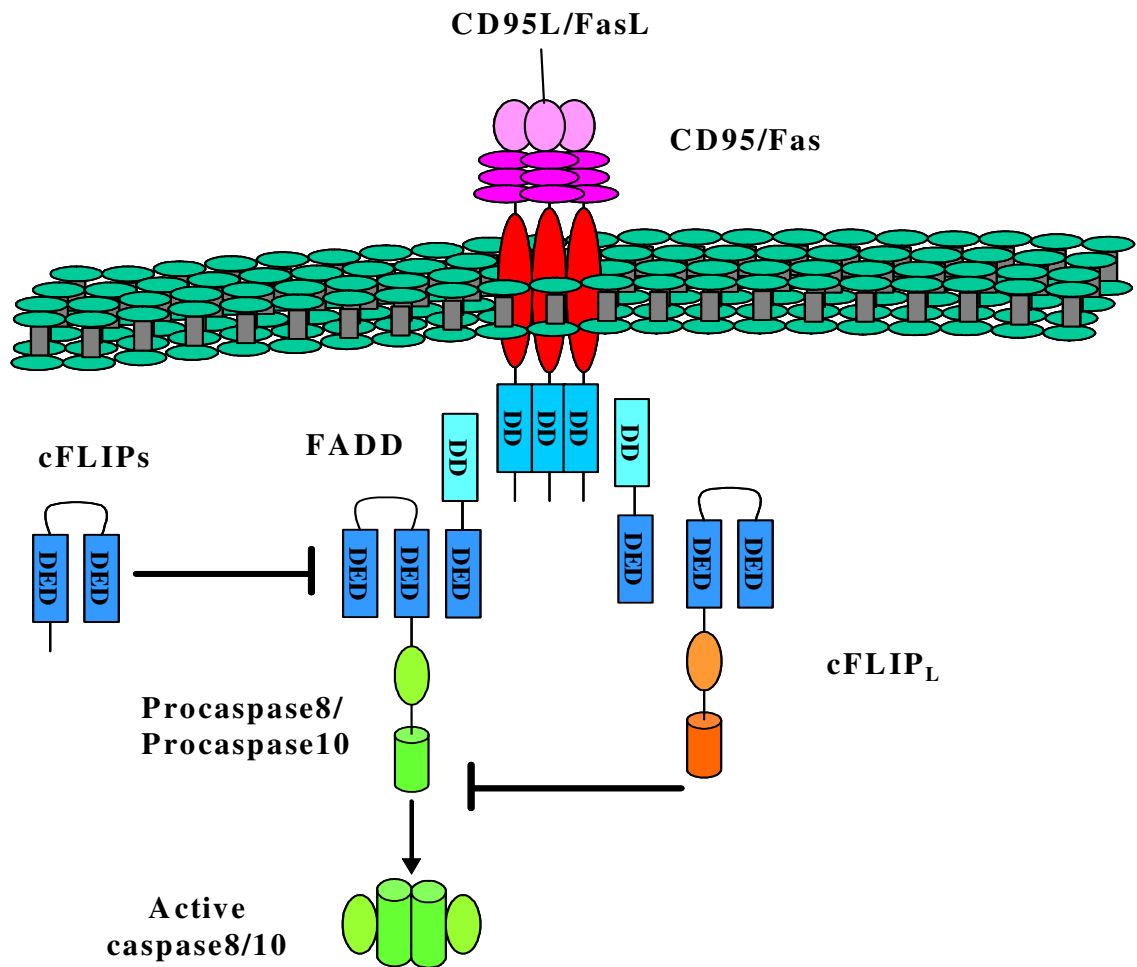


Figure 4: The CD95/Fas death inducing signaling complex.

Schematic representation of the DISC formed after the engagement of CD95L/FasL to CD95/Fas. The DD on the adapter protein FADD interacts with the receptor's DD, whereas the DED binds the correspondent death effector domain in the pro-domain of the inactive initiator caspase-8/-10. The short form of the inhibitor of apoptosis cFLIP (cFLIP_s) binds to the DED of FADD and prevents the recruitment of the initiator pro-caspases to the DISC, whereas the long form of the cFLIP (cFLIP_L) allows the recruitment of the procaspase-8 to the DISC, but inhibits its processing into the active form.

Recruitment and accumulation of procaspase-8/-10 at the DISC result in self processing and spontaneous activation of the caspase via autoproteolytic cleavage. Active caspase-8 (FLICE) is released from the DISC into the cytoplasm and cleaves various proteins in the cell such as caspase-3, -6 and -7, which are ultimately responsible for the degradation of key cellular components resulting of the completion of the cell death programme.

Additional DED-containing proteins have been found in a certain class of herpes viruses. These proteins are called FLIP and bind to the CD95-FADD complex. It exists in a short

(cFLIP_S) and a long isoform (cFLIP_L), as a result of different splice variants. Both forms of cFLIP are recruited and bind to the DISC upon stimulation [Scaffidi et al., 1999] and inhibit procaspase-8 recruitment to the DISC (Figure 4).

2.1.2 TRAIL (Apo2L) and its receptor

TRAIL/Apo2L (Apo 2 Ligand or tumour necrosis factor related apoptosis inducing ligand) was discovered by two independent groups, based on its sequence homology to TNF and CD95L [Pitti et al., 1996;Wiley et al., 1995]. Like most other members of the TNF superfamily of ligands, TRAIL/Apo2L is primarily expressed as a type II membrane protein of about 33-35 kDa [Liabakk et al., 2002] and is capable to induce apoptosis [Walczak and Krammer, 2000].

The receptor system for TRAIL/Apo2L is complex and comprises four exclusive receptors. Two of these- DR4 (TRAIL-receptor-1 (TRAIL-R1)) and DR5 (TRAIL-receptor-2 (TRAIL-R2), KILLER, TRICK2) - are death receptors which contain a cytoplasmic death domain that is able to transduce an apoptotic signal. By contrast, two other receptors DcR1 (TRAIL-receptor-3, (TRAIL-R3), TRIDD, LIT) and DcR2 (TRAIL-receptor-4 (TRAIL-R4), TRUNDD) are unable to initiate apoptotic cell death and believed to act as decoys. Finally, a soluble receptor called osteoprotegerin (OPG) also exists that binds with low affinity to TRAIL at physiological temperatures [Truneh et al., 2000].

In particular, CD95 and TRAIL share a similar pattern of intracellular signal transduction: binding of TRAIL to TRAIL-R1 and/or TRAIL-R2 results in recruitment of the adaptor protein FADD. FADD recruits procaspase-8 via its death effector domain (DED) to form the membrane protein complex DISC [Sprick et al., 2000]. In turn, active caspase-8 cleaves downstream caspases as the effector caspase 3, leading to cleavage of cellular proteins, DNA and subsequent programmed cell death [Salvesen and Abrams, 2004;Earnshaw et al., 1999].

2.1.3. Tumour necrosis factor pathway

TNF (tumour necrosis factor) binds to two different receptors, TNF receptor 1 and 2 (TNF-R1/TNF-R2). TNF-R1 assembles a DISC similar to CD95, TRAIL-R1 and TRAIL-R2 with TRADD (tumour necrosis factor receptor 1 associated death domain protein) as additional adapter for the FADD/ procaspase-8 complex [Chen and Goeddel, 2002].

In contrast to other death receptors, TNF-R1 has pleiotropic activities such as induction of apoptosis and activation of the transcription factor NF- κ B, leading to the induction of a number of antiapoptotic proteins. Activation of the NF- κ B axis proceeds via a multi protein complex containing TRADD, RIP1, TRAF2 and cIAP1, binding to TNF-R1 (complex 1). Upon biochemical modifications in RIP1, TRAF2 and TRADD, complex 1 dissociates from TNF-R1 and a complex consisting of FADD, procaspase 8, cFLIP and procaspase-10 binds to the receptor (complex 2). Thus, antiapoptotic and apoptotic pathways are subsequently activated from the same receptor [Micheau and Tschopp, 2003] (Figure 5).

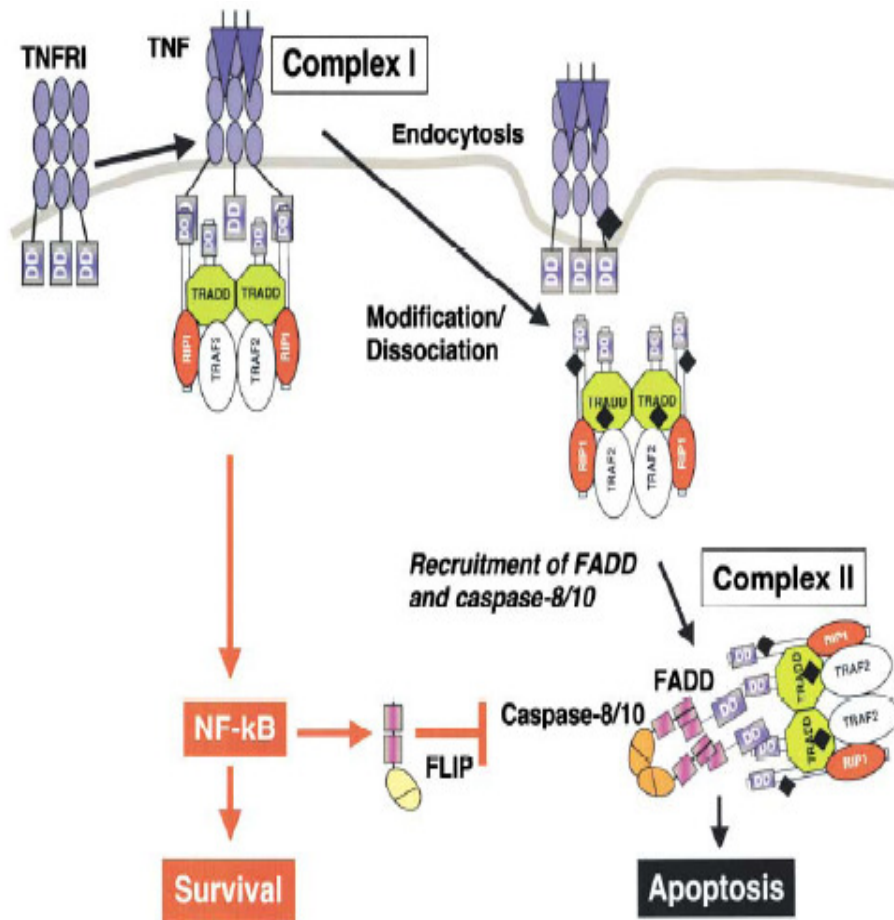


Figure 5: Model of TNFR1- Mediated Apoptosis.

After binding of TNF to TNFR1, rapid recruitment of TRADD, RIP1, and TRAF2 occurs (complex I). Subsequently TNFR1, TRADD, and RIP1 become modified and dissociate from TNFR1. The liberated death domain of TRADD (and/ or RIP1) now binds to FADD, resulting in caspase 8/10 recruitment (forming complex II) and resulting in apoptosis. If NF-κB activation triggered by complex I is successful, cellular FLIP L levels are sufficiently elevated to block apoptosis and cell survive.

2.2. Mitochondrial mediated apoptosis (intrinsic pathway)

In contrast to the *extrinsic* apoptosis pathway, the *intrinsic* pathway is stimulated from inside the cell. Various stimuli (e.g., UV- irradiation, reactive oxygen species [ROS], growth factor withdrawal or chemotherapeutic drugs) activate mitochondria through Bcl-2 family members- Bax and Bak. From mitochondria, caspase-dependent or –independent apoptosis signalling pathways are initiated. Participation of mitochondria in apoptosis induction mainly involves

the release of caspase activating proteins into the cytosol. The release of cytochrome c from the mitochondria results in the activation of the apoptotic protease activating factor-1 (Apaf-1). In the presence of cytochrome c and ATP, the CARD domain of Apaf-1 binds with the CARD domain of procaspase 9, and this forms the mitochondrial DISC, also designated as apoptosome [Zou et al., 1997]. Following activation, the apoptosome-associated caspase-9 will in turn activate downstream caspases like caspase 3, 6 and 7 [Li et al., 1997].

Common to all these events is that they lead to efflux of cytochrome c, usually located in the lumen between the inner and outer membrane from the mitochondria. Key to regulating the integrity of the mitochondrial membrane is proteins of the Bcl-2 family which can be divided into three subfamilies according to the presence of the Bcl-2 homology domains (BH): BH3-only, BH1-BH4 and BH1-BH3 family members. Bcl-2, Bcl-xL, Mcl-1, A1, Boo/Diva and Bcl-w belongs to the second subgroup and act as antiapoptotic proteins. The third subgroup includes Bax, Bak and, Bok and the proteins Bad, Bid, Bim, Bod, Bik, Puma and Noxa which act proapoptotically belongs to the BH-3 only proteins.

(see Figure 6).

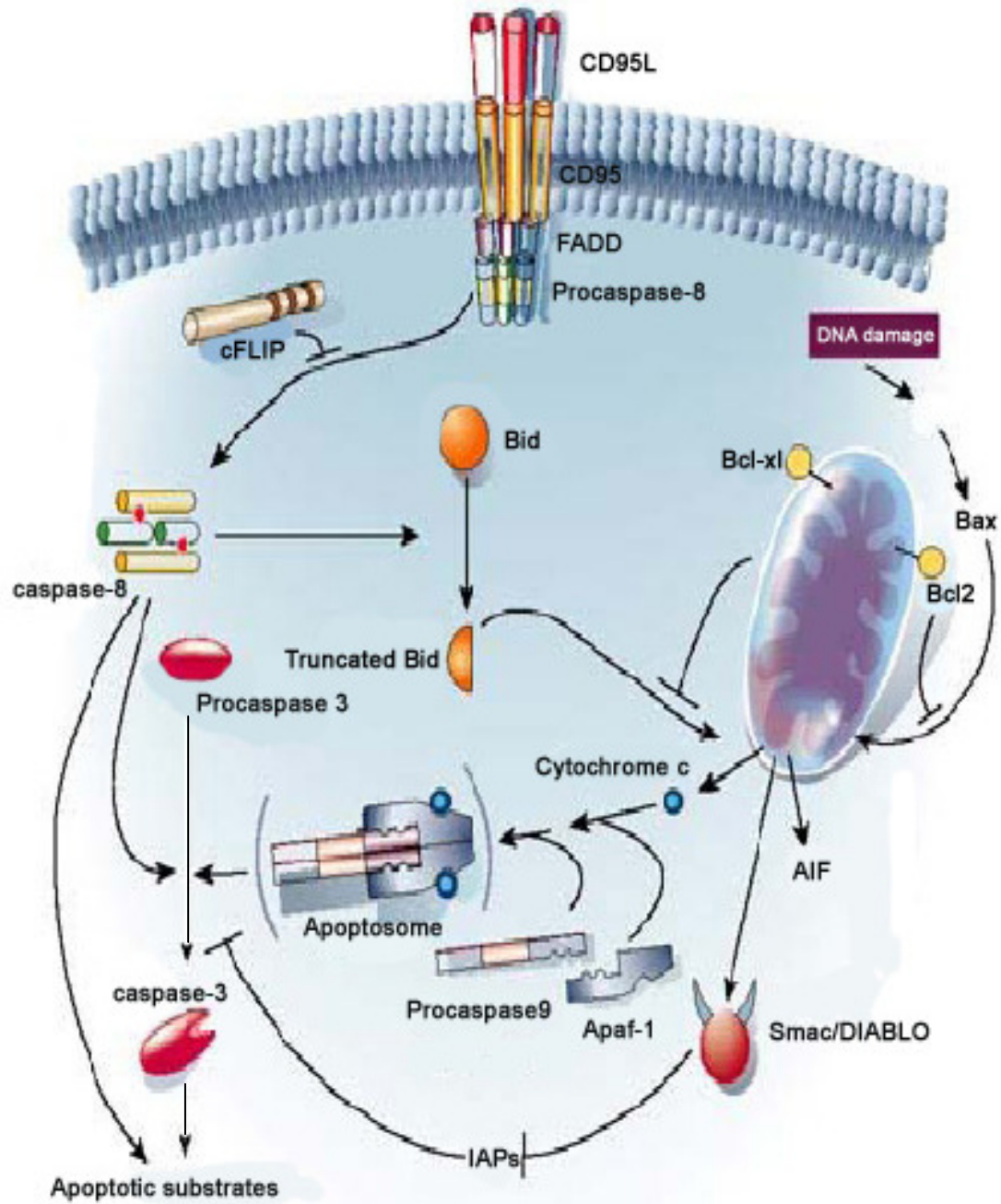


Figure 6: The major apoptotic pathways in mammalian cells
 (Adapted from: Hengartner, MO. The biochemistry of apoptosis. Nature 2000; 407:770- 776)

2.3. Caspases

Caspases are aspartic acid-specific cysteine proteases, which become activated in most forms of apoptosis. In cells, they localise in nucleus, cytoplasm and mitochondrial intermembrane space, and can also be translocated to the plasma membrane receptors via adapter proteins [Boldin et al., 1996; Colussi et al., 1998; Muzio et al., 1996; Thornberry et al., 1992].

