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Induktion der Apoptose in TRAIL-resistenten Krebszellen

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This thesis is dedicated to my everlasting love, my husband, who gave me all the support I needed.

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LIST OF ABBREVATIONS

°C	centigrade
aa	amino acids
AIF	apoptosis inducing factor
Apaf-1	apoptotic protease-activating factor-1
ATP	adenosine triphosphate
AV-FITC	fluorescein isothiocyanate conjugate of Annexin V
Bad	Bcl-2-antagonist of cell death
Bak	Bcl-2-antagonist killer 1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphomas
Bcl-X_L	Bcl-2-like 1 long
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting mediator of cell death
Bip	immunoglobulin binding protein
BIR	Baculovirus IAP repeat
bp	base pairs
BSA	Bovine serum albumin
$CaCl_2$	calcium chloride
CAD	caspase activated deoxyribonuclease
CARD	caspase recruitment domain
Caspase	cysteinyl aspartate specific proteinases
CD-95	cluster of differentiation 95
c-DNA	complementary DNA
CED	cell death abnormal
c-$FLIP_L$	cellular-FLICE-like inhibitory protein long
c-$FLIP_S$	cellular-FLICE-like inhibitory protein short
c-IAPs	cellular-IAPs
cm	centimetre
CO_2	carbon dioxide
COX	cytochrome *c* oxidase

CRD	cysteine rich domain
CrmA	cytokine response modifier A
C-terminus	Carboxy-terminus
DcR	decoy receptor
DDs	death-domains
DED	death effector domain
dH_2O	distilled water
Diablo	direct IAP binding protein with low p*I*
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DR	death receptor
dsRNA	double stranded RNA
E1A	early region 1A
FACS	Fluorescence Activated Cell Sorting
FADD	Fas associated death domain
Fas-L	Fas-ligand
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
Fig	Figure
FLICE	Fas-associated death-domain-like IL-1β-converting enzyme
g	gram
G	guanine; glycine
GTP	guanosine triphosphate
h	hours
IκB	inhibitor of κB
IFN	interferon
kb	kilobase
kDa	kiloDalton
JNK	c-Jun N-terminal kinase
l	litre
µg	microgram
µl	microliter

μm	micrometer
μM	micromolar
Mcl-1	myeloid cell leukemia sequence-1
MEM	modified Eagle's medium
mg	milligram
min	minutes
ml	millilitre
mm	millimeter
mM	millimolar
moi	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
MOM	mitochondrial outer membrane
mRNA	messenger RNA
miRNA	micro RNA
MW	molecular weight
NF-κB	Nuclear Factor-κB
nM	nanomolar
nt	nucleotides
NTP	Nucleoside Triphosphate
NTPase	Nucleoside Triphosphatase
N-terminus	Amino-terminus
O/N	overnight
ORF	open reading frame
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pfu	plaque forming units
PI	propidium iodide
PUMA	p53 upregulated modulator of apoptosis
RING	really interesting gene
RIP-1	receptor-interacting protein-1
RISC	RNA induced silencing complex
R.T.	room temperature
RNA	ribonucleic acid
RNAi	RNA interference

Rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrilamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small inhibitory RNA
stRNA	small temporal RNA
TSA	trichostatin A
t-Bid	truncated-Bid
TEMED	N, N, N', N'-tetramethylethylenediamine
TNF-α	tumor necrosis factor-α
TNFR-1	tumor necrosis factor receptor-1
TRADD	tumour necrosis factor receptor-associated death domain
TRAF-2	tumor necrosis factor receptor-associated factor-2
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
U/ml	units/millilitre
UV	ultraviolet
V	volt
v/v	volume/volume
VPA	valproic acid
X-IAP	X-chromosome-linked IAP
w/v	weight/volume
z-VAD-fmk	benzyloxycarbonil-Val-Ala-DL-Asp-fluoromethylketone

1. INTRODUCTION

Cancer is the second leading cause of death, behind heart disease in the European Union and the USA. In some countries such as the UK cancer has already taken the position of the main death cause of males. The development of cancer results from deregulated proliferation or an inability of cells to undergo apoptotic cell death (Thomson, 1985; Steller, 1995). Complex organisms have evolved at least two cellular mechanisms to suppress the proliferation of cells to prevent oncogenic transformation: **apoptosis and cellular senescence**.

Senescent cells (CS) have a slowed down cell cycle and do not divide as fast as normal cells. They do not penetrate the surrounding tissues, metastasize, and respond to chemotherapeutic treatments. Senescence ("growing old") originally described that normal human cells grown in culture would only go through a finite number of division before permanent growth arrest (Hayflick & Moohead, 1961). Cells that underwent senescence cannot divide even if stimulated by mitogens, but they remain metabolically and synthetically active and show a characteristic change in morphology, such as enlarged and flattened cell shape and increased granularity (Campisi, 2001). The most widely used marker with considerable (but not absolute) specificity to senescent cells is the SA-beta-galactosidase, which is detectable by X-gal staining at pH 6.0 (Dimri et al., 1995). Consistent with its role in suppressing cancer, cellular senescence is controlled by several tumor-suppressor genes. The most crucial of these encode the p53 and pRB proteins. Together, p53 and pRB are the most commonly lost functions in mammalian cancers (Bringold & Serrano, 2000). Cellular senescence can be induced by DNA damage - double strand breaks or oxidation (Campisi, 2000). The other cellular mechanisms to suppress proliferation is apoptosis.

1.1 APOPTOSIS

1.1.1 Apoptosis

The term "programmed cell death" was first used in 1961 by Lockshin and Williams who showed that this process occurs during insect development and that it is energy and gene transcription dependent (Vaux, 2002). In 1965, Kerr introduced the criteria to distinguish between necrosis and programmed cell death (reviewed in Kerr, 2002). He noted that cell death similar to that described by Gluckmann occurred in individual hepatocytes after the

induction of hepatic ischemia and that this form of cell death was different to necrosis of cells. Although this process was at first described by Kerr as “shrinkage necrosis”, it was only in 1972 that Kerr, Wyllie and Curie proposed the term apoptosis, a Greek word describing the process of leaves falling from a tree or petals from a flower, to depict this specific type of cell death (Kerr et al., 1972). Since then, enormous progress has been made in the field and what only started as a morphological description, has now become a central concept in biology.

Apoptosis is a term used to describe the most typical and frequent phenotype of programmed cell death. It is a genetically controlled, energy-dependent process of cell death conserved through evolution in all metazoans (Raff, 1992; Ameisen, 2002; Vaux, 2002). It is now clear that apoptosis is an essential component of animal development participating in morphogenesis, sexual differentiation, deleting superfluous structures and in the epigenetic self organization of the immune and nervous systems (reviewed in Jacobson et al., 1997; Vaux & Korsmeyer, 1999; Krammer, 2000; Yuan & Yankner, 2000). Apoptosis also occurs in adult tissue to control cell numbers and to remove non-functional, damaged cells (Raff, 1992). It represents a quality control procedure and a defense mechanism to eliminate potentially harmful cells (auto-reactive lymphocytes), to eliminate infected cells, to prevent the emergence of genetic heterogeneity, and to stop the development of cancer (Raff, 1992; Vaux & Korsmeyer, 1999).

Deregulation of the apoptotic process is responsible for many diseases in humans. Here it is remarkable that the first component of an apoptotic system was recognized in a human disease. The human *bcl-2* (B-cell lymphomas) gene was found to be over-expressed in follicular lymphoma, one of the most common cancer of blood cells in humans, as a result of a t(14:18) chromosome translocation (Vaux et al., 1988). It was shown that *bcl-2* did not act, like other oncogenes (*c-myc* or *abl*), to promote cell proliferation, instead it prevented cell death after growth factor withdrawal: this was the first experimental evidence that inhibition of apoptosis could lead to cancer in humans (Vaux et al., 1988). On the other hand, excessive apoptosis is related to neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease (Yuan & Yanker, 2000), and many other diseases like acquired immuno-deficiency syndrome (AIDS) and stroke.

1.1.2 Morphological and biochemical hallmarks of apoptosis

Initially, analysis of apoptosis was based mainly on well-defined morphologic features, which are essentially those described by Kerr (Kerr et al., 1972). Dying apoptotic cells present shrinkage of the cytoplasm and a condensed nucleus in which the chromatin appears pyknotic (condensed) and compacted into dense masses (reviewed in Saraste & Pulkki, 2000; Geske & Gerschenson, 2001). As apoptosis continues, the nucleus becomes fragmented. In the final stage of apoptosis, the cell detaches from the surrounding tissue and breaks up into several membrane-bound vesicles containing intact organelles and nuclear fragments.

In vivo these apoptotic bodies are engulfed by neighboring cells and macrophages and removed from the tissue. If these apoptotic bodies are not phagocytosed, as it is the case in an *in vitro* system, they will undergo a necrosis-like degradation called secondary necrosis. Activation of endonucleases during apoptosis yielding a ladder of degraded DNA during biochemical analyses was the first marker that did not rely on morphology. The activation of caspase-activated deoxyribonucleases (CAD), the murine homologue of the human DNA fragmentation factor subunit 40 (DFF-40), cleaves the DNA into internucleosomal fragments of high molecular weight that produce the classical DNA ladder in agarose gel electrophoresis specifically associated with apoptosis (Liu et al., 1997).

Another biochemical characteristic of apoptotic cells is the exposure of phosphatidyl-serine from the inner to the outer surface of the plasma membrane that serves as a recognition signal for the engulfment of dying cells by macrophages and neighboring cells (Fadok et al., 1992). Probably the best known apoptotic feature, also responsible for almost all of the biochemical and morphological changes in apoptosis, is the activation of a proteolytic system involving a specific family of proteases: the cysteinyl aspartate specific proteinases (caspases) (Alnermi et. al, 1996).

1.1.3 Caspases

Caspases are a class of cysteinyl proteases that specifically cleave on the C-terminal side of an aspartic acid residue in the scissile bond (Alnermi et al., 1996). Despite all the information and the amount of experimental evidence on the central role of caspases in the execution of apoptosis, it is very surprising that the first caspase to be identified, named ICE or caspase-1, was not involved in apoptosis but in the proteolytic maturation of the pro-interleukin 1β to its biological active form (Cerretti et al., 1992). The first clue for a possible role of ICE or related family members in apoptosis came from the discovery that CED-3, a pro-apoptotic

protein in *Caenorhabditis elegans*, is a homologue of the mammalian ICE protein (Yuan et al, 1993).

Several observations indicate the role of caspases in apoptosis. Natural or synthetic inhibitors that abrogate caspase activity can effectively prevent or delay the apoptotic phenotype. Gene deletion studies have shown that caspases (in particular caspases 3, 8 and 9) are required for apoptosis during mammalian development (reviewed in Vaux & Korsmeyer, 1999). *Caspase-8 -/-* mouse embryos die at day 11 with abnormal formation of the heart (Varfolomeev et al., 1998). Similarly, *caspase-3* or *caspase-9* knockout mice die early in development and show profound defects in apoptosis in the nervous system resulting in brain overgrowth (Kuida et al., 1996; 1998). Finally, specific caspase cleavage sites have been found in the majority of the proteins proteolytically processed during apoptosis.

1.1.3.1 Structure of mammalian caspases (Zymogen organization)

All caspases share common features that characterize this family of proteases (reviewed in Cohen, 1997; Earnshaw et al., 1999, Fuentes-Prior & Salvesen, 2004). Caspases are synthesized as an inactive single chain pro-enzyme - zymogen. Each zymogen (procaspase) contains an N-terminal prodomain, a large central and a C-terminal domain yielding the large and the small subunit, respectively, of the active protease. The large subunit contains the active site cysteine, the signature motif of this family of proteases (Wolf & Green, 1999). An aspartic acid cleavage site, a short linker (about 10 amino acids), separates each the prodomain from the "large subunit" and the "large" from the "small subunit" although in some caspases an interdomain linker containing two aspartate cleavage sites separates the large and the small subunits (Cohen, 1997). The presence of these caspase cleavage sites in the polypeptide chaing of procaspases is consistent with the ability of caspases to auto-activate or to be activated by other caspases suggesting a signal transduction pathway of proteolytically activated proteases (Nicholson, 1999).

Based on phylogenetic amino acid sequence analyses, caspases have been classified into three major subfamilies: the ICE, CED-3 and ICH-1 (*Ice* and *ced-3* homologue) subfamilies (Cohen, 1997; Donepudi & Grütter, 2002). The ICE subfamily mostly consists of inflammatory caspases (Lippens, 2000) and the CED-3 and ICH-1 subfamilies exclusively contain caspases involved in apoptosis.

The individual caspases have two major structural differences that are correlated with their biological functions: the substrate specificity and the length as well as the amino acids

sequence of the N-terminal prodomain (Nicholson, 1999; Wolf & Green, 1999; Donepudi & Grütter, 2002; Shi, 2002). Caspases recognize at least four contiguous amino acids, named P4, P3, P2, P1, and cleave after the C-terminal P1 residue. All mammalian caspases have an absolute requirement for an aspartic acid residue in the P1 position, while the amino acid residues in the P2-P3-P4 positions are not conserved and determine the substrate specificities of the different caspases (Shi, 2002; Fuentes-Prior & Salvesen, 2004). Despite these simple requirements, caspases are extremely stringent indicating that the three-dimensional context is very important for the recognition and the proteolysis of the substrate (Nicholson, 1999).

The N-terminal prodomain is the other major structural difference between the individual caspases (Wolf & Green, 1999; Donepudi & Grütter, 2002). Caspase-3, -6 and -7 are characterized by a short N-terminal prodomain of about 20-30 residues whereas caspase-8, -10 and the members of the ICH-1 and the ICE subfamily have an extended prodomain (>90 amino acids). Based on these structural differences and their biological role, caspases can be divided in two groups: the inflammatory caspases (caspase-1, -5, -11, -13) and the apoptotic caspases (caspase-2, -3, -6, -7, -8, -9, -10). In addition, according to the order and the mechanisms of activation, the apoptotic caspases can be further classified into initiator (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, -7) (Earnshaw et al., 1999; Nicholson 1999; Chang & Yang, 2000) (Summarized in **Figure 1.1**)

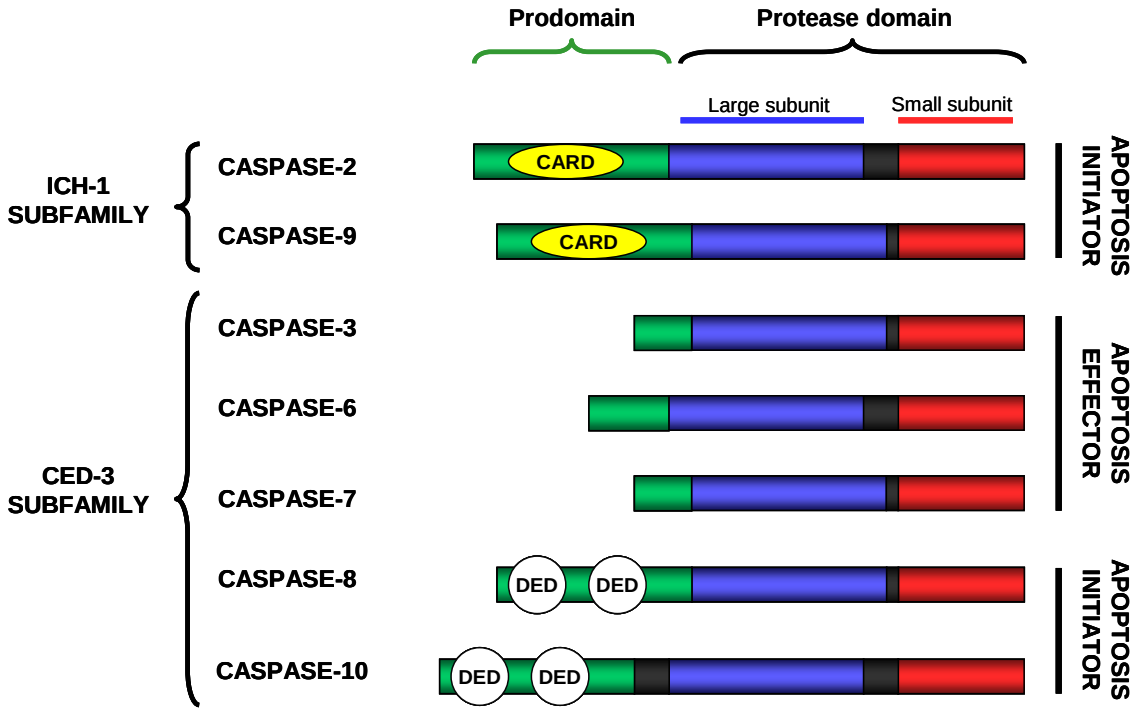

Figure 1.1: Caspase Structures.
The diagram shows the structure of human caspases involved in apoptosis. Each caspase possesses an N-terminal peptide (green box) or prodomain and two additional domains called "large" (blue box) and "small" (red box) subunit. The domains are separated by a linker peptide (black box). Apoptotic effector caspases have a short prodomain while apoptotic initiator caspases are characterized by a long prodomain, in which two types of elements can be found, the CARD and DED domain. These domains mediate the interaction between caspases and specific adapter molecules essential for caspase activation. Adapted from Cohen (1997), Chang & Yang (2000), and Fuentes-Prior & Salvesen (2004).

1.1.3.2 Caspase activation

The initiator caspases are the first to be activated in a particular death signal transduction pathway (Shi, 2002). They contain long N-terminal prodomains important for their activation. Among these prodomains, two different types of elements are known: the caspase recruitment domain (CARD) and the death effector domain (DED) (Wolf & Green, 1999). Although they are unrelated, CARDs and DEDs have similar structures composed of six closely packed antiparallel amphipathic α-helices (Fuentes-Prior & Salvesen, 2004). These elements mediate the interaction between the long prodomains of initiator caspases and specific adapter molecules, which drive their activation (Green, 1998; Stennicke & Salvesen, 2000). A major difference between these elements is the nature of their interaction: the DED-DED and CARD-CARD associations are mediated by charge-charge and hydrophobic interactions, respectively (Kaufmann & Hengartner, 2001). Two copies of DED elements are found in caspase-8 and -10, associated with death receptor-initiated cell death (extrinsic pathway). In contrast, Caspase-9 contains a CARD and is associated with mitochondrial-initiated cell death (intrinsic pathway). The other important feature of initiator caspases is the substrate specificity, which allows them to recognize their targets: the effector caspases (Nicholson, 1999). The effector caspases are usually activated by the initiator caspases and they are responsible for the majority of the proteolytic events that lead to the apoptotic phenotype (Green, 2000).

To achieve the active form, the caspases undergo a complex structural rearrangement including proteolytic cleavage between the large and the small subunit and the large subunit and the N-terminal prodomain (Shi, 2002). Although these cleavages are not necessary for the activation of initiator caspases (Boatright & Salvesen, 2003), all the active caspases display the same arrangement. The only exception is caspase-9 which does not require the removal of the N-terminal prodomain for its activation (Renatus et al., 2001).

The active caspase is a dimer of two catalytic domains (monomers) composed of the large and small subunits derived from a single caspase molecule (review in Fuentes-Prior & Salvesen, 2004). The activation of caspases can be divided into two mechanisms known as homo-activation and hetero-activation (Stennicke & Salvesen, 2000). The first mechanism is involved in the activation of the initiator caspases and reflects the important property of these caspases to activate themselves. In contrast, the hetero-activation mechanism occurs during the activation of the effector caspases since these caspases are activated by the initiator caspases or other proteases.

The activation of procaspase-8 proceeds in a specific order and requires dimerization as well as cleavage processing (Chang et al., 2003). Within the DISC, procaspase-8 dimerizes to acquire its catalytically active conformation (Boatright et al., 2003; Boatright & Salvesen, 2003, Chang et al., 2003). It has been shown that these newly formed procaspase-8 dimers are able to cleave neighboring dimers in a specific order: first, cleavage in the interchain domain separates the large and the small subunit and a second cleavage removes the N-terminal prodomain. The mature caspase-8 is then released to the cytosol where it can activate the effector caspases. After activation, the trimeric state of the death receptor theoretically supports the binding of three procaspase-8 molecules. Two of these procaspase-8 molecules dimerize within the same complex while the third procaspase-8 associates with the unpaired procaspase-8 of an adjacent DISC complex (Chang et al., 2003). This model is supported by the observation that clustering of death receptor complexes is an important step in CD95-induced apoptosis (Algeciras-Schimnich et al., 2002). In this context, it has been proposed that high levels of death receptor clustering induce a massive release of mature caspase-8 and inevitably apoptosis (reviewed in Algeciras-Schimnich et al., 2002; Peter & Krammer, 2003).

The activation of both caspase-8, by the death receptor pathway, and caspase-9, by the cytochrome *c*/Apaf-1 pathway, converge on the activation of the effector caspases-3, -6 and -7. While the effector caspases exert the same type of effects in response to various apoptotic stimuli, different initiator caspases mediate distinct sets of signals. This model explains how different apoptotic signals induce the same biochemical and morphological changes.

Effector caspases are activated by the initiator caspases or other proteases (hetero-activation) (Stennicke & Salvesen, 2000). The hetero-activation is a two step process (reviewed in Chang & Yang, 2000). The first cleavage is carried out by initiator caspases (or other proteases) and occurs in the interdomain linker region between the small and the large subunit to generate a partially processed p20/p12 form. In the second step, this active intermediate form processes itself to produce the fully active, mature p17/p12 caspase.

Caspase-3 and -7 have the same substrate and inhibitor specificity. In contrast, the N-terminal domain presents the largest difference and this region could be involved in distinct subcellular localization (Fuentes-Prior & Salvesen, 2004). Based on the N-terminal prodomain, caspase-6 is considered to be an effector caspase but the substrate specificity differ from that of caspase-3 and -7 and is more similar to that of the initiator caspases (Nicholson, 1999).

Once activated, the effector caspases can initiate a positive feedback loop to amplify the activation of initiator caspases. It has been shown that caspase-3 activates caspase-6, which in turn cleaves and activates caspase-8 (Slee et al., 1999). In a similar way, caspase-3 directly performs a feedback cleavage of caspase-9 to generate a p35/p10 or p37/p10 form which seem to be more active then the p35/p12 generated by autocatalytic processing (Zou et al., 2003).

The effector caspases are responsible for the cellular changes that occur during apoptosis. To date, more than 60 proteins have been shown to be cleaved *in vivo* and *in vitro* by effector caspases and this list is growing (reviewed in Cohen, 1997; Earnshaw et al., 199; Fuentes-Proor & Salvesen, 2004; Na et al., 2007). Caspase death substrates include proteins involved in DNA metabolism, cellular signaling, cellular repair, macromolecular synthesis and structural proteins such as actin, gelsolin and fodrin (reviewed in Chang & Yang, 2000). The cumulative effects resulting from the cleavage of these death substrates are to disable homeostatic and repair processes, cell cycle arrest, inactivate inhibitor of apoptosis, mediate the structural disassembly and morphological changes and mark the dying cell for engulfment.

The activation complex involved depends on the pathway triggered by a specific apoptotic stimulus. There are two well defined apoptotic pathways: the death receptor (extrinsic) and the mitochondrial (intrinsic) pathway.

1.1.4 The death receptor pathway

The extrinsic (or death receptor) pathway is responsible for elimination of unwanted cells during development, immune system education and immunosurveillance. This pathway is triggered by the engagement of cell surface death receptors with their specific ligands (reviewed in Peter & Krammer, 2003; Lavrik et al., 2005). The death receptors (DRs) are a family of transmembrane proteins that belong to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. Mammalian death receptors include: Fas/APO-1/CD-95, TNF-R1, DR-3/Apo-3/WSL-1/TRAMP, DR-4/TRAIL-R1, DR-5/TRAIL-R2/TRICK-2/KILLER and DR-6 (Ashkenazi & Dixit, 1998; Lavrik et al., 2005). These receptors share conserved cysteine rich domains (CRD) at their extracellular domains, which define their ligand specificity (Bodmer et al., 2002). The cytoplasmatic domains contain a homotypic recruitment element of 60-70 amino acids, termed death domain (DD), necessary for signal transduction (Ashkenazi & Dixit, 1998).

The activation ligands, also called death ligands, are structurally related homotrimeric molecules that belong to the TNF gene superfamily: Fas ligand (Fas-L) binds to Fas receptor, TNF-α to TNF-R1, Apo-3 ligand (Apo-3L) to Apo-3 and Apo-2 ligand (Apo-2L or TRAIL) binds to DR-4 and DR-5 (Ashkenazi & Dixit, 1998).

1.1.4.1 Fas-mediated death receptor pathway

The Fas (CD95/Apo-1) apoptotic pathway has been extensively studied and has become the model for the mammalian extrinsic pathway (Peter & Krammer, 2003; Lavrik et al., 2005). Fas is constitutively present in various tissues but its ligand is expressed mainly in activated T lymphocytes and natural killer cells. Fas-induced apoptosis plays an important role in lymphoid tissue homeostasis and removal of viral-infected and tumor cells. Unstimulated Fas exists as a pre-associated homotrimer in the plasma membrane (Peter & Krammer, 2003). Upon ligation to Fas-L, Fas receptor forms microaggregates at the cell surface leading to recruitment of the adapter protein FADD (Fas associated death domain) (Algeciras-Schimnich et al., 2002). This protein contains a DD at its C-terminus and binds to the cytoplasmatic tail of Fas via DD homotypic interactions. FADD also contains a DED in its N-terminal region. The DED of FADD interacts with the DED in the prodomain of caspase-8 leading to the recruitment of procaspase-8 (Peter & Krammer, 2003). Fas, FADD and pro-caspase-8 do not associate under non-apoptotic conditions. The complex formed by Fas, FADD and pro-caspase-8 (and possibly other proteins) is known as the death-inducing-signaling complex (DISC) (Ashkenazi & Dixit, 1998; Peter & Krammer, 2003).

1.1.4.2 TNF-R1-mediated death receptor pathway

The TNF-R1 (tumor necrosis factor receptor-1) mediates the pleiotropic effects of the inflammatory cytokine TNF-α. The TNF-R1 is expressed ubiquitously while its ligand is only expressed by activated macrophages and T cells in response to infection (Chang & Yang, 2000). TNF-α can induce either apoptosis or the expression of pro-inflammatory, immunomodulatory and anti-apoptotic genes through the activation of NF-κB (nuclear factor κB) and AP-1 (reviewed in Budihardjo et al., 1999; Barnhart & Peter, 2003; Chang & Yang, 2000). An elegant model to explain the mechanism governing the decision to induce one of these pathways has been proposed (Micheau & Tschopp, 2003). This model involves the formation of two sequential complexes. The first complex (complex I) is generated in the plasma membrane by the binding of TNF-α to the TNF-R1. This event induces the

association between the adapter molecule TRADD (tumor necrosis factor receptor-associated death domain), and TNF-R1 via DD interaction; in turn, TRADD recruits TRAF-2 (tumor necrosis factor receptor-associated factor type-2) and RIP-1 (receptor interacting protein 1) leading to the activation of NF-κB. In contrast to the Fas or TRAIL signaling pathway, FADD and caspase-8 are not recruited to the activated receptor, thus this complex does not induce apoptosis (Harper et al., 2003; Micheau & Tschopp, 2003).

The pro-apoptotic signal depends on the formation of a second complex (complex II) in the cytoplasm. After internalization of complex I, TRADD, RIP-1 and TRAF-2 soon dissociate from TNF-R1 and the DDs of TRADD, which were previously engaged in the interaction with the DDs of TNFR-1, become available to bind the DDs of FADD leading to the formation of complex II (Micheau & Tschopp, 2003). The ability of the newly formed complex II to induce apoptosis depends on the signal previously triggered by complex I: if the activation of NF-κB is productive then the expression of anti-apoptotic genes, such as c-$FLIP_L$, inhibits complex II; in contrast, if the activation of NF-κB is not productive, the complex II recruits caspase-8 and induces apoptosis (Micheau & Tschopp, 2003).

The signal pathway through Apo-3/DR-3 is similar to TNF-R1, however the expression pattern of Apo-3 and its ligand Apo-3L/DR-3L differs from those of TNF-R1 and TNF-α: Apo-3 is expressed mainly in the immune tissues while its ligand is present constitutively in many tissue (Chang & Yang, 2000).

1.1.4.3 TNF-related apoptosis-inducing ligand and its receptors

TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L) was identified in 1995 based on its sequence homology to other members of the TNF super family and is constitutively expressed in a wide range of tissues (LeBlank & Ashkenazi, 2003). TRAIL is a membrane protein that can be cleaved from the cell surface to form a soluble ligand. Both full-length membrane - expressed TRAIL and the soluble ligand can rapidly induce apoptosis in a wide variety of human cancer cells (Wiley et al., 1995). In contrast to other members of TNF family, whose expression is tightly regulated and are often only transiently expressed on activated cells, TRAIL mRNA is expressed constitutively in a wide range of tissue (Peter & Krammer, 2003; Lavrik et al., 2005). Another important and unique characteristic of TRAIL is its ability to selectively trigger receptor-mediated apoptosis in cancer cells but not in normal cells (Chang & Yang, 2000; Ashkenazi et al., 1999; Lawrence et al., 2001). For this reason, TRAIL has been extensively studied as an anticancer reagent.

TRAIL interacts with an intricate receptor system consisting of two apoptosis-inducing agonistic death receptors, death receptor 4 (DR4/TRAIL-R1) and death receptor 5 (DR5/TRAIL-R2), and three antagonistic or decoy receptors, decoy receptor 1 (DcR1/TRAIL-R3), decoy receptor 2 (DcR2/ TRAIL-R4), and the soluble receptor osteoprotegerin (OPG) (LeBlanc & Ashkenazi 2003). TRAIL binds as homodimer to DR4 and DR5, which results in trimerisation of the receptors and this leads to the recruitment of Fas-associated death domain (FADD), which in turn allows binding and activation of the initiator caspase-8. Mature caspases-8 are capable to activate downstream effector caspases, which execute apoptosis (Bodmer et al., 2000; Kischkel et al., 2000; Sprick et al., 2000). DcR1 lacks a death domain and DcR2 contains a truncated death domain, so the binding of TRAIL to these receptors does not induce apoptosis but could instead prevent apoptosis by sequestering available TRAIL or by interfering with TRAIL-DR4 or –DR5 signaling complex (Kimberley & Screaton, 2004). The existence of decoy receptors and their widespread expression on normal cells was initially seen as the explanation for protection of normal cells against TRAIL-inducing apoptosis (Budihardjo et al., 1999; Chang & Yang, 2000).

Apoptosis induced by death receptors can be modulated at several levels. Intracellular anti-apoptotic molecules can block the apoptotic signaling pathway. Such molecules include cellular FLICE – like inhibitory protein (c-FLIP), which competes with caspase-8 for binding to FADD (Irmler et al., 1997), or XIAP, c-IAP-1 and c-IAP-2, that can act to inhibit active caspases. Another mechanism of TRAIL resistance is through activation of the transcription factor NF-kB (Jeremias et al., 1998; Hu et al., 1999; Harper et al., 2001).

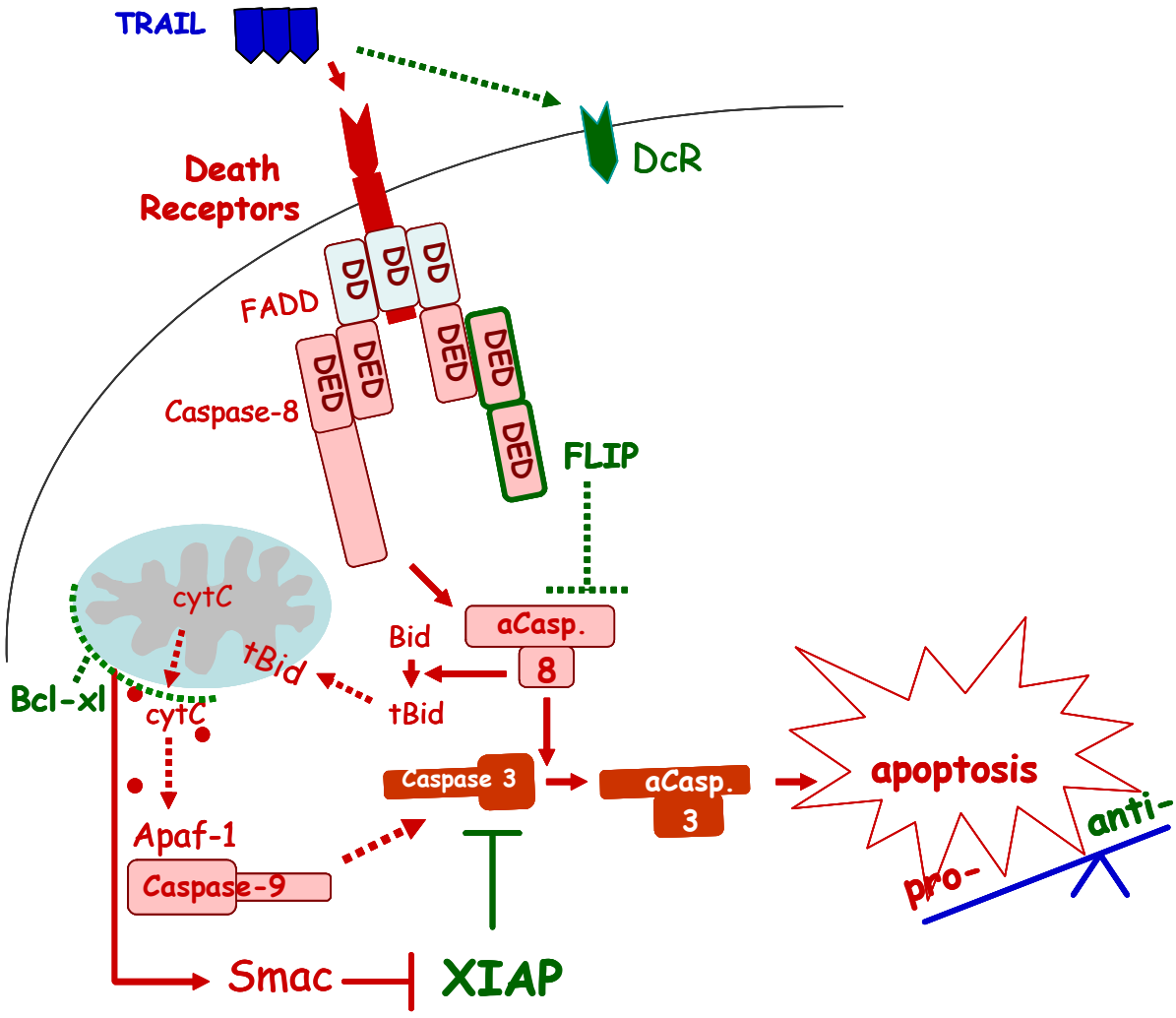

Figure 1.2: Schematic illustration of TRAIL-induced apoptosis signalling pathway with factors that modulated the TRAIL responsiveness.
Highlighted in green the anti-apoptotic factors and in red and orange the pro-apoptotic factors. Activation of apoptosis is induced through trimerization of DR5 or DR4 by the ligand TRAIL, DcR1 and DcR2 acting as decoys competing with DR5 and DR4. FLIP, IAP proteins and Bcl-xl can prevent TRAIL-induce apoptosis.

1.1.5 The mitochondrial pathway

The mitochondrial (or intrinsic) pathway is used to eliminate cells following mitochondrial damage, ionizing radiation and chemotherapeutic drugs (reviewed in Green & Reed, 1998; Desagher & Martinou, 2000; Green & Kroemer, 2004; Jiang & Wang, 2004). These and other apoptogenic stimuli promote the release of several pro-apoptotic proteins from the mitochondria to the cytosol, including cytochrome *c*, second mitochondria-derived activator of caspases (Smac/DIABLO), and apoptosis inducing factor (AIF) (reviewed in Van Loo et al., 2002; Van Gurp et al., 2003). This release happens through the permeabilization of the outer mitochondrial membrane. Once in the cytosol, cytochrome *c* binds and induces oligomerization of Apaf-1 and an essential component of the vertebrate apoptosome (reviewed in Wang, 2001). Cytochrome *c* binds and allows the Apaf-1 to stretch out into a more linear structure. In the presence of ATP, Apaf-1 oligomerizes, forming a heptameric complex, which then recruits procaspases-9. This complex is called apoptosome (reviewed in Wang, 2001; Cain et al., 2002).

1.1.5.1 The IAP family proteins

The enzymatic activity of caspase-3, -7 and -9 can be inhibited by endogenous proteins named inhibitors of apoptosis (IAP). The IAP family proteins were originally identified as baculovirus proteins capable of inhibiting apoptosis in infected cells (review in Deveraux & Reed, 1999). Homologues of the viral IAP (vIAP) have been found and characterized in mammals including XIAP (X-linked IAP), c-IAP1 (cellular-IAP1) and c-IAP2, ML-IAP (melanoma-IAP), N-IAP (neuronal-IAP), Bruce/Apollon and survivin (Deveraux & Reed, 1999; Vaux & Silke, 2003).

The IAP family proteins are characterized by one or more ~70 amino acid zinc-binding motifs called baculoviral IAP repeat (BIR) domains and, in some cases, a C-terminal RING zinc finger domain (**Figure 1.3**) (review in Deveraux & Reed, 1999).

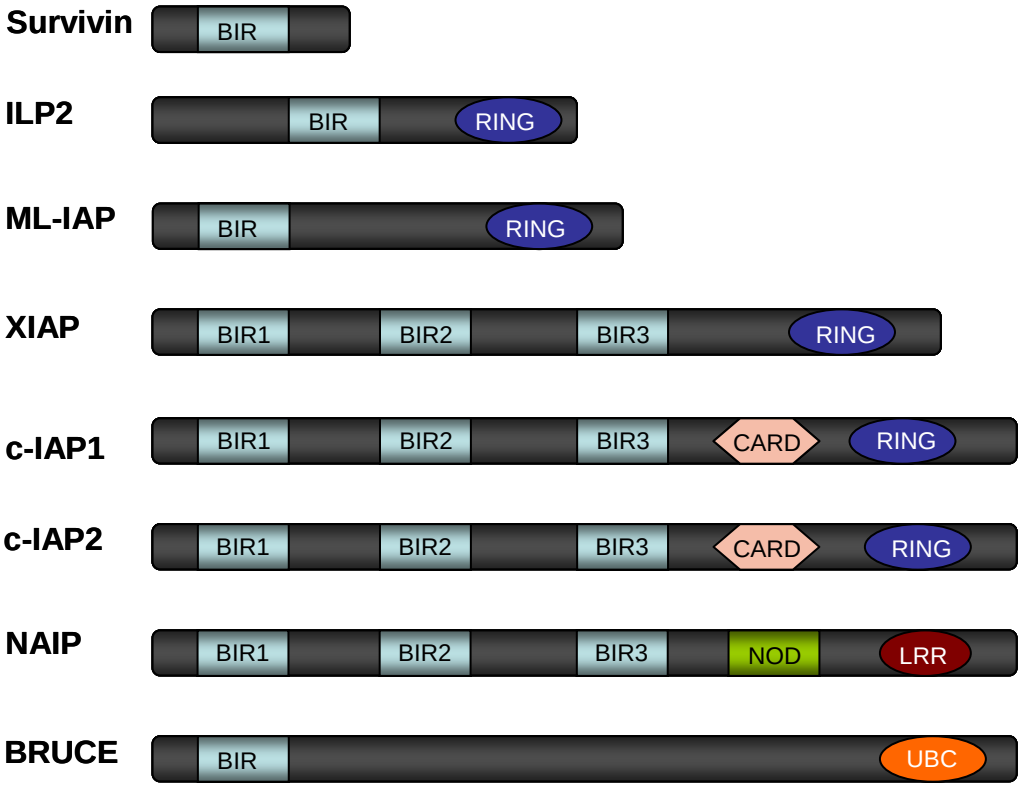

Figure 1.3: Comparison of IAP family of proteins.
Adapted from Srinivasa et al., 2008. For more details please see text.

The anti-apoptotic activity has been demonstrated only for subset of IAPs, in particular for XIAP, c-IAP1, c-IAP2 and ML-IAP (Vaux & Silke, 2003). Among these IAPs, the most extensively studied is XIAP. To prevent apoptosis, XIAP binds directly to and inhibits processed caspase-9, -3 and -7. The ability of XIAP to inhibit caspase-9 resides on the BIR-3 domain (Takahashi et al., 1998). Within the apoptosome, procaspase-9 undergoes a rapidly autocatalytic cleavage generating the large p35/34 and the small p12 subunits. This cleavage exposes an ATPF motif at the N-terminal of the p12 subunit, which can bind the BIR-3 of the XIAP. This interaction is essential to XIAP mediated inhibition of caspase-9 (Srinivasula et al., 2001). XIAP inhibition of processed caspase-3 and -7 is mediated by a distinct domain, in particular by the BIR-2 and a few residues in the linker region between the BIR-1 and BIR-2 domains (Huang et al., 2001). It has been shown that XIAP can inhibit both processed caspase-9 and caspase-3 forming a complex (XIAP/caspase-9/caspase-3) that is retained in the apoptosome (Bratton et al., 2002; Twiddy et al., 2004).

Two mitochondrial proteins can overcome the anti-apoptotic activity of XIAP: Smac/Diablo and HrtA2 (high temperature requirement serine protein A2, also known as Omi). Both Smac/Diablo and HrtA2/Omi are encoded by nuclear genes and after their synthesis they are sequestered into mitochondria through a classical mitochondrial targeting sequence at the N-terminus (reviewed in Van Loo et al., 2002; Van Gurp et al., 2003; Vaux & Silke, 2003). In the mitochondria, the targeting sequence is proteolytically removed so that the mature Smac/Diablo exposes a tetrapeptide motif in its N-terminal domain, which interacts with the BIR-3 domains of XIAP.

During the intrinsic pathway, Smac/Diablo is released from the mitochondria to the cytosol where it binds the BIR-3 domain of XIAP thereby competitively displacing caspase-9 (Riedl & Shi, 2004). Since the inhibition of caspase-3 by XIAP is mediated by another domain, the binding of Smac/Diablo to the BIR-3 domain probably induces the release of caspase-3 due to steric hindrance (Huang et al., 2003). HrtA2/Omi functions in a similar manner to Smac/Diablo to remove XIAP from caspases (Van Loo et al., 2002). In conclusion, activation of caspase-9 requires its recruitment to the apoptosome and the removal of XIAP inhibition. These two events depend on the release of apoptogenic proteins (cytochrome *c*, Smac/Diablo and HrtA2/Omi) from the mitochondria to the cytosol, a process that is highly regulated by the members of the Bcl-2 family. In this context XIAP has been well study in cancer cells and related to chemotherapeutic resistance.

1.1.5.2 Bcl-2 family proteins

The mitochondrial outer membrane permeabilization (MOMP) is a necessary step for the release of apoptogenic factors that lead to the activation of caspase-9. Several death signals converge on mitochondria to induce MOMP (reviewed in Green & Reed, 1998; Bratton & Cohen, 2001; Green & Kroemer, 2004). It is clear that the release of pro-apoptotic proteins from the mitochondria is controlled by members of the Bcl-2 family (Gross et al, 1999; Degli Espositi & Dive, 2003; Scorrano & Korsmeyer, 2003; Danial & Korsmeyer, 2004).

The Bcl-2 family of proteins represent a critical intracellular checkpoint that resides immediately upstream of mitochondria (Scorrano & Korsmeyer, 2003; Danial & Korsmeyer, 2004). Members of this family direct apoptotic signals from either intrinsic or extrinsic stimuli towards the mitochondrial surface and control, directly or indirectly, the integrity of the mitochondrial outer membrane (Degli Espositi & Dive, 2003; Danial & Korsmeyer, 2004).

The Bcl-2 family proteins are characterized by four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4 (reviewed in Danial & Korsmeyer, 2004). Based on their pro-survival and pro-apoptotic nature and on the occurrence of the different BH domains, Bcl-2 family members are classified in three groups. First, anti-apoptotic Bcl-2-like proteins including Bcl-2, Bcl-x_L, Mcl-1 and Bcl-w display amino acid sequence homology in all BH domains. Secondly, multidomain pro-apoptotic members, Bax, Bak and Bok, show amino acid sequence conservation in BH1-3 domains. Finally, the BH3-only proteins such as Bid, Bik, Bim and NOXA have amino acid sequence homology only in the BH3 domain. Besides the BH domains, some Bcl-2 family members possess a C-terminal hydrophobic domain, which is probably responsible for their membrane localization **Figure 1.4** (Schinzel et al., 2004).

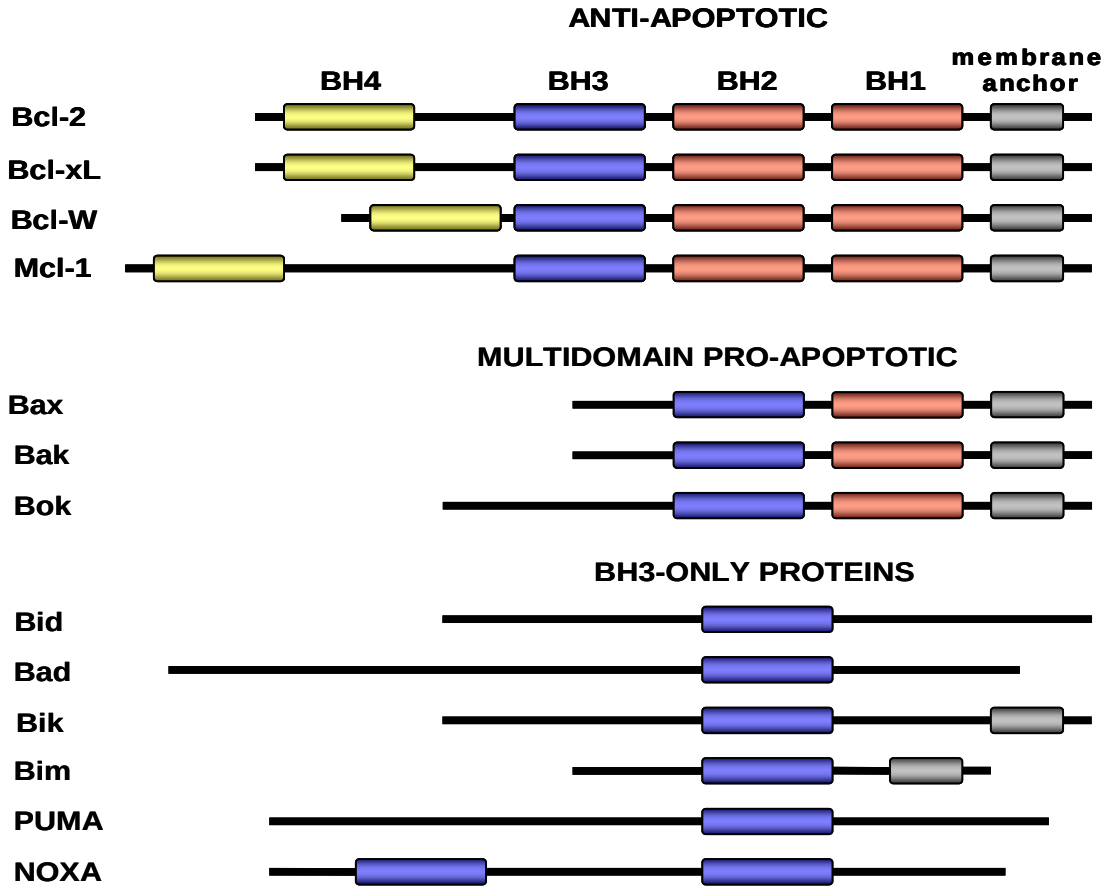

Figure 1.4: The *Bcl-2 family*
In this schematic drawing of Bcl-2 and its related proteins, the members of the Bcl-2 family protein are divided into three categories, anti-apoptotic, multidomain pro-apoptotic and the BH3-only proteins. The Bcl-2 homology (BH) domains are highlighted. Adapted from Gross et al. (1999) and Danial & Korsmeyer (2004).

The anti-apoptotic members inhibit the release of apoptogenic factors from mitochondria whereas the pro-apoptotic members induce it. Pro- and anti-apoptotic proteins have different distributions in the cell (Degli Espositi & Dive, 2003; Petros et al., 2004). The anti-apoptotic proteins are localized in the mitochondria, endoplasmic reticulum and nuclear membrane. In

contrast, the pro-apoptotic members mostly reside in the cytosol and only after a death signal they undergo a conformational change that enables them to integrate in membranes, especially the mitochondrial outer membrane (Schinzel et al., 2004).

The BH3-only proteins reside upstream of anti- and multidomain pro-apoptotic members and act as sensors that selectively respond to specific death signals. The pro-apoptotic activity of BH3-only proteins depends in particular on the BH3 domain as mutations in this region block their ability to induce apoptosis (Danial & Korsmeyer, 2004). Following a death stimulus, the BH3-only proteins are activated (controlled/regulated) by transcriptional up-regulation (NOXA and PUMA), by post-translational modification (e.g. cleavage (Bid) and dephosphorylation (Bad)), and by translocation (Bim) (reviewed in Gross et al., 1999). The activated BH3-only proteins are able to translocate to the mitochondrial outer membrane and activate the multidomain members Bax and Bak (Gross et al., 1999; Sharpe et al., 2004; Lucken-Ardjomande and Martinou, 2005).

In healthy cells, Bax exists as a monomer in the cytosol or loosely attached to the mitochondrial outer membrane while Bak already resides in the mitochondrial outer membrane. Approximately 10-15% of Bax and Bak are also localized in the endoplasmic reticulum (Scorrano & Korsmeyer, 2003; Schinzel et al., 2004). All the BH3-domain-only proteins tested and several types of apoptotic stimuli that induce the intrinsic pathway, even those that operate at the ER level, require Bax and Bak activation, suggesting that these two multidomain pro-apoptotic proteins represent an essential gateway to the intrinsic pathway (Scorrano et al., 2003; Danial & Korsmeyer, 2004).

The anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xl share all the conserved BH domains (BH1-4) and can interact with pro-apoptotic Bcl-2 family proteins. In these proteins BH4 domain located in the N terminal region is an essential domain for inhibition of apoptosis. Several studies have indicated that Bcl-2 and Bcl-xl efficiently suppresses the permeabilization and breakdown of the outer mitochondrial membrane (Shimizu et al., 1998; Marzo et al., 1998). Bcl-xl blocks apoptosis by sequestering BH3-domain only protein in stable mitochondria complexes and/or by preventing cytochorome c release from mitochondria (Saelens et al., 2004). Overexpression of Bcl-xl protein has been associated with a reduced sensitivity of cancer cells to drugs (Zhu et al., 2008; Conticello et al., 2008). Altered expression of Bcl-2 family proteins has been reported in a variety of human cancers and has been linked to treatment response (Cory et al., 2002)

1.1.6 Crosstalk between the intrinsic and extrinsic pathway

The death signal transduction cascade downstream of the DISC is not the same among all types of cells. In particular, in type I cells such as thymocytes, the activation of caspase-8 rapidly leads to the maturation of caspase-3 and apoptosis in a mitochondria-independent pathway. In contrast, in type II cells such as hepatocytes, the activation of caspase-3 requires a mitochondrial amplification loop and it is inhibited by Bcl-2 and Bcl-x_L (**Figure 1.5**) (Yoon & Gores, 2002; Bratton & Cohen, 2003).

The crosstalk between the death receptor and mitochondrial pathway is mediated by the activation of Bid (reviewed in Gross et al., 1999). Following its activation by a death receptor signal, caspase-8 cleaves Bid at its N-terminal domain generating a p15 C-terminal fragment. The truncated p15 Bid (t-Bid) then translocates to the MOM where it activates Bax/Bak, inducing the release of apoptogenic factors from the mitochondria and the activation of mitochondrial pathway (Gross et al., 1999; Danial & Korsmeyer, 2004).

It has been proposed that in type II cells the DISC is formed quite poorly and there is not enough active caspase-8 to induce the maturation of caspase-3. Therefore, type II cells require both caspase-8 and the apoptosome to activate caspase-3 (Peter & Krammer, 2003).). In type II cells the apoptosome formation is not essential to induce apoptosis (Yin et al., 1999). Bid induces the release of cytochrome *c* and other apoptotic proteins such as Smac/Diablo, which antagonizes the inhibitory effects of XIAP. It has been demonstrated that XIAP prevents CD95-mediated apoptosis through the inhibition of caspase-3, and Bcl-2/Bcl-x_L exerts the same effect through the inhibition of Smac/Diablo release from the mitochondria (Bratton et al., 2002). In this context, the type I/II phenotype could be determined by the ratio between the level of XIAP expression and the amount of active caspase-3 produced. In type I cells, a high level of active caspase-3, due to an efficient DISC formation and caspase-8 activation, overcomes the inhibitory effects of XIAP. In contrast, in type II cells, inefficient activation of caspase-8 leads to low production of active caspase-3, which is efficiently inhibited by XIAP. The activation of Bid and the release of Smac/Diablo from the mitochondria remove the XIAP inhibition inducing an efficient activation of caspase-3 (Bratton & Cohen, 2003). In addition, the caspase-3→caspase-6→caspase-8 feedback loop provides an amplification pathway for high production of active caspase-8 (Slee et al., 1999).

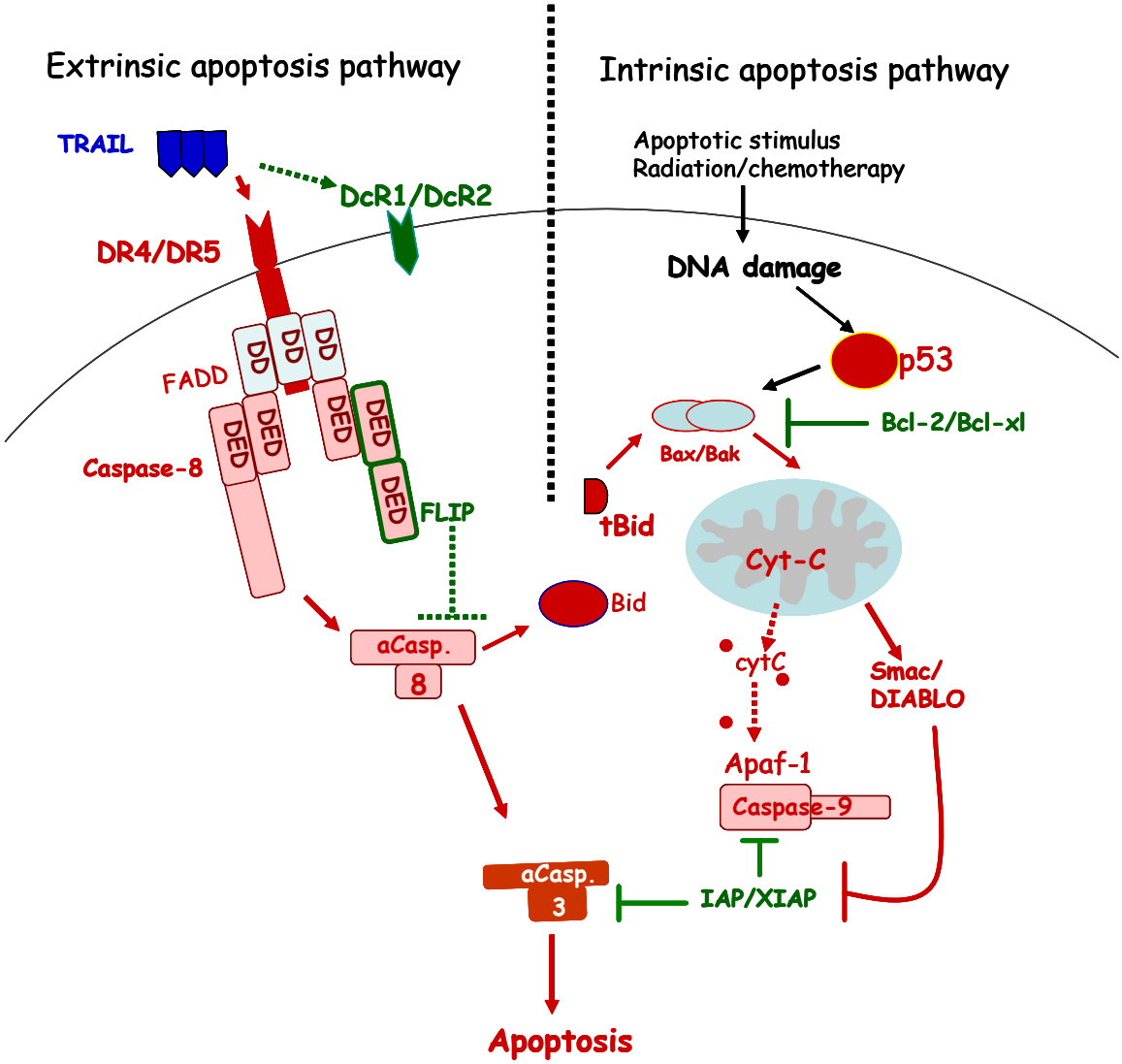

Figure 1.5: Schematic drawing of the crosstalk between the intrinsic and extrinsic apoptotic signalling pathaws.
In sometypes of cells (type II) the crosstalk is necessary for the induction of apoptosis. Adapted from Takeda et al. (2007).