Although the first mammalian caspase (caspase-1) was identified as an important regulator of the inflammatory response [Thornberry et al., 1992], at least 7 of the 14 known mammalian caspases have important roles in apoptosis. The apoptotic caspases are generally divided into two classes: the initiator caspases, which include caspase-2, -8, -9 and -10 and the effector caspases, which include caspases-3, -6, -7 [Riedl and Shi, 2004] (see Figure 7).

All caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. Following a proapoptotic stimulus, they are sequentially processed into a precursor, noncatalytic domain and two catalytic subunits: a large subunit 20 kDa containing the active site Cys, and a smaller 10 kDa subunit [Yamin et al., 1996; Muzio et al., 1998]. Two small and two large subunits are assembled together to yield an active caspase enzyme with two active sites [Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994]. Proapoptotic caspases can be generally divided into subclasses with either long prodomains or short prodomains present on the nonactivated precursors. The long prodomains in the first class contain protein motifs, such as CARD (caspase recruitment domains, found in caspase-9 and -2) or DED (death effector domains, found in caspase-8 and -10).

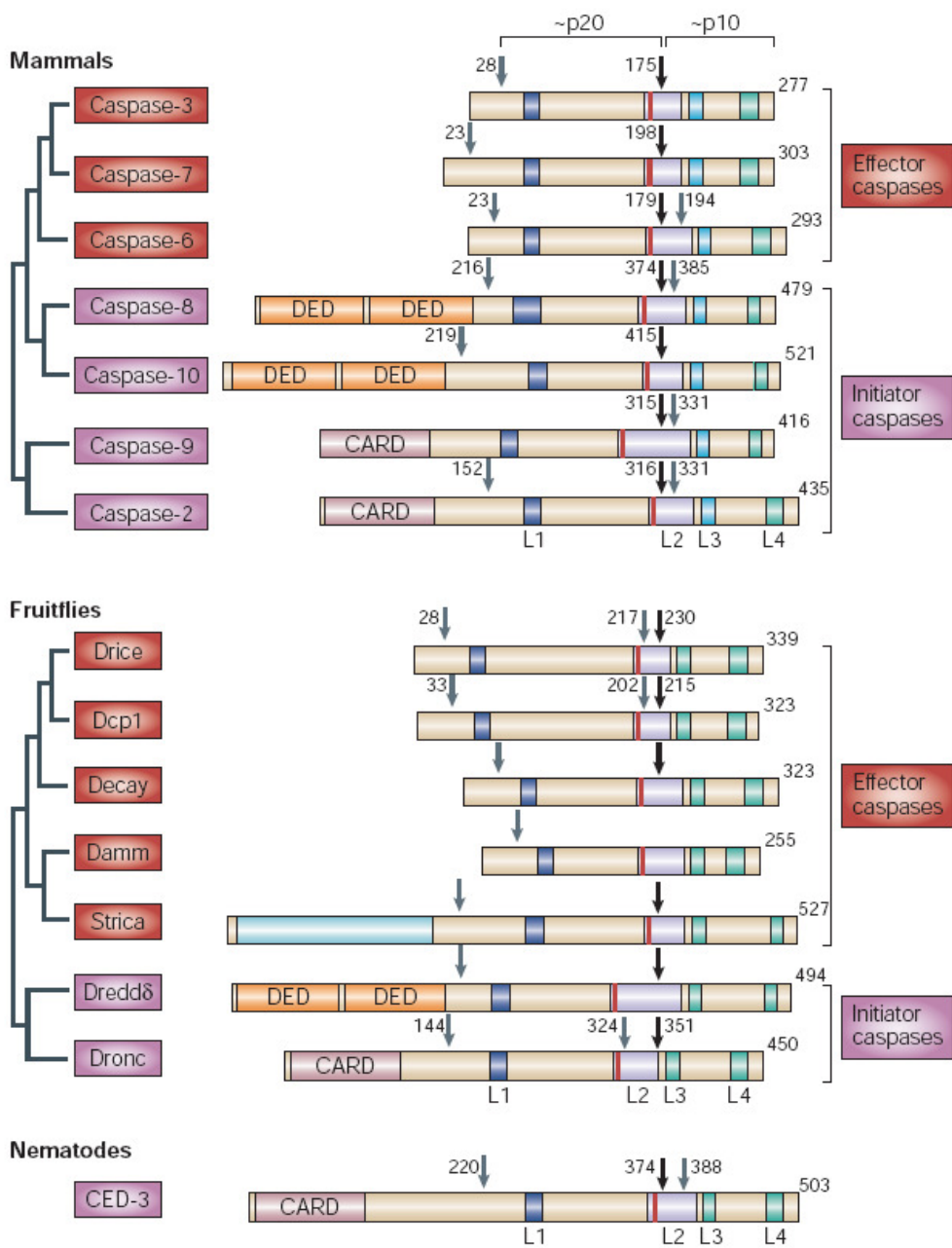


Figure 7: Diagrammatic comparison of all effector and initiator caspases in mammals, fruitflies and nematodes. Adapted from <http://www.bath.ac.uk/bio-sci/hejmadi/apoptosis%20&%20caspases%20nm%20nov04.pdf>

2.4. FADD/ MORT1

FADD/MORT1 has been originally identified in a yeast two hybrid screen with the CD95 death domain as a bait [Chinnaiyan et al., 1995; Boldin et al., 1995]. It contains both DD and a DED and act as an adapter between DD- containing receptors and the DED- domain-containing caspases, namely caspase-8 and caspase-10. It is generally believed that FADD binds directly to the death domains of CD95, TRAIL-R1 and TRAIL- R2 by interaction of its death domain with the death domains of the receptors. The central role of FADD as an adaptor linking death receptors to caspase activation is further supported by experiments using cell lines or mouse embryonic fibroblasts (MEFs) deficient in FADD. In these systems, FADD has been shown to be essential for recruitment of caspase-8 and caspase-10 not only to the CD95 DISC, but also to the TRAIL-R1 and TRAIL- R2 DISCs [Kischkel et al., 2001; Sprick et al., 2000]. In addition, cells lacking FADD are resistant to CD95-, TNF- and TRAIL- mediated apoptosis [Yeh et al., 1998; Juo et al., 1999; Zhang et al., 1998; Kuang et al., 2000]. Blockage of CD95- and TNF-mediated killing could also be observed when a dominant mediated version of FADD was overexpressed [Chinnaiyan et al., 1996].

Among the death receptors, TNF-R1 differs from CD95 and TRAIL-R1 and -R2. Recruitment of FADD to TNF-R1 does not seem to be mediated by direct interacting of the DD of FADD with the DD of TNF-R1. Instead it has been proposed that an additional molecule , the TNF-R1- associated DD- containing protein (TRADD) act as a bridge between TNF-R1 and FADD [Boldin et al., 1996; Hsu et al., 1995].

In addition to its function in pro- apoptotic pathways, another possible function for FADD was determined by the analysis of mice that either lack FADD in their T cell compartment or overexpress a dominant negative version of FADD under a T cell specific promoter [Zhang et al., 1998; Kabra et al., 2001; Newton et al., 2000; Newton et al., 1998]. Interestingly, lymphocytes derived from these mice are not only resistant to death-receptor-mediated

apoptosis, but also show a defect in lymphocyte proliferation. In line with these results, FADD has been shown to be phosphorylated by a cell regulated kinase also suggesting a connection between FADD and the regulation of cell proliferation [Scaffidi et al., 2000]. This idea is further corroborated by a recent publication showing that indeed the reversible phosphorylation of FADD is necessary for proper cell- cycle progression in T cells [Hua et al., 2003].

3. Mitogen activated protein kinase family (MAPK)

The highly conserved mitogen activated protein kinase (MAPK) family is one of the major kinase families that regulate cells by transducing extracellular signals into cellular response [Robinson and Cobb, 1997]. In mammalian systems, these responses include cellular proliferation, differentiation, development, the inflammatory response and apoptosis. The classic MAPK cascade consists of three sequential intracellular protein kinase activation steps and is initiated when the first member, MAPK kinase kinase (MAPKKK) is activated (Figure 8). MAPKKK is a protein kinase that phosphorylates and activates MAPK kinase (MAPKK), and this activation is followed by activation of the specific MAPK.

MAPKs activate numerous protein kinases, nuclear proteins, and transcription factors, leading to downstream signal transduction. Activation of the MAPK cascade is rapid and enables to respond to environmental changes in a regulated fashion.

Three important members of this family are the extracellular signal- regulated kinase (ERK), p38 and c-JUN NH₂- terminal kinase (JNK). JNK was originally identified as a 54 kDa stress-activated protein kinase (SAPK) and is activated in response to many of the stimuli that activate p38 such as lipopolysaccharide, TNF, IL-1, osmotic stress and ultraviolet radiation [Chen et al., 2001].

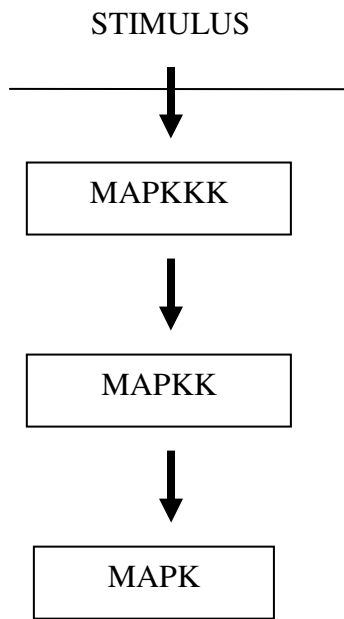


Figure 8: Diagrammatic representation of the mitogen activated protein kinase (MAPK) signaling pathway.

The JNK protein kinases are encoded by three genes: JNK1, JNK2 and JNK3. JNK1/2 is ubiquitously expressed, whereas JNK3 appears to be expressed only in brain, heart and testis.

3.1. Activator protein- 1 (AP- 1)/ c-jun

AP- 1 was one of the first mammalian transcription factors to be identified [Angel and Karin, 1991], but its physiological function is still being unraveled. AP- 1 activity is induced by a plethora of physiological stimuli and environmental insults. In turn, AP- 1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. However, despite increasing knowledge regarding the physiological function of AP- 1, the target genes mediating these functions are not always obvious.

AP- 1 is not a single protein, but a menagerie dimeric basic region- leucin zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2), Maf (c- Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ ATF3, B- ATF, JDP1,

JDP2) sub- families, which recognize either 12- O- tetradecanoylphorbol- 13- acetat (TPA) response elements (5'- TGAG/ CTCA- 3') or cAMP response elements (CRE, 5'- TGACGTCA- 3') [Chinenov and Kerppola, 2001].

C-Jun is the most potent transcriptional activator in its group [Ryseck and Bravo, 1991] whose transcriptional activity is attenuated and sometimes antagonized by JunB [Chiu et al., 1989;Schutte et al., 1989]. It is activated in chemically induced murine liver tumours and HCCs of humans [Yuen et al., 2001], suggesting an important oncogenic function for this gene in liver tumours of mammals.

Recently, we demonstrated that the MEKK/JNK pathway operates upstream of the CD95 pathway to mediate chemotherapeutic drug- induced apoptosis in liver cells via a c-jun responsive element [Eichhorst et al., 2000].

3.2. c-jun and apoptosis in tumour cells

The regulation of cell proliferation by AP- 1 might be of crucial importance for the multi-stage development of tumours [Liu et al., 2002;Park et al., 1999]. However, AP- 1 does not always promote cell proliferation- it also has anti- proliferative activities. The limiting components that guide the decision seem to be the Jun proteins, with the Fos proteins has a little effect [Shaulian and Karin, 2001].

C-jun is primarily a positive regulator of cell proliferation, as c-jun- deficient fibroblasts have a marked proliferation defect *in vitro* [Schreiber et al., 1999;Wisdom et al., 1999] and the proliferation of c-jun- deficient hepatocytes is severely impaired during liver regeneration *in vivo* [Behrens et al., 2002].

Early *in vitro* studies indicated that increased AP- 1 activity can lead to apoptosis in specific cell types, including human tumour cells [Shaulian and Karin, 2002]. However, oncogenic AP- 1 can antagonize apoptosis in liver tumours [Eferl et al., 2003]. The differential activity

of AP-1 in apoptosis is best exemplified by the dual role of c-jun in neuronal cells and hepatocytes. Increased c-jun activity promotes apoptosis in neuronal cells *in vitro*. When the activation of c-jun is impaired, either in *Jnk3*- null or *c-jun*^{AA/AA} mice, which express a c-jun mutant that cannot be activated by JNKs, hippocampal neurons are protected from KAINATE- induced cytotoxicity [Behrens et al., 1999;Zhang et al., 2002;Yang et al., 1997b]. By contrast c-jun is required for the survival of fetal hepatocytes which undergo apoptosis in c-jun- deficient mouse embryos [Eferl et al., 1999]. The cell specific consequence of AP- 1 activity for apoptosis is probably due to differential regulation of pro- apoptotic and anti- apoptotic target genes.

In neuronal cells c-jun regulates the expression of Bim, a pro- apoptotic BCL2 family member that is crucial for neuronal apoptosis [Whitfield et al., 2001]. In T cells, c-jun and c-fos regulate the gene that encode Fas Ligand (FasL/CD95L), which can trigger apoptosis through the Fas receptor (CD95R) [Kasibhatla et al., 1998]. The crucial pro- apoptotic target gene that is repressed by c-jun in tumours might be Trp53 (p53). This has been shown for liver tumours that lack c-jun, in which p53 is upregulated and consequently induces apoptosis [Eferl et al., 2003].

The differential regulation of pro- apoptotic and anti- apoptotic genes indicates that AP- 1 can promote apoptosis in some tumour types, whereas it induces survival in others.

4. Aim of study

The Hepatocellular carcinoma (HCC) also called hepatoma is one of the most common malignancies and has the fourth highest mortality rate worldwide [Coleman, 2003].

The major factor for the development of HCC is well defined and some of the steps involved in hepatocarcinogenesis have been elucidated in recent years. However, no clear picture of how and in what sequence these factors interact at the molecular level has emerged yet.

Malignant transformation of hepatocytes occurs in the context of chronic viral hepatitis, chronic liver injury, regeneration and as many as 80% of cases occur in cirrhotic livers [Ganem and Prince, 2004;Moradpour and Blum, 2005;Wu and Yu, 2007]. The alcoholic liver cirrhosis is the leading cause of liver failure. The liver damage progresses gradually from hepatic steatosis over steatohepatitis to liver cirrhosis and finally hepatocellular carcinoma.

Other factors such as the activation of cellular oncogenes and/or inactivation of tumour suppressor genes such as the p53 gene affect the genomic instability and may also contribute to the development of HCC [Moradpour and Blum, 2005].

Current available therapeutic modalities for HCC are largely inadequate. Surgical approaches such as resection and transplantation are the treatment of choice for HCC; however, because of the underlying liver disease, only a minority of patients are suitable for resection, and access to transplantation is limited by organ availability. Local tumour ablation is effective for early HCC, and chemoembolization is of benefit in intermediate-stage disease. So far, no first-line therapy has emerged for advanced HCC. Therefore, research efforts are focused on novel targeted therapies.

Failure to induce cell death in response to apoptotic stimuli is a hallmark of cancer: loss of CD95 expression or impairment of the CD95 and TRAIL-receptor pathways is associated with dedifferentiation, larger tumour size and poor prognosis, and is a predictor of tumour size recurrence [Okano et al., 2003;Ito et al., 2000;Shin et al., 2001;van Noesel et al., 2002;Ozoren and El Deiry, 2003]. Intervention to influence the sensitivity to programmed cell death is therefore a promising therapeutic strategy.

The significance of c-jun as a major regulator of hepatocyte proliferation and apoptosis prompted us to investigate the function of this gene in liver cancer. Previous publications have shown that c-jun downregulation sensitizes cells to apoptotic stimuli by inducing the p53 activity [Eferl et al., 2003]. To better understand how c-jun interferes with hepatocyte proliferation/ apoptosis through the CD95 receptor pathway and develop new strategies to

prevent liver carcinogenesis, we studied the importance of c-jun loss for hepatocarcinogenesis. We developed new siRNA target sequences and showed that:

- 1.) **20nM of siRNA effectively downregulates c-jun in HepG2 cells**
- 2.) **C-jun downregulation sensitizes HepG2 cells to apoptosis**
- 3.) **The death receptors are relevant in c-jun downregulated HepG2 cells**
- 4.) **Downregulation of c-jun in HepG2 cells increases apoptosis by upregulating of CD95 receptor level and preventing FADD phosphorylation**

II. Material and Methods

1. Material

Except where stated differentially, all chemicals were of at least analytical grade and purchased from by Sigma-Aldrich (Taufkirchen, Germany), Amersham Biosciences (Freiburg, Germany), Serva (Heidelberg, Germany), Merck (Darmstadt, Germany), Invitrogen/Gibco (Karlsruhe, Germany) or Roth (Karlsruhe, Germany)

1.1. Common Buffers

PBS (Phosphate buffered Saline):	NaCl	137 mM
	Na ₂ HPO ₄	8,1 mM
	KCl	2,7 mM
	KH ₂ PO ₄	1,5 mM
	pH = 7,4	
TBE (10X):	Tris	0,45 M
	Boric Acid	0,45 M
	EDTA	10 mM
	pH = 8,3	
Western Running Buffer (10X)	Tris	0,25 M
	Glycin	1,9 M
	SDS	1%

Towbin (10X)	Tris	250 mM	
	Glycin	190 mM	
	MetOH	20%	
TBST (10X)	Tris-base	24,2 g	
	NaCl	80 g	
	pH= 7,6		
	Tween 20	1ml/L	
Loading Buffer (5X)	SDS	1 g	
	Bromphenolblue	5 mg	
	2 M Tris-HCl	1,5 ml	pH= 6,8
	2- Mercaptoethanol	1,25 ml	
	100 mM EDTA	0,5 ml	
	Glycerol	4,73 ml	
	H ₂ O	1,12 ml	
Stacking	Acryamid 30%	6,5 ml	
	Bis 2%	2,5 ml	
	Tris-HCl	12,5 ml	pH= 6,8
	SDS 10%	0,5 ml	
	H ₂ O	28 ml	
12,5% SDS-PAGE	Acryamid 30%	41,6 ml	
	Bis 2%	16 ml	
	Tris-HCl	25 ml	pH= 8,8

	SDS 10%	1 ml
	H ₂ O	16,4 ml
Protein Lysis Buffer:	Tris-HCl	20 mM pH = 7,4 (4°C)
	NaCl	130 mM
	Glycerol	10% (w/v)
	Triton X-100	1% (w/v)
	1 x Complete Protease Inhibitors (Roche)	
	EDTA	2 mM
	NaF	500 mM
	Na ₃ VO ₄	10 mM
Homogenization Buffer:	Saccharose	250 mmol/l
	Tris	20 mmol/l
	EGTA	2 mmol/l
	pH = 7,5 (4°C)	
	PMSF	200 mM (in 2-Propanol)
	Aprotinin	5 mg/ml (in water)
	Leupeptin	10 mmol/l (in water)
	2- Mercaptoethanol	0,7 µl/ml
Stripping Buffer:	62,5 mM Tris-HCl	250 ml pH= 6,7
	SDS	5 g
	2- Mercaptoethanol	1760 µl

Nicoletti Buffer:	Sodiumcitrat	0,1 % (w/v)
	Triton X-100	0,1 % (w/v)
	Propidiumiodid	50 µg/ml
Freezing medium	FCS	90%
	Dimethylsulfoxid	10%

1.2. Real Time PCR- Primer

All primers were purchased from Metabion (Martinsried), MWG Biotech or Thermo.

Name	forward	Reverse	Product
c-jun	5' TGG AAA CGA CCT TCT ATG ACG AT 3'	5' CTG TAG CCA TAA GGT CCG CTC T 3'	70 bp
CD95	5' GCC CAA GTG ACT GAC ATC AA 3'	5' CAG GCC TTC CAA GTT CTG AG 3'	84 bp
FasL	5' GGC CTG TGT CTC CTT GTG AT 3'	5' TGC CAG CTC CTT CTG TAG GT 3'	99 bp
Gapdh	5' ACC CAC TCC TCC ACC TTT GA 3'	5' CTG TTG CTG TAG CCA AAT TCG T 3'	100 bp

1.3 RNAi Primer

Name	sense	Antisense	Purchase
jun4 siRNA	5' AAA AAA GGA AGC TGG AGA GAA T 3'	5' ATT CTC TCC AGC TTC CTT T 3'	Ambion AM1620
jun siRNA	5' AAA AAA GGA AGC TGG AGA GAA T 3'	5' ATT CTC TCC AGC TTC CTT T 3'	Ambion AM16104
ctrl siRNA	5' AAG GCT ACG TCC AGG AGC GCA 3'	5' AAT GCG CTC CTG GAC GTA GCC 3'	Ambion AM16104
Caspase-8 siRNA	Sequence unknown	Sequence unknown	Dharmacon M-003466-04
jun _s siRNA	Sequence unknown	Sequence unknown	Dharmacon M-003268-02
jun1 siRNA	5' AAC GCC TCG TTC CTC CCG TCC 3'	5' AAG GAC GGG AGG AAC GAG GCG 3'	Ambion AM1620
jun11 siRNA	5' AAC TTC AAC CCA GGC GCG CTG 3'	5' AAA CTG CGT TAG CAT GAG 3'	Ambion AM1620
jun20 siRNA	5' AAC TCA TGC TAA CGC AGC AGT 3'	5' AAA CTG CTG CTG TAG CAT GAG 3'	Ambion AM1620

1.4 Antibodies

Name	kDa	Purchase	Order Number
c-jun	39	BD Biosciences Pharmingen	610326
phospho- cjun (Ser 63)	48	Cell signalling (New England Biolabs)	9261
CD95 (Fas C-20)	48	Santa Cruz Biotechnology	sc-715
FasL	45	BD Biosciences Pharmingen	556387
phospho- FADD (ser 194)	28	New England Biolabs	2781S
FADD (518)	30	Santa Cruz Biotechnology	sc-6035
Actin	40	ImmunO (MP Biomedicals)	691001
Gapdh antibody [6C5]	36	abcam	ab8245
E- Cadherin	120	BD Biosciences	610181
caspase 8		were a kind gift from Peter Krammer [Krueger et al., 2001;Scaffidi et al., 1998].	

1.5 Reagents

Leucine zipper tagged CD95 Ligand (LZCD95L) was a kindly gift from Prof. Dr. Peter H. Krammer (DKFZ Heidelberg) and have been described previously [Kuntzen et al., 2005]. The death receptor ligands TRAIL (APO-2L) and TNF- α , to stimulate the specific receptors were purchased from Sigma and 5-FU and Actinomycin D were purchased from Sigma-Aldrich

1.6 Cell lines

The HepG2 cell line derived from hepatocellular carcinoma and is sensitive to CD95- induced apoptosis. Huh7 cell line is also derived from hepatocellular carcinoma and shown to express p53 with half- life as a result of point mutation at codon 220 [Hsu et al., 1993]. All cell lines are a kindly gift from Peter Krammer.

All cells were cultured in RPMI 1640 medium (PAA laboratories) supplemented with 10% FCS (PAA laboratories) and 1% penicillin/ streptomycin (Gibco/ BRL) in a humidified atmosphere at 5% CO₂ and 37 °C.

For transfection experiments OptiMEM I + Glutamax I (Gibco) were used.

2. Methods

2.1 Cell culture techniques

2.1.1 Freezing and thawing

For long- term storage, passages 1-10 were stored in liquid nitrogen while higher passages were kept at -80 °C. Since ice crystals forming during the freezing and process could rupture the cells, a special freezing medium was used (see section 1.1 Common Buffers).

Cells were centrifuged at 1200 rpm for 5 min, resuspended in freezing medium at a concentration of 5×10^5 cells (1 ml per cryovial) and frozen at -20 °C for one day. Afterwards, they were transferred to -80 °C and, if desired, after another day to liquid nitrogen.

For thawing: cells were thawed in a warm water bath (37 °C) and transferred to a centrifuge tube with 10 ml prewarmed complete medium. Centrifugation was performed at 1200 rpm for 5 min. Afterwards, the cell pellet was dissolved in 30 ml prewarmed complete medium. The culture was left to grow for at least three days before any experiments.

2.1.2 Determination of cell concentration

To determine the cell number, cells were washed once with 5ml PBS and 1 ml of trypsin was added to the cells. The cells were incubated for approx. 5 min in the incubator, and resuspended in prewarmed medium. Cells were diluted 1:2 with Trypan blue (Gibco/ BRL) and were counted in a Neubauer counting chamber to determine the number of cells per ml suspension.

2.1.3 Splitting and seeding

Cells were split every two to three days and diluted to 1:2.

For experiments, cells were seeded a day before transfection in 6- well plates at a density of $1, 5 \times 10^5$ cells. The cell suspension was centrifuged at 1200 rpm for 5 min and resuspended in prewarmed medium. After determining the cell density, the appropriate concentration was adjusted by dilution in prewarmed medium.

2.2. Light microscopy

Morphologic changes of apoptotic cells such as shrinking, blebbing and formation of apoptotic bodies can be detected by visualization in a light microscope.

Cells at a concentration of $1, 5 \times 10^5$ (6- well plate) were either transfected with ctrl. siRNA or jun siRNA for 30 hours. After incubation, probes were viewed with light optical microscope (Zeiss) at 10 X magnification and pictures were taken with the connected camera.

2.3. Measurement of apoptosis by flow cytometry

Flow cytometry allows one to measure, with high accuracy, relative quantities of a variety of cell constituents simultaneously. When the measurements are recorded in a list mode, it is possible to attribute each of the several measured features to a particular cell and thus to obtain correlated measurements of these features on a cell by cell basis. Cellular heterogeneity can thus be estimated and subpopulations with distinct characteristics can be discriminated. Thus, multiparameter flow cytometry offers improved opportunities to describe the complex

relationships between cell activation, proliferation, differentiation, maturation and decomposition within heterogeneous cell populations as e.g., blood and bone marrow where different differentiation lineages are mixed together, or tumours where malignant cells may be discriminated by clonal characteristics.

Cells were scraped with a rubber policeman and washed once with cold PBS (1200 rpm, 5 min). The pellets were resuspended in 200µl nicoletti buffer and left overnight at 4 °C in the dark. Untreated cells were used for adjustment of instrument settings. The fluorescence intensity (FL-2) as well as scatter parameters was acquired. The percentage of apoptotic cells was determined from the region of the subG₁ peak.

Measurement was performed in a flow cytometer (FACS Calibur Becton Dickinson, Heidelberg, Germany) using Cell Quest as software 10,000 events were counted. Specific rate of apoptosis was calculated as (rate probe-rate control)/ (100%- rate control).

2.4. RNAi

RNA interference (RNAi) is a well- recognized pathway involved in cellular defense against viral invasion, transposon expansion, and post- transcriptional regulation [Zamore, 2001;Tuschl, 2001;Sharp, 2001;McManus and Sharp, 2002;Hannon, 2002;Hutvagner and Zamore, 2002]. As a novel biological pathway, RNAi has quickly distinguished itself as a valuable reverse genetic tool. The functional intermediates in the RNAi pathway, small interfering RNA (siRNAs), may be synthesized by a variety of means and introduce intracellularly to effect changes in gene function.

For transient transfection, OligofectamineTM (Invitrogen) was used according to the protocol of the manufacturer. 1.2×10^5 cells were seeded in 12 wells 24 hours prior to transfection in RPMI with FCS without antibiotics. Transfection was carried out in OptiMEM (Gibco) without FCS, which was added after 4 hours.

One and a half days later (approx. 30 h), cells were washed with cold PBS and either harvested for analysis or stimulated for further 16 h. Cells were pooled and 50% were used for flow cytometry analysis and Western blot respectively.

2.5. Western blot analysis

Agarose gels are best for separating larger molecules, like DNA, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is better suited for running smaller ones, like proteins. Sodium dodecyl sulphate is an anionic detergent that binds quantitatively to proteins, denaturing them linearly and uniform charge, so that they can be separated solely on the basis of their size.

Western analysis was carried out as follows. Briefly, proteins were separated by SDS-PAGE and transferred to PVDF (Millipore) membranes. Membranes were blocked for one hour in 1 x TBS solution containing 0,1% Tween 20 (TBS-T) and 5% milk powder. Membranes were then incubated with primary antibodies in TBS-T and 5% milk powder at 4°C overnight, followed by the appropriate HRP-conjugated secondary antibody for one hour at room temperature. Western blots were developed using the ECL system from Amersham.

2.5.1 Protein determination

The method developed by Bradford [Bradford, 1976] makes use of the feature of Coomassie Brilliant Blue G- 250 to change its absorption maximum from 465 nm to 595 nm on binding to proteins. The protein content of the lysates can thus be quantified by comparing the absorption with a calibration curve prepared with increasing concentrations of BSA.

Measurement was performed in Photometer Ultraspec 3100 pro (Biochrom) Cell lysates were diluted 1:500 in H₂O containing Bradford solution (1:5 diluted). 5 minutes were allowed for the color shift to develop. Samples were measured at 592 nm.

Cells were seeded in 6- well plates (1, 5 x 10⁵) and were incubated over night. The next day cells were transfected with siRNA (see section 2.4.) incubated for 30 hours and either left untreated or stimulated for 16 hours. For harvest, three wells were pooled and cells centrifuged at 1400 rpm, 4 °C for 5 minutes. The pellet was washed in cold PBS. After another centrifugation step, the pellets was resuspended in approx. 50 µl protein lysis buffer (depending on pellet size) and were at regular intervals shake at 4 °C for 30 minutes. The solution was cleared by centrifugation (1400 rpm, 4 °C, 5 minutes) and the protein solution was transferred to fresh tubes. Protein determination was performed following the protocol of Bradford (see section 2.5.1.). The protein solution was diluted with 5 x loading buffer, boiled at 95 °C for 3 minutes and used directly for electrophoresis or stored at – 20 °C.