1.2 TARGETING APOPTOSIS PATHWAYS

2.1 Concept

Conventional cytotoxic drugs have useful activity but lack tumor selectivity due to indiscriminate inhibition of cell division in tumor and normal tissue. Therefore, new therapies for cancer are extremely necessary. Targeted proteins involved in apoptosis are agents designed to exploit the increasing understanding of the molecular and cellular pathology of cancer. In the development of a new cancer therapy the stages of target identification and validation are the most important and challenging.

To target a specific protein in a cancer pathway two important tools were used, the use of recombinant adenovirus and gene silencing. In this way we were able to identify and validate a new therapy for pancreatic cancer. Over the years a number of gene transfer vehicles have been developed that can roughly be divided into two categories: synthetic and virus-based gene delivery systems. Synthetic gene delivery systems depend on direct delivery of genetic information into a target cell and include direct injection of naked DNA and encapsulation of DNA with cationic lipids (liposomes). Although these delivery systems exhibit low toxicity, gene transfer in general is inefficient and often transient. Viral delivery systems are based on viruses that have the ability to deliver genetic information into the host cell. Viral vectors currently available for gene therapy are based on different viruses and can roughly be categorized into integrating and non-integrating vectors. Vectors based on retrovirus (including lentivirus and foamy virus) have the ability to integrate their viral genome into the chromosomal DNA of the host cell, potentially resulting in lifelong gene expression. Vectors based on adenovirus (Ad), Herpes Simple Virus type 1 (HSV-1) and Adeno-Associated Virus (AAV) represent non-integrating vectors. These vectors deliver their genomes into the nucleus of the target cell, where they remain episomal.

1.2.2 Adenoviral Vector

1.2.2.1 Adenovirus Structure and Replication

Adenoviruses (Ads) are non-enveloped, double-stranded DNA viruses under the family *Adenoviridae* (Berk, 2007; Wold and Horwitz, 2007). Viral particles are 80–120 nm in diameter with icosahedral symmetry and contain a linear genome of 26–44 kb in size (Shenk 1996). The linear viral dsDNA genome coding region is flanked by inverted terminal repeats (ITRs), and contains five early transcription regions (E1A, E1B, E2, E3, E4) and one late transcription region from which five families of late mRNAs (L1-5) are generated (Ferrari et

al., 1996). The Adenovirus genome is intimately associated with viral proteins (core) and is packaged in the viral capsid, which consists primarily of three proteins: hexon, penton base, and fibers (Shenk, 2001).

The Adenovirus replication cycle can be divided in two phases, early and late. During the early phase of the replication cycle, the viral DNA is transported to the nucleus and transcription of early viral genes is initiated (E1A). Early gene products interfere with antiviral host cell defense mechanisms and direct the host cell to enter the cell cycle, supporting transcription and DNA replication. As soon as DNA replication is initiated by the E2 gene product, late events in the viral replication start. During this phase, gene expression of mRNA regulated by the major late promoter increases, which results in high production of structural proteins that assemble together with viral genomes in the nucleus. The newly synthesized virions are released with viral genomes in the nucleus. Then the newly synthesized virion are released from the cell by the induction of cell lysis (Shenk, 2001).

1.2.2.2 Adenoviral Vector Development and Production

The first recombinant Adenovirus vector has been generated by deleting the E1 and/or E3 gene regions in the viral genome, allowing the introduction of promoter and transgene sequences up to 6.5-8.3 kb (Berkner & Sharp, 1983; Haj-Ahmad & Graham, 1986). The early region (E1A) gene products are the first adenoviral proteins expressed following infection and function primarily to activate transcription of the other viral early gene products (reviewed in Branton et al., 1985). The E1A proteins also force changes to host cell growth that are thought to allow more efficient production of viral progeny (reviewed in Mymryk and Bayley, 1994). So the removal of E1 gene from the vector markedly hampers transcription of E2 genes and, consequently, DNA replication and production of structural viral proteins. Therefore, E1-deleted Adenovirus vectors have to be propagated in cells, which have been engineered to provide E1 functions *in trans,* which is especially important with respect to safety for human gene therapy applications. The standard complementing cell line is the human embryonic kidney cell line 293, which carries a chromosomally integrated segment of Ad5 DNA from the left end of the genome, encompassing the E1 region (Graham et al., 1977; Amalfitano et al., 1998). Thus, E1-deleted Adenovirus vectors are efficiently propagated in 293 cells, complementing the E1 gene products. Isolation, purification and concentration of adenoviral vectors is uncomplicated. The conventional method is to extract virions from infected cells by cycles of freezing and thawing, followed by purification and concentration of virions by CsCl equilibrium centrifugation.

1.2.2.3 Adenoviral vector tropism and transduction

Adenovirus serotype 5 is commonly used for gene therapy studies, and consequently the Adenovirus vector host range is similar to that of the wild-type virus. The viral receptor and coreceptor of this serotype have been identified, the virus bind to the cellular receptor CAR (coxsackie virus and adenovirus receptor) (Bergelson et al., 1997), a member of the immunoglobulin superfamily, through the knob of the fiber, and virus entry occurs through clathrin-mediated endocytosis after binding of the penton base to integrins (Wickhmam et al., 1993). Although the receptors for Adenovirus are ubiquitously expressed, inefficient transduction due to low level expression of the receptors has been observed in some tissues (Zabner et al., 1997). Differences in the host-range of Adenovirus serotypes indicate that beside CAR other cellular receptors are involved and consequently the cellular tropism of Adenovirus vectors can also be altered by use of alternative serotypes (Zabner et al., 1999; Shayakhmetov et al., 2000; Chillon et al., 1999; Dharmapuri et al., 2009). Several cell surface molecules have been identified as receptors for Adenovirus attachment and entry. Tissue tropism of Adenovirus vectors is greatly influenced by their receptor usage. A variety of strategies has been investigated to modify Adenovirus vector tropism (Sharma et al., 2009).
Adenovirus can be used as vector in gene therapy as they infect a wide variety of cells types, efficiently transduce dividing as well as non-dividing cells and carry up to 8 kb of foreign DNA, a size that is more than adequate for most expression cassettes (Shabram et al., 1997). They are stable and can easily be purified to high titers; adenovirus DNA do not integrate into the host genome, thereby avoiding the possibility of insertional mutagenesis but having the disadvantage that expression of the introduced gene is usually transient in proliferating cells. In addition, the synthesis of viral proteins from the vector elicits a strong cellular and humoral immune response (Kafri et al., 1998) resulting in the elimination of cells that express viral proteins as well as the transgene. Therefore, only transient transgene expression is observed. Despite this problems Ad-based gene transfer has been successfully utilized in gene therapy and vaccine applications (Potthoff et al., 2009; Osada et al., 2009; Kim et al., 2009). In an increasing number of human clinical trials based on recombinant Ad-based vectors as a gene transfer platform, progress has been made utilizing Ad-based vectors as a vaccine platform in HIV, cancer immunotherapy approaches and in vaccination for other infections (Lemiale et al., 2003; Boyer et al., 2005; Holman et al., 2007; Tucker et al., 2008; Zhou et al., 2008; Gabitzsch et al., 2009; Sheets et al., 2009; Sorensen et al., 2009; Tuve et al., 2009).

Although remarkable progress has been made in the development of viral vectors, the ideal vector remains elusive. The wide variety of diseases that may benefit from gene therapy will mandate specific requirements of viral vectors, such as tissue-specific transduction and regulated gene expression. Therefore, it is unlikely that one single vector system will suffice for all gene therapy purposes. Nevertheless, gene therapy approaches have great promise to influence human health in the future.

1.2.3 Gene silencing

In the past few years gene silencing has become the most widely used technology to abolish the expression of a specific gene. RNA interference (RNAi) was first observed by plant biologists in the late 1980s, but its molecular mechanism remained unclear until the late 1990s when RNAi was discovered in the nematode worm *Caenorhabditis elegans* as an evolutionary conserved gene-silencing mechanism (Fire et al., 1998; Reinhart et al., 2000). Since then RNAi has become a powerful tool to uncover gene function and has been evolving as a new therapeutic modality.

The mediator of RNAi is double stranded (ds) RNA. RNAi can be induced in mammalian cells by the introduction of synthetic (delivered exogenously) double-stranded small interfering RNAs (siRNAs) 21–23 base pairs (bp) in length or by plasmid and viral vector systems that express double-stranded short hairpin RNAs (shRNAs) (expressed endogenously from RNA polymerase II or III promoters) that are subsequently processed to siRNAs by the cellular machinery **Figure 1.6** (Brummelkamp et al., 2002; Sui et al., 2002; Yu et al., 2002).

Those double-stranded RNA (dsRNAs) larger than about 30 bp are recognized by the highly conserved RNase III enzyme Dicer (Bernstein et al., 2001; Dykxhoorn et al., 2003; Zamore, 2002) and cleaved into ~21–23 nt small interfering (si) RNAs characterized by symmetric 2–3 nt 3’ overhangs with a hydroxyl and a phosphate group at the 3’ and 5’ ends, respectively. siRNAs are considered to trigger RNAi upon recruitment a “RNA-induced silencing complex” (RISC). The endogenous nuclease activity of RISC eventually induces endonucleolytic cleavage of the target mRNA that is recognized by hybridization with the RISC-incorporated siRNA antisense strand (Elbashir et al., 2001; Kisielow et al., 2002).

The limitations of using plasmid vectors in terms of efficiency and difficulty in transfecting non-dividing cells and primary cells have resulted in investigates developing retroviral (Brummelkamp et al., 2002; Rubinson et al., 2003) and adenoviral (Arts et al., 2003) vector systems for shRNA delivery (Clayton, 2004). Viral vectors permit the efficient delivery and

stable expression of shRNA constructs in a range of mammalian cells including in primary cells. Double-stranded RNA (dsRNA) reagents are used to bind to and promote the degradation of target RNAs, resulting in knockdown of the expression of specific genes. RNAi has been widely used in mammalian cells to define the functional roles of individual genes, particularly in disease. In addition, siRNA and shRNA libraries have been developed to allow the systematic analysis of genes required for disease processes such as cancer (Mohr et al., 2008; Chen et al, 2009). In this context we used the siRNA technology to analyzed proteins functions involved in TRAIL induced apoptosis pathway of pancreatic cancer.

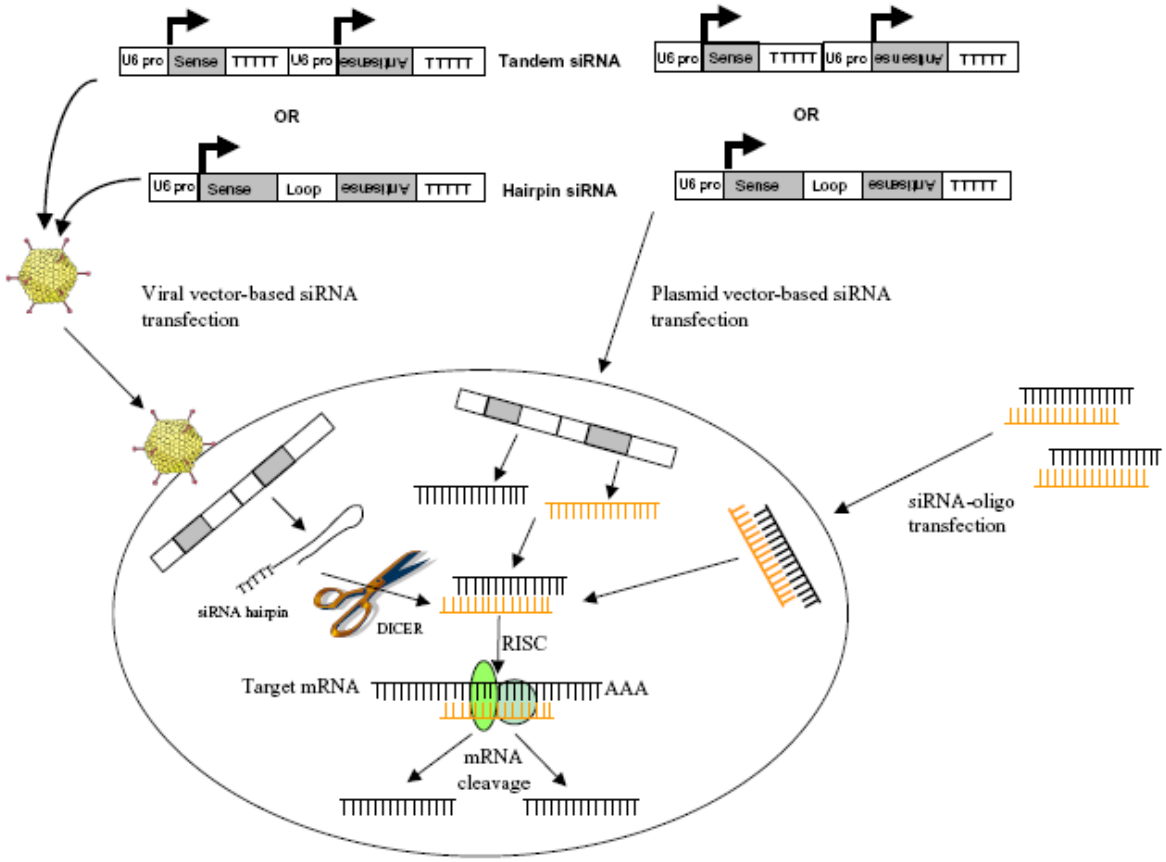

Figure 1.6: **Different forms to express siRNA into the cells**.
Modified from Ray et al. (2005).

1.3 RESEARCH OBJECTIVES

From all cancer types pancreatic cancer is one of the most aggressive human tumors with a 5-year survival of only 3% and a striking resistance to chemotherapy and radiotherapy (Li et al., 2004; Yao et al., 2007). The search for new therapeutic approaches includes strategies exploiting the deregulation of apoptotic pathways commonly found in cancer cells. One new approach for cancer therapy involves the use factor-related apoptosis-inducing ligand (TRAIL), which shows to predominantly kill cancer cells, while sparing normal cells. Although it displays promising activity in other tumor models, the effects of TRAIL on human pancreatic cancer cells is limited, pancreatic cancer cells fail to undergo apoptosis in response to TRAIL treatment resulting in tumor resistance (Braeuer et al., 2006; Karikari et al., 2007; Mori et al., 2007; Vogler et al., 2007; Vogler et al., 2008; Vogler et al., 2009).

In order to develop novel strategies to overcome TRAIL resistance of cancer cells I wanted to elucidate the factors and mechanisms that attenuate TRAIL-induced apoptosis. In this context I wanted to determine which factors are involved in TRAIL resistance of Panc-1 cells, which factors would need to be inhibited or overexpressed to sensitize TRAIL-induced apoptosis and also which strategy may be optimal to use with TRAIL in combination therapy to overcome this resistance. Moreover, the down regulation and over-expression of anti-apoptotic genes in cells causes changes in cell proliferation and the analysis of the mechanism of these changes will be examined.

2. MATERIAL AND METHODS

2.1 MATERIALS

2.2.1 Buffers and solutions

- **Phosphate-buffered saline (PBS):** NaCl 137 mM, Na_2HPO_4 7 mM, KH_2PO_4 1.5 mM, KCl 2.7 mM, pH 7.4
- **PBS- Dulbecco 1x:** Gibco/Invitrogen, Carlsbad, USA
- **Tris buffer saline (TBS) :** Tris-HCl 25 mM pH 8.0, NaCl 137 mM
- **TBS-Tween**: Tris-HCl 25 mM pH 8.0, NaCl 137 mM, in dH_2O, 1% Tween 20
- **Tris-EDTA (TE**) : Tris-HCl 10 mM; EDTA 1 mM; pH 8.0
- **Lysis Buffer:** 50 mM Tris pH 7.4, 10% glycerol, 0.5% NP40, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KCl and Complete Mini Protease Inhibitors (*Roche, Basel, Switzerland)*
- **Tris-Acetate EDTA buffer (TAE):** 1 mM EDTA; 40 mM Tris-Acetate pH 8.0
- **DNA-loading buffer (10x):** 100 mM EDTA; 50 % glycerol; 1 % SDS; 0,25 % Bromphenolblue; 0,25 % Xylenecyanol; pH 8,0.
- **Anode I Buffer:** 300 mM Tris, 20% methanol,
- **Anode II Buffer:** 25 mM Tris, 20% methanol,
- **Cathode Buffer:** 25 mM Tris, 40 mM 6-aminodexanoic acid, 20% methanol
- **SDS-PAGE running buffer:** 24.8 mM Tris/HCl; 20 mM Glycine; 0.1% (w/v) SDS; add dH_2O to 1 l.
- **4x SDS Stacking gel buffer:** 500 mM Tris/HCl pH 6,8; 0.4% (w/v) SDS.
- **4x SDS gel buffer:** 1.5 M Tris/HCl pH 8,8; 0.4% (w/v) SDS.
- **Protein sample buffer:** 65 mM Tris/HCl pH 6,8; 10 % glycerol (v/v); 4 % SDS (w/v); 4 % β- mercaptoethanol (v/v); 0,2 % Bromphenolblue (w/v) in dH_2O.
- **Antibody dilution buffer:** 3% bovine serum albumin (BSA) in TBS-Tween
- **Blocking buffer:** 3-5% (w/v) dry milk in TBS-Tween
- **5×Annealing Buffer:** 500 mM Potassium Acetate pH 7,4; 10 mM Magnesium Acetate, 150 mM HEPES-KOH

- **Nicoletti Buffer:** 0.1 % (w/v) sodium citrate; 0,05 - 0.1 % (v/v) Triton X-100; 50 µg/ml Propidium Iodide.
- **4x Annexin V binding buffer:** 10 mM HEPES pH 7.4; 140 mM NaCl; 25 mM $CaCl_2$
- **TBS Buffer:** 25 mM Tris/HCl; 137 mM NaCl; 27 mM KCl, pH 7,4
- **2x HBS:** 140 mM NaCl, 1,5 mM Na_2HPO_4; 50 mM HEPES, pH 7.2; sterilized and stored at -20ºC
- **$CaCl_2$-solution:** 250 mM in H_20; sterilized

2.2.2 Reagents

- **Acrylamide-Bis-Acrylamide,** 30% ratio 29:1: *Sigma-Aldrich, UK*
- **1,2-Bis-(dimethylamino)-ethane (TEMED):** *Sigma-Aldrich, UK*
- **Ammoniumperoxidedisulfate (APS),** 10% (w/v) : *Sigma-Aldrich, UK*
- **Agarose:** *Sigma-Aldrich, UK*
- **Annexin V-FITC:** *Roche, Mannheim, Germany*
- **BSA (bovine serum albumin) :** *Gibco/Invitrogen, UK*
- **Ponceau S-solution:** *Sigma-Aldrich, UK*
- **CsCl:** Roche, Mannheim, Germany
- **Caspase Inhibitor zVADfmk**: *Santa Cruz, CA, USA*
- **DNA Standard: 100bp, 1kb**: *New England Biolabs, UK*
- **Enhanced chemiluminescent (ECL) Detection Reagent**: *GE Healthcare, UK*
- **Milk powder:** *Carl Roth, Karlsruhe, Germany*
- **Propidium Iodide:** *Sigma-Aldrich, UK*
- **Protease Inhibitor Cocktail (Tabletts):** *Sigma-Aldrich, UK*
- **Antibiotics Ampicillin and Kanamycin,** for bacteria strain selection: *Sigma-Aldrich,UK*
- **Antibiotics Penicillin/Streptomycin solution**, 100x for cell culture: *Gibco/Invitrogen, Carlsbad, USA*
- **Antibiotic Blastocydin™,** for eukaryotic selection: *InvivoGen, France*
- **Fetal Calf Serum (FCS):** *Gibco/Invitrogen, UK*

- **Trypsin/EDTA:** *Gibco/Invitrogen, UK*
- **T4 DNA Ligase:** *New England Biolabs, UK*
- **Restriction enzymes:** *New England Biolabs*
- **Gateway LR Clonase™ II enzyme:** *Invitrogen*
- **SYBR-Green:** *Gibco/Invitrogen, UK*
- **Transfection reagent Fugene:** *Roche, Mannheim, Germany*
- **Recombinant human TRAIL**: *R&D Sytems, Minneapolis, MN, USA*; Reconstitute in 1xPBS with 0,1 % BSA to a concentration of 50 µg/µl and stocked at -80 °C
- **Recombinant human Interferon-γ**: *PeproTech, Rocky Hill, NY, USA;* Reconstitute in 1xPBS to a concentration of 1mg/ml, stocked at - 20 °C
- **Valproic acid sodium salt (VPA):** *Sigma-Aldrich;* Reconstituted in 1xPBS to a concentration of 1M and stocked at -80 °C.
- **Trichostatin A (TSA*): Sigma-Aldrich;* Reconstituted in 1xPBS to a concentration of 3,3mM and stocked at -80 °C.

2.2.3 Media

- **LB Media**: *Sigma-Aldrich*
- **OptiMem**: *Gibco/Invitrogen*
- **Dulbeccos Modified Eagle *M*edium (DMEM):** *Gibco/Invitrogen*
- **Mc Coy´s 5A:** *Cambrex; New Jersey, USA*
- **RPMI 1640:** *Gibco/Invitrogen*

2.3.4 Antibodies

ANTIGEN	MW (kDa)	ANTIBODY	ISOTYPE	DILUTION
Bak	25	Anti-Bak polyclonal antibody Cell Signaling Technology	Rabbit IgG	1/1000
Bax	20	Anti-Bax polyclonal antibody Cell Signaling Technology	Rabbit IgG	1/1000
BCl-xl	30	Anti-Bcl-xl polyclonal antibody Cell Signaling Technology	Rabbit IgG	1/1000
Bid	29	Anti-Bid polyclonal antibody R&D Systems, Minneapolis, MN, USA	Rabbit IgG	1/1000
Caspase-3	Proform 35 Active form 19/17	Anti-caspase-3 Monoclonal antibody (3G2) Cell Signaling Technology	Mouse IgG	1/1000
Caspase-8	Proform 57 Active form 43/18	Anti-caspase-8 monoclonal antibody (1C12) Calbiochem	Mouse IgG	1/1000
Caspase-9	Proform 47 Active form 32/17	Anti-caspase-9 monoclonal antibody (LAP6 96-2-22) Novus Biologicals, CO, USA	rabbit IgG	1/1000
c-Flip	Flip long 55 Flip short 30	Antic-Flip monoclonal antibody (NF6) Alexis Biochemicals	Mouse IgG	1/200
CuZnSOD	19	Antic-CuZnSOD monoclonal The BindingSite, Birmingham, England	Sheep IgG	1/2000
DR4	56	Anti-DR4 polyclonal antibody (H-130) Santa Cruz Biotechnology	Rabbit IgG	1/1000

DR5	40 (mature) 48 (precursor)	Anti-DR5 polyclonal antibody (#3696) Cell Signaling Technology	Rabbit IgG	1/1000
FADD	28	Anti-Fadd polyclonal antibody Cell Signaling Technology	Rabbit IgG	1/1000
XIAP	53	Anti-XIAP monoclonal antibody (48/hILP/XIAP) BD Pharmingen, San Diego, CA, USA	Mouse IgG	1/1000

HRP-conjugated secondary antibodies: Santa Cruz Biotechnology

- **Anti-sheep** IgG (rabbit);
- **Anti-rabbit** IgG (goat);
- **Anti-mouse** IgG (goat).

2.2.5 DNA plasmids

- **pEGFP-N1** (Clontech, Mountain View, USA);
- **pcDNA3** (Invitrogen, Carlsbad, USA);
  pcDNA3.DR4 (Genentech, San Francisco, CA, USA)
- **pU6Entry** (Invitrogen)
  (for more details of cloning such as strategy see 2.2.1.7 and 2.2.1.8)
  pU6Entry.shcas-9
  pU6Entry.shBcl-xl
  pU6Entry.shXIAP
  pU6Entry.shc-Flip
  pU6Entry.shEGFP
  pU6Entry.shScramble
- **pENTR1A** (Invitrogen)

pENTR.DsRed
pENTR.DR4
pENTR.EGFP

- **pAd/CMV/V5- DEST™** (Invitrogen)
  pAd.CMV.DR4
  pAd.CMV.DsRed
  pAd.CMV.EGFP

- **pAd/BLOCK-iT™6- DEST** (Invitrogen)
  pAd.shcas-9
  pAd.shc-Flip
  pAd.shBcl-xl
  pAd.shXIAP
  pAd.shEGFP
  pAd.shScramble

- **pDrive cloning vector** (Qiagen)

- **pEF-DEST51** (Invitrogen)
  pEF.DR4

- **pBlock-iT™** (Invitrogen)
  pBlock-iT.U6.shXIAP
  pBlock-iT.U6.shBcl-xl

2.2.6 Adenovirus vectors

- **Ad.DR4** (CMV promoter-dependent DR4 expression)
- **Ad.DsRed** (CMV promoter-dependent DsRed expression)
- **Ad.EGFP** (CMV promoter-dependent EGFP expression)
- **Ad.shcas-9** (CTTCGTTTCTGCGAACTAAC)
- **Ad.shc-Flip** (TGTCGGGGACTTGGCTGAACT)
- **Ad.shBcl-xl** (GGAGATGCAGGTATTGGTGAG)
- **Ad.shXIAP** (GTGGTAGTCCTGTTTCAGC)

- **Ad.shEGFP**
- **Ad.shScramble**

2.2.7 Cell lines

- **A459**: human lung cancer cell line
- **AsPC-1:** human pancreatic carcinoma
- **Caco-2:** epithelial human colon carcinoma
- **CAKI-2:** human bladder carcinoma
- **DLD-1:** epithelial human colon carcinoma
- **HCT-116:** epithelial human colon carcinoma
- **HEK293A:** Human Embryonic Kidney *293* cells
- **HeLa:** epithelial human cervix adenocarcinoma
- **HT29:** colon cancer cell line
- **N52:** Human amniocyte cell line
- **Panc-1:** epithelial human pancreatic carcinoma

2.2 METHODS

2.2.1 MOLECULAR BIOLOGY METHODS

2.2.1.1. Transformation of competent cells

Plasmid DNA was transformed into competent *E. coli*. An aliquot of 25-100 µl competent bacteria stored at -80ºC was thawed on ice and added to 10-50 ng of plasmid mixture. Cells were incubated for 30 min on ice, and then heat-shocked for 30 sec to 2 min at 42ºC. Afterwards 200-500 µl of LB was added and the cultures were incubated shaking at 37ºC for 45 min to allow cell growth. Cells were plated onto agar medium containing appropriate antibiotics. Plates were incubated at 37ºC overnight and checked for colonies.

2.2.1.2 Preparation of plasmid DNA from bacteria

Small and large-scale plasmid purifications were performed using plasmid mini- and maxiprep kits from Sigma-Aldrich or QIAGEN according to the manufacturer´s recommendation.

2.2.1.3 Estimation of DNA quantity and purity

DNA quantification was measured using a spectrophotometer at 260 nm and 280 nm. The reading at 260 nm allowed the calculation of the concentration of nucleic acid in the sample with A of 1 corresponding to approximately 50 µg/ml of dsDNA or 40 µg/ml of ssDNA. The ratio A_{260}/A_{280} provides an estimation of the purity of the nucleic acid. Pure preparation of dsDNA have values of about 1.8.