2.5.2 Membrane Stripping

In order to detect different proteins on a given PVDF membranes, bound antibodies from previous experiments have to be removed. For that purpose, membranes were immersed in stripping buffer and treated for 30 minutes at 50 °C on a shaking platform. Subsequently, remnants of stripping buffer were washed away by three washing steps in TBS-T (10 min at room temperature). Stripping efficiency was confirmed by applying ECL solution (see section 2.5). When no bands became apparent the membrane was blocked again to cover unspecific binding sites with 5 % non-fat dry milk in TBS- T for 1 h at RT. Afterwards, the next primary antibody was applied.

2.6. RNA-Isolation and Reverse transcription

Obtaining high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments, including Northern analysis, nuclease protection assays, RT-PCR, RNA mapping, in vitro translation and cDNA library construction.

1 µg of total RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (MMLV) (Life technologies) with oligo(dT)15 primers (Roche) in a 20 µl reaction tube containing 10 mM DTT and 500 µM desoxynucleotide triphosphates. One µl aliquots were amplified in a DNA thermocycler (MJ Research) with 0, 5 units of Taq DNA polymerase (Roche) in a 50 µl reaction. Thirty reaction cycles were performed. Each cycle consisted of a denaturation step (94°C for 30 s), an annealing step (54°C for 30 s) and an elongation step (72°C for 10 min). PCR products were analysed in agarose gels.

2.7. Real Time PCR

Real-time RT-PCR was performed on a Rotorgene 3000 (Corbett Research, Sydney, Australia) using heat-activated Taq DNA polymerase (Amplitaq Gold; Applied Biosystems). After an initial activation phase (for the Taq polymerase) of 5 min at 95°C, the samples were cycled 40 times at 95°C for 10 s and 60°C for 10 s and 72°C for 30 s. Target gene forward and reverse primers and probes were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA). Commercially available predeveloped TaqMan assay reagents were used for the internal standards human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 18S ribosomal RNA (18 S rRNA). All primers and probes were obtained from

Applied Biosystems. All primers were cDNA specific, not amplifying genomic DNA. The following sequences of oligonucleotide primers were used (see Primer list 1.2).

3.15. Statistics

Statistical analysis was performed using the SPSS software (version 11.0; SPSS, Inc., Chicago, IL, USA). Data are generally given as mean \pm standard deviation, if not otherwise stated. Student t test was used for paired data. Mean differences of non-parametric data were analyzed by the Mann-Whitney U test. Significance was assessed using a Monte Carlo approach with a post-hoc Bonferroni correction. A difference was considered to be statistically significant at $p < 0.05$.

2.8. Densitometrie software

Densitometrie analysis was carried out using TINA software Version 2.0 (Raytest, Germany)

2.9. Membrane protein isolation

After siRNA transfection (see section 2.4) cells were scraped with a rubber policeman and washed once with cold PBS (200 x g, 2 min, 4°C). The pellets were resuspended in homogenisation buffer and sonicated for 10 s. After a second centrifugation step (800 x g for 2 min at 4°C) the supernatant were transferred in a new tube which stored at -80°C over night. The next day, homogenate was thawed and transferred in a ultracentrifugation tube and ultracentrifuged (Beckman Airfuge Rotor A110) at 25 psi for 8 min. After centrifugation the supernatant (the cytosolic fraction) were transferred in a new tube and the pellet (membrane

fraction) were refilled with homogenisation buffer, resuspend. Protein measurement was made for both fractions using the photometer.

2.10 Luciferase Reporter Assays

The purpose of the reporter gene assay is to measure the regulatory potential of an unknown DNA-sequence.

HepG2 cells were transiently transfected with siRNA. After 24 h cells were transfected again with 0,4 µg of Vector DNA (pCH110) and 1 µg of firefly- Luciferase Vectors. The pCH110 vector encodes the β-galactosidase gene under the control of the SV40 promoter (Amersham Biosciences), which was used as an internal control for transfection efficiency. After 24 h, cells were scraped and washed with cold PBS. The pellet were resuspended in 100 µl Reporterlysisbuffer and stored at -80°C for at least 24 h. The next day, cells were thawed at RT, vortexed and centrifuged (14.000 rpm, 10 min, 4°C). Supernatant transferred in a new tube and luciferase activities were determined in duplicate samples as described by the manufacturer (Promega GmbH). Luciferase values (RLU) were calculated by dividing the luciferase activity by the β-galactosidase activity. The data represent means ± SD of three independent experiments performed in duplicates.

III. Results

1. 20 nM of siRNA effectively downregulates c-jun in HepG2 cells

Up to now, mainly two techniques were used for knock-out experiments. One is based on the replacement of a specific wildtype gene by a mutated and often non-functional allele on the genomic level by means of homologous recombination. This technique is very stable but relatively time consuming (>1 year). Another approach which yields much faster results makes use of antisense oligodeoxynucleotides. Advantages of the antisense method are that it is easy, high-throughput and applicable to genes essential for cell growth. However, the method had a disadvantage that silencing efficacy is sometimes too low to analyze gene function.

In this context, RNAi seems to be a good alternative to study gene functions. Recently published protocols provide information for the production of siRNA using simple molecular biology techniques [Elbashir et al., 2001a; Elbashir et al., 2001b].

Blast search was used to find potential siRNA target sequences targeting c-jun and the same method was used to create a negative control (ctrl siRNA) not containing any mammalian-gene coding sequence (For sequence see section 1.3. in Materials).

In order to show that self synthesized siRNA is able to downregulate c-jun in vitro, HepG2 cells were separately transfected as described in the method section, with four different self synthesized in vitro transcribed siRNA (designed as: jun1-, jun11-, jun20- and jun4 siRNA) and with a pool of the same siRNA sequences. For creating self synthesized siRNA we used 29-mer DNA oligonucleotide with 21 nt encoding the siRNA and 8 nt complementary to the T7 Promoter Primer.

Western Blot analysis shows that only jun4 siRNA was able to decrease the expression level of c-jun to a significant amount in vitro (Figure 1A). Western blot analysis was also used to compare the effect of cells transiently transfected with the in vitro synthesized jun4 siRNA sequence with the transfection of the same sequence produced by chemical synthesis

(designed as jun – Ambion cat. AM16104) or with a pool of different chemically synthesized sequence commercially available (designed as jun_s – Dharmacon cat. M-003268-02). Western Blot analysis shows that jun siRNA as well as jun_s siRNA was able to decrease the expression level of c-jun (**Figure 1B**) and proved also to abolish the amount of phosho- c-jun, showing that siRNA transfection is effective in reducing the active form of jun (**Figure 1C**). Effective silencing effect of jun siRNA transfection was also assessed at the transcription level by Real Time-PCR (**Figure 1D**) which confirmed abolished levels of c-jun in cells transfected with the aforementioned sequences but not in cells transfected with the ctrl siRNA sequence.

Figure 1

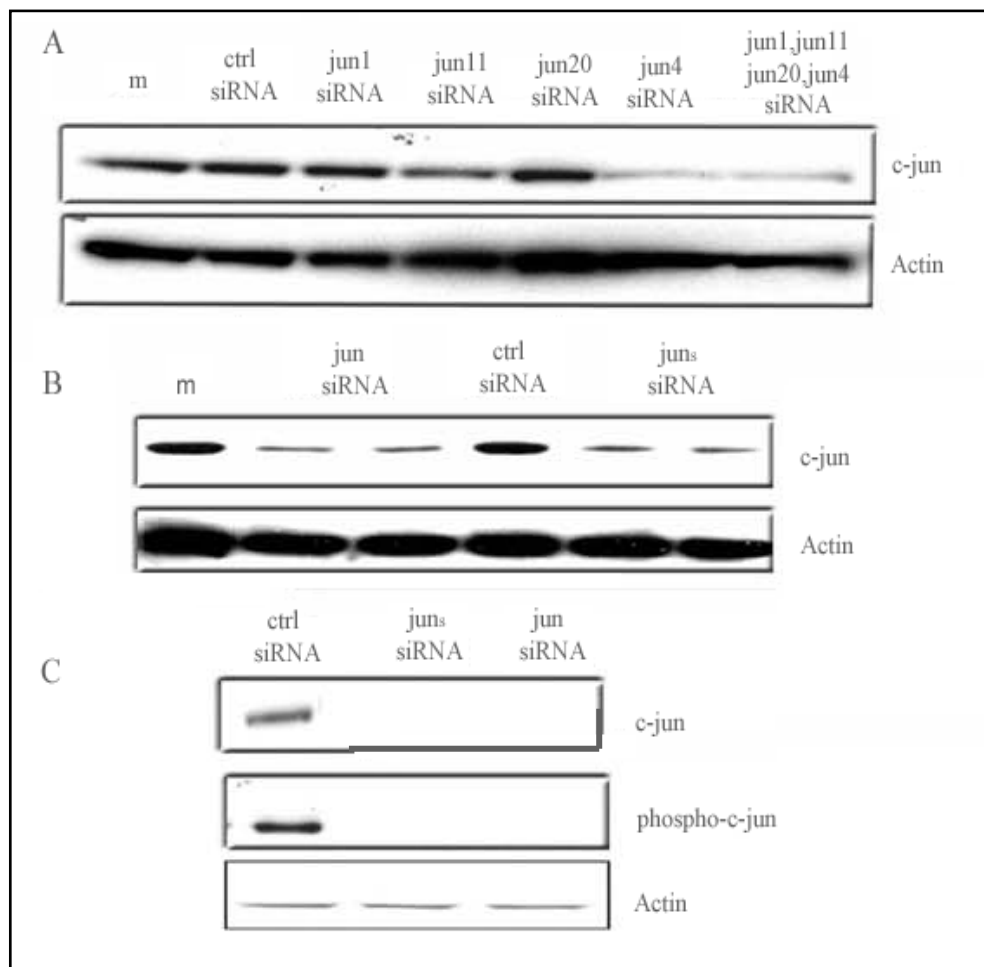


Figure 1A: siRNA downregulates c-jun in HepG2 cells and affect phospho c-jun.

HepG2 cells were transfected with siRNA targeting c-jun compared to 20 nM control siRNA (ctrl siRNA). The first band indicates mock (m) transfected HepG2 cells. Western blot analysis shows the downregulation of c-jun in HepG2 cells by jun4 siRNA and by the pool of siRNA (last band) transfected cells. β -Actin was used as loading control. **B:** HepG2 cells were transfected with either 20 nM jun siRNA (chemically synthesized) or with a pool of siRNA targeting c-jun (jun_s siRNA (smartpool (Dharmacon): 5 nM each, jun_s siRNA) to compare the difference between both. No difference was shown between both variants of siRNA. **C:** HepG2 cells were transfected with 20nM siRNA against c-jun (jun_s siRNA, jun siRNA) compared with 20 nM control siRNA (ctrl.siRNA) transfected cells. siRNA targeting c-jun shows also to abolish the amount of phospho-c-jun.

To further assess also at the functional level the effect of c-jun inhibition, a reporter assays for the AP1-transcriptional activity was performed (**Figure 2**). This investigation showed a remarkable decrease of the AP1-transcriptional activity only in cells treated with coding siRNA sequences, hereby confirming the efficacy of our self-synthesized siRNA to investigate the functional effects of c-jun inhibition.

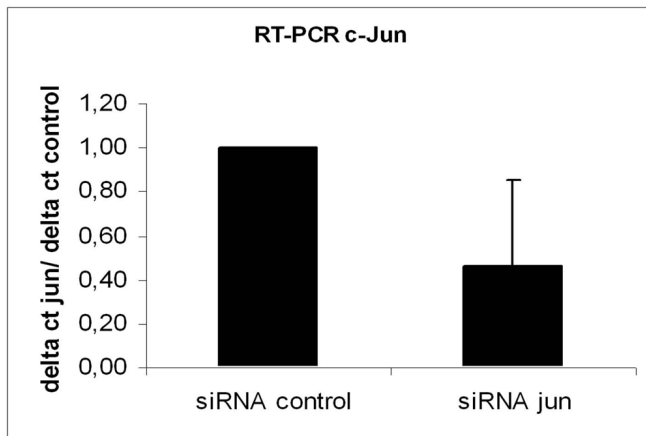


Figure 2: Downregulation of c-jun by siRNA on mRNA level.

mRNA level of c-jun were determined by realtime-PCR. The expression levels are given as ratio to control primer (Gapdh). The experiment confirmed the downregulation of c-jun on a transcriptional level using siRNA

As no difference could be observed between the both sequences (jun siRNA and jun_s siRNA) the known siRNA sequence (jun siRNA) was chosen for further experiments.

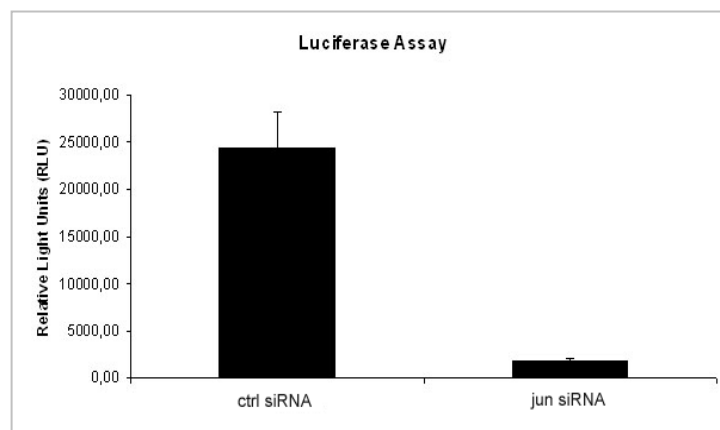


Figure 3: Luciferase Reporter assay shows a functional repression of c-jun in HepG2 cells.

HepG2 cells were cotransfected with a luciferase reporter plasmid under the control of four repeat sequence of TRE DNA element followed by a collagenase promoter and pCH110 vector harboring the β -galactosidase cDNA (internal standard) using the Fugene Reagent according to manufacturer protocol (Roche Diagnostics, Indianapolis, IN). Twenty-four h after transfection, cells were lysed. Luciferase expression was measured using the luciferase assay system (Promega) according to the manufacturer's protocol. Reporter activity was normalized for transfection efficiency using β -galactosidase.

To assess the stability of c-jun inhibition in our system by assessing c-jun expression by Western blot in HepG2 cells, a time-kinetic study of c-jun inhibition was performed over seven days after transfection. As shown in Fig. 4, expression levels of c-jun were completely abolished 12 hours after transfection (**Figure 4**). The expression level of c-jun in jun siRNA transfected HepG2 cells increases between days 5 to 7 (120-168 h after transfection) in comparison to ctrl siRNA-transfected HepG2 cells and achieved control level at day 7 (168 h). Therefore, all experiments were realized in this time frame (up to 7 days). In summary, we could successfully synthesize a form of c-jun-targeting siRNA which proved effective and stable in HepG2 cell line abolishing the function of c-jun over 5 days. Also, we showed siRNA transfection resulted in 12,4-fold decrease of c-jun expression as judged by Western blot analysis.