2.2.1.4 Restriction endonuclease digestion

Restriction enzyme reactions were set up using the appropriate amount of enzyme as recommended by the manufacturer and ~1.0 µg DNA was used for each digestion. The reactions were performed in the suitable reaction buffer complemented with BSA (final concentration 100 µg/ml), if required. The reaction mix was incubated for 1 h at 37ºC or as recommended by the manufacturer. For double digestions involving two non-compatible enzymes, a purification step was carried out prior to subsequent digestion.

2.2.1.5 Horizontal DNA gel electrophoresis and extraction of DNA

To control restriction endonuclease digestion or ligation of DNA fragments a horizontal DNA gel electrophoresis with 0.8% agarose gel containing ethidium bromide or SYBR-Green was performed using TAE buffer. The applied current was 60-120 mA. Prior to loading samples were mixed with gel-loading buffer (stock solution 6-fold). To estimate the size of a DNA fragment a DNA marker was analyzed in parallel. The DNA bands were visualized with ultraviolet light. When necessary the desired bands were excised form the gel with a scalpel and DNA was isolated. DNA was recovered from agarose gel slices with the Gel extraction Kit (Sigma-Aldrich) following the manufacturer´s instructions.

2.2.1.6 DNA ligation

T4 DNA ligase was employed to covalently connect vector DNA (backbone) with the insert DNA (fragment). Appropriate molar ratios (1:3) between linearized vector (50-200 ng) and insert DNA were used in a 10 µl reaction with 1 unit T4 DNA ligase and 1xT4 ligation buffer. The reaction mixture was incubated at 17ºC for 3-12 h.

2.2.1.7 siRNA design and short hairpin expression vector

The most efficient known RNAi triggers are double stranded RNAs containing 21–23 nt sense and antisense strands hybridized to have 2 nt overhangs at both 3’ ends. The sense strand is homologous whereas the antisense strand is complementary to the target mRNA. Suitable siRNAs must fulfill two criteria to allow functional analysis of a given target gene in mammalian cells: they must first be effective in triggering RNAi and second specific to exclusively inhibit the expression of the target gene. The design of specific siRNAs requires sequence information of the target gene (at least 19 nt), which can be readily obtained from databases. However, the efficacy of any given siRNA cannot yet been predicted from sequence information alone, since secondary and tertiary structures of the target RNA are believed to play an important role for potential hybridization between target RNA and the siRNA antisense strand. It has been suggested that sequences located within introns should not be used as target sites for siRNAs as the gene silencing machinery operates only in the cytoplasm and not within the nucleus. Also,

sequences encompassing the translation initiation site and potential protein binding sites located in the 5' and 3' untranslated region (UTRs) should be avoided. Therefore, the selection of the targeted mRNA sequence seems more or less to be a question of trial and error as theoretically any coding subsequence can be chosen if no information on RNA structure *in vivo* is available. Therefore, a screening strategy for individual siRNAs is necessary to select functional siRNAs (Lee et al., 2002; Scherr et al., 2003).

The small interfering RNAs (siRNAs) are delivered to cells as synthetic duplexes or as short hairpin RNAs (shRNA) by plasmids or viral vectors. The first step to produce a plasmid that delivers a shRNA that successfully silences a gene is to choose a good shRNA motif. This was performed by using the website of the company Invitrogen and the motif with the highest score was chosen. For the cloning strategy into the pU6entry vector nt sequence for the recognition sites of the BbsI and EcoRI enzymes needed to be added. For processing shRNA into siRNA nt sequence for a loop were added between the sense and antisense nt from the target RNA (siRNA motif) as shown below.

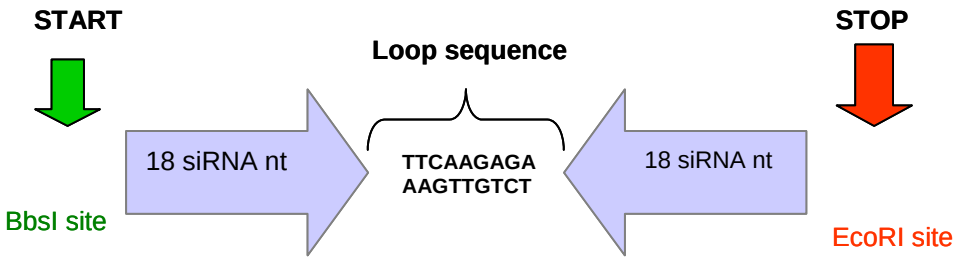

Figure 2.1. Cloning strategy used to clone the short hairpin into the backbone plasmid

The primers (sense and antisense for each motif) were eluted at the concentration of 2 µg/µl and 13 µl of each primer was then hybridized with annealing buffer (see section 2.2.1) and cloned into the pU6.Entry vector linearized with BbsI and EcoRI. The plasmid resulting form the ligation was then analyzed for the short hairpin insert by cleavage with NdeI and SalI enzymes. The expression vectors were then tested for the functionality of the short hairpin by transfecting HEK 293 cells. 72 h after transfection the cells were harvested and tested by western blot.

2.2.1.8 Adenovirus vector

2.2.1.8.1 Cloning Adenovirus vectors expressing a short hairpin

For the production of the adenoviral vectors carrying the short hairpin expression cassettes the BLOCK-iT™ Adenoviral RNAi Systems (*pAd/BLOCK-iT™Dest*) from the company *Invitrogen* were used. These plasmids have the sequence of the Adenovirus serotype 5 with E1/E3 region deleted. The cloning of the insert cassette (U6 promoter and short hairpin sequence) into the pAd/BLOCK-iT™Dest has been done using the Gateway® Technology from Invitrogen. The Gateway Technology is based in site-specific recombination, *attachment* (*att*) *sites*, attL sites on the pENTR plasmid and attR sites on the destination vector (*pAd/ BLOCK-iT™)***.** Once the Entry clone is ready, the sequence of interested is easily shuttled to the destination vector. This reaction is mediated by a robust enzyme mixture called LR Clonase®, which contains the necessary protein activity to excise the gene of interest from the Entry clone and integrate it into the Destination vector (see below). The Destination vector has a *ccdB* gene a Chloramphenicol-resistance (*cmR*). These factors get swapped during the recombination process that also selects positively the clones that grow after the recombination under ampicillin selection. CcdB Protein inhibits *E. coli* DNA Gyrase and avoid the growth of almost all *E. coli* strains (Bernard and Couturier, 1992). The clones were testing by cleaving them with NdeI enzyme.

2.2.1.8.2 Cloning Adenovirus vector expressing a transgene

In the same way as described above once the Entry vector is done the gene of interest is shuttled to the destination vector. One important difference here is the destination that has been used, *pAd/CMV/V5- DEST*™ (Invitrogen) that has a CMV promoter to control the expression of transgene. For the cloning of the Ad.CMV.EGFP the EGFP fragment was isolated from the pEGFP.N1 plasmid with NotI and BamHI enzymes and ligated into the pEntry vector. For the Ad.CMV.DsRed vector a PCR generated the fragment which was subsequently cloned into pDrive and then subcloned into pEntry to generate pEntry.DsRed. The DR4 fragment was cut out from pCDNA3.DR4 with KpnI and NotI and cloned into pEntry for the generation of pAd.CMV.DR4.

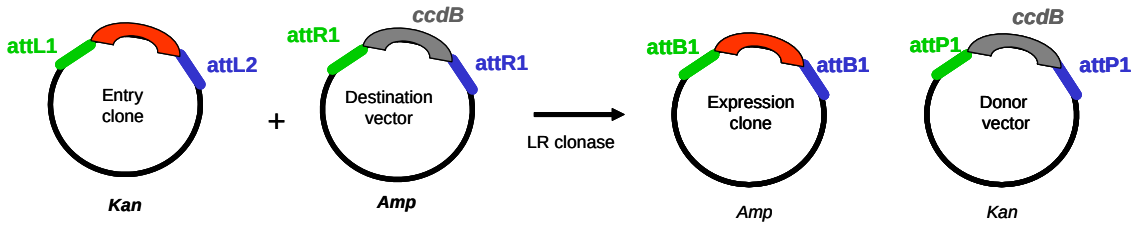

Figure 2.2. Scheme of a clonase reaction

2.2.1.8.3 Adenovirus production

Once the destination plasmid (*pAd.CMV.transgene* or *pAd.sh.*) was ready and tested at the DNA level for containing the insert by cleavage with appropriate enzymes the next step was a PacI cleavage to linearize the plasmid. After cleavage, the DNA was purified before calcium phosphate-mediated transfection into a 10 cm plate of N52 cells (Schielner et al., 2000). The cells were daily examined for signs of Cytopathic Effect (CPE) and once a complete CPE was observed (most cells rounded but not all detached) cells were harvested followed by four steps of freezing-thawing to release the virus from the cells. These crude lyses were used then to re-infect larger quantities of cells to produce the adenovirus vector in a large scale. The last amplification was performed in 15 flasks (75 cm^2). After full CPE was observed cells were harvested and the crude extract, after freezing-thawing, was resuspended into 10 ml of TBS buffer (see section 2.2.1). 5 g of CsCl was added to the crude lysate. The solution was then transferred to a 14x89 mm *Beckman* tube and centrifuged for about 20 h at 32000rpm and 4ºC in a Sorval 100 SE centrifuge using a Sorval TH-641 rotor. The adenovirus vectors were collected from the CsCl gradient with a syringe and then purified with PD10 columns (GE Healthcare). To the final two ml of purified adenovirus vector solution glycerol were add to a final concentration of 10% to allow the virus to be stored safely at -80º.

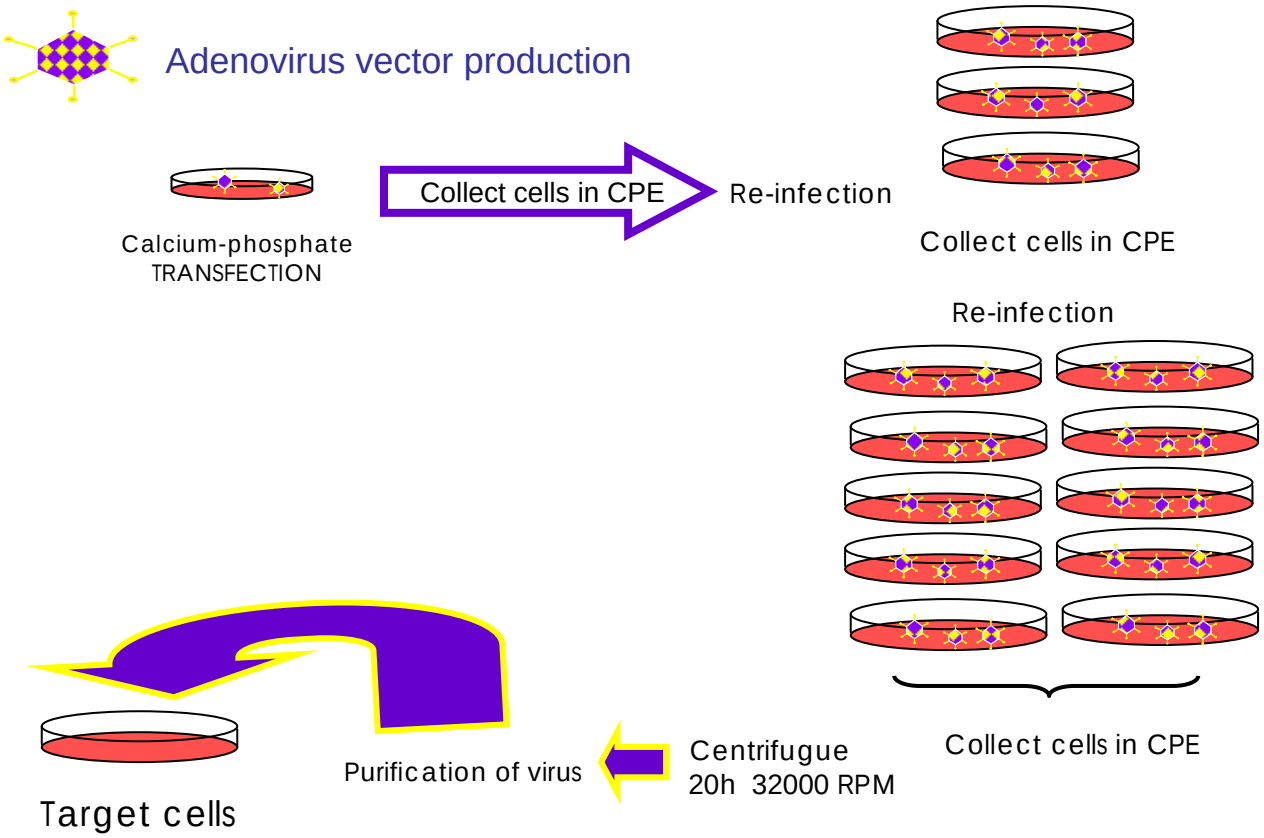

Figure 2.3. Scheme of Adenovirus vector production

2.2.1.843 Adenovirus vector titration and transduction

To estimate the amount of the virus particles the capsid amount were measured (determined by protein measurement) and for the transduction MOI (*M*ultiplicity *O*f *I*nfection) were used. MOI is based on the fact that from every 25 particles only about one is infectious, so the number of virus particles/cell used were multiplied by 25.

2.2.1.9 pBLOCK-iT cloning

For the production of stable clones the RNAi motifs were subcloned from the Entry vectors (Invitrogen) into pBLOCK-iT6-DEST plasmids (Invitrogen) according to the manufacturer's instruction**.**

2.2.2 BIOCHEMICAL METHODS

2.2.2.1 Preparation of cells extracts

Cells were trypsinised and harvested. All following steps were carried out at 4ºC or on ice. The cells were washed once with cold PBS and resuspended in ice-cold lyses buffer (see section 2.2.1). Cells were then centrifuged at 14.000 rpm for 15 min at 4ºC. The clear supernatant was aliquoted and frozen and stored at -20ºC.

2.2.2.2 Protein quantification.

According to the manufacturer's instruction, the BCA^{TM} protein assay kit (*PIERCE*) was used to determine the concentration of proteins in cell extracts.

2.2.2.3 Sodium DodecylSulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis.

SDS-PAGE was performed following a protocol adapted from (Laemmli 1970) using 4% stacking and 12% separating gels (1.5 mm thickness) at 80-120 V and constant current. Fifty µg of protein from each sample were mixed with an appropriate volume of loading buffer (see section 2.2.1) to give a final volume of 20-30 µl for loading. The samples were boiled for 5 min and then loaded on the stacking gel (see section 2.2.1) along with a prestained protein marker (broad range 6-175 kDa, Biolabs) to determine the molecular mass of proteins. Protein transfer onto PVDF (*GE Healthcare*) membrane: Prior to protein transfer, the PVDF membrane was soaked in methanol (pre-activation) followed by dH_2O and pre-chilled blot buffer. Transfer was carried out at 40-50mA constant current for 1 h. Transfer and loading of protein was routinely checked by Ponceau S staining. After the protein transfer, the PDVF membrane was blocked by shaking in blocking buffer for 30 min at room temperature. The membrane was then incubated with the primary antibody diluted in antibody dilution buffer. To remove excess of primary antibody, the membrane was washed with TBST, three times for 10 min. Next, the membrane was incubated for one h at RT with the secondary antibody in TBST 3% BSA solution. Blots were then washed with TBST three times for 10 min and finally with enhanced chemiluminescence (ECL) solution for 1 min. The signal was recorded with

MultiSpectral Imanging System (BioSpectrum®) camera using Image Reader and Adobe Photoshop CS3 Extended analysis software.

2.2.3 CELL BIOLOGY METHODS

2.2.3.1 Cell Culture

All steps were performed in a laminar flow hood. The human cells were routinely cultivated in 25 cm^2 or 75 cm^2 tissue culture flasks in DMEM with glutamine added supplemented with 10% fetal calf serum (FCS) and antibiotics Penicillin/Streptomycin (100 IU/ml and 100 mg/ml, respectively) at 37ºC with 5% CO2 in a humidified incubator. When cells reached 80-90% confluence, they were washed with PBS, trypsinised and resuspended in fresh medium. An appropriate fraction of cell solution was transferred into a new culture flask with fresh medium for further cultivation or seeded for further experiments.

2.2.3.2 Drug treatments

TRAIL (10 ng/ml) treatment was carried out 24 h after cells were seeded, 24 h after interferon-gamma co-treatment, 24 h after adenovirus transdcution expressing a transgene or 48 h after adenovirus transduction expressing a short hairpin RNA.

Interferon-gamma (1 μg/ml) treatment was carried out 24 h after cells were seeded or 48 h after adenovirus transduction expressing a short hairpin RNA.

HDAC inhibitor VPA treatment was carried out 24 h after seeding the cells at different concentrations of 0.5 mM, 1 mM, 2 mM and 5 mM for 24 h and 48 h incubation time.

HDAC inhibitor TSA treatment was carried out 24 h after seeding the cells at two different concentrations of 0.5 μM and 1 μM for 24 h.

2.2.3.3 Flow cytometry analysis

For the three different assays using flow cytometry the cells were first trypsinized for harvesting. After trypsinization, the cells were mixed with their previous medium and centrifuged at 1500 rpm for 5 min. The cells were washed once with 1xPBS and the samples were treated according to the type of analysis described below.

There are many ways of detecting apoptosis by flow cytometry. Apoptotic cells can be recognized by a characteristic pattern of morphological, biochemical and molecular changes. The methods of detecting apoptosis by flow cytometry are based on the measurement of DNA content, altered membrane permeability or the detection of endonucleolysis as characterized by DNA strand breaks.

2.2.3.3.1 Annexin V Binding Assay

In normal cells, phosphatidylserine (PS) residues are found in the inner part of the cytoplasmic membrane. During apoptosis, the PS residues are translocated in the outer membrane and are externalized. In general, though not always, this is an early event in apoptosis and is thought to be a signal to neighboring cells that a cell is ready to be phagocytose. Annexin-V is a specific PS-binding protein that can be used to detect apoptotic cells. Annexin-V is available conjugated to a number of different fluorochromes. We used Annexin-V in combination with PI. After the procedure described above, the supernatant was discarded and the pellet was resuspended in 1 ml of Annexin V binding buffer (see 2.2.1) to get a cell density of $1x10^6$ cells/ml. Five hundred µl of the cell suspension were transferred into a clean Eppendorf tube and incubated with 1.25 µl of Annexin V-FITC in the dark for 15 min at RT. After incubation, 10 µl of propidium iodide was added to each sample in the dark. The samples were placed on ice, wrapped with aluminium foil and analyzed (10,000 events) immediately by flow cytometry using FL1 (Annexin V-FITC detection) and FL3 (propidium iodide detection) channels.

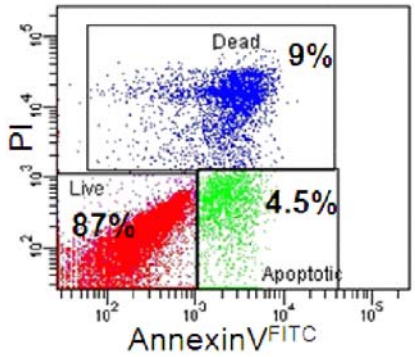

Figure 2.4. An example of Annexin V binding assay quadrant dot blot analysis. In red double negative cell population, live cells; in green Annexin V positive cells, apoptotic cells; and in blue the double positive cell population that represents dead cells.

2.2.3.3 Nicoletti Assay

For the detection of apoptotic cell death, standard DNA fragmentation assays according to Nicoletti (Nicoletti et al., 1991) were used. These methods take advantages of DNase activation and internucleosomal DNA-degradation that becomes clearly visible in advanced apoptotic processes. Apoptotic cells, due to a change in membrane permeability, show an increased up-take of PI. Cells were harvested with the supernatant, washed once with 1xPBS and lysed in Nicolleti buffer (see section 2.2.1) and stained with PI. At this point cells have been converted into DNA clumps. The volume of Nicoletti buffer was about 1 ml for $5x10^5$ cells. Before measuring by flow cytometry the cells were incubated for 2-16 h at 4ºC in dark. The fluorescence signal was measured using a laser that supplies light at 488 nm in the FL3 (PI detection) channel. Cells with normal DNA content show typical G1-M-G2 cell-cycle specific DNA content histograms. Cells with decreased (hypodiploid) DNA content are considered apoptotic cells. 5000 to 10000 events were measured.

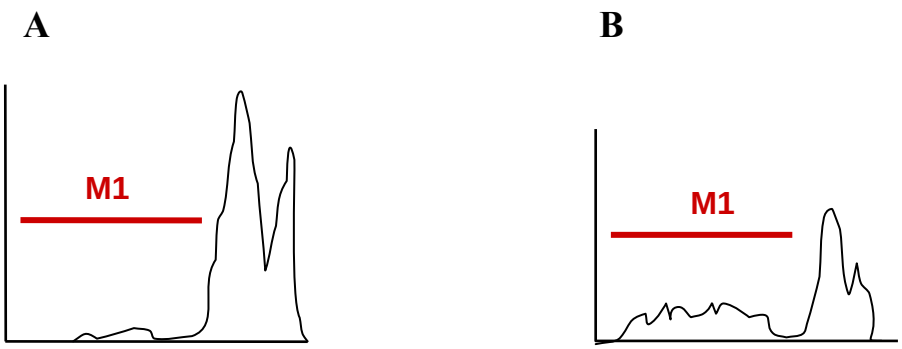

Figure 2.5. Nicoletti method-DNA histograms

A) Typical control DNA histogram. **M1** (statistical marker) has been placed to mark sub-diploid DNA that is almost absent on this histogram. The diploid (G1), and tetradiploid (G2) DNA is clearly visible in the form of 2 peaks in the far-right part of the histogram.

B) Example of a typical apoptotic DNA histogram with most cells undergoing apoptosis. Therefore, G1 and G2 peaks are fused together and shifted to the left and wide sub-G1 DNA content is clearly visible in the middle right part of the histogram.

2.2.3.3.3 EGFP measurement

Flow cytometry can be used also for measuring eGFP *(*Enhanced Green Fluorescence Protein*)* expression in cells. Through this measurement transfection efficiency and virus transduction can be verified.

Cells are harvested as described above and resuspended in 1xPBS. EGFP fluorescence is measured at the channel 1 (FL1-High), the flow cytometry provides the percentual and the count of green fluorescence cells (EGFP positive).

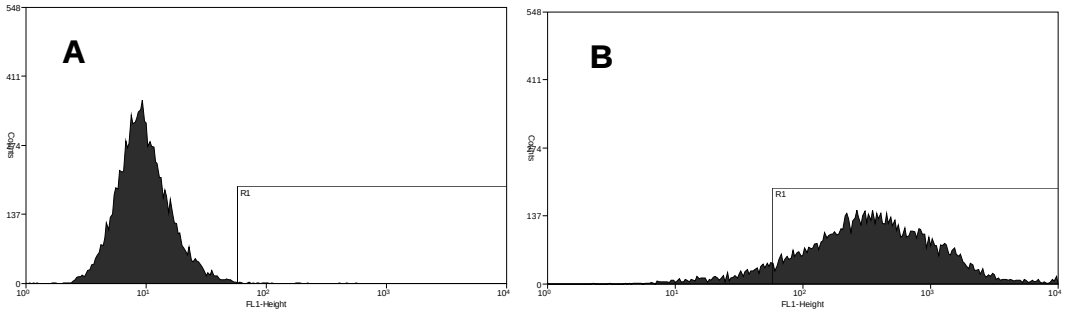

Figure 2.6. Histogram of flow cytometry analysis of EGFP expression. **A)** Non-transfected cells, R1=0,3% of total cells counted. **B)** Cells transfected with EGFP, clearly show a shift to fluorescence, R1=92% of the cells are GFP-positive.

2.2.3.3.4 Proliferation Assay (BrdU incorporation)

The most accurate method for determining a cell´s ability to proliferate is to directly measure DNA synthesis. For this purpose PI staining in combination with BrdU incorporation and FACS was performed (Natoni et al., 2005; Pollok et al., 2007). BrdU (final concentration 10 µM) was added to cells for 1 h. After the incubation, the cells were washed in PBS, suspended by trypsinization and resuspended in 1 ml PBS. Then cells were fixed by the addition of 100% ethanol to a final concentration of 70% ethanol. For PI/BrdU staining the cells were collected by centrifugation and resuspended in 1 ml PBS. Then 1 ml of 4 M HCl was added and cells were incubated for 15 min at room temperature. Next cells were washed twice in PBS-0.5% BSA-0.1% Tween 20. After resuspension in 200 µl of this solution, anti BrdU antibody (1/40 dilution) was added and incubated for 30 min. In the following anti-mouse FITC-conjugated secondary antibody was added and incubated 30 min in the dark. These cells were then resuspended in 0.5 ml of PI/RNAse staining solution (BD Pharmingen Cat. N° 550825) and incubated for 30 min at RT in the dark. The cells were then analyzed by flow cytometry.

2.2.3.4 Transfection of eukaryotic cells

For transfection experiments cells were prepared a day before and the medium was changed 4 h or 24 h after the transfection. To check the efficiency of the tranfection pEGFP.N1 plasmid was also transfected and the percentage of green fluoresence cells was measured 24 or 48 h after transfection, either by microscopy or by flow cytometry.

2.2.3.4.1 Calcium phosphate transfection

Cells were splitted to about 50-70% confluency a day prior transfection. For each 10 cm dish, 0.5 ml 2xHBS were aliquoted into a sterile 1.5 ml microfuge tube and in a separate tube 5-15 μg of DNA (dependent of the plasmid) were mixed with 2.5 M $CaCl_2$ and enough distilled water to bring the total volume to 0.5 ml (see table below for different area dishes). The $CaCl_2$/DNA mix was then added to the HBS drop-wise with vortexing. After 20 min of incubation, the DNA $CaPO_4$ co-precipitate was added directly to the surface of the media containing the cells by dropping slowly. Dishes were then placed into the incubator for 4-24 h. The media was then removed and cells were washed once with PBS, next fresh complete medium was added and incubation was resumed for 24-48 h prior to assaying.

While the Calcium phosphate transfection method is a very efficient means of introducing DNA into cells in many cell systems, it is very inefficient in many others. In our experience, this method works best in HEK293 cells, in which we obtain from 80% to 95% transfection efficiency. We use this transfection method for transient experiments but this method is quite sensitive to the amount of input plasmid.

Table 2.2. Amounts of transfection media used for different surface areas.

Format	**Culture Media**	**Transfection Media**
6 well plate	1.5 ml	0.5 ml
10 cm plate	6 ml	1 ml
15 cm plate	15 ml	3 ml

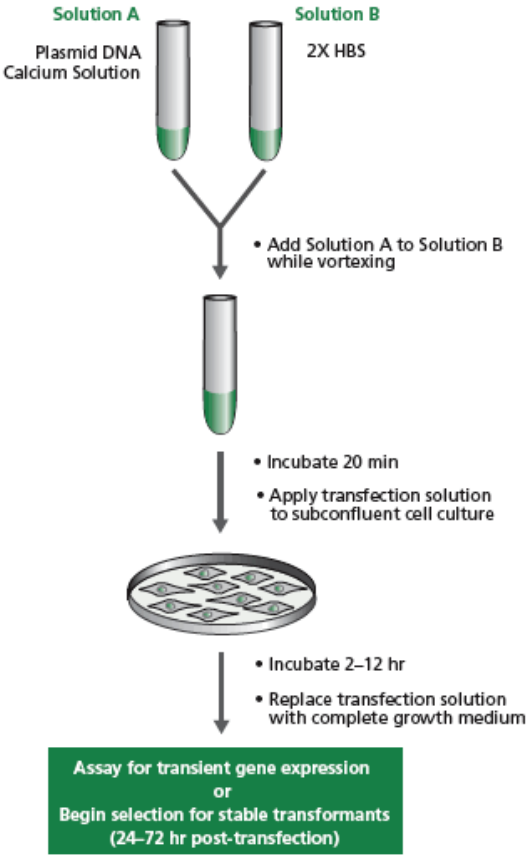

Figure 2.7. Scheme of the calcium phosphate transfection

2.2.3.4.2 Lipofection transfection with FuGENE6

Lipofection (or liposome transfection) is a lipid-based transfection technique used to introduce genetic material into a cell by means of liposomes (DNA and liposome form a complex), which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer. The main advantages of lipofection are its high efficiency, its ability to transfect all types of nucleic acids in a wide range of cell types, its ease of use, reproducibility and low toxicity. In addition this method is suitable for all transfection applications. Fugene 6 transfection was carried out according to the manufacturer's instruction (Roche) in 50-80% confluent cells.

2.2.3.4.3 Electroporation

By electroporation a significant increase in the electrical conductivity and permeability of the cell membrane caused by an externally applied electrical field allows the plasmid to enter the cell. The application is performed with electroporators which create an electro-magnetic field in the cell solution. 70-80% confluent cells were trypsinized and centrifuged at 1500 rpm for 5 min and resuspended in 200µl medium. The cell suspension was then pipetted into a glass or plastic cuvette which has two aluminum electrodes on its sides. DNA plasmid was also added into the cuvette with the cell solution and the electroporation was done in a Gene Pulser Xcell *BIO-RAD* under 160V and with a capacity of 1000 µF. After the electric pulse the cells were immediately washed with fresh medium and kept in normal cell culture conditions for 3 days.

2.2.3.5 Generation of stable clones

3 days after electroporation of Panc-1 cells with the plasmid expressing a short hairpin the cells were split in five 15 cm dishes and kept under positive selection with Blasticidin (10 µg/ml). After about 3 weeks, isolated clones were visible, picked with cloning discs and transferred to 24-well plates until confluence was achieved. The clones were scaled-up and tested by western blot analysis for XIAP and Bcl-xl silencing.

2.2.3.6 Colony forming assay

Cells were treated with 10 ng/ml TRAIL for 1 h and then counted. A total of $5x10^5$ cells were seeded into a 50 ml tissue culture flask (T-25) and cultured for 12 days. The cells were then fixed in 100% ethanol for 10 min before they were stained with crystal violet and counted.

2.2.3.7 Senescence Staining

Limited capacity to replicate is a defining characteristic of most normal cells and culminates in senescence, an arrested state in which cells remain viable but display altered patterns of gene and protein expression (Godstein 1990). An enlarged cell size and expression of pH-dependent β-galactosidase activity further characterize senescent cells. For the detection of senescence the Senescence-associated β-Galactosidase Staining Kit (Cell Signaling) was used according to the manufacturer's instruction. The kit is designed to histochemically detect β-galactosidase activity at pH 6, a known characteristic of senescent cells.

2.2.3.7 Statistical Analyses.

If not otherwise stated, three independent experiments were performed in triplicate. Experimental values are expressed as mean value ± standard error (S.E.). For significance analyses Student's t-test was used and $p < 0.05$ was considered significant and $p < 0.001$ as highly significant.

3. RESULTS

3.1 TRAIL responsiveness and NF-κB target genes

Apoptosis is regulated by balanced activities of several anti-apoptotic factors (e.g. c-Flip, Bcl-xl, XIAP), which are mostly controlled by the transcription factor NF-κB (Braeuer et al., 2006; Song et al., 2006; Whang et al., 2007), and pro-apoptotic factors (e.g. caspases, death receptors etc.).

To investigate the relative contribution of pro- and antiapoptotic factors in cells and to analyze TRAIL-responsiveness in human cell lines data from altogether 11 cell lines were collected (Buneker et al., 2009). It is apparent that the apoptotic response of all these cell lines differed greatly. For example the colon cancer cell line HCT116 and the cervix carcinoma cell line HeLa were TRAIL-sensitive while the pancreatic carcinoma cell line Panc-1 was TRAIL-resistant. TRAIL in high doses (10 ng/ml) only induced less than 14% of specific cell death in Panc-1 cells. In contrast, the TRAIL-sensitive cancer cell lines HeLa and HCT116 underwent between 50-70% apoptosis with 10 ng/ml TRAIL after 24 h. Then these three cell lines were studied in great details about their levels of c-Flip, Bcl-xl and XIAP, three anti-apoptotic NκB-regulated factors (**Figure 3.1**). This analysis revealed a remarkable difference in the expression of Bcl-xl by western blot analysis, with the highest levels detected in Panc-1 cells and no detectable levels in HeLa, and intermediate levels in HCT116. XIAP levels also appeared to be higher in Panc-1 cells compared to the other two cell lines whereas c-Flip was hardly detectable in any of the three cell lines. In all western blot experiments performed in this study the CuZnSOD protein, a housekeeping protein, was used as a control for the protein loading.

Fig. 3.1

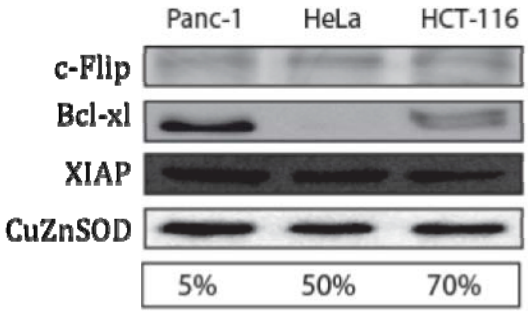

Figure 3.1: Western blot analysis showing the levels of anti-apoptotic proteins. c-Flip$_L$, Bcl-xl and XIAP levels were compared in three different selected cell lines displaying variations in response to TRAIL. CuZnSOD protein levels served as the control for protein loading. The lowest panel displays the levels of apoptosis in these cells after incubation with 10 ng/ml of TRAIL. The experiment was performed at least twice and the western blots are representative for these experiments.

3.2 Production of Adenovirus vectors expressing shRNA

For the production of Adenovirus vectors the Gateway system (Invitrogen) was used. The system requires an "entry" and a "destination" plasmid. After the fragment coding for the short hairpin was cloned into the pU6.ENTR entry plasmid the resulting plasmid was cleaved with NdeI and SalI to check for the presence of the insert. The small difference in DNA size from the short hairpin was visible in an agarose gel (**Figure 3.2A**). This plasmid was then first tested for expression and functionality of the short hairpin RNA. Subsequently the pU6ENTR.sh plasmid was then used as starting (entry) vector for the "clonase" reaction (recombination reaction kit, Invitrogen), whereas the "destination" vector was pAd.BLOCK-iT. The resulting plasmid was checked for NdeI cleavage sites. The U6 promoter with the small short hairpin insert cassette contained one NdeI cleavage site whereas the pAd.BLOCKiT has two NdeI cleavage sites. Therefore, the resulting positive plasmid must contain 3 cleavage sites for NdeI. This way the number of NdeI cleavage sites were used to show which plasmid had the insert after the clonase reaction. (**Figure 3.2B**). The positive plasmid then was used to produce a recombinant Adenovirus vector expressing a short hairpin RNA.

Fig. 3.2A **Fig. 3.2B**

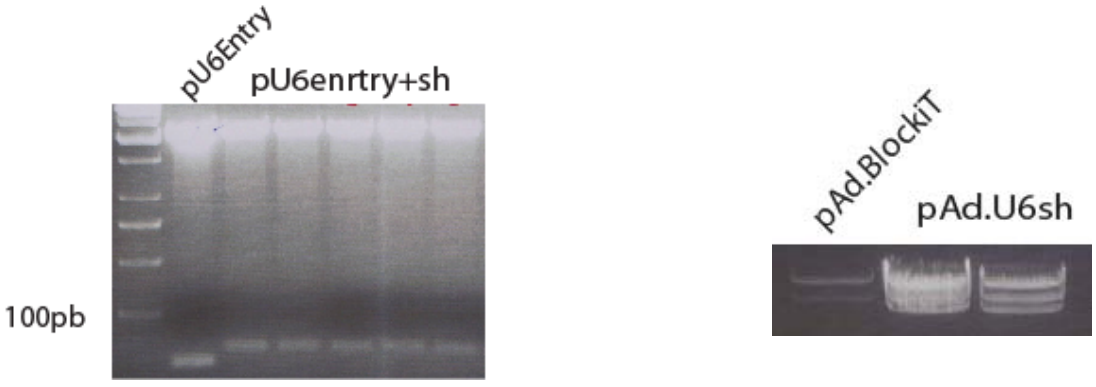

Figure 3.2: Determination of the hairpin containing plasmids. (A) The 1.5% agarose gel shows the DNA fragments produced after cleavage of the pU6.Entry empty backbone and pU6.Entry+sh plasmid with SalI and NdeI to compare with the plasmid before and after the cloning of the short haipin into the pU6.Entry vector. **(B)** The 0.8% agarose gel shows the DNA fragment of NdeI cleavage of the resulting plasmids after the clonase reaction between the pU6.Entry.sh and the pAd.BLOCKiT.

3.3 Down-regulation of NF-κB target genes and TRAIL sensitization

To assess the role of the proteins gene silencing technology was used. siRNAs motives that previously have been described to specifically down-regulate their intended targets Bcl-xl (Whang et al., 2006), c-Flip (Song et al., 2006) and XIAP (Braeuer et al., 2006) were cloned into an Adenovirus vector. As shown in **Figure 3.3A** all three specific shRNA motives efficiently silenced the corresponding protein expression. All siRNA experiments in this study were carried out with a siRNA knocking down EGFP as a control using Ad.shEGFP to ensure that the results are not attributable to an RNA interference-induced interferon response.

To determine whether Bcl-xl and c-Flip are also involved in TRAIL resistance in Panc-1 cells, the latter were transduced with Adenovirus vector expressing a hairpin against these genes and cells were then treated with TRAIL. Cells infected with Ad.shEGFP were used in parallel as negative control and Ad.shXIAP-transduced cells were used as positive control for comparison. The exposure of phosphatidylserine (PS) on the cell surface is an early feature of apoptotic cells (Fadok et al., 1992). Therefore, the time course of apoptosis was first analyzed by measuring PS exposure on the cell surface using FACS. *In vivo*, phagocytes recognize PS as a signal to remove apoptotic cells preventing an inflammatory response. *In vitro*, detection of PS can be achieved using the anticoagulant Annexin V (AV), which, in the presence of calcium, interacts specifically with PS. The fluoresceine isothiocyanate conjugate of Annexin V can be used to detect apoptotic cells by flow cytometry. Since membrane permeabilization is observed in necrosis, AV can also interact with PS in the inner layer of plasma membrane of necrotic cells. In the absence of phagocytosis, this effect occurs *in vitro* during the late stage of apoptosis due to the necrotic-like disintegration of the cells. In combination with propidium iodide (PI), a nucleic acid stain that cannot penetrate the membrane of viable cells, AV binding assay is a powerful method to distinguish between viable cells (AV –, PI –), early apoptotic cells (AV +, PI –), late apoptotic and necrotic cells (AV +, PI +).

AV-FITC and PI analysis of the cells, shown in **Figure 3.3B** as a diagram, clearly demonstrated that after TRAIL treatment apoptosis increased in absent of the anti-apoptotic protein Bcl-xl (about 30%) but silencing XIAP expression result in even higher

levels of apoptotic cells (about 40% increase). In contrast, silencing of c-Flip did not sensitize the cells to TRAIL-induced apoptosis.

Fig. 3.3 A

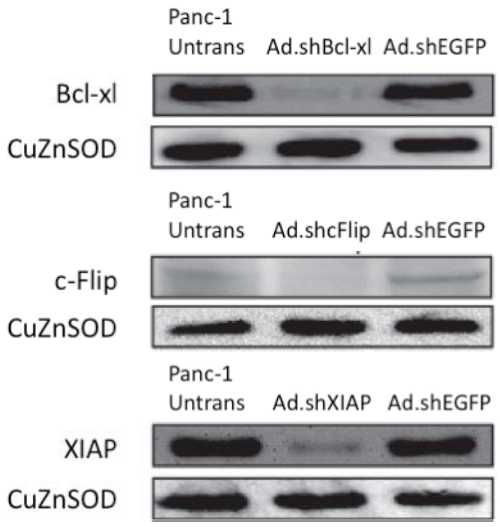

Fig. 3.3 B

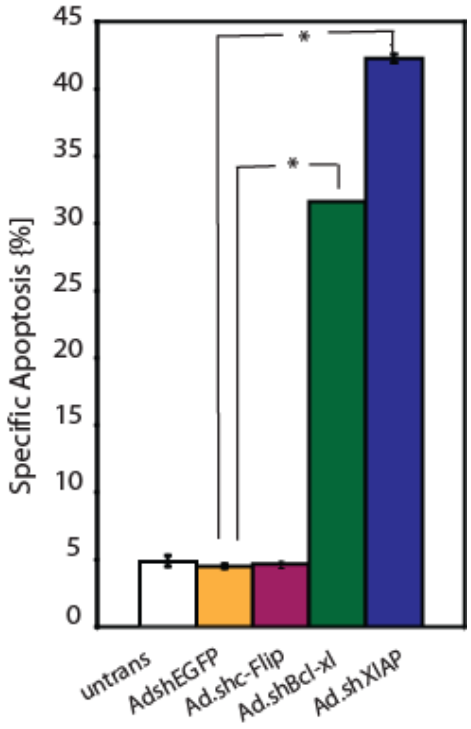

Figure 3.3: Down-regulation of Bcl-xl, c-Flip and XIAP proteins due siRNA treatment. A) Western blot analysis was performed to determine the remaining levels of the proteins after shRNA expression. The experiment was performed at least twice and the western blots are representative for these experiments. **B)** Specific apoptosis induced by TRAIL treatment after gene silencing was measured by Annexin-FITC and FACS. All experiments panel A were independently performed at least three times and a western blot is presented.The apoptosis of TRAIL-treated cells was detected at least three times using Nicoletti assays and once by Annexin-FITC and FACS. The latter is also representative in regard to the Nicoletti (data not shown). Annexin-FACS was repeated three times. The means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments $P < 0.05$. Untrans: untransduced. The Annexin-FACS was carried out in collaboration with Dr. A. Mohr.

3.4 Clonogenic growth assay

A Colony Forming Unit assay was performed from cells transduced with Ad.shEGFP, Ad.shc-Flip, Ad.shBcl-xl, Ad.shXIAP after TRAIL treatment to further analyze the sensitization of cells in a long-term study. Only knock down of XIAP result in a reduction of cell growth.



Figure 3.4. Clonogenic survival. Clonogenic survival was determined by a clonogenic assay and colony formation is shown in percentage of colonies of treated compared to untreated cells. The experiment was performed twice and the presented results are representative for both experiments.

3.5 Down-regulation of XIAP in combination with Bcl-xl

In order to further analyze the role of Bcl-xl in TRAIL sensitization Bcl-xl expression was knocked down in combination with inhibition of XIAP to better understand the TRAIL mechanisms involved in lack of apoptosis induction in Panc-1 cells. The down-regulation of Bcl-xl was first shown by western blot analysis (**Figure 3.5A**) and apoptosis levels were measured by FACS (**Figure 3.5B**). When both proteins, Bcl-xl and XIAP, are down-regulated in Panc-1 cells the apoptotic levels increased slightly but still remained below the apoptotic levels in the TRAIL-sensitive cancer cells such as HCT116 or HeLa cells treated with the same concentration of TRAIL. Therefore, I asked which other factors might determine the TRAIL responsiveness in Panc-1 cells and pro-apoptotic factors were analyzed that could regulate TRAIL-induced apoptosis.

Fig. 3.5A

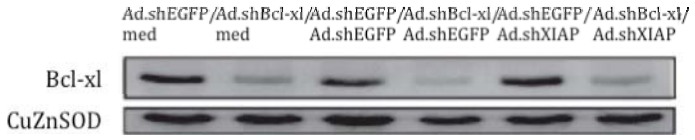

Fig. 3.5B

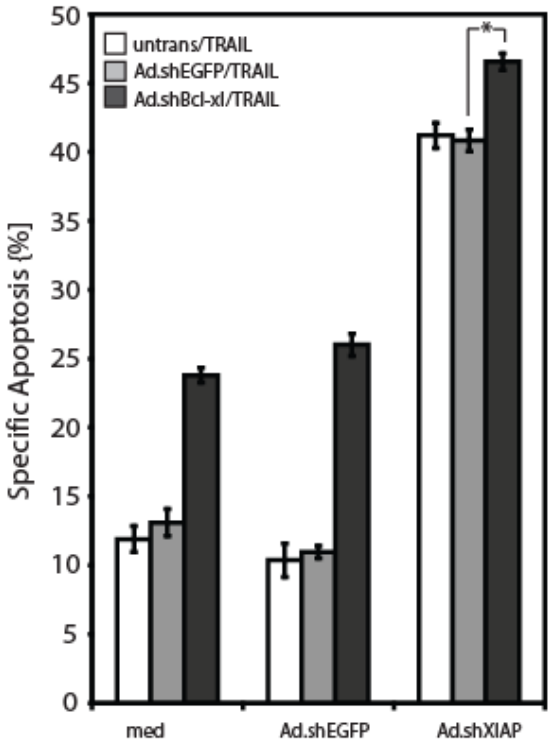

Figure 3.5. Down-regulation of XIAP in combination with Bcl-xl. **(A)** Analyses of Bcl-xl levels remaining after Ad.shBcl-xl in combination with Ad.shEGFP and Ad.shXIAP. The experiment was performed at least twice and the western blots are representative for these experiments. **(B)** Apoptosis levels after transduction with Ad.shBcl-xl in combination with Ad.shXIAP and Ad.shEGFP were determined using Nicoletti. All experiments were independently performed at least three times and the means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments $P < 0.05$. Untrans: untransduced cells

3.6 Analyses of proapoptotic factors

To address the other side of apoptosis regulation, pro-apoptotic factors including, death receptors, caspases and other downstream pro-apoptotic proteins were analyzed and compared.

Apoptotic signaling is initiated by binding of TRAIL to the receptors TRAIL-R1 or TRAIL-R2. Both receptors are able to activate the apoptotic pathway by forming the cytoplasmatic DISC that results in the activation of caspase-8. In contrast, TRAIL-R3 and TRAIL-R4 cannot transmit an apoptotic signal and have most likely an inhibitory function. So functional surface expression of TRAIL-R1 and/or TRAIL-R2 is a prerequisite for the induction of cell death by TRAIL. Therefore, the expression of the four membrane-bound TRAIL receptors was determined in TRAIL-resistant Panc-1 cells in parallel to TRAIL-sensitive HeLa (cervix carcinoma) and HCT116 (colon carcinoma) cells (Buneker et al., 2009). The pro-apoptotic TRAIL-receptors TRAIL-R1 and TRAIL-R2 are highly expressed on the surface of the TRAIL-sensitive HCT116 cells. HeLa cells have lower expression levels of TRAIL-R1 and TRAIL-R2, but both receptors are present on the cell surface. Interestingly, in Painc-1 cells all four TRAIL-receptors are expressed at very low levels with TRAIL-R2 showing a very moderate expression level compared to HeLa and HCT116 cells and with TRAIL-R1 being nearly absent. TRAIL-activated cell death is dependent on the expression of these pro-apototic TRAIL-receptors and these data suggest that TRAIL-responsiveness may also be a function of the expression of pro-apoptotic receptor. As Panc-1 cells express the pro-apoptotic TRAIL-receptors only at very low levels (Zou et al., 2002) it was interesting to study to which extent TRAIL sensitivity can be influenced by increasing the pro-apoptotic potential of Panc-1 cells, that means to which extent TRAIL sensitivity can be increased by over-expression of one of the pro-apoptotic TRAIL-receptors.

In addition to the analysis of the TRAIL receptors, the expression levels of caspase and other important pro-apoptotic protein such as Bax, Bak, and Bid were investigated (**Figure 3.6**). No significant differences were seen with the exception of the expression of pro-caspase 9 and Bak. In contrast to the pro-enzyme, Caspase-9 was detected at very low levels in Panc-1 cells and Bak levels were very low in HCT116 cells.

Fig. 3.6

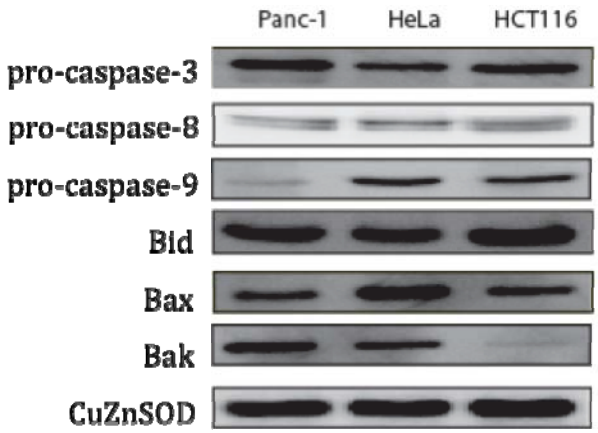

Figure 3.6: Analyses and comparison of pro-apoptotic factors. A representative western blot of three repeats shows the levels of pro-apoptotic proteins Bak, Bax, Bid, and caspases in Panc-1, HeLa and HCT116 cells. CuZnSOD serves as a loading control.

3.7 Over-expression of TRAIL-R1 receptor (DR4)

One pro-apoptotic factor that is very important in cell death signaling by TRAIL is the receptor TRAIL-R1(DR4). Its expression has been shown to be especially low in Panc-1 cells, and among the four TRAIL receptors it is the receptor expressed at the lowest level. Therefore, DR4 was over expressed using an adenovirus vector to investigate the importance of this receptor in the TRAIL resistance of these cells. The cells were first transduced with Ad.DR4 at different MOI to confirm DR4 expression induced by the vector (**Figure 3.7A**). Then the cells were double infected with adenoviruses expressing shRNA against a chosen anti-apoptotic protein and subsequently with Ad.DR4 followed by TRAIL treatment. The apoptosis levels increased after combining the down-regulation of XIAP with DR4 over-expression. In contrast, no higher sensitization was detected when combining DR4 with Bcl-xl down-regulation. In correlation with the data below c-Flip silencing did not induce apoptosis, not even in combination with DR4 over-expression (**Figure 3.7B**).

When TRAIL binds to either of the DRs the executing apoptosis pathway is triggered by activation of caspase-8, which can directly cleave and activate downstream executer caspases such as caspase-3. Therefore, cells were checked in parallel for apoptosis levels for activation of executer caspases by western blot analysis. Because the highest apoptosis levels were detected in cells transduced with short hairpin RNA against XIAP in combination with DR4 over-expression only in these cells caspase-8 activation was determined. Subsequently caspase-3 activation was tested in samples transduced with a control adenovirus vectors (Ad.DsRed). As shown in **Figure 3.7C** caspase-8 is strongly activated after DR4 over-expression. Due to the higher levels of DR4 more TRAIL ligand can be bound and therefore more caspase-8 is recruited and processed leading to higher activation of caspase-3 after release from XIAP binding (**Figure 3.7D**). Detailed analyses revealed that the apoptosis data correlated with the appearance of activated caspase-8 and caspase-3.

Fig. 3.7A

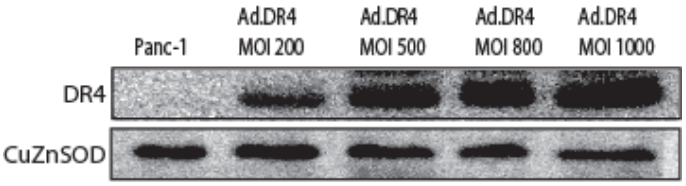

Fig. 3.7B

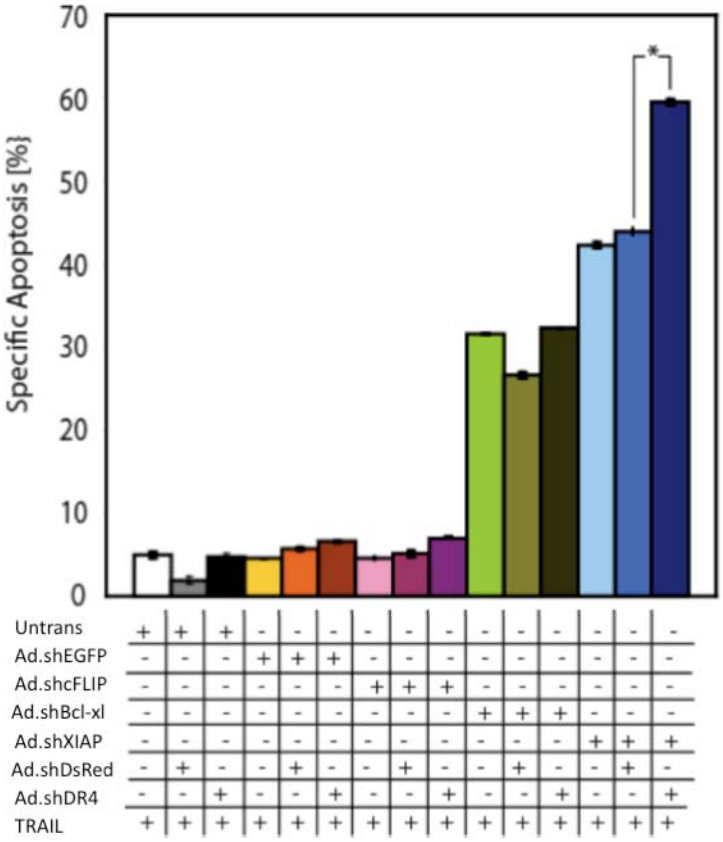

Fig. 3.7C

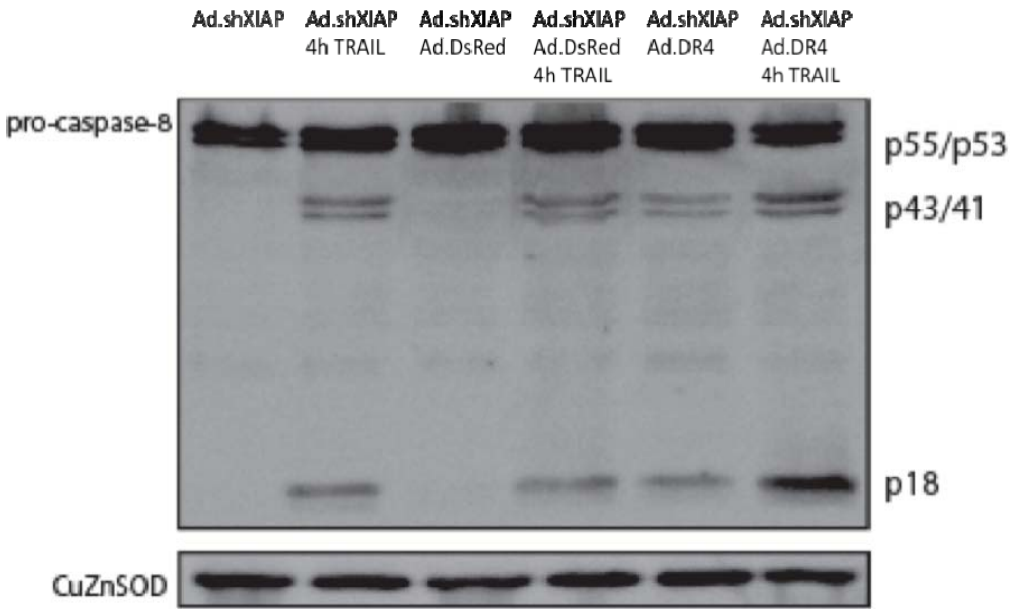

Fig. 3.7D

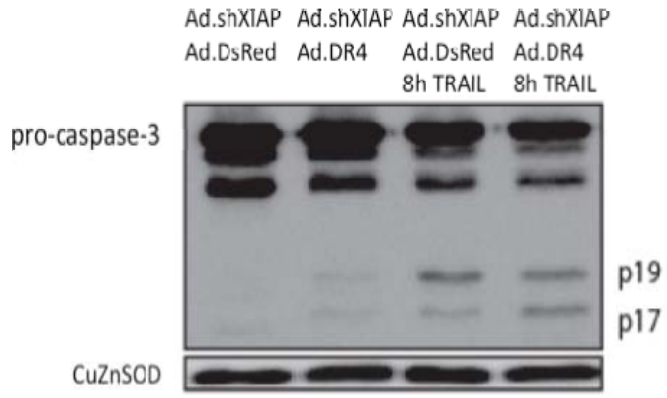

Figure 3.7: Contribution of DR4 to apoptotic signaling. (A) Panc-1 cells were transduced with Ad.DR4 at different MOIs and the expression level of DR4 was determined by western blot analysis. **(B)** Panc-1 cells were transduced with different adenovirus vector expressing short hairpin RNA to silence Bcl-xl, c-Flip and XIAP. 48 hours after infection they were then double transduced with Ad.DR4 for 24 hours and subsequently treated with TRAIL for additional 24 hours. Adenovirus vector expressing DsRed protein was used as a negative control for Ad.DR4 transduction. The amount of apoptotic cells in the experiment was measured by FACS. All experiments were independently performed at least three times and the means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments $P < 0.05$. **(C)** Caspase-8 is stronger activated after XIAP silencing and over-expression of DR4 as detected by western blot analysis**. (D)** Subsequently to the activation of casapse-8, also caspase-3 is slightly stronger activated in the same sample, when XIAP is silenced and DR4 is over expressed. The caspase-3 was shown by western blot using an anti-caspase-3 antibody that recognized the p17/p12 fragment as a result of the cleavage and activation of the pro-caspase-3. Untrans: untransduced cells
Panels A, C & D show representative western blots of more than three experiments.
The Annexin-FACS experiment in panel B was carried out in collaboration with Dr. A. Mohr. The experiment was performed at least three additional times using Trypan blue staining and microscopic analyzes, which were consistent with Annexin data.