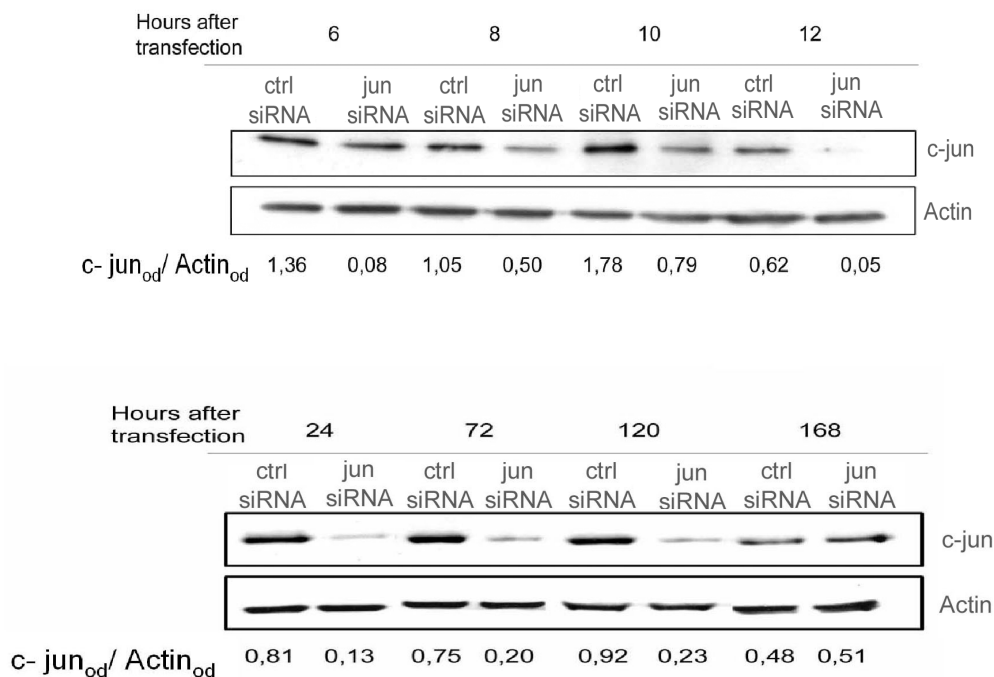


Figure 4: Downregulation of c-jun via siRNA in HepG2 cells completely abolished the function over 5 days.

HepG2 cells were transiently transfected with siRNA targeting c-jun or ctrl. siRNA. Expression of c-jun was analyzed by western blot at the indicated time points. β -Actin was used as as loading control. The intensity of the signals was measured densitometrical and the ratios of c-jun vs. actin signals are displayed.

2. C-jun downregulation sensitizes HepG2 cells to apoptosis

C-jun has been shown to have positive, negative or no influence on apoptosis under different experimental conditions [Leppa and Bohmann, 1999; Mak and Yeh, 2002]. Increased c-jun activity promotes apoptosis in neuronal cells *in vitro*. In contrast, c-jun is required for the survival of fetal hepatocytes, which undergo apoptosis in c-jun deficient mouse embryos [Eferl et al., 1999; Behrens et al., 2002]. Therefore the function of c-jun in relation to apoptosis needed further investigation.

To assess how c-jun inhibition affects apoptosis in our system, HepG2 cells were transfected with jun siRNA or ctrl siRNA as described, and the apoptosis rate was measured by flow cytometry [Nicoletti et al., 1991]. As shown in Fig. 5, c-jun-transfected cells showed a mild but significant increase in apoptosis compared to untreated cells (**Figure 5**).

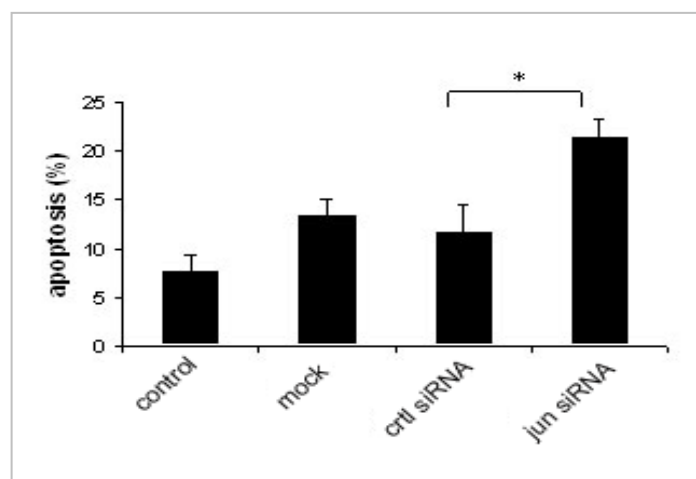


Figure 5: Downregulation of c-jun in HepG2 cells shows a significant higher apoptosis rate than control cells

HepG2 cells were transfected with control and jun siRNA 30 hours before harvest. Measurement of apoptosis were performed after nicoletti treatment by flow cytometry. Untreated (control) and mock treated cells were also measured for apoptosis, * P= 0,006

Consistently, as we analyzed the cell morphology, apoptotic features could be seen (**Figure 6A,B**). Therefore downregulation of c-jun in HepG2 cells lead to spontaneous apoptosis up to 20% compared to control cells. This experiment suggests that c-jun has a protective function against apoptosis in HepG2 cells.

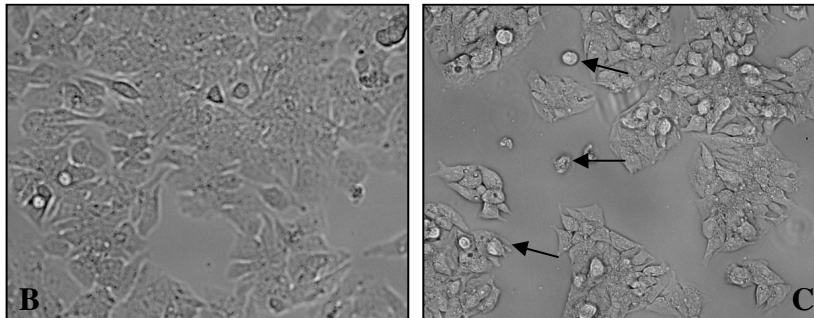


Figure 6A,B: Downregulation of c-jun in HepG2 cells show apoptotic features

Microscope analyses show HepG2 cells **B** treated with ctrl.siRNA and **C** treated with jun siRNA. Both probes were viewed with light optical microscope at 10 X magnification.

3. The death receptors are relevant in c-jun downregulated HepG2 cells

Loss of sensitivity to apoptosis is a hallmark of cancer leading to cell immortalization [Eichhorst and Krammer, 2001]. The way how cells lose their ability to undergo apoptosis is thought to occur through several mechanisms which include loss of the death receptors CD95 or TRAIL-receptor. To study the influence of c-jun on death receptor mediated apoptosis we stimulate several membrane receptors in hepatoma cells, known to be able to induce apoptosis. To this aim leucine zipper-tagged CD95 ligand (LZCD95L) TRAIL or TNF α was used. While stimulating cells with TNF α , Actinomycin D was added to enhance its pro-apoptotic effect. To assess the influence of c-jun inhibition on the sensitivity to receptor-mediated apoptosis in these cells substances were administered in different concentrations in cells transiently transfected with jun siRNA or with ctrl siRNA. Initially, cells were treated

with different concentrations of the leucine zipper-tagged CD95 ligand (LZCD95L) and TRAIL 30 hours after transfection of these cells with jun siRNA (**Figure 7 and 8**).

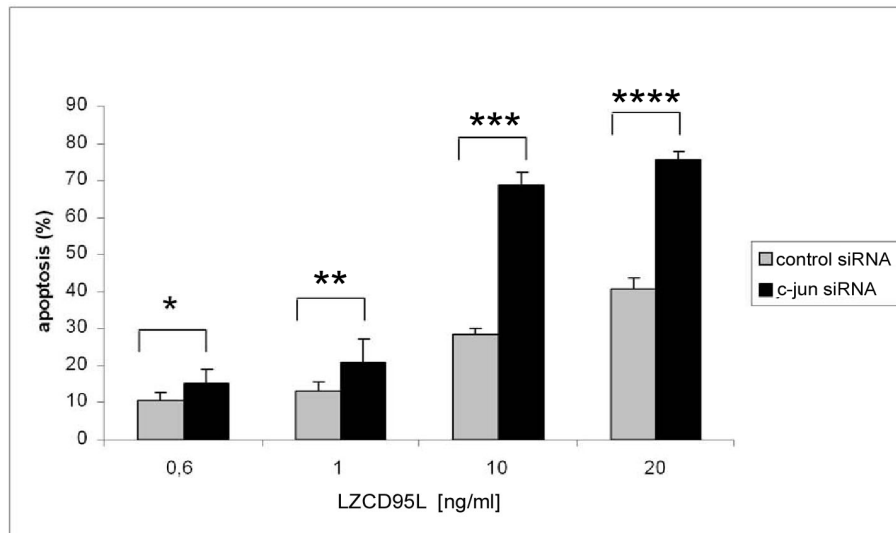


Figure 7: Effect of c-Jun inhibition and concomitant stimulation of CD95 in cancer cells on apoptosis.

HepG2 cells were transfected either with ctrl- or c-jun siRNA. 30 hours later, cells were stimulated with the indicated dose of LZCD95L. The apoptosis rate was measured by flow cytometry using propidium iodide, * P=0,016; ** P=0,014; *** P=0,001; **** P=0,002.

Addition of TRAIL or CD95L led to a dose-dependent increase of apoptosis in ctrl siRNA transfected cells. However, the increase of apoptosis rates in jun siRNA transfected cells after stimulation with CD95L or TRAIL was higher than that observed in ctrl siRNA transfected cells (**Figure 7 and 8**). The difference between apoptosis rates in ctrl- and jun- siRNA transfected cells after stimulation was higher than that achieved by jun siRNA transfection alone. These findings suggested, that c-jun inhibition facilitates the transmission of the pro-apoptotic signal in the cells, hereby synergistically increasing apoptosis after membrane receptor stimulation. Since this effect was better to be seen at a concentration of 10 ng/ml LZCD95L or 2,5 ng/ml TRAIL, this concentrations were used for further experiments.

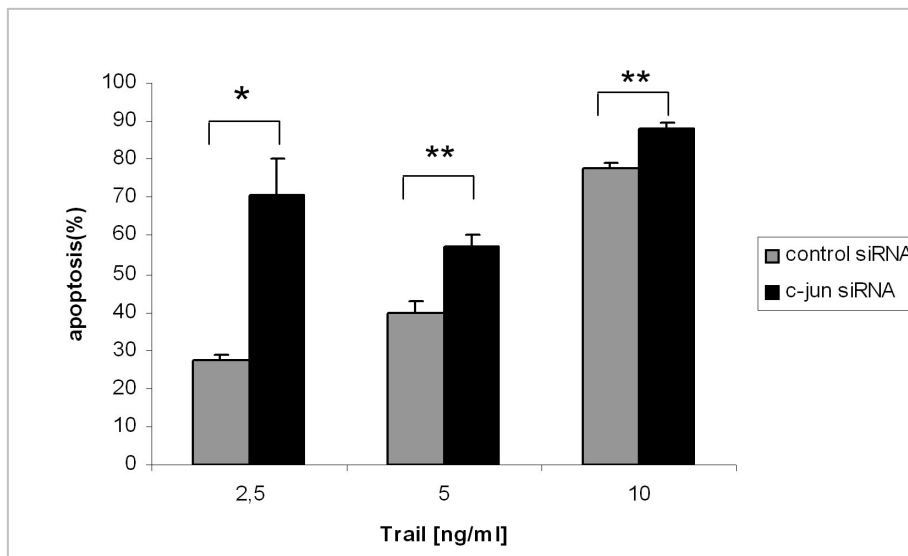


Figure 8: Effect of c-jun inhibition and concomitant stimulation of TRAIL in cancer cells on apoptosis.

HepG2 cells were transfected either with ctrl- or jun siRNA. 30 hours later, cells were stimulated with the indicated dose of TRAIL. The apoptosis rate was measured by flow cytometry using propidium iodide, * P=0,02; ** P<0,001.

SiRNA transfection without any administration of death receptor stimulating substances (control) for 16 hours induced 10% apoptosis in cells transfected with ctrl siRNA and 23% in jun siRNA transfected cells (**Figure 9**). Downregulation of c-jun in HepG2 cells with concomitant application of LZCD95L and TRAIL however, dramatically increased apoptosis rates up to 70% indicating that c-jun acts to suppress the CD95- and TRAIL mediated apoptosis signalling pathways (**Figure 9**).

The TNF receptor stimulation is known to activate both, a survival and a death signalling pathway, resulting in most cancer cells, in no effect on apoptosis. The survival pathway is represented by the TNF α mediated activation of NF-kappa B, which is known to activate antiapoptotic signalling. The death signalling pathway is recruited by FADD, procaspase8, cFLIP and procaspase 10.

To obtain an overall proapoptotic effect by the stimulation of cells with TNF α , Actinomycin D (Act.D), an inhibitor of nucleic acid synthesis which is thought to shift the balance of TNF α toward a proapoptotic effect by inhibiting the transcriptional activity bound to NF κ B activation, was administered in a concentration of 100 ng/ml. The TNF α +Act.D treated cells do not show a significant difference between ctrl and jun siRNA transfected cells (**Figure 9**).

To further investigate whether the apoptosis-enhancing effect of c-jun downregulation in liver cells is specific for death receptor mediated signaling pathway, cells transfected with jun siRNA were stimulated with the chemotherapeutic agent 5-FU, a reagent that causes apoptosis *in vitro* that is partially mediated via the CD95 system and is caused by up-regulation of CD95 and CD95L [Muller et al., 1997;Eichhorst et al., 2001;Tillman et al., 1999]. On the bases of these data, HepG2 cells after c-jun downregulation, were treated with 100 μ g/ml 5-FU and the apoptosis rate was determined by flow cytometry analysis. We observed, that c-jun downregulation and concomitant application of 5-FU also showed a significantly higher apoptosis rate than the control cells, suggesting that the transcription factor AP-1 acts to suppress the CD95 mediated apoptosis signaling pathway trough the anticancer drug 5-FU (**Figure 9**).

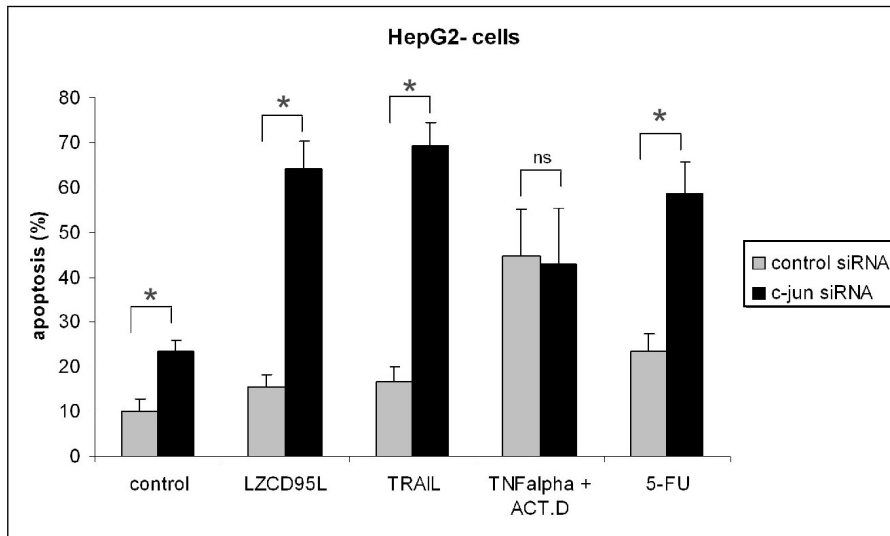


Figure 9: c-jun downregulation and concomitant application of apoptosis inducing substances in HepG2 cells induces significant higher apoptosis rate than the control cells

HepG2 cells were transfected with either control- or jun siRNA and were stimulated 30 hours after siRNA transfection with indicated dose of LZCD95L, TRAIL, and 5-FU. The apoptosis rate was measured by flow cytometry. HepG2 cells treated with TNF α +Act.D do not show a difference between control- and c-jun siRNA treated cells, ns P=0,802; * P<0,001.