3.8 HDAC inhibitors do not affect DR4 and DR5 levels

Histone deacetylases (HDACs) constitute a new class of agents for apoptosis-targeting therapy, have been proven to induce apoptosis, differentiation and cell cycle arrest in a variety of tumor cell lines, and therefore have anticancer potential. To express its genes, a cell must control the interaction of DNA with histones and the nucleosome formation. This is modulated by activation and inactivation of histone acetylases (HAT), which acetylate lysine residues in core histones leading to a less compact and more transcriptionally active chromatin. Conversely the actions of HDACs, which remove the acetyl groups from the lysine residues, lead to the formation of a condensed and transcriptionally inactive chromatin. Reversible modification of the terminal tails of core histones constitutes the major epigenetic mechanism for remodeling higher order chromatin structure and controlling gene expression. HDAC inhibitors (HDI) block this action and can result in hyperacetylation of histones, therefore affecting gene expression (Thiagalingam et al., 2003)

To show the importance of DR4 in Panc-1 TRAIL resistance another approach avoiding the use of adenovirus was performed using the putative capacity of Valproic Acid (VPA) and Trichostatin A (TSA) to upregulate the surface expression of both agonistic TRAIL receptors. Treatment of Panc-1 cells with VPA for 24 and 48 hours (**Figure 3.8A**) or with TSA for 24 hours (**Figure 3.8B**) did not upregulate DR4/DR5 receptors. Even further treatment for up to 48 hours of VPA and higher doses of TSA (1μM) did not result in any change in the expression of DR4 and DR5. Because of low basic levels of DR4 and DR5 proteins in Panc-1 cells, not detectable by western blot analysis, the colon cancer cell line HT29 that has higher levels of the proteins was used as a positive control for DR4 Panc-1 cells transduced with Ad.DR4 and for DR5.

Fig. 3.8 A

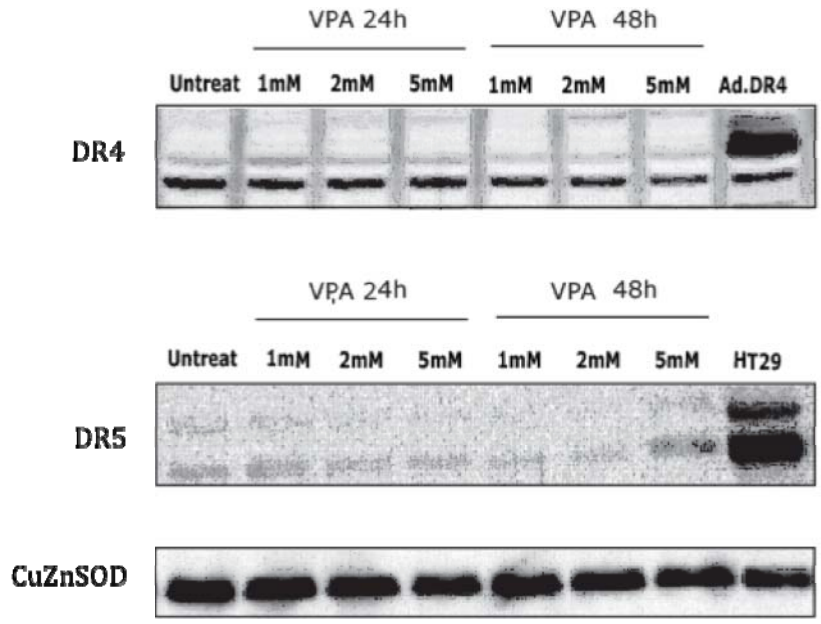

Fig. 3.8 B

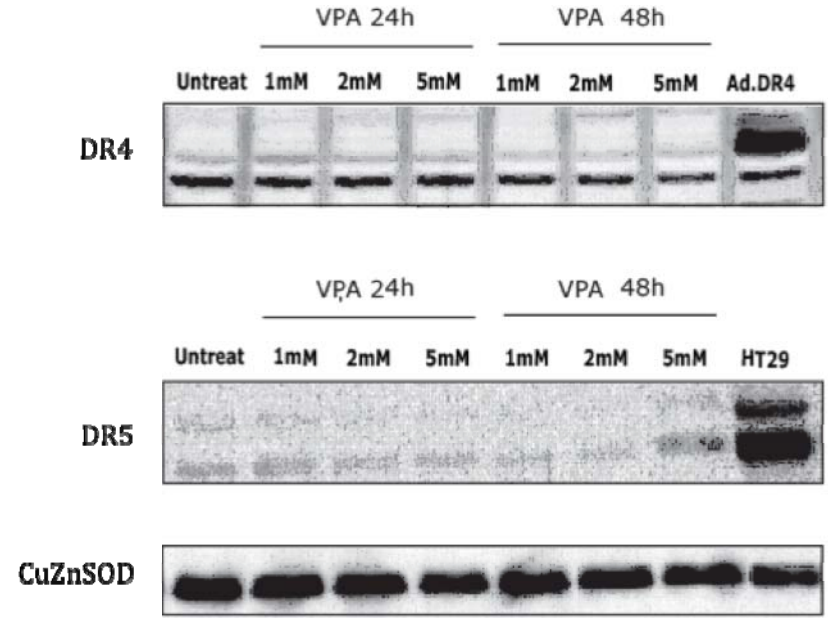

Figure 3.8: Panc-1 cells treated with HDCA inhibitors. (A) Protein extractions of untreated cells and treated cells with different concentrations of VPA were analyzed for DR4 and DR5 expression. **(B)** Cells were also treated with TSA and in the same way and western blots were performed to detect the changes on DR4 and DR5 protein levels. CuZnSOD serve as loading control in both panels. Untreat: untreated cells. The experiment was performed more three times. The western blot shown here is representative for both experiments and was performed in collaboration with a summer student Branko Cirovic.

3.9 IFN-γ enhances TRAIL-induced apoptosis

Caspase-8 is a key molecule involved in DISC formation, which is a molecular event that occurs at an early stage of TRAIL-induced apoptosis. On TRAIL binding, activated death receptors recruit and activate caspase-8 at the DISC level. Caspase-8 is a pro-apoptotic factor present at low levels in Panc-1 cells, therefore I focused on caspase-8.

Interferons are able to block viral replication and additionally induced a variety of other effects, including immune modulation, differentiation, apoptosis, and inhibition of proliferation and angiogenesis. IFN-γ alone can be sufficient to induce apoptosis in some cells, but often sensitizes cells for death receptor-induced apoptosis without being apoptotic per se (Chawla-Sarkar et al., 2003). This is underlined by the findings that proapototic genes, including those encoding caspase-8, TRAIL and FasL have been identified as transcriptional targets of IFN-γ (Ruiz-Ruiz et al., 2000; Shin et al., 2001; Yang et al., 2003). To induce the expression of caspase-8 the cells were pre-treated with IFN-γ prior to the experiments described below.

The effect of IFN-γ on the expression level of caspase-8 was confirmed by western blot analysis (**Figure 3.9A**) and cells were then pre-treated with IFN-γ after down-regulation of c-Flip, Bcl-xl, XIAP and before TRAIL addition. Finally, the apoptotic cells were determined by FACS (**Figure 3.9B**). IFN-γ pre-treatment in combination with Bcl-xl down-regulation triggered an increase of apoptosis and in combination with down-regulation of XIAP a significant enhancement of TRAIL-induced apoptosis was detected, yielding an almost 2.0 fold increase. However, in combination with c-Flip siRNA no differences in apoptosis levels were measured. The degree of sensitization was well surpassed by increasing the pro-apoptotic potential through over-expression of caspase-8 in comparison to the sensitization levels by targeting XIAP in combination with Bcl-xl (**Figure 3.5B**) or from the over-expression of the pro-apoptotic DR4 receptor. The apoptotic data suggest that caspase-8 levels are even more important than those of the DR4 in TRAIL-induced apoptosis sensitization of Panc-1 cells.

Next, it was explored whether targeting of XIAP and caspsase-8 in combination also had an effect on long-term survival by performing clonogenic assays. Importantly, shRNA-mediated silencing of XIAP after IFN-γ pre-treatment significantly reduced clonogenic

growth of Panc-1 cells upon TRAIL addition compared to control cells silenced with shRNA against EGFP, c-Flip and Bcl-xl.

In fact, balance of pro-apoptotic and anti-apoptotic factors is necessary to increase TRAIL responsiveness in Panc-1 cells and this may be used as an effective approach to overcome TRAIL resistance in pancreatic cancer.

Fig. 3.9A

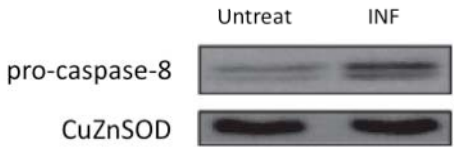

Fig. 3.9B

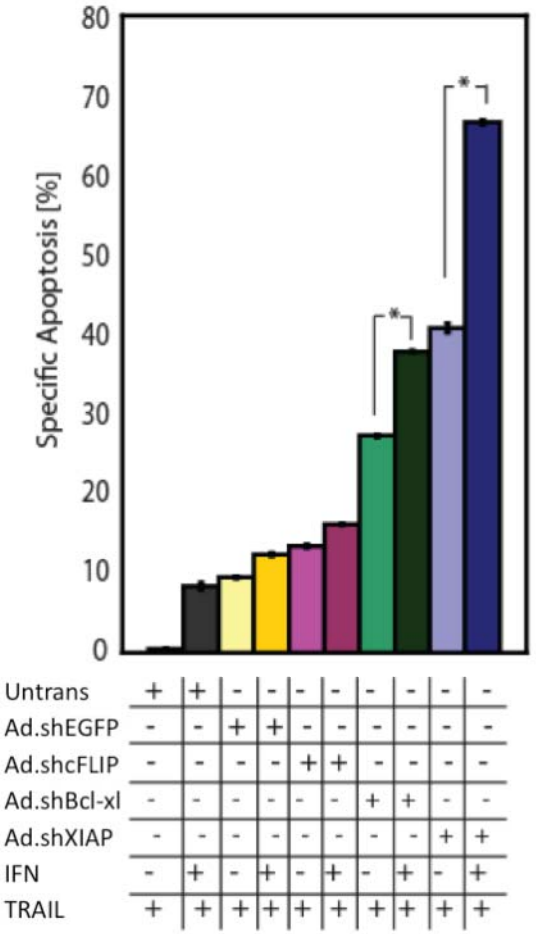

Fig. 3.9C



Figure 3.9: Effect of IFN-γ treatment on TRAIL sensitization. **(A)** An increase in caspase-8 levels after IFN-γ treatment detected by western blot analysis. The experiment was performed at least twice and the western blots are representative for these experiments. **(B)** Diagram of specific apoptosis that shows the effect of IFN-γ pre-treatment in combination with knock down of c-Flip, Bcl-xl and XIAP after TRAIL treatment. All experiments were independently performed at least three times and the means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments $P < 0.05$. **(C)** Clonogenic assays were performed to confirm the positive long-term effect of the strategy based on knocking down XIAP combined with up-regulation of caspase-8 for increased TRAIL responsiveness. Colony formation was performed once and is expressed as percentage of colonies of treated compared to untreated and **i** means IFN-γ treatment. Untreat: untreated cells, Untrans: untransduced cells, IFN: IFN-γ treatment

3.10 Silencing of caspase-9 does not block TRAIL sensitization induced by IFN-γ

Caspase-9 levels in Panc-1 cells are very low and because of that this pro apoptotic factors could also play an important role in the TRAIL resistance. To address the role of caspase-9 in TRAIL sensitization down-regulation of XIAP and increase of caspase-8 due to IFN-γ treatment was combined with shRNA mediated knock down of caspase-9 using adenovirus vector Ad.shcas9. In control experiments the levels of caspase-9 remained unchanged after IFN-γ treatment (**Figure 3.10A**). The shRNA against caspsase-9 efficiently down-regulated caspase-9 protein expression (**Figure 3.10B**). In cells transduced with Ad.shcas9 and pre-treated with IFN-γ the addition of TRAIL lead to cell death and apoptosis was not blocked or significantly reduced in these cells (**Figure 3.10C**). These findings suggested that caspase-9 did not influence the TRAIL sensitization achieved by down-regulation of XIAP in combination with caspase-8 over-expression.

Fig. 3.10A

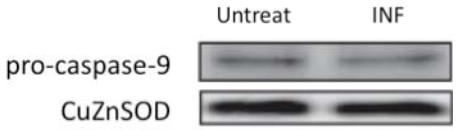

Fig. 3.10B

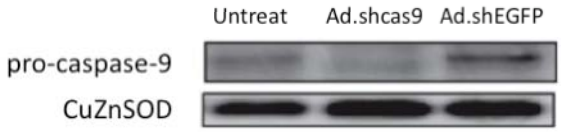

Fig. 3.10C

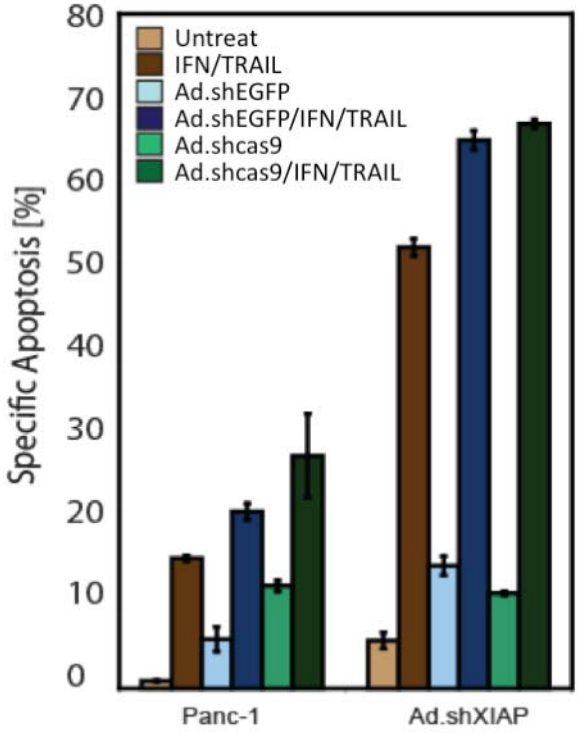

Figure 3.10: Effect of caspase-9 silencing on TRAIL sensitization after IFN-γ pre-treatment. (A) Western blot analysis shows pro-caspase-9 levels after IFN-γ treatment. **(B)** Western blot analysis presents down-regulation of caspase-9 after use of Ad.shcas9. The experiments in panels A and B were performed at least twice each and the western blots are representative for these experiments. **(C)** Apoptosis level after down-regulation of caspase-9 reveal no significant influence of caspase-9 in IFN-γ-dependent TRAIL sensitization of Panc-1 cells (left panel). In contrast, knock down of XIAP enhanced apoptosis of Panc-1 cells (right panel). All experiments were independently performed at least three times and the means and the standard deviations are presented. Untreat: untreated cells, IFN: IFN-γ pre-treatment

3.11 Use of shXIAP stable cell lines to validate the results obtained by adenovirus transduction

To reconfirm and validate the experiments presented above stable shXIAP-expressing Panc-1 cells lines were generated. For the further analysis three clones with down-regulation of XIAP (shXIAP 5, 9, 10) below the level of detection and one clone with XIAP levels (shXIAP 7) remaining unchanged as control were selected. **Figure 3.11A** shows the down-regulation of XIAP in all three cell lines in comparison to the parental Panc-1 and shXIAP 7 cell lines. The cells were treated with TRAIL and apoptosis levels ranged from 35-45%, as shown in **Figure 3.11B**. To evaluate also the long term effects of down-regulation of XIAP after TRAIL treatment a clonogenic assay was performed with all stable cell lines and in parallel with the parental cell line Panc-1. The experimental set up allowed the investigation of long time effects of the down-regulation of XIAP upon TRAIL treatment (**Figure 3.11C**).

Since sensitization for TRAIL induced apoptosis was observed in three clones of stable cell lines of Panc-1, in which XIAP was knocked down by shRNA, it is unlikely that sensitization for TRAIL-induced apoptosis provided by XIAP down-regulation was simply a result of clone selection.

Fig. 3.11A

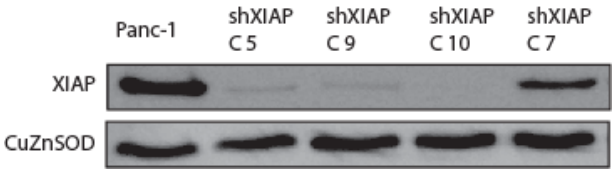

Fig. 3.11B

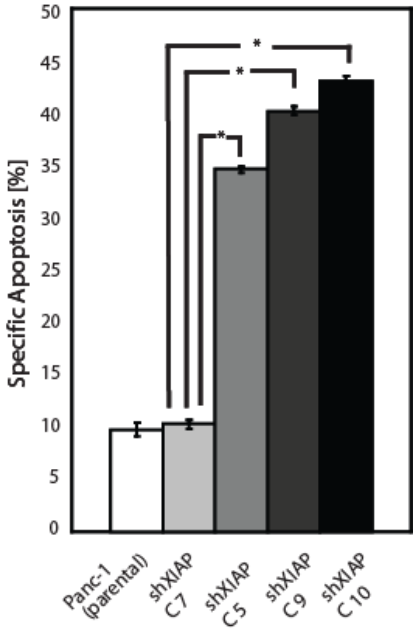

Fig. 3.11C

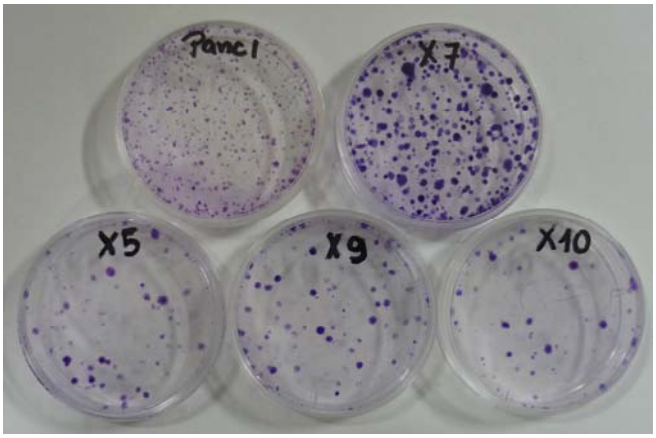

Figure 3.11. XIAP knock down in stable cell lines and apoptosis. (A) Western blot analysis indicating the efficiency of the down-regulation of XIAP. The experiment was performed at least twice and the western blots are representative for these experiments. **(B)** Apoptosis assays confirming the influence of down-regulation of XIAP on the TRAIL sensitivity, all stable cell lines are TRAIL-sensitive. All experiments were independently performed at least three times and the means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments P ≤0.001. **(C)** Clonogenic assays of shXIAP stable cell lines shXIAP 5, 9, and 10 (marked as X5, X9 and X10), which expressed low levels of XIAP were compare to a stable Panc-1-derived cell line shXIAP 7 (highlighted as X7) expressing normal levels of the protein and the parental cell line Panc-1 after treatment with TRAIL. The experiment was performed two times and the presented result is a representative for both experiments.

3.12 Over-expression of DR4 in shXIAP stable cell lines

After unsuccessful attempts to increase expression of the agonist TRAIL receptors with HDCAi, DR4 was expressed with the Adenovirus vector Ad.DR4 as an alternative to enhance the level of DR4 in Panc1 shXIAP stable cell lines. The parental and the four stable cell lines were transduced with Ad.DR4 at MOI 800 (**Figure 3.12A**) and then treated with TRAIL for 24 hours. The apoptosis levels were the same in all three shXIAP stable cell lines shXIAP 5, 9, and 10 showing a reduced XIAP level with a small but significant increase in apoptosis when transduced with Ad.DR4.

Fig. 3.12A

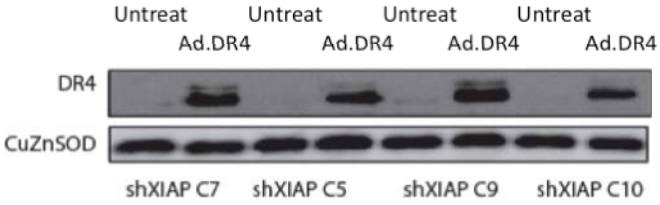

Fig. 3.12B

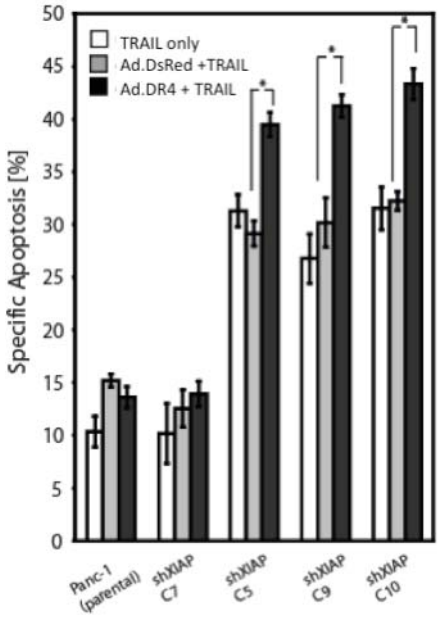

Figure 3.12. DR4 over-expression in shXIAP stable cell lines. (A) Western blot analysis presenting the levels of DR4 expression in the shXIAP stable cell lines shXIAP 5, 9, and 10. Detection of CuZnSOD served as a loading control. The experiment was performed at least twice and the western blots are representative for these experiments. **(B)** After TRAIL treatment, apoptosis was measured and the levels were significantly increased by shXIAP and DR4 over-expression. All experiments were independently performed at least three times and the means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments $P < 0.05$. Untreat: untreated cells

3.13 Combining DR4 over-expression with IFN-γ and TRAIL treatment

The shXIAP stable cell lines shXIAP 5, 9, and 10 were first pre-treated with IFN-γ for 24 hours before they were incubated with TRAIL. The levels of apoptosis were similar to the levels generated when cells were transduced with Ad.shXIAP instead using stable cell lines (**Figure 3.13A**).

Since HDCAi did not increase the level of the agonist TRAIL receptor DR4 (**Figure 3.8**) Ad.DR4 was used to over express DR4 in the shXIAP stable cell lines. After transduction with Ad.DR4 for 24 hours, the cells were pre-treated with IFN-γ for additional 24 hours before incubation with TRAIL. The amounts of cells undergoing apoptosis are shown in **Figure 3.13B**. A significant increase of apoptosis was measured by over expressing DR4 in combination with an increase of caspase-8 after pre-treatment with IFN-γ in the shXIAP stable cell line shXIAP 5, 9 and 10, resulting in apoptosis rate of 80-90%.

Fig. 3.13A **Fig. 3.13B**

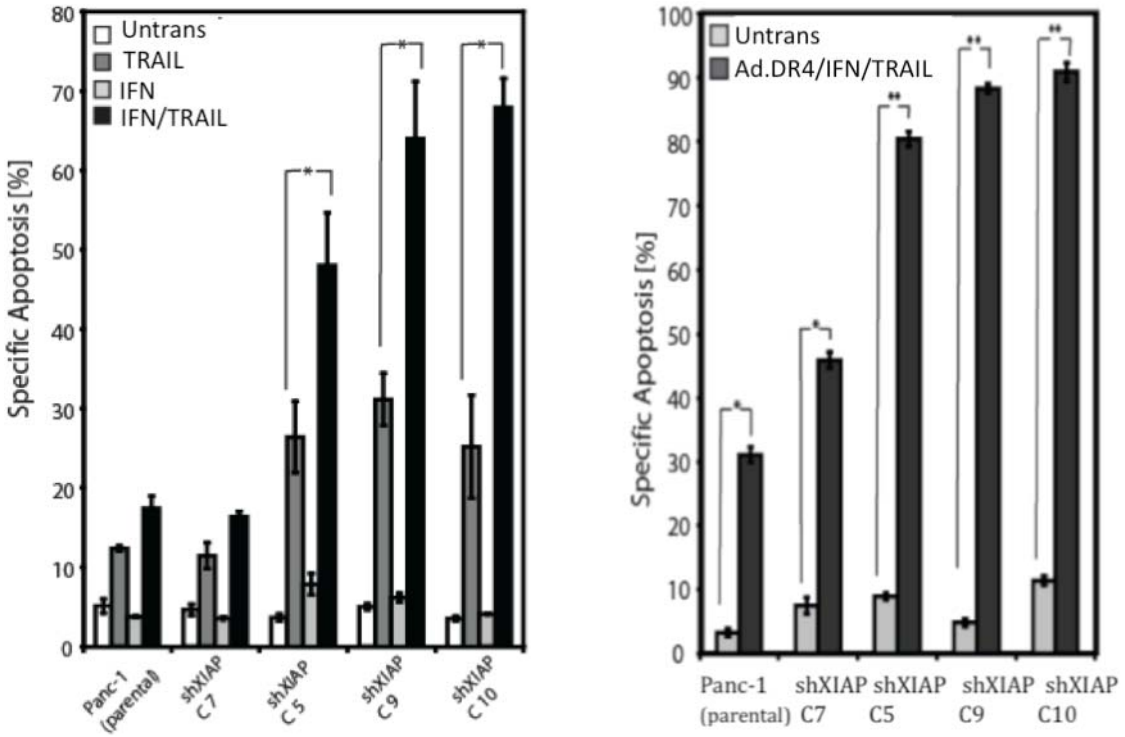

Figure 3.13. TRAIL sensitization of Panc-1 after IFN-γ pre-treatment. (A) Apoptosis levels on shXIAP stable cell lines by pre-treatment of IFN-γ **(B)** Apoptosis levels by combining DR4 over-expression and IFN-γ pre-treatment in shXIAP stable cell lines. All experiments were independently performed at least three times and the means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments P <0.001. Untrans: untransduced cells, IFN: IFN-γ pre-treatment

3.14 Sensitization to TRAIL-induced apoptosis by down regulating XIAP in combination with IFN-γ treatment is not restricted in multiple cell lines

To determine whether these results were restricted to a single cell line, the study was expanded and another pancreatic cancer cell line, ASPC-1, was included. This cell line was chosen because of its XIAP levels, its ability to be infected with Adenoviruses and its TRAIL-resistance. **Figure 3.14A** shows that XIAP can be silenced with Adenovirus Ad.shXIAP in ASPC-1 cell line. The western blot analysis reveals the protein levels which did not change after transduction of Ad.shXIAP. In order to validate the treatment in this pancreatic cancer cell line cells were pre-treated with IFN-γ after down-regulation of XIAP. TRAIL treatment showed a remarkable sensitization after IFN-γ incubation, resulting in a rate of apoptotic cells that ranged from 70-75% dead cells in untransduced cells and cells transduced with the control adenovirus (Ad.shEGFP) and that yielded a significant increase (80-85% of dead cells) when the IFN-γ treatment was combined with knock down of XIAP (**Figure 3.14B**).