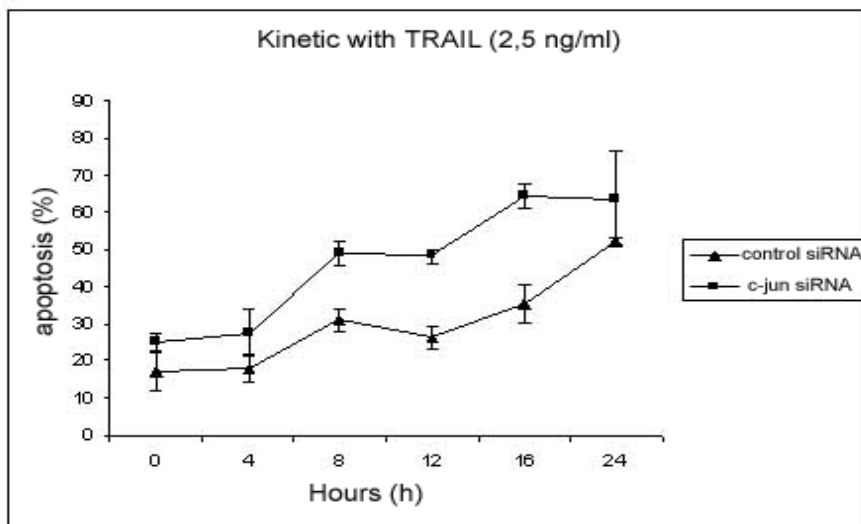


Figure 10: Kinetic analysis showed a specific effect of apoptosis in HepG2 cells after downregulation of c-jun and concomitant application of TRAIL

HepG2 cells were transfected with either ctrl- or jun siRNA and were stimulated 30 hours after transfection with indicated dose of TRAIL. The apoptosis rate was measured by Facs after indicated time.

Kinetic analysis of the apoptosis rate with ctrl- and jun siRNA and concomitant receptor specific ligand TRAIL treated cells showed that the effect of apoptosis is specific. It can be detected after 8 hours with a difference of approx. 20% (by TRAIL application). The apoptosis level of ctrl- and jun siRNA treated cells reached the same level after 24 hours (**Figure 10**). We assume, after 24 h the apoptosis effect is not specific, because the effect of c-jun-/ctrl siRNA prevails over the application of TRAIL.

To elucidate the role of CD95 receptor for c-jun dependent sensitization to apoptosis, we used Huh7 cells, a hepatocellular carcinoma cell line. These cells can not express the CD95 receptor due to mutated p53 as a result of a point mutation at codon 220 [Hsu et al., 1993] and therefore represent a cell line to address this question [Muller et al., 1998].

Huh7 cells were treated with jun siRNA as described for HepG2 cells. We observed a significantly higher apoptosis rate in c-jun downregulated cells both in CD95L, TRAIL and 5-FU stimulated cells compared to ctrl siRNA treated cells (**Figure 11**). The unstimulated (control) cells showed the same effect. The apoptosis rate is significantly higher in c-jun downregulated Huh7 cells compared to ctrl siRNA treated cells. In contrast to HepG2 cells the apoptosis rate was generally lower. Huh7 cells with concomitant application of siRNA and TNF α + Act.D did not show a difference in apoptosis rate (**Figure 11**), thus confirming, that TNF α death receptor pathway uses a different signalling pathway than CD95 or TRAIL.

We also observed a significant increase in apoptosis rate in Huh7 cells, which were concomitantly, treated with 5-FU and jun siRNA compared to ctrl siRNA and 5-FU treated cells. However, the apoptosis rate was also lower in Huh7 cells compared to HepG2 cells. A functional lack of CD95 receptor in TNF α treated Huh7 cells however, does not affect the apoptosis rate. This shows again, that the TNF α induced death pathway act completely different than the CD95 or the TRAIL pathways.

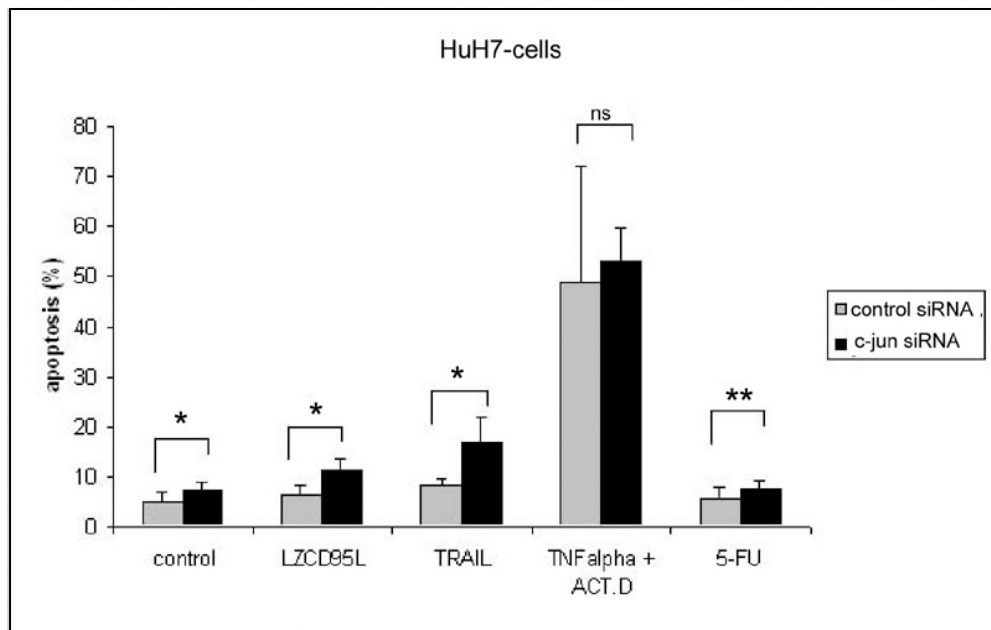


Figure 11: c-jun downregulation and concomitant application of apoptosis inducing substances in HuH7 cells induces significant higher apoptosis rate than the control cells

Huh7 cells were transfected with either control- or jun siRNA and were stimulated 30 hours after siRNA transfection with indicated dose of LZCD95L, TRAIL, and 5-FU. The apoptosis rate was measured by flow cytometry. HuH7 cells treated with TNF α +Act.D do not show a difference between control- and c-jun siRNA treated cells, ns P=0,6334; * P<0,001; ** P=0,017

According to this observation protein isolation has been carried out in c-jun downregulated HepG2 cells and the level of p53 was determined (**Figure. 12**)

Unlike earlier observations [Kuntzen et al., 2005;Eferl et al., 2003], p53 protein level was not increased in HepG2 cells, suggesting that p53 is not responsible for the increase in sensitivity to receptor induced apoptosis.

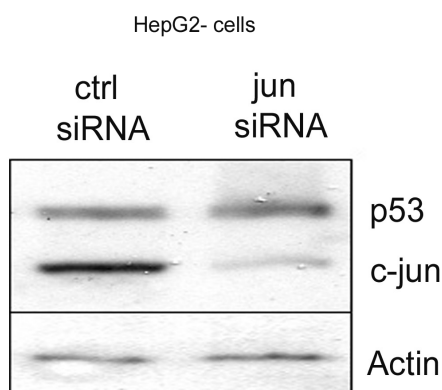


Figure 12: c-jun downregulation in HepG2 cells do not show an increase of p53 level

HepG2 cells were transfected with either ctrl- or jun siRNA and protein isolation with westernblot was performed. Actin was used as loading control.

Taken together, these results suggest that c-jun enhances not only the apoptosis ratio in HepG2 cells, but is specific for death receptor-mediated apoptosis. Furthermore, p53 protein level was not increased in these cells, suggesting that the increased sensitivity to receptor mediated apoptosis is not p53 dependent.

4. Downregulation of c-jun in HepG2 cells increases apoptosis by upregulating of CD95 receptor level and preventing FADD phosphorylation

In order to investigate the involvement of caspase-8 in signal transduction, cleavage products of caspase-8 were analyzed by Western blotting, after c-jun downregulation and concomitant stimulation with leucine zipper-tagged CD95 Ligand (LZCD95L) [Kuntzen et al., 2005]. Cleavage of caspase-8 produces two intermediates (p42 and p44) which are further processed to the active 20 kDa fragment. The increase of apoptosis as measured by flow cytometry in c-jun downregulated cells, corresponded to an increase of caspase-8 activation, as judged by its cleaved active subunits (**Figure 13**).

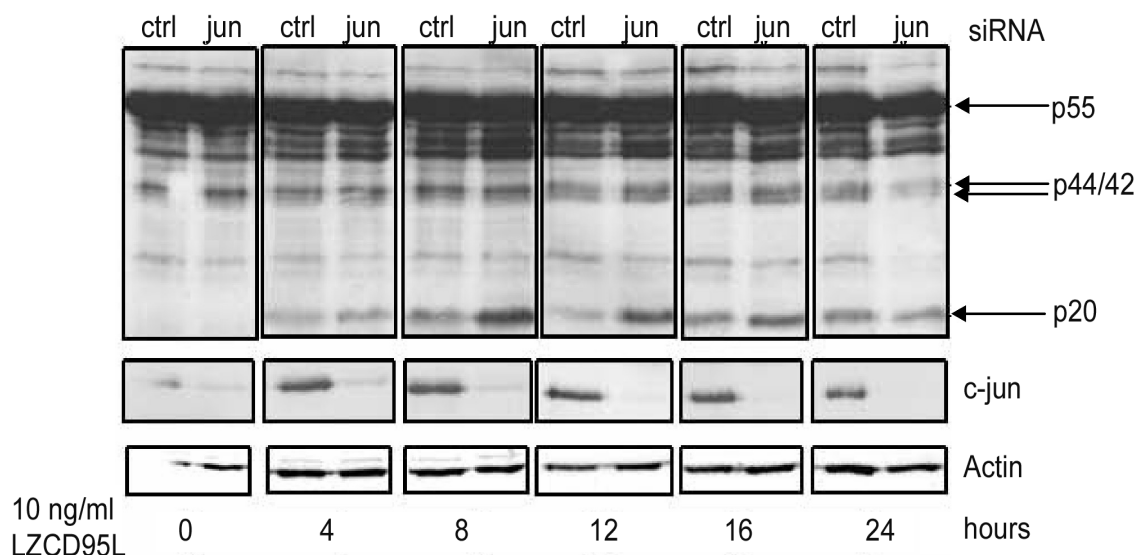


Figure 13: Western blot analysis shows an increase of caspase-8 activation in c-jun downregulated HepG2 cells after 8 hours

HepG2 cells transfected with either ctrl- or jun siRNA and stimulated 30 hours after transfection with the indicated dose of LZCD95L. Total cell lysates were prepared at the indicated time points and caspase-8 expression was analyzed by immunoblotting. Westernblot analysis has been carried out. β -Actin was used as a loading control.

To investigate whether c-jun inhibits the CD95 signalling pathway upstream or downstream of caspase 8, we co-transfected HepG2 cells with jun and caspase-8 (casp8) siRNA (**Figure 14**).

Co- transfection of HepG2 cells with casp8 siRNA abolished the effect of c-jun downregulation after receptor stimulation, suggesting that c-jun acts upstream of caspase 8, likely on the CD95 or DISC level (**Figure 14**).

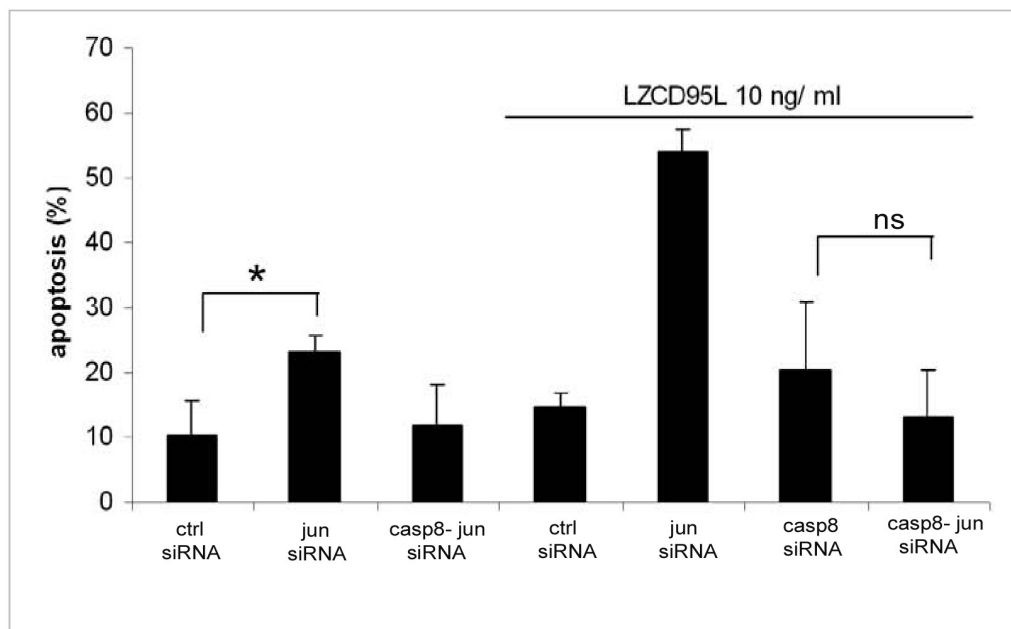


Figure 14: Increased apoptosis rate after downregulation of c-jun in HepG2 cells is mediated by caspase-8 HepG2 cells were transfected with either ctrl- or jun siRNA and were stimulated 30 hours after siRNA transfection with indicated dose of LZCD95L. Flow cytometry analysis was performed, * P<0,001; ns P=0,225

Moreover, the cleaved fragment of caspase-8 (p20) is visible in c-jun silenced HepG2 cells, which confirmed our assumption, that c-jun inhibits CD95 signalling pathway upstream of caspase-8 (**Figure 15**).

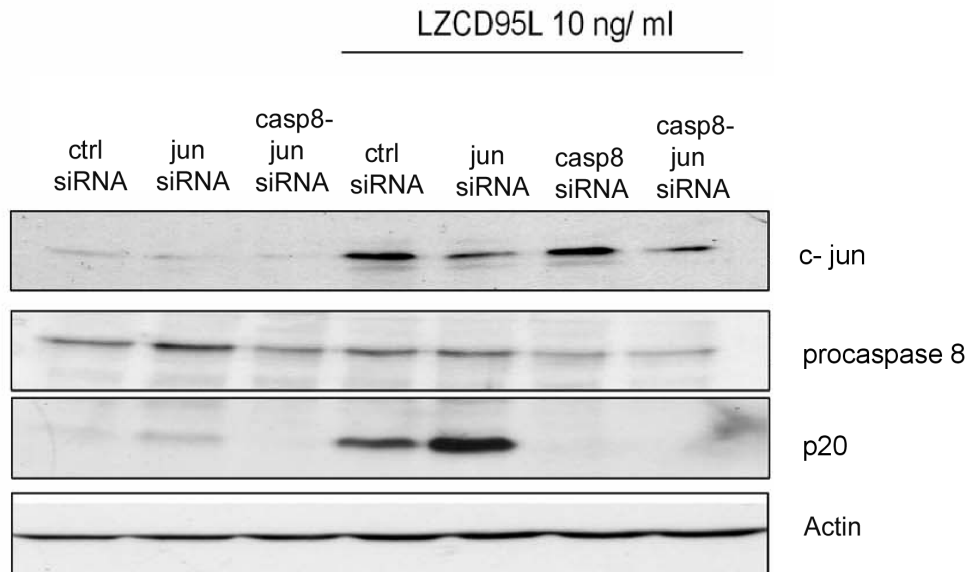


Figure 15: c-jun inhibits CD95 signalling pathway upstream of caspase-8

HepG2 cells were co- transfected with either ctrl-, jun- or casp8 siRNA and were stimulated 30 hours after siRNA transfection with indicated dose of LZCD95L. Western blot analysis was performed. β -Actin was used as a loading control.

Since c-jun caused an increase of caspase-8 cleavage product, we evaluated whether this might be due to increased expression of CD95 receptor. First, we verified the mRNA level of CD95 receptor using RT-PCR method. This clearly showed an upregulation of CD95 in HepG2 cells treated with c-jun siRNA (**Figure 16**). An upregulation of CD95 on the transcription level does not necessarily increase the translational level; therefore protein isolation was performed to confirm this result.

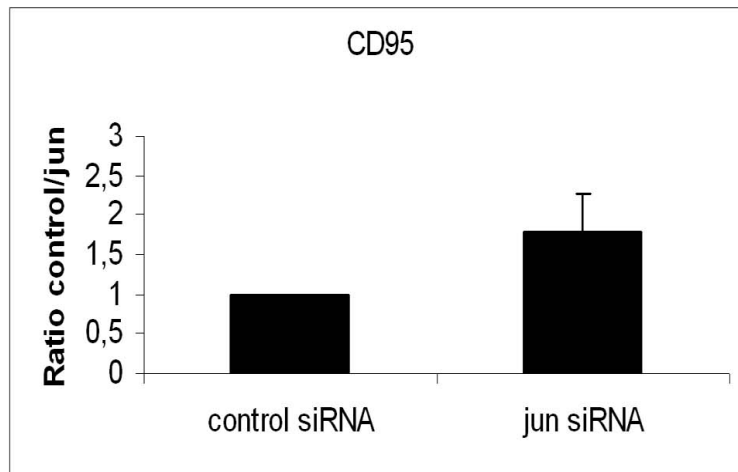


Figure 16: HepG2 cells treated with c-jun siRNA showed an upregulation of CD95 mRNA level

HepG2 cells were transfected with either ctrl- or jun siRNA. After transfection, cDNA was isolated and realtime PCR analysis were performed against CD95 receptor.

A total protein isolation followed by western blot analysis, however showed only a minimal change in the level of CD95 protein expression after c-jun- downregulation by siRNA transfection. The densitometry analysis showed an increase of approximately two times of CD95 receptor protein in c-jun downregulated HepG2 cells (**Figure 17**).

Since CD95 receptor is a transmembrane protein, total protein isolation is insufficient to detect active CD95 receptor protein located at the cell membrane. C-jun downregulation could affect not only the translation level of CD95, but also rapid transport of presynthesized CD95 receptor molecules stored in vesicles. Therefore, downregulation of c-jun may contribute to the upregulation of CD95 cell surface expression.

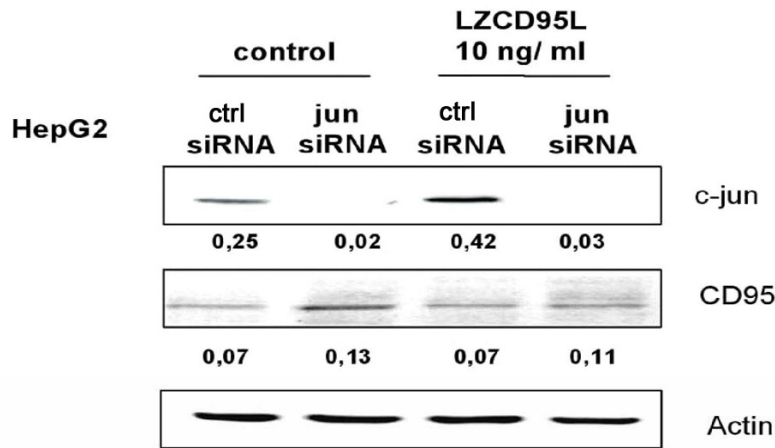


Figure 17: c-jun- downregulation by siRNA transfection shows a minimal change in the level of CD95 expression in total cell lysates.

HepG2 cells were transfected with either ctrl- or jun siRNA and were stimulated 30 hours after siRNA transfection with indicated dose of LZCD95L. Western blot analysis was performed at indicated time points.

Optical density was calculated as follows: For c-jun Western blot (first lane) $od1 = \text{ctrl siRNA} + \text{c-jun band density} = \text{A}$; $od2 = \text{ctrl siRNA} + \text{Actin band density} = \text{B}$; $\text{A/B} = \text{X}$; $od3 = \text{jun siRNA} + \text{c-jun band density} = \text{C}$; $od4 = \text{jun siRNA} + \text{actin band density} = \text{D}$; $\text{C/D} = \text{Y}$; $\text{Y/X} = 0,25$. Same calculation was performed for CD95 antibody.

Therefore, we isolated membrane proteins and analyzed the expression of CD95 in the membrane and cytosolic fraction using Western blot analysis.

The results of membrane protein purification in **Figure 18** confirmed earlier observations. In c-jun downregulated HepG2 cells the level of CD95 receptor was increased compared to control siRNA transfected cells.

As a loading control of membrane proteins we used E- Cadherin, a transmembrane glycoprotein that mediates epithelial cell-to-cell adhesion. The loading control of cytosolic proteins was accomplished with GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (**Figure 18**).

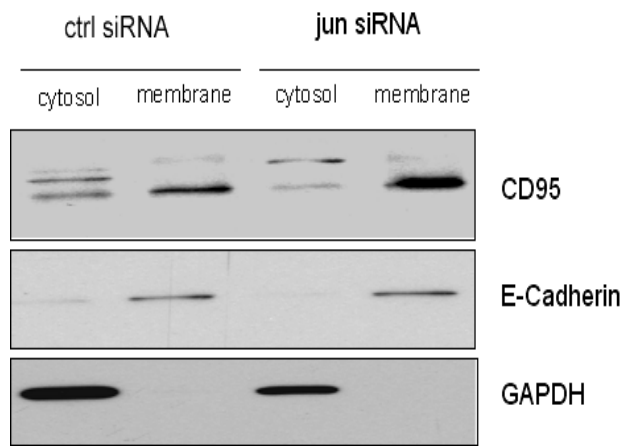


Figure 18: Membrane protein purification showed an increase of CD95 receptor levels in c-jun downregulated HepG2 cells

HepG2 cells were treated with ctrl- and jun siRNA. Western blot analysis of membrane and cytosolic fraction of siRNA treated HepG2 cells was performed. E- Cadherin were used as loading control for membrane fraction and GAPDH were used as loading control for cytosolic fraction.

So far, we could show that a downregulation of c-jun in HepG2 cells sensitizes for death receptor mediated apoptosis. These results showed an increase of CD95 receptor both at the transcriptional and the translational level in c-jun downregulated HepG2 cells. Further c-jun downregulation in HepG2 cells increased the membrane protein level of CD95.

Other components, which play a crucial role in CD95 receptor mediated apoptosis, may also be regulated by c-jun downregulation.

A number of proteins are recruited to the cytoplasmic domain of CD95 receptor protein. These include FADD, which functions primarily as an adaptor protein and is a key component of the intracellular signalling cascade, which leads to caspase-8 activation.

In this section, we investigated the phosphorylation status of FADD in HepG2 cells after c-jun downregulation. Indeed, we could show in c-jun knockdown HepG2 cells that the adaptor protein FADD is unphosphorylated compared to control cells, indicating that c-jun might interfere with apoptosis by modifying the phosphorylation status of FADD (**Figure 19**).

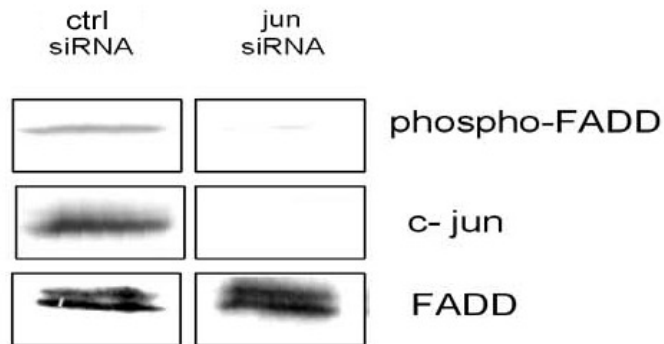


Figure 19: HepG2 cells treated with jun siRNA shows an unphosphorylated FADD

HepG2 cells were transfected with either ctrl- or jun siRNA. Western blot analyses were used to identify the phosphorylation status of c-jun downregulated HepG2 cells. FADD were used as loading control and c-jun was used to confirm c-jun downregulation.

A number of FADD-interacting kinases have been identified and one kinase that has been shown to associate with and phosphorylate FADD *in vivo* is HIPK3 and its splice variant FIST [Curtin and Cotter, 2004]. Therefore we hypothesized that HIPK3 and FIST expression are regulated through the transcription factor c-jun.

Taking together, these results showed an unphosphorylated FADD in HepG2 cells treated with jun siRNA.

IV. Discussion

HCC is one of the most common cancers in the world, accounting for 6 percent of all cancers worldwide and has the fourth highest mortality rate worldwide [Coleman, 2003]. There are multiple factors involved for why some people develop HCC and others do not. The most significant known factor is the presence of cirrhosis. Other factors may include genetic potential, hormonal influences, advancing age, state of general health and nutrition, lifestyle habits (especially alcohol abuse), and exposure to viruses and chemicals.

Apoptosis has also become the focus of many researchers since it became apparent that deregulation of the apoptotic programme is pathophysiologically involved in liver diseases [Patel et al., 1998]. Evidence indicates that extrinsic and intrinsic apoptosis pathways are downregulated in hepatocellular carcinoma. The CD95 receptor, which is normally expressed on hepatocytes, is downregulated in liver tumour cells, resulting in double resistance to cell death: cells cannot kill themselves via autocrine suicide and the immune system surveillance via attacking T- lymphocytes cannot eliminate newly occurring aberrant cells. In an analysis of 22 hepatocellular carcinomas, 9 showed no CD95 expressions and 13 had reduced CD95 expression [Strand et al., 1996]. CD95 also correlated with differentiation of tumour: highly differentiated tumours express more CD95 than less differentiated ones [Ito et al., 1998].

Therefore, the re-sensitisation of tumour cells is a promising therapeutic strategy. Indeed, re-introduction of CD95 into liver tumours cells showed encouraging results in mice [Shimizu et al., 1996]. A different approach is the activation of cell death effector molecules, such as caspases. Recently, alpha-(trichloromethyl)-4-pyridineethanol was found to facilitate apoptosome formation and induce caspase 3 activation in a panel of tumour cells [Jiang et al., 2003]. For HCC treatment, either proapoptotic pathways can be activated or overactivated antiapoptotic mechanisms, such as FLIP or survivin, may be shut down. Small components that reactivate caspases or agents, such as siRNA, for downregulation of overactivated antiapoptotic pathways are likely to enter the clinic. Numerous methods allow researchers to deliver siRNA to the target cells, including nonviral and viral vectors.

Alternatively, upstream modifying signalling pathways such as MAPK may be targeted with the outcome of apoptosis inhibition or promotion.

The present study demonstrates, that downregulation of c-jun via siRNA sensitizes hepatoma cells to receptor mediated apoptosis. In this context, FADD phosphorylation seems to be critical. In addition, c-jun downregulation lead to upregulation of CD95 cell surface protein. Therefore, c-jun is a potent target to treat cancer.

1. c-jun is a potent candidate for apoptosis induction in HepG2 cells

As the MAPK signalling pathway not only promotes cell proliferation, but also mediates cell survival and is upregulated in cancer cells, it seems to be a good therapeutic target. Especially the JNK pathway has been implicated in apoptotic responses to DNA damage, cell stress and cytotoxic drugs. It appears that c-Jun N- terminal kinase (JNK) activation is a proapoptotic event in non- malignant cells that results in the activation of proapoptotic members of the Bcl-2 family and cytochrome c release from mitochondria [Nijhawan et al., 2000]. In cells with dysregulated proliferation or cell cycle, the role of JNK and c-jun is more controversial. In certain cell types, c-jun overexpression is clearly the basis for resistance to DNA-damaging drugs, and reversal of resistance has been observed using c-jun antisense RNA. In contrast, the proposed role of c-jun as a mediator of apoptosis is further supported by recent data describing the regulation and function of components of the mitogen- activated protein kinase (MAPK) cascade regulating AP-1 activity. Two types of MAPK, JNK and p38, as well as common upstream kinases, including MEKK, are activated by genotoxic agents, such as UV and the alkylating agent such as methyl methanesulfonate (MMS), to phosphorylate and thereby activate the transcription factor c-jun [Ip and Davis, 1998; Minden and Karin, 1997]. Persistent activation of JNK by dominant active MEKK-1 resulted in hyperphosphorylation

and activation of c-jun and increased apoptosis in PC12 cells [Le Niculescu et al., 1999]. Vice versa, inhibition of JNK activity by transdominant negative mutant conferred resistance to apoptosis by various genotoxic agents [Zanke et al., 1996]. In mice lacking the neuron specific JNK isoform, JNK3, stimulation of the glutamate receptors does not result in excitotoxicity and apoptosis of hippocampal neurons [Yang et al., 1997b]. In line with this data, neuronal apoptosis induced by the excitatory amino acid kainate is absent in mice expressing a mutant form of c-jun, which contains amino acid substitutions at the critical JNK phosphorylation site [Behrens et al., 1999].

Despite these different lines of evidence, suggesting an important role of AP-1 proteins in cell death, a direct link between AP-1 activity and the induction of specific initiators or executors of apoptosis has not yet been identified.

To investigate the role of c-jun in hepatocellular carcinoma cell line (HepG2), c-jun was downregulated by siRNA. Light microscopic analyses revealed the formation of apoptotic bodies. Usually, shedding of apoptotic fragments was accompanied by a condensation of the cell. A flow cytometry measurement of cell size and granularity clearly demonstrated that downregulation of c-jun in HepG2 cells lead to cell shrinkage and increase granularity.

These results confirmed earlier studies done in liver cells: Mouse embryos lacking c-jun, due to homologous recombination/knock out, died at midgestation and exhibited defects in hepatogenesis. There was a dramatic increase in apoptosis in fetal liver in the absence of c-jun [Eferl et al., 1999]. Therefore, fetal hepatocytes require c-jun for cell survival. Hepatocellular cancer cells like HepG2 behave like fetal liver cells. They also require c-jun for cell survival, since c-jun downregulation via siRNA in HepG2 cells leads to apoptosis shown in Figure 2A. Differentiated hepatocytes, however, require c-jun for cell cycle progression, since conditional deletion of c-jun in adult livers mainly reduced the proliferation capacity of hepatocytes after partial hepatectomy, a strong inducer of cell- cycle reentry [Behrens et al.,

2002]. These liver specific functions of c-jun for cell survival and cell- cycle progression imply that this gene is a major regulator in the development of hepatic carcinomas.