Fig. 3.14A

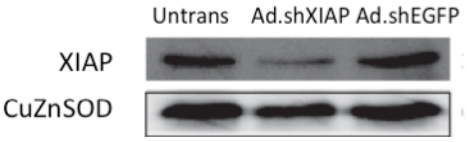

Fig. 3.14B

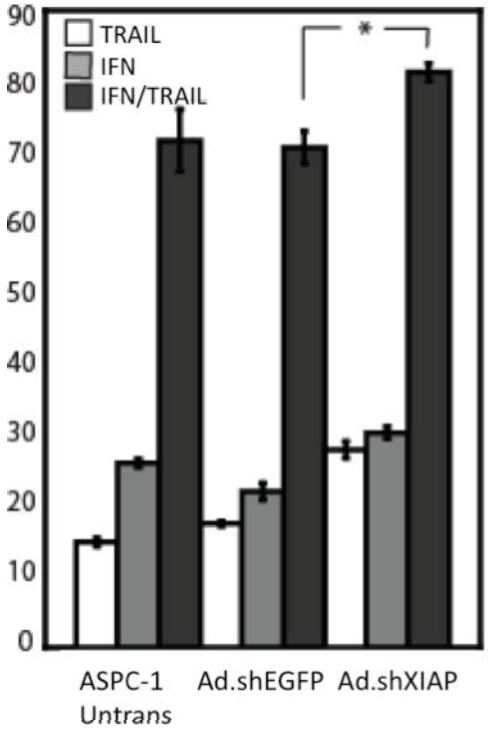

Figure 3.14. Pancreatic cancer cells ASPC-1 can be sensitized to TRAIL-induced apoptosis. (A) Western blot analysis performed to detect levels of XIAP after transduction with Ad.shXIAP. The experiment was performed at least twice and the western blots are representative for these experiments. **(B)** Diagram shows effect of XIAP down-regulation and IFN-γ pre-treatment after TRAIL treatment on apoptosis levels in ASPC-1 cells. All experiments were independently performed at least three times and the means and the standard deviations are presented. The asterisks highlights the comparison of significant experiments $P < 0.05$. Untrans: untransduced cells, IFN: IFN-γ pre-treatment

3.15 Down-regulation of XIAP-induced growth retardation in Panc-1 cells

During the experiments described above it was observed that down-regulation of XIAP can also have a positive long-term effect on the growth of these cells. In addition, microscopic morphological differences and changes were visualized in the cells with stable XIAP down-regulation. Cell proliferation was analyzed comparing the growth of Panc-1 shXIAP stable cell line clone 5, 9 and 10 with the parental cell line during 7 days in culture (**Figure 3.15A**). The stable shXIAP cell lines showed a small retardation in comparison to the parental Panc-1 cell line shXIAP clone 7.

After a longer period in culture, the absence of XIAP protein seemed to lead to an increase in cell size to a flattening of the cells. I speculated that senescence might be a reason for that and senescence-specific histology of the shXIAP stable cell line 5 was carried out. The number of SA-β-Gal-positive cells was quantified by microscopy. The blue senescence-positive cells were counted and are presented in **Figure 3.15B**. Almost all cells were stained positively for senescence.

Another method to detect senescent cells is based in measuring Bromodeoxyuridine (BrdU) incorporation in proliferating cells. This method can differentiate between senescence (<5% BrdU positive) and crisis (>30% BrdU positive) based on the number of the cells that have actively incorporated BrdU. BrdU is a synthetic thymidine analog that is incorporated into cellular DNA when the cell is dividing (during the S-phase of the cell cycle). Antibodies against BrdU that are conjugated to fluorescent markers are commercially available and can be used to detect proliferating cells passing through S phase, thereby providing visual evidence of cell division. **Figure 3.15C** shows proliferation analysis using BrdU incorporation from the shXIAP stable cell line 5 and 7 (highlighted in blue). In shXIAP stable cell line 7 (XIAP-positive control cell line) about 20.4% BrdU-positive cells were detected compared to just 2.8% in shXIAP cell line 5, in addition shXIAP 5 mostly cells are arrested at G2 phase as determined by FACS confirming a cell growth arrest in shXIAP stable cell line 5 (summarized in table 3.1).

The shRNA targeting XIAP used in the experiments described above was previously characterized by Braeuer et al. (2006). Nevertheless a second shRNA-targeting XIAP was designed to further validate these results. These two independent non-overlapping siRNAs were tested for their ability to diminish XIAP and influence TRAIL-induced

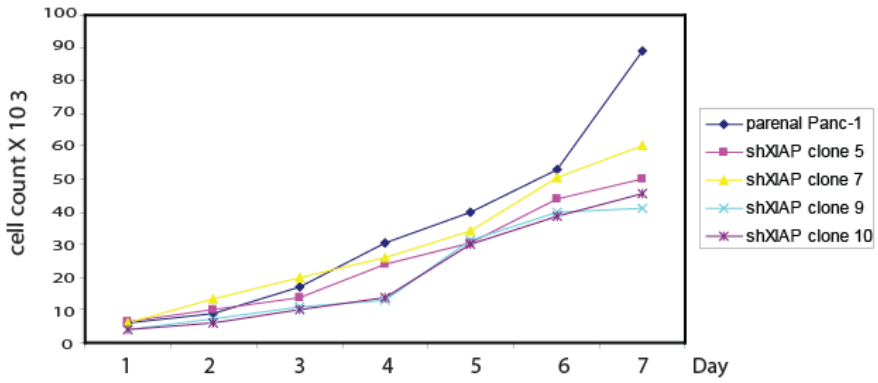

Figure 3.15A. Cell growth curve. Cell growth of Panc-1 shXIAP stable cell line clone 5, 9 and 10 in comparison to the parental Panc-1 cells during 7 days in culture. The experiment was performed twice and the presented diagram is representative for both experiments.

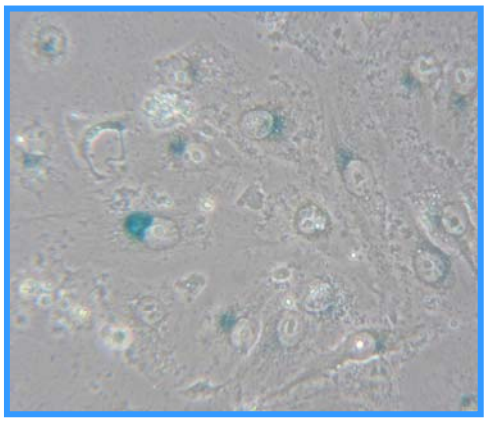

Figure 3.15 B. SA-β-Gal senescence staining. The shXIAP stable cell line 5 of the parental cell line Panc-1 was analyzed by light microscopy as described in Materials and Methods.
The knock-down was independently carried out twice. Once in Panc-1 and once in HeLa. A representative picture for both experiment is presented here.

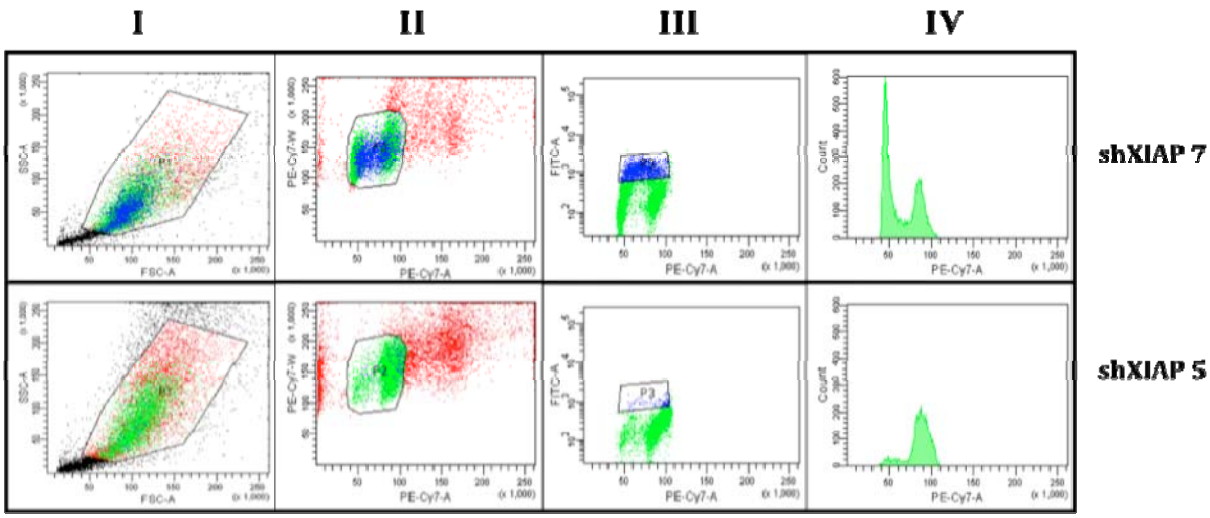

Figure 3.15C: BrdU incorporation proliferation assay. BrdU incorporation in shXIAP stable cell lines 5 and 7, which are XIAP-negative and positive, respectively. The complete shXIAP knockdown clone 5 shows reduced incorporation (less than 3%) in comparison to the cell line clone 7 that still has XIAP protein levels (20%). I: forward and site scatter (setting P1 gate: exclusion of cell debris seen in black), II: DNA versus DNA diagram of cycling cells (setting P2 gate: exclusion of cell aggregates and additional cell debris both seen in red), III: BrdU incorporation (FITC) versus DNA (PE) (setting P3 gate: BrdU-positive cells in blue and BrdU-negative cells in green), and IV: cell numbers versus DNA detection (PE); the gates are according to I and II. All gates were selected for clone 7 and then applied to clone 5 cells. These are the representative data of two experiments. They were performed in collaboration with Dr. Alessandro Notani (NCBES).

Table 3.1: Quantificaton of the BrdU incorporation proliferation assay

	XIAP-positive clone 7		**XIAP-negative clone 5**	
	No. of events	Percent of P1	No. of events	Percent of P1
Cycling cells: P2	8,962	82.2	4,641	48.6
BrdU-positive cells: P3	1,824	20.4	129	2.8

apoptosis. Both shRNA directed toward XIAP significantly reduced XIAP expression and changed cell morphology which confirming the results described above.

Moreover, each XIAP-specific shRNA efficiently silenced XIAP protein expression but not the expression of other proteins that were analyzed. These data indicate that the results most likely are not attributed to siRNA "off-target" effects or an RNA interference-induced interferon response, because two distinct non-overlapping siRNA knocked down XIAP whereas the control shRNA did not show these effects.

4. DISCUSSION

In order to maintain homeostasis and host defense in multicellular organisms, a subset cells within an organism must undergo apoptosis, a process of programmed cell death (PCD). Dysregulation of this process is implicated in various diseases including cancer (Thompson, 1995). From all types of cancers, pancreatic cancer is the fourth leading cause of cancer related deaths in the western world (Jemal et al., 2009) and the prognosis is the worst among all cancers due to a lack of improvement in its detection, diagnosis and treatment strategies (Hirata et al., 2007). Due to the delay of clinical diagnosis pancreatic cancer is often detected only at an advanced stage with survival of 4 to 6 months and a 5 years survival remaining at <4% (Li et al., 2004; Yao et al.. 2007). Several treatments have been tried but unfortunately chemotherapy still relies on only few drugs and has produced unsatisfactory results with only few chemotherapeutic agents having beneficial effects (Neoptolemos et al., 2004; Louvet et al., 2005; Arumugam et al., 2006; Larbouret et al., 2007; Azria et al., 2008; Wolpin et al., 2009; Fukada et al., 2009; Sasajima et al., 2009; Mackenzie & McCollum, 2009; Yoo et al., 2009). Determination of mechanisms implicated in intrinsic and acquired resistance to chemotherapy is considered to play a key role to explain the limited benefit of most treatments employed in pancreatic cancer. Key molecular factors which have been implicated in the inefficiency of pancreatic cancer treatment include: deficiencies in drug uptake, lack of activation of DNA repair pathways, tumor microenvironment and resistance to apoptosis due to lack of apoptotic factors or over-expression of anti-apoptotic proteins. To gain an improved understanding of factors involved in drug resistance of pancreatic cancer, apoptotic pathways were investigated at a molecular level in this thesis.

TRAIL has received considerable interest in oncology since it displays specific antitumor activity against a wide range of tumor cells (Gura, 1997). Although TRAIL has the potential to kill cancer cells in a selective manner most of cancer are TRAIL resistant and this resistance represents a barrier for the development of efficient therapies. The resistance to TRAIL can occur at multiple steps in the TRAIL signaling pathway (Zhang and Fang 2005). Some studies (Braeuer et al., 2006; Khanbolooki et al., 2006) found a NF-κB mediated survival of cells treated with TRAIL and that in fact, NF-κB target gene

XIAP is over expressed in those cells and this anti-apoptotic protein plays a major role in TRAIL resistance. Several other studies have confirmed this theory by showing that down-regulation or inhibition of XIAP yielded an increase in apoptosis after TRAIL treatment in several types of cancer cell lines (Chawla-Sarkar et al., 2004; Spee et al., 2006; Shrader et al., 2007; Pan et al., 2008; Makhov et al., 2009; Connolly et al., 2009; Fakler et al., 2009; White-Gilbertson et al., 2009) and in TRAIL-resistant pancreatic cell lines (Braeuer et al., 2006; Karikari et al., 2007; Mori et al., 2007; Vogler et al., 2007; Vogler et al., 2008; Vogler et al., 2009). Albeit the studies showed a significant increase in the sensitivity of cancer cells after blocking XIAP none of the studies showed an increase of apoptosis higher than 50%, levels expected if XIAP would be the only factor playing a role behind the TRAIL resistance. Normally Panc-1 cells are extremely resistant to TRAIL and by diminishing the constitutive levels of NF-κB in these cells they undergo apoptosis almost at the same level as XIAP was reduced (Braeuer et al., 2006). This led to the hypothesis that from the NF-κB target genes XIAP might be the most relevant in this context. Because Bcl-xl (Khanbolooki et al., 2006; Song et al., 2007; Siegelin et al., 2008; Song et al., 2009) and c-Flip (Lin et al., 2008; Park et el., 2009; Siegelin et al., 2009; Lee et al., 2009) are known to confer TRAIL-resistance to cells these two proteins were analyzed in the work presented here. Immunoblot analyses revealed that c-Flip is almost absent in Panc-1 cells and also in the other 2 TRAIL-sensitive cell lines whereas Bcl-xl is present in significant amounts in Panc-1 cells, at low levels in HCT116 and almost absent in HeLa cells, an indication that Bcl-xl, together with XIAP, could play a role in the TRAIL resistance of Panc-1 cells.

c-Flip$_L$ is one regulatory mechanism of the intrinsic pathway that prevents pro-caspase-8 recruitment and/or inhibits its activation within the DISC. Although c-Flip$_L$ can induce activation of pro-caspase-8, its role in apoptosis remains elusive (Peter et al., 2004). It has been shown that c-Flip$_L$ can inhibit or activate procaspase-8 depending on the level of its expression; at physiological low concentration c-Flip$_L$ can enhance Fas-mediated apoptosis but at high concentration blocks apoptosis by inhibiting caspase-8 (Chang et al., 2002). In contrast to this result, Micheau and co-workers described that even at low concentration c-Flip$_L$ inhibits apoptosis (Micheau et al., 2002). However, in TRAIL-induced apoptosis role of c-Flip has been controversial. Wang et al. (2007) reported that

increased levels of expression of c-Flip$_L$ in TRAIL-resistant pancreatic cancer cell lines in comparison to sensitive cells whereas I did not OBTAIN the same result as shown in this thesis. It also has been shown that siRNA knock-down of endogenous c-Flip$_L$ sensitizes various human breast tumor cell lines (Palacios et al., 2006), many TRAIL-resistant (Ricci et al., 2004; Bellail et al., 2009; Zong et al., 2009; Chen et al., 2009), and pancreatic cancer cells lines (Wang et al., 2007) to TRAIL-induced apoptosis. In order to find out if the low levels of c-Flip$_L$ in Panc-1 cells are related to the strong resistance of those cells to TRAIL-induced apoptosis I targeted c-Flip$_L$ using a short hairpin RNA but I did not detect any increased apoptosis levels in these cells. These results were confirmed in a long-term survival experiment performed by a clonogenic assay. To prove that my findings are reproducible I additionally used two different independent short hairpin RNA motives described in the literature (Palacios et al., 2006; Song et al., 2006). I failed to find any evidence for an involvement of c-Flip$_L$ in Panc-1 TRAIL resistance suggesting that c-Flip is not involved and therefore my further analyses did not include c-Flip$_L$.

Bcl-xl is known to confer resistance to various chemotherapeutic agents and multiple studies using Bcl-xl siRNA approaches revealed that Bcl-xl can be associated with TRAIL resistance in cancer cells (Zangemeister-Wittke, 2003; Zhu et al., 2007; Zhu et al., 2008; Hetschko et al., 2008). However, Bcl-xl is not always found to be responsible for TRAIL-induced apoptosis resistance (Yoshida et al., 2008; Kim et al., 2008; Ndozangue-Touriguine et al., 2008). In pancreatic cancer cells down-regulation of Bcl-xl by inhibiting NF- κB has been described to restore the TRAIL sensitivity in TRAIL-resistant cells (Khanbolooki et al., 2006) and that, after TRAIL treatment, an increase in the intracellular level of Bcl-xl is responsible for development of acquired TRAIL resistance (Song et al., 2007). Hylander et al., 2005 analyzed patient-derived pancreatic tumors and determined heterogeneity in TRAIL responsiveness, sensitive and resistant pancreatic tumors. Moreover, Bcl-xl was detected at higher levels in TRAIL-resistant in comparison to TRAIL-sensitive tumors. In the same way other studies have found over-expression of Bcl-xl in pancreatic cancer TRAIL-resistant cells (Hinz et al., 2000; Ibrahim et al., 2001; Bai et al., 2005). Blocking Bcl-xl expression increases mitochondrial membrane depolarization and cytochrome-c release from mitochondria and

subsequently leads to activation of caspase-9 and -3 inducing apoptosis. To prove and analyze Bcl-xl involvement in Panc-1 TRAIL resistance western blot analyses were performed. These investigations confirmed the down-regulation of the protein after exposing cells to shBcl-xl and changes in apoptotic levels were measured after TRAIL treatment. Despite the increase in the apoptotic levels when Bcl-xl is down-regulated the levels are not as high as when XIAP is down-regulated. Experiments combining a down-regulation of Bcl-xl and XIAP revealed that the number of apoptotic cells was significantly increased but the effect was small. The release of caspase-3 caused by XIAP silencing is essential for TRAIL-induced apoptosis in Panc-1 cells. Gene silencing experiments confirmed that both proteins, XIAP and Bcl-xl, contributed to TRAIL resistance in Panc-1 and these data agree with Khanbolooki et al., 2006. Caspase-9 does not play a central role in Panc-1 TRAIL resistance and the Bcl-xl survival signal is not the main player.

The over-expression of anti-apoptotic proteins and the survival signal given by the transcription factor NF-κB are definitively not the only factors interfering and modulating the TRAIL-resistance in pancreatic cancer. Since the mechanisms underlying TRAIL resistance are very complex I analyzed the possible apoptotic factors involved in the sensitization to TRAIL. Bak, Bax, Bid, caspase-3, -9, -8. The basic levels were analyzed by immunoblotting in Panc-1, HeLa and HCT116 cells. In addition, flow cytrometry showed the expression of the four TRAIL receptors on these cells.

Ibrahim et al., 2001 studied the importance of the TRAIL receptor on pancreatic adenocarcinomas and demonstrated that these cells expressed high levels of apoptosis-inducing receptors and ligands. Nevertheless, despite expressing intact receptors and signaling machineries they showed differential susceptibility to cell death induced by TRAIL. This could be explained by the fact that these cells expressed differentially high levels of decoy receptors, as well as molecules known to be inhibitors of apoptosis. Furthermore, another report (Ozawa et al., 2001) analyzed DR4 and DR5 in the normal and cancerous human pancreas and the sensitivity of pancreatic cancer cells to TRAIL-induced cytotoxicity. Independent of the presence of DR4 and DR5 pancreatic cancer cells showed a significantly lower sensitivity in comparison to other cancer cell lines and these findings reveal that pancreatic cancers are insensitive towards TRAIL-mediated

apoptosis despite expression of DR4 and DR5 suggesting the presence of mediators which inhibit the TRAIL cell-death-inducing pathway in pancreatic cancer cells. I therefore also analyzed the receptors and found that Panc-1 cells have all four receptors expressed at low levels with the death receptor DR4 being expressed at the lowest level. The TRAIL sensitive cells HeLa and HCT116 presented higher death receptor levels than Panc-1 cells. These results agree with Khanbolooki et al., (2006), who showed that TRAIL-resistant Panc-1 and HS766T cells expressed lower levels of DR4 and DR5 than TRAIL-sensitive cells. The expression of DR4 and DR5 correlates with the TRAIL sensitivity as recently published by Buneker et al. (2009). In this study protein levels and mRNA from the four TRAIL receptors from eleven cells lines were analyzed and found to correlate with TRAIL responsiveness in those cells. In addition, an increasing number of publications have now proposed that TRAIL resistance is mediated in part or completely by the absence or low levels of DR4 and DR5 expression and the sensitivity is restored when those receptors are over expressed (Sussman et al., 2007; David et al., 2008; Iacomino et. al, 2008; Goda et al., 2008; Gomez-Benito et al., 2007; Cui et al., 2009; Yeh et al., 2009; Aroui et al., 2009; Ganten et al., 2009; Yodkeeree et al., 2009; Oikonomou et al., 2009; Miyagawa et al., 2009; Shenoy and Pervaiz et al., 2009; Aguilera et al., 2009; Falschlehner et al., 2009). In this work I investigated the effect of the very low DR4 expression on the TRAIL resistance of Panc-1 cells by over expressing this receptor. Pro-apoptotic receptor agonist-induced cell death depends on amplifying the apoptotic signal via caspase-8-mediated activation of Bid and subsequent activation of the caspase-9-dependent mitochondrial apoptotic pathway. My results show a significant increase of apoptosis levels, and an increase in caspase-8 and caspase-3 activity was observed when an over-expression of DR4 was combined with down-regulation of XIAP. The caspase-3 released from XIAP binding by down-regulation of XIAP needs to be activated because caspase-3 is not spontaneously activated. In this context the up-regulation of DR4 induced an increase of caspase-8 activation, which subsequently activated caspase-3. Although in my studies a DR4 over-expression was combined with down-regulation of Bcl-xl I did not observe any increase of the apoptotic levels in comparison to the levels when only Bcl-xl was silenced. This data indicated again that the activation of caspase-3 by caspase-9, which happens due to the

cytochrome-c release by blocking Bcl-xl, is not enough to induce high levels of apoptosis, even when the upstream death signal is increased. The caspase-3 release blocked by XIAP is the key event. In all experiments I used adenovirus-mediated DR4 gene transfer to achieve high expression levels of DR4 in Panc-1 cells.

The lack or dysfunction of DR4 expression in cancer cells can be due to mutations (Kim et al., 2000; Shin et al., 2001; Kuraoka et al., 2005) or occur through epigenetic modulation as gene promoter methylation (Horak et al, 2005; Lee et al., 2009) or promoter acetylation (Ishdorj et al., 2008; Aguilera et al., 2009), two types of modifications that are responsible for chromatin plasticity. The field of histone deacetylation represents a second area receiving much interest in respect to transcriptional control. This field centers on the now generally accepted view that chromatin structure is plastic and that histone (de)acetylation regulates genome structure and hence transcription. Modifying this process by histone deacetylase (HDAC) inhibitors can therefore regulate transcription (Thiagalingam et al., 2003). Because of the pharmaceutical potential of HDAC inhibitors I tried to induce the expression DR4 and DR5 in Panc-1 cells as an alternative to the use of adenovirus as an expression vector. In a recent publication (Aguilera et al., 2009) the histone deacetylase inhibitor MS-275 was used to reactivate the expression of DR4 and sensitize medulloblastoma cell lines to TRAIL. In addition, several other studies (Inoue et al., 2004; Kim et al., 2004; Guo et al., 2004; Insinga et al., 2005; MacFarlane et al., 2005; Tamer et al., 2005; Fandy et al., 2005; Singh et al., 2005; Inoue et al., 2006; Earel et al., 2006; Iacomino et al., 2008; Frew et al., 2008) have shown that restoration of the expression of the agonistic TRAIL receptors by histone deacetylase inhibitor (HDACi) led to increased TRAIL-dependent cell death (Susumu et al., 2004; Inoue et al., 2004; Kim et al., 2004; Guo et al., 2004; Insinga et al., 2005; MacFarlane et al., 2005; Tamer et al., 2005; Fandy et al., 2005; Singh et al., 2005; Inoue et al., 2006; Earel et al., 2006; Iacomino et. al, 2008; Frew et al., 2008). However, Du et al., 2009, described no change in DR4 mRNA in leukemia cells after treatment with SB (sodium butyrate), which is consistent with an earlier study (Kim et al., 2004). The latter is in agreement with my findings since I also failed to enhance expression of DR4 and DR5 in Panc-1 cells using TSA (Trichostatin A) and VPA (Valproic Acid). TSA was already demonstrated to strongly suppress growth of pancreatic

adenocarcinoma cell lines including Panc-1 cells (Donadelli et al., 2003; Piacentini et al., 2006) by altering expression of pro- and anti-apoptotic genes with the exclusion the TRAIL receptors (Moore et al., 2004). VPA has been shown recently to sensitize pancreatic cancer cells to etoposide-induced apoptosis (Fritsche et al., 2009) and induced growth arrest (Jones et al., 2008). Some reports considered the involvement of the intrinsic pathway on the HDACi (Rosato et al., 2003; Moore et al., 2004; Sonnemann et al., 2006; Fandy et al., 2005; Chen et al., 2009; Dai et al., 2008) and contrary to literature I do not find any change in the levels of other death pathways-related proteins such as c-Flip, Bcl-xl, XIAP, Bak and Bax (data not shown).

Over-expression of DR4 recruits more TRAIL ligand and consequently activates more caspase-8, which plays a pivotal role in this apoptotic signaling pathway. Being able to sensitize cells by over expressing DR4 in combination with down-regulation of XIAP and since I analyzed caspase-8 basic levels in Panc-1 cells and low levels were detected I speculate that increasing in these levels might induce high levels of apoptosis. IFN-γ is known to up-regulate caspase-8 (Ruiz-Ruiz et al., 2000) and it alone can be sufficient to induce apoptosis in some cells but often sensitizes cells for death receptor-induced apoptosis without being apoptotic per se (Chawla-Sarkar et al., 2003). IFN-γ is also able to inhibit the growth of numerous tumors (Kominsky et al., 2000; Detjen et al., 2001; Sasagawa et al., 2000; Zhao et al., 2007). In the literature many reports have shown that resistance to TRAIL is linked to deficient expression or deregulation of caspase-8 in several types of cancer (Teitz et al., 2000; Eggert et al., 2000; Grotzer et al., 2000; Fulda et al., 2001; Yasui et al., 2005; Zhang et al., 2005; Gomez-Denito et al., 2007; Hacker et al., 2009). Hacker et al., 2009, combining VPA and INF-γ treatment, restored caspase-8 expression and the sensitivity to TRAIL in primary medulloblastoma samples. Tong et al., 2008 also use INF-γ to sensitize neuroblastoma cells to TRAIL induced apoptosis which was possibly due to the up-regulation of caspase-8. Lissat et al., 2007 demonstrated that up-regulation of caspase-8 was sufficient to restore TRAIL sensitivity in resistant Ewing's sarcoma cells and this was achieved by pre-treatment with INF-γ providing a rationale for the inclusion of IFN-γ in upcoming clinical trials with TRAIL.