2. c-jun inhibition sensitizes HepG2 cells to receptor induced apoptosis (Targeting death receptors)

The hepatoma cell line HepG2 was used to study the function of c-jun in death receptor mediated apoptosis at a molecular level. The JNK pathway has been shown to be closely linked to apoptosis [Davis, 2000]. Studies in this area have also shown, that the MEKK-1/ JNK kinase/ JNK/ c-jun axis acts proapoptotically upstream of the CD95 pathway to mediate chemotherapeutic drug-induced apoptosis [Eichhorst et al., 2000]. Similarly, deletion of single components of the JNK pathway also results in apoptosis and c-jun knockout mice as well as MAPK kinase 4 knockout mice die of increased apoptosis in the liver [Yang et al., 1997a]. The role of c-jun in CD95- mediated apoptosis is less clear. A link between JNK axis and the CD95 pathway is provided by the finding that c-jun exerts AP-1-mediated suppression of CD95 promoter [Eferl et al., 2003;Ivanov et al., 2002]. Downregulation of CD95 is frequently observed during tumour progression. Ivanov *et al.* demonstrated that ectopic expression of dominant- negative mutant of c-jun (TAM67) led to increased CD95 promoter activity of CD95 mRNA level in melanoma cells. They also showed that *c-jun*^{-/-} fibroblasts exhibit an increase in CD95 expression in these cells [Ivanov et al., 2003;Ivanov et al., 2002]. Similarly, we observed the same in HepG2 cells. C-jun downregulation via siRNA led to an increase of CD95 receptor expression, both at the transcriptional and translational level. Preliminary results showed an increased level of CD95 receptor after downregulation of JNK. Our study is the first one to show that c-jun downregulation, the member of AP-1 transcription factor, increases CD95 expression level in HepG2 cells. One possible explanation is the fact, that the CD95 promoter contains three AP-1-binding elements [Ivanov

et al., 2003] and binding of AP-1 results in CD95 suppression. Mutations within the promoter that abrogate the binding of c-jun are sufficient to increase the CD95 expression. Therefore, we conclude, an increase of CD95 expression lead to apoptosis. Through a concomitant treatment with death receptor specific ligand, the leucine zipper-tagged CD95 ligand (LZCD95L), the apoptosis rate was dramatically increased.

In the case of the TRAIL-R1 (DR4) and TNFR we saw the same effect. Downregulation of c-jun and a concomitant application of its specific ligand in HepG2 cells increased the apoptosis rate up to 70 % compared to ctrl siRNA transfected cells. Interestingly, the TRAIL-R1 promoter also contains three putative AP- 1 binding sites. However, Guan et al. showed that the human death receptor 4 is upregulated by AP- 1 [Guan et al., 2002]. They showed that only one binding site is active and activation of AP- 1 induces a rapid TRAIL-R1 expression through binding to the TPA- response element, resulting in apoptosis in H1792 human lung adenocarcinoma cells and PC-3 human prostate cancer cells. But c-jun has various functions in different tissues. Therefore, downregulation of c-jun via siRNA in HepG2 cells could increase the TRAIL-R1 level and a concomitant application of TRAIL results in an increased apoptosis rate. Indeed our experiments showed that downregulation of c-jun and concomitant application of TRAIL causes increased apoptosis in HepG2 cells. Westernblot analyses could show, if downregulation of c-jun increases the expression level of TRAIL-R1.

Of critical importance during apoptosis through death receptors, is the activation of caspases, which are common in both (CD95 and TRAIL) pathways. In this setting, Fas-associating protein with death domain (FADD) is a central adaptor molecule to recruit and activate the initiator caspase-8 upon ligand-mediated aggregation.

The caspase-8 is the first caspase, which is activated after activation of the receptor. Parallel to the increase of apoptosis as measured by flow cytometry, in c-jun downregulated cells, we saw an increase of caspase-8 activation as judged by appearance of its cleaved active subunits in the immunoblot. This experiment shows again, that c-jun affects directly the death receptor

pathway. Moreover, a co-transfection of casp8 siRNA abolished the effect of c-jun downregulation after receptor stimulation, suggesting that c-jun acted upstream of caspase 8, likely at the level of CD95 or DISC.

Recently, researchers could shown, that the phosphorylation of FADD at serine 194 (S191, murine FADD) in different tissues is important for regulating apoptosis [Shimada et al., 2004;Scaffidi et al., 2000;Curtin and Cotter, 2004;Alappat et al., 2005].

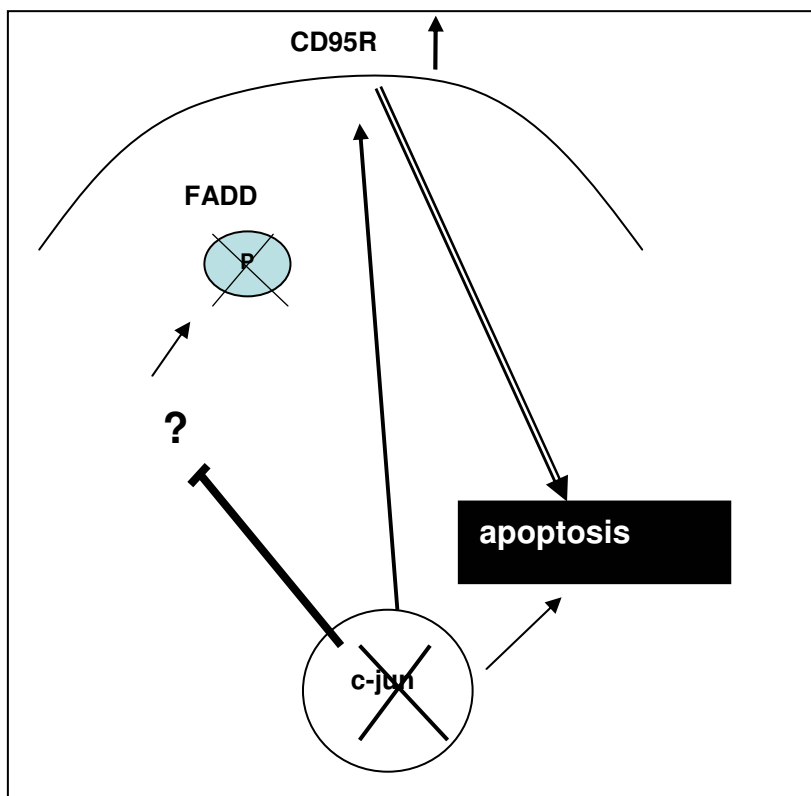
In this context, we showed that FADD phosphorylation was dependent on c-jun activity in hepatoma cells, and inhibition of c-jun using RNAi prevented FADD phosphorylation. Recently, Curtin and Cotter identified the kinase HIPK3 and its splice variant FIST the kinase that is responsible for the phosphorylation of FADD in prostate carcinoma cells [Curtin and Cotter, 2004]. We speculate in our system this kinase could also be important for FADD phosphorylation. Further experiments could give some answers.

Interestingly, no significant difference was observed in apoptosis rates between ctrl- and jun siRNA transfected cells, after concomitant stimulation with TNF α . TNF α signalling shares many features with CD95 signalling in the liver, specifically the activation of caspase-8 including FADD, Bid cleavage and mitochondrial signalling to apoptosis. Oligomerized TNFR1 internalizes and via the adaptor protein FADD activates caspase-8 [Hsu et al., 1995]. Additionally, other signals emanate from TNFR activation. Activated death receptors recruit adaptor proteins, such as tumour necrosis factor receptor associated protein 2 (TRAF2) and receptor interacting protein (RIP), that stimulate I κ B kinase (IKK) and proteosomal I κ B degradation, leading to activation and nuclear translocation of NF κ B [Barnhart and Peter, 2003]. NF κ B induces increased expression of survival genes, including BclXL, A1, XIAP, cFLIP and iNOS [Yin and Ding, 2003;Hatano et al., 2001]. Thus, mouse hepatocytes in culture do not undergo apoptosis after exposure to TNF α unless NF κ B-dependent gene expression is blocked, for example with cycloheximide or introduction of an I κ B superrepressor [Hatano et al., 2000;Ni et al., 1994]. The ability of TNF α to stimulate both,

pro- and antiapoptotic pathways, at least accounts in part for its pleiomorphic and seemingly opposing effects. Some reports suggest NFκB-dependent pathways inhibit cell death by inactivating JNK pathways, and JNK inhibition attenuates liver injury with preservation of hepatic architecture following warm ischemia/reperfusion and cold storage/reperfusion injury [Uehara et al., 2005;Uehara et al., 2004]. In our study, c-jun seems to be independent of TNFα induced apoptosis.

In conclusion, we found that the inhibition of c-jun expression in hepatoma cell line HepG2 reduced FADD phosphorylation and therefore increases the sensitivity of these cells to CD95 receptor-mediated apoptosis.

Furthermore, an increase of CD95 receptor both transcriptional and translational level in c-jun downregulated HepG2 cells was shown. Therefore, the sensitivity in c-jun downregulated HepG2 cells could be due to upregulation of CD95 and also through the reduced FADD phosphorylation.



3. c-jun and p53

It is tempting to speculate about the relationship between AP-1 and the tumour suppressor protein p53 in the context of apoptosis. Both AP-1 and p53 are nuclear transcription factors, both are regulated post-transcriptionally by stress signalling pathways, both can induce apoptosis in response to genotoxic agents, and both activate extrinsic and mitochondrial death pathway components. Furthermore, JNK can phosphorylate p53 and induces protein stabilisation. Several recent publications have linked AP-1 to modulation of the p53 pathway to explain the role of AP-1 in cell survival.

C-jun was described to suppress the transcription of the p53 gene in fibroblasts, which is also a known regulator of CD95 expression [Schreiber et al., 1999;Schreiber et al., 1999]. Mouse fibroblasts lacking c-jun exhibit upregulated p53 mRNA and protein levels, leading to decreased proliferation [Shaulian et al., 2000]. A recent study has provided compelling evidence that the oncogenic properties of c-jun may be linked to the antagonism of the pro-apoptotic activity of the p53 gene. Liver-specific inactivation of the c-jun gene demonstrated an essential role in the development of chemically induced hepatocellular carcinomas (HCC) [Eferl et al., 2003]. Tumours that developed in the absence of hepatic c-jun expression had increased levels of apoptosis. And c-jun null hepatocytes were sensitized to TNF α - induced apoptosis. The absence of c-jun in HCC tumours was linked to elevated levels of p53 and its pro-apoptotic target gene, *Noxa* (without effecting other pro- apoptotic p53 targets) [Eferl et al., 2003]. In agreement with these observations, we recently found upregulation of p53 and CD95 upon treatment HepG2 cells with the JNK inhibitor SP600125 [Kuntzen et al., 2005]. In contrast to this data, we could not see an upregulation of p53 protein in c-jun downregulated HepG2 cells. However, experiments in the Huh7 cell line [Nakabayashi et al., 1982] showed an increase of apoptosis rate both in unstimulated and concomitant death receptor stimulated cells. But compared to HepG2 cells the apoptosis rates were generally

lower. Presumably p53 is required in liver cells to increase the apoptosis rate. Therefore, we conclude that c-jun downregulation sensitizes hepatoma cells to death receptor induced apoptosis. p53 is needed to further increase the apoptosis rate.

V. Summary

C-jun is a proto-oncogene and member of the AP-1 transcription factor family. Although c-jun was shown to be involved in many different cellular functions including cell proliferation, differentiation, transformation and apoptosis in different cell types, its role in carcinogenesis remains elusive.

In this report we investigated the hypothesis that c-jun played a role in liver tumourigenesis by inhibiting the CD95 signaling pathways. To this aim, we investigated apoptosis rates in HepG2 and Huh7 hepatoma cells after inhibition of c-jun and concomitant incubation with several apoptosis-inducing substances.

We showed an effective suppression of c-jun both at the RNA and protein level after transfection with siRNA directed against c-jun. Functional suppression was confirmed by Luciferase- reportergen assays. Flow cytometry analysis showed a significant increase in the apoptosis rate in c-jun downregulated HepG2 cells. Moreover, incubation of HepG2 with the death receptor ligands CD95L and TRAIL and the chemotherapeutic agent 5- FU causes up to 60% apoptosis compared to control siRNA treated cells. This effect was not observed in cells treated with TNF- α . In contrast to HepG2 cells, experiments in Huh7 cells also showed an increase of the apoptosis rate in c-jun downregulated cells, but compared to HepG2 cells, the apoptosis rates were generally lower. Presumably, the mutated p53 gene played a role in Huh7 cells. Moreover, in correlation with c-jun downregulation an increase of the non-phosphorylated active form of FADD, which mediated caspase activation downstream of CD95 and TRAIL receptors, could be observed

Therefore we conclude c-jun downregulation inhibits the FADD phosphorylation and causes the sensitization of HepG2 cells to CD95L, TRAIL and 5-FU-mediated apoptosis. Pharmacologic inhibition of c-jun may therefore serve as effective cancer therapy.

VI. Zusammenfassung

c-jun ist ein Mitglied der AP-1 Transkriptionsfaktorfamilie und wurde als Protoonkogen beschrieben. Bisherige Arbeiten konnten zeigen, dass c-jun in vielen verschiedenen zellulären Funktionen wie Proliferation, Differenzierung, Transformation und Apoptose involviert ist. Die Funktion von c-jun ist abhängig von der zellulären Differenzierung und vom Zelltyp. Der Mechanismus jedoch, über den c-jun zur Karzinogenese beiträgt, ist noch zu klären.

In dieser Arbeit haben wir die Rolle von c-jun in der Karzinogenese und seine Rolle in Bezug auf Apoptose über den CD95 vermittelten Signalweg untersucht. Dazu wurde die Apoptoserate in den Hepatomzelllinien HepG2 und HUH7 nach Inhibierung von c-jun und nachträglicher Inkubation mit verschiedenen Apoptose auslösenden Substanzen, gemessen.

Wir konnten zeigen, dass eine siRNA-Transfektion zur erfolgreichen Herabregulation des c-Jun Transkriptionsfaktors sowohl auf RNA- als auch auf Proteinebene, führt. Dieser Effekt konnte, wie eine kinetische Analyse zeigte, über fünf Tage beobachtet werden. Die funktionale Suppression von c-jun konnte mit Hilfe eines Luciferase-Reporterassays bestätigt werden. Durchflusszytometrische Analysen zeigten eine signifikante Erhöhung der Apoptoserate nach siRNA-Transfektion. Darüber hinaus führte eine Herunterregulation von c-jun und eine anschließende Stimulation mit CD95L und TRAIL sowie mit dem Chemotherapeutikum 5-FU, verglichen mit Kontrollen, zu einer weiteren Steigerung der Apoptoserate auf bis zu 60%. Weiterhin konnte gezeigt werden, dass eine Stimulation mit dem Tumornekrosefaktor alpha (TNF- α) nach c-jun Herunterregulierung keine signifikanten Unterschied zwischen den Kontrollzellen aufwies. Ähnliche Ergebnisse wurden in Huh7 Zellen beobachtet, die experimentell gleich behandelt wurden. Jedoch zeigte sich hier, dass die Apoptoserate insgesamt niedriger war als in HepG2 Zellen. Mögliche Erklärung könnte das p53 Gen sein, welches in diesen Zellen in mutierter Form vorliegt. Weiterhin konnte gezeigt werden, dass in c-jun herunterregulierten Zellen die nicht phosphorylierte Form von FADD vorlag, welches die caspase Aktivierung downstream von CD95 and TRAIL

Rezeptoren vermittelt. Somit kann Zusammengefasst werden, dass eine Herabregulierung von c-jun eine Inhibierung der FADD Phosphorylierung zu Folge hat und dieses dazu führt, dass HepG2 Zellen auf CD95L, TRAIL and 5-FU vermittelte Apoptose sensitiviert werden.

Eine pharmakologische Inhibierung des c-jun in diesen Zellen, könnte somit als ein wichtiger Faktor in der Krebstherapie eingesetzt werden.

VII. Literature

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Career profile

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10.1993 – 09. 1996 Secondary School preparing for higher education in Bremen, Germany
10.1996 – 07.2001 Biology study, University of Bremen, Germany
03. 2003 – 02. 2006 Ph.D. at the Medical Policlinic II, Ludwig-Maximilians University, Munich, Germany, Dissertation: C-Jun downregulation sensitizes hepatoma cells to receptor induced apoptosis through preventing FADD phosphorylation

Conferences attended and Poster presentations

1. “Untersuchung der Rolle von c-jun im CD95 Signalweg”. 60. Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten mit Sektion Gastroenterologische Endoskopie, Köln 14.–17. September 2005 Deutschland (poster presentation).
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2. Kuntzen C, **Sonuc N**, De Toni EN, Opelz C, Mucha SR, Gerbes AL, Eichhorst ST (2005)

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