In addition to the IFN-γ-mediated up-regulation of caspase-8, Yasui et al., 2005 showed increased TRAIL-induced apoptosis levels of approximately 30% after transfecting Panc-

1 cells with a caspase-8 expression vector. My western blot analyses of caspase-8 levels in Panc-1 cells suggest that TRAIL resistance of Panc-1 cells can be related to a partial loss of pro-caspase-8 in those cells. Western blot analyses showed an increase in caspase-8 level after IFN-γ pre-treatment. These results are consistent with published data, which demonstrated IFN-γ mediated up-regulation of caspase-8 in cancer cell lines derived from neuroblastoma (Fulda et al., 2002), medulloblastoma (Meister et al., 2007; Hacker et al., 2009), Ewing`s sarcoma (Kontny et al., 2001), colon carcinoma (Langaas et al., 2001), breast carcinoma (Ruiz-Ruiz et al., 2000), cholangiocarcinoma (Ahn et al., 2002) and pedriatic tumors (Harada et al., 2002). My survival assay data from an experiment involving down-regulation of XIAP combined with an IFN-γ pre-treatment after TRAIL treatment showed a highly significant increase in apoptosis of about 70%, which is the same as observed in the TRAIL sensitive cells. Also in combination with down-regulation of Bcl-xl I observed increased apoptosis induced by TRAIL, confirming that Bcl-xl also plays a role in TRAIL resistance. Due to the very low levels of caspase-9 detected in Panc-1 cells and due to the fact that INF-γ may indirectly increase the expression of caspase-9 (Janardhanan et al., 2008) I silenced caspase-9 in combination with the XIAP, and pre-treatment of IFN-γ. The results showed that TRAIL-induced apoptosis due to the absence of caspase-9 was not blocked and western blot analyses showed that IFN-γ pre-treatment did not affect the levels of caspase-9. Confirming that caspase-9 did not play a crucial role in this resistance and that in this scenario the up-regulation of caspase-8 was the factor that sensitized Panc-1 cells to TRAIL-induced apoptosis. These findings demonstrate that caspase-8 expression acted as key determinant for TRAIL sensitivity in Panc-1 cells. Furthermore, the combination of XIAP down-regulation and DR4 over-expression with IFN-γ pre-treatment gave rise to extreme high levels of apoptosis, ranging from 85-95% of cell death, which is even above the apoptosis levels observed in TRAIL-sensitive cells.

The use of recombinant adenovirus as an expression vector is a good tool to investigate and validate some protein or gene targets involved in cell signaling pathways. To evaluate the use of those set-ups and confirm the findings I needed to use an alternative approach. Therefore, I generated stable XIAP knock-down cells and used them in combination with Ad.shBcl-xl, Ad.DR4 and also with IFN-γ pre-treatments. All the

results using these stable cells were in accordance with the previous results supporting my theory that to sensitize Panc-1 cells to TRAIL I need to restore a balance of anti- and pro-apoptotic factors.

Finally, I demonstrated that this sensitization strategy worked in another TRAIL-resistant pancreatic cancer cell line, ASPC-1. In these cells sensitization to TRAIL by blocking XIAP has previously been shown (Mori et al., 2007). After TRAIL treatment, down-regulation of XIAP in combination with IFN-γ pre-treatment induced high levels of apoptosis. In these cells the apoptosis effects of IFN-γ and TRAIL were higher in comparison to Panc-1 cells indicating that caspase-8 up-regulation in this pancreatic cancer cell line is more crucial for TRAIL resistance than in Panc-1 cells. Since there was a significant difference in the apoptosis levels in ASPC-1 pre-treated with IFN-γ only and in combination with XIAP down-regulation I concluded that sensitization to TRAIL-induced apoptosis by down-regulating XIAP in combination with IFN-γ treatment is not restricted to only one pancreatic cell line. Here I provide convincing evidence that XIAP and caspase-8 are involved in regulating sensitivity of pancreatic carcinoma cells towards TRAIL.

My findings demonstrate that in order to evaluate the possible efficacy of TRAIL on tumor cells not only the levels of anti-apoptotic gene products have to be considered but also the activity of the proteins acting in a pro-apoptotic manner have to be taken into account. Impairing the balance between anti- and pro-apoptotic systems from both sides by inhibition of apoptosis-antagonist and stimulation of pro-apoptosis factors provides the best way to enhance the anti-cancer effect of TRAIL. Taken together, death receptors, caspase-8 and NF-κB signaling adjust and modulate the sensitivity and threshold levels of checkpoint-mediated TRAIL action. These findings may provide useful information for the development of TRAIL-based therapeutic strategies.

That XIAP acted as a key determinant in TRAIL sensitivity is well supported by many reports. But during my study I observed that XIAP might be also involved in cell proliferation. By silencing XIAP and treating the cells with TRAIL a significant inhibition in cell growth was observed by a clonogenic assay. In contrast, silencing of c-$Flip_L$ and Bcl-xl did not affect clonogenic growth. Zhang et al. (2005) described in breast cancer that siRNA against XIAP not only promoted cell apoptosis but also arrested the

cells in the G1 phase of the cell cycle. Interestingly Vogler et al. (2005) demonstrated that Smac, an inhibitor of XIAP, prevented proliferation and increased cell cycle arrest or senescence. Several reports have recently shown similar results for pancreatic carcinomas. Collins et al. (2007) also reported a significant growth inhibition in Panc-1 cells using XIAP small-molecule antagonists in combination with TRAIL. Giagkousiklidis et al., 2007 showed that silencing XIAP significantly reduced clonogenic growth in PaTuII cells upon γ-irradiation and Vogler et al., 2008 demonstrated in a tumor regression model in xenograft mice that XIAP inhibition acts in concert with TRAIL to cause regression of established pancreatic carcinoma. More recently Ma et al., 2009 reported that XIAP RNAi knock-down inhibited the proliferation of ovarian carcinoma cells and Wang et al. (2009) demonstrated the induction of cell arrest in laryngeal cancer cell line by inhibiting XIAP. In addition, Dai et al. (2009) reported the antitumor effect of Adv-XIAP-shRNA by down-regulating XIAP, promoting apoptosis and inhibiting proliferation of colon cancer cells.

Further experiments with my shXIAP stable cell lines were carried out after the observation that in long term cell culture analyses, at about passage 15-20, these cells stopped to grow and showed morphological changes such as enlarged cytosol, flattened cell shape and increased granularity. A clonogenic assay upon TRAIL treatment proved that the silencing of XIAP in these cells restrained clonogenic tumor growth whereas the parental Panc-1 cells and the control cell line with a low XIAP level (clone 7) showed normal growth. These initial observations suggested that XIAP-silenced Panc-1 cells may have turned into senescent cells and one shXIAP stable cell line was analysed by senescence staining. All cells of this cell line lacking XIAP were highly positive for senescence-associated β-galactosidase, which demonstrated that these cells stop to grow after a finite number of cell divisions as a consequence of replicative senescence. In addition to these histochemical findings, I also performed a proliferation assay using BrdU incorporation to monitor the replication active cells. This assay also allowed to gain more details of the cell cycle in these shXIAP-silenced stable cell lines. In these analyses less than 3% of the cells were replicating and still proliferating whereas the rest of them were arrested in G2 phase of the cell cycle. In contrast to these results, the stable cell line with low XIAP levels showed normal proliferation confirming this observed lack of

proliferation was replicative senescence and depended on the absence of XIAP and not the culture conditions of these cells. However, in the literature it has been described that one of the characteristics of replicative senescence is a stable, irreversible arrest of cell proliferation with a G1 DNA content (reviewed in Goldstein, 1990; Campisi et al., 1996). Another feature of senescence cells is the lack of susceptibility to apoptosis, although some cells may die during passage in culture (Pignolo et al., 1994) and senescent cells are substantially more resistant to dying in response to apoptotic stimuli (Wang et al., 1994). These later features were also observed, the shXIAP clones turned to be less TRAIL-sensitive for apoptosis response (data not shown). Although the stable shXIAP-silenced cells presented multiple important characteristics of senescent cells they revealed a significant difference to common senescent cells and their cell cycle arrest, they were arrested in G2. Therefore, I call them senescence-like growth-arrested cells.

Furthermore, I also generated XIAP-down-regulated stable cell lines based on HeLa and MCF-7 cells and the same morphological changes were observed (data not shown). The mechanisms that regulate or inhibit proliferation linked to XIAP-silencing have not been described yet but senescence has been mostly described to be under the control of p53 or pRb proteins. Panc-1 cells express mutant p53 (homozygote R273H) which supports the idea that observed lack of proliferation in stable shXIAP-silenced cell lines derived from Panc-1 might not show a classical replicative senescence as generally described in the literature. However, Campaner et al. (2010) describe that inhibition of Cdk2-induced Myc-dependent senescence in a human tumor cell line lacking functional p53. CDKs govern the entry of cells into mitosis and they play critical roles in both the commitment to cell division and the quality control mechanisms that safeguard genome integrity representing therapeutic targets in human cancer (Shapiro, 2006). In summary, these newly established G2-arrested cells lacking XIAP indicated the requirement to investigate the role for XIAP in pathways controlling proliferation of cells. Moreover, the ability to induce a senescence-like phenotype in p53-deficient and aggressive pancreas cancer cells reveals a putative novel therapeutic treatment of patients and therefore, it is worthwhile to investigate the effect and its molecular mechanism in the future.

5. ABSTRACT

For cancer therapy TRAIL is of special interest because TRAIL has been shown to preferentially kill cancer cells but sparing normal cells. Importantly, combined treatment of cells with TRAIL together with chemotherapy or γ-irradiation has been demonstrated to synergize and to achieve higher anticancer activity in cancer cell lines and *in vivo* tumor models. However, pancreatic cancer a commonly known very aggressive cancer type has been shown to be frequently TRAIL-resistant. In the study presented here the TRAIL-resistant cell line Panc-1 was used as a model and Panc-1 cells were investigated whether they could be sensitized to TRAIL-induced apoptosis.

In order to characterize TRAIL resistance in Panc-1 cells analyses of factors that may contribute to TRAIL resistance in Panc-1 cells were carried out. Adenovirus vectors were established to express shRNA to down-regulate anti-apoptotic proteins such as c-FLIP, Bcl-xl, XIAP or alternatively to over express pro-apoptotic factors such as the death receptor TRAIL-R1. Moreover, the expression of caspase-8 was induced in these targeted cells by the addition of interferon-γ. These treatments were verified to yield a balance between over-expression of pro-apoptotic factors and down regulation of anti-apoptotic factors. This optimization of the combined treatments was proven to be necessary to overcome TRAIL resistance in Panc-1 cells. Thus, over-expression of TRAIL-R1 with an increase in caspase-8 levels combined with down-regulation of XIAP led to a substantial increase of the TRAIL-dependent apoptotic response of Panc-1 cells. This effect in regard to the TRAIL responsiveness was outstanding compared to that by inhibition of NF-κB or XIAP in Panc-1 cells. Finally, by analyzing the long-term effect of down-regulation of XIAP in those cells we observed a change in phenotype and in proliferation. The analyses of these cells revealed that these stable XIAP knock-out Panc-1 cells yielded cells with a phenotype, which is senescence-like. Thus these cells are no longer capable to proliferate and the phenotype of these aggressive pancreatic cancer cells is dramatically changed since they lost their proliferation capabilities. Forcing such aggressive pancreatic cells into senescence may reveal a new avenue for future pancreatic cancer patient treatment.

6. REFERENCES

Aguilera, D. G., C. M. Das, N. D. Sinnappah-Kang, C. Joyce, P. H. Taylor, S. Wen, M. Hasselblatt, W. Paulus, G. Fuller, J. E. Wolff and V. Gopalakrishnan (2009). "Reactivation of death receptor 4 (DR4) expression sensitizes medulloblastoma cell lines to TRAIL." J Neurooncol **93**: 303-318.

Ahn, E. Y., G. Pan, S. M. Vickers and J. M. McDonald (2002). "IFN-gammaupregulates apoptosis-related molecules and enhances Fas-mediated apoptosis in human cholangiocarcinoma." Int J Cancer **100**: 445-451.

Algeciras-Schimnich, A., S. R. Vlahakis, A. Villasis-Keever, T. Gomez, C. J. Heppelmann, G. Bou and C. V. Paya (2002). "CCR5 mediates Fas- and caspase-8 dependent apoptosis of both uninfected and HIV infected primary human CD4 T cells." Aids **16**: 1467-1478.

Ameisen, J. C. (2002). "On the origin, evolution, and nature of programmed cell death: a timeline of four billion years." Cell Death Differ **9**: 367-393.

Aroui, S., S. Brahim, J. Hamelin, M. De Waard, J. Breard and A. Kenani (2009). "Conjugation of doxorubicin to cell penetrating peptides sensitizes human breast MDA-MB 231 cancer cells to endogenous TRAIL-induced apoptosis." Apoptosis.

Arts, G. J., E. Langemeijer, R. Tissingh, L. Ma, H. Pavliska, K. Dokic, R. Dooijes, E. Mesic, R. Clasen, F. Michiels, J. van der Schueren, M. Lambrecht, S. Herman, R. Brys, K. Thys, M. Hoffmann, P. Tomme and H. van Es (2003). "Adenoviral vectors expressing siRNAs for discovery and validation of gene function." Genome Res **13**: 2325-2332.

Arumugam, T., V. Ramachandran and C. D. Logsdon (2006). "Effect of cromolyn on S100P interactions with RAGE and pancreatic cancer growth and invasion in mouse models." J Natl Cancer Inst **98**: 1806-1818.

Ashkenazi, A. (2002). "Targeting death and decoy receptors of the tumour-necrosis factor superfamily." Nat.Rev.Cancer **2**: 420-430.

Ashkenazi, A. and V. M. Dixit (1998). "Death receptors: signaling and modulation." Science **281**: 1305-1308.

Azria, D., C. Seblain-El-Guerche, N. Girard, C. Hennequin and F. Huguet (2008). "[The value of chemoradiotherapy in the management of locally advanced pancreatic adenocarcinoma: systematic review]." Bull Cancer **95**: 1116-1130.

Bai, J., J. Sui, A. Demirjian, C. M. Vollmer, Jr., W. Marasco and M. P. Callery (2005). "Predominant Bcl-XL knockdown disables antiapoptotic mechanisms: tumor necrosis

factor-related apoptosis-inducing ligand-based triple chemotherapy overcomes chemoresistance in pancreatic cancer cells in vitro." Cancer Res **65**: 2344-2352.

Behrend, L., A. Mohr, T. Dick and R. M. Zwacka (2005). "Manganese superoxide dismutase induces p53-dependent senescence in colorectal cancer cells." Mol Cell Biol **25**: 7758-7769.

Bellail, A. C., L. Qi, P. Mulligan, V. Chhabra and C. Hao (2009). "TRAIL agonists on clinical trials for cancer therapy: the promises and the challenges." Rev Recent Clin Trials **4**: 34-41.

Bellail, A. C., M. C. Tse, J. H. Song, S. Phuphanich, J. J. Olson, S. Y. Sun and C. Hao (2009). "DR5-mediated DISC controls caspase-8 cleavage and initiation of apoptosis in human glioblastomas." J Cell Mol Med.

Berkner, K. L. and P. A. Sharp (1983). "Generation of adenovirus by transfection of plasmids." Nucleic Acids Res **11**: 6003-6020.

Bernard, P. and M. Couturier (1992). "Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes." J Mol Biol **226**: 735-745.

Bernstein, E., A. M. Denli and G. J. Hannon (2001). "The rest is silence." Rna **7**: 1509-1521.

Boatright, K. M., M. Renatus, F. L. Scott, S. Sperandio, H. Shin, I. M. Pedersen, J. E. Ricci, W. A. Edris, D. P. Sutherlin, D. R. Green and G. S. Salvesen (2003). "A unified model for apical caspase activation." Mol Cell **11**: 529-541.

Boatright, K. M. and G. S. Salvesen (2003). "Caspase activation." Biochem Soc Symp: 233-242.

Boatright, K. M. and G. S. Salvesen (2003). "Mechanisms of caspase activation." Curr Opin Cell Biol **15**: 725-731.

Bodmer, J. L., P. Schneider and J. Tschopp (2002). "The molecular architecture of the TNF superfamily." Trends Biochem Sci **27**: 19-26.

Boyer, J. L., G. Kobinger, J. M. Wilson and R. G. Crystal (2005). "Adenovirus-based genetic vaccines for biodefense." Hum Gene Ther **16**: 157-168.

Braeuer, S. J., C. Buneker, A. Mohr and R. M. Zwacka (2006). "Constitutively activated nuclear factor-kappaB, but not induced NF-kappaB, leads to TRAIL resistance by up-regulation of X-linked inhibitor of apoptosis protein in human cancer cells." Mol Cancer Res **4**: 715-728.

Branton, P. E., S. T. Bayley and F. L. Graham (1985). "Transformation by human adenoviruses." Biochim Biophys Acta **780**: 67-94.

Bratton, S. B., J. Lewis, M. Butterworth, C. S. Duckett and G. M. Cohen (2002). "XIAP inhibition of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95- and Bax-induced apoptosis." Cell Death Differ **9**: 881-892.

Bringold, F. and M. Serrano (2000). "Tumor suppressors and oncogenes in cellular senescence." Exp Gerontol **35**: 317-329.

Brummelkamp, T. R., R. Bernards and R. Agami (2002). "A system for stable expression of short interfering RNAs in mammalian cells." Science **296**: 550-553.

Budihardjo, I., H. Oliver, M. Lutter, X. Luo and X. Wang (1999). "Biochemical pathways of caspase activation during apoptosis." Annu Rev Cell Dev Biol **15**: 269-290.

Buneker, C., A. Mohr and R. M. Zwacka (2009). "The TRAIL-receptor-1: TRAIL-receptor-3 and -4 ratio is a predictor for TRAIL sensitivity of cancer cells." Oncol Rep **21**: 1289-1295.

Cain, K., S. B. Bratton and G. M. Cohen (2002). "The Apaf-1 apoptosome: a large caspase-activating complex." Biochimie **84**: 203-214.

Campaner, S., M. Doni, P. Hydbring, A. Verrecchia, L. Bianchi, D. Sardella, T. Schleker, D. Perna, S. Tronnersjo, M. Murga, O. Fernandez-Capetillo, M. Barbacid, L. G. Larsson and B. Amati (2010). "Cdk2 suppresses cellular senescence induced by the c-myc oncogene." Nat Cell Biol **12**: 54-59

Campisi, J. (1996). "Replicative senescence: an old lives' tale?" Cell **84**: 497-500.

Campisi, J., G. P. Dimri, J. O. Nehlin, A. Testori and K. Yoshimoto (1996). "Coming of age in culture." Exp Gerontol **31**: 7-12.

Chang, D. W., Z. Xing, Y. Pan, A. Algeciras-Schimnich, B. C. Barnhart, S. Yaish-Ohad, M. E. Peter and X. Yang (2002). "c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis." Embo J **21**: 3704-3714.

Chang, H. Y. and X. Yang (2000). "Proteases for cell suicide: functions and regulation of caspases." Microbiol Mol Biol Rev **64**: 821-846.

Chawla-Sarkar, M., S. I. Bae, F. J. Reu, B. S. Jacobs, D. J. Lindner and E. C. Borden (2004). "Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis." Cell Death Differ **11**: 915-923.

Chillon, M., A. Bosch, J. Zabner, L. Law, D. Armentano, M. J. Welsh and B. L. Davidson (1999). "Group D adenoviruses infect primary central nervous system cells more efficiently than those from group C." J Virol **73**: 2537-2540.

Clancy, L., K. Mruk, K. Archer, M. Woelfel, J. Mongkolsapaya, G. Screaton, M. J. Lenardo and F. K. Chan (2005). "Preligand assembly domain-mediated ligand-independent association

between TRAIL receptor 4 (TR4) and TR2 regulates TRAIL-induced apoptosis." Proc Natl Acad Sci U S A **102**: 18099-18104.

Cohen, G. M. (1997). "Caspases: the executioners of apoptosis." Biochem J **326 (Pt 1)**: 1-16.

Connolly, K., R. Mitter, M. Muir, D. Jodrell and S. Guichard (2009). "Stable XIAP knockdown clones of HCT116 colon cancer cells are more sensitive to TRAIL, taxanes and irradiation in vitro." Cancer Chemother Pharmacol **64**: 307-316.

Cui, D. D., Y. Huang, S. H. Mao, S. C. Chen, M. Qiu, L. L. Ji and C. Yi (2009). "Synergistic antitumor effect of TRAIL and adriamycin on the human breast cancer cell line MCF-7." Braz J Med Biol Res **42**: 854-862.

Dai, Y., L. Qiao, K. W. Chan, M. Yang, J. Ye, R. Zhang, J. Ma, B. Zou, C. S. Lam, J. Wang, R. Pang, V. P. Tan, H. Y. Lan and B. C. Wong (2009). "Adenovirus-mediated down-regulation of X-linked inhibitor of apoptosis protein inhibits colon cancer." Mol Cancer Ther **8**: 2762-2770.

Danial, N. N. and S. J. Korsmeyer (2004). "Cell death: critical control points." Cell **116**: 205-219.

David, E., R. Sinha, J. Chen, S. Y. Sun, J. L. Kaufman and S. Lonial (2008). "Perifosine synergistically enhances TRAIL-induced myeloma cell apoptosis via up-regulation of death receptors." Clin Cancer Res **14**: 5090-5098.

Desagher, S. and J. C. Martinou (2000). "Mitochondria as the central control point of apoptosis." Trends Cell Biol **10**: 369-377.

Detjen, K. M., K. Farwig, M. Welzel, B. Wiedenmann and S. Rosewicz (2001). "Interferon gamma inhibits growth of human pancreatic carcinoma cells via caspase-1 dependent induction of apoptosis." Gut **49**: 251-262.

Deveraux, Q. L. and J. C. Reed (1999). "IAP family proteins--suppressors of apoptosis." Genes Dev **13**: 239-252.

Dharmapuri, S., D. Peruzzi and L. Aurisicchio (2009). "Engineered adenovirus serotypes for overcoming anti-vector immunity." Expert Opin Biol Ther **9**: 1279-1287.

Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith and et al. (1995). "A biomarker that identifies senescent human cells in culture and in aging skin in vivo." Proc Natl Acad Sci U S A **92**: 9363-9367.

Donepudi, M. and M. G. Grutter (2002). "Structure and zymogen activation of caspases." Biophys Chem **101-102**: 145-153.

Donepudi, M., A. Mac Sweeney, C. Briand and M. G. Grutter (2003). "Insights into the regulatory mechanism for caspase-8 activation." Mol Cell **11**: 543-549.

Dower, S. K., J. E. Sims, D. P. Cerretti and T. A. Bird (1992). "The interleukin-1 system: receptors, ligands and signals." Chem Immunol **51**: 33-64.

Dykxhoorn, D. M., C. D. Novina and P. A. Sharp (2003). "Killing the messenger: short RNAs that silence gene expression." Nat Rev Mol Cell Biol **4**: 457-467.

Earnshaw, W. C., L. M. Martins and S. H. Kaufmann (1999). "Mammalian caspases: structure, activation, substrates, and functions during apoptosis." Annu Rev Biochem **68**: 383-424.

Eggert, A., M. A. Grotzer, T. J. Zuzak, B. R. Wiewrodt, N. Ikegaki and G. M. Brodeur (2000). "Resistance to TRAIL-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression." Med Pediatr Oncol **35**: 603-607.

Elbashir, S. M., W. Lendeckel and T. Tuschl (2001). "RNA interference is mediated by 21- and 22-nucleotide RNAs." Genes Dev **15**: 188-200.

Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton and P. M. Henson (1992). "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages." J Immunol **148**: 2207-2216.

Fakler, M., S. Loeder, M. Vogler, K. Schneider, I. Jeremias, K. M. Debatin and S. Fulda (2009). "Small molecule XIAP inhibitors cooperate with TRAIL to induce apoptosis in childhood acute leukemia cells and overcome Bcl-2-mediated resistance." Blood **113**: 1710-1722.

Falschlehner, C., T. M. Ganten, R. Koschny, U. Schaefer and H. Walczak (2009). "TRAIL and Other TRAIL Receptor Agonists as Novel Cancer Therapeutics." Adv Exp Med Biol **647**: 195-206.

Fandy, T. E., S. Shankar, D. D. Ross, E. Sausville and R. K. Srivastava (2005). "Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma." Neoplasia **7**: 646-657.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**: 806-811.

Fuentes-Prior, P. and G. S. Salvesen (2004). "The protein structures that shape caspase activity, specificity, activation and inhibition." Biochem J **384**: 201-232.

Fukada, I., H. Ikeda, K. Yamaguchi, M. Okabe, A. Tsuruta, Y. Morimoto, K. Kawamoto, K. Sano, T. Paku, S. Imai, Y. Yoshida, T. Ito and K. Ogasahara (2009). "[A case of recurrent pancreatic cancer with lung metastasis responding to s-1 combined gemcitabine chemotherapy]." Gan To Kagaku Ryoho **36**: 1733-1736.

Fulda, S. and K. M. Debatin (2002). "IFNgamma sensitizes for apoptosis by upregulating caspase-8 expression through the Stat1 pathway." Oncogene **21**: 2295-2308.

Fulda, S., M. U. Kufer, E. Meyer, F. van Valen, B. Dockhorn-Dworniczak and K. M. Debatin (2001). "Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer." Oncogene **20**: 5865-5877.

Gabitzsch, E. S., Y. Xu, L. H. Yoshida, J. Balint, R. B. Gayle, A. Amalfitano and F. R. Jones (2009). "A preliminary and comparative evaluation of a novel Ad5 [E1-, E2b-] recombinant-based vaccine used to induce cell mediated immune responses." Immunol Lett **122**: 44-51.

Ganten, T. M., J. Sykora, R. Koschny, E. Batke, S. Aulmann, U. Mansmann, W. Stremmel, H. P. Sinn and H. Walczak (2009). "Prognostic significance of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor expression in patients with breast cancer." J Mol Med **87**: 995-1007.

Geske, F. J. and L. E. Gerschenson (2001). "The biology of apoptosis." Hum Pathol **32**: 1029-1038.

Giagkousiklidis, S., S. H. Vellanki, K. M. Debatin and S. Fulda (2007). "Sensitization of pancreatic carcinoma cells for gamma-irradiation-induced apoptosis by XIAP inhibition." Oncogene **26**: 7006-7016.

Goda, A. E., T. Yoshida, M. Horinaka, T. Yasuda, T. Shiraishi, M. Wakada and T. Sakai (2008). "Mechanisms of enhancement of TRAIL tumoricidal activity against human cancer cells of different origin by dipyridamole." Oncogene **27**: 3435-3445.

Goldstein, S. (1990). "Replicative senescence: the human fibroblast comes of age." Science **249**: 1129-1133.

Gomez-Benito, M., P. Balsas, X. Carvajal-Vergara, A. Pandiella, A. Anel, I. Marzo and J. Naval (2007). "Mechanism of apoptosis induced by IFN-alpha in human myeloma cells: role of Jak1 and Bim and potentiation by rapamycin." Cell Signal **19**: 844-854.

Gomez-Benito, M., M. J. Martinez-Lorenzo, A. Anel, I. Marzo and J. Naval (2007). "Membrane expression of DR4, DR5 and caspase-8 levels, but not Mcl-1, determine sensitivity of human myeloma cells to Apo2L/TRAIL." Exp Cell Res **313**: 2378-2388.

Green, D. R. (1998). "Apoptotic pathways: the roads to ruin." Cell **94**: 695-698.

Green, D. R. and G. Kroemer (2004). "The pathophysiology of mitochondrial cell death." Science **305**: 626-629.

Green, D. R. and J. C. Reed (1998). "Mitochondria and apoptosis." Science **281**: 1309-1312.

Grotzer, M. A., A. Eggert, T. J. Zuzak, A. J. Janss, S. Marwaha, B. R. Wiewrodt, N. Ikegaki, G. M. Brodeur and P. C. Phillips (2000). "Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression." Oncogene **19**: 4604-4610.

Guo, F., C. Sigua, J. Tao, P. Bali, P. George, Y. Li, S. Wittmann, L. Moscinski, P. Atadja and K. Bhalla (2004). "Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells." Cancer Res **64**: 2580-2589.

Gura, T. (1997). "How TRAIL kills cancer cells, but not normal cells." Science **277**: 768.

Hacker, S., A. Dittrich, A. Mohr, T. Schweitzer, S. Rutkowski, J. Krauss, K. M. Debatin and S. Fulda (2009). "Histone deacetylase inhibitors cooperate with IFN-gamma to restore caspase-8 expression and overcome TRAIL resistance in cancers with silencing of caspase-8." Oncogene **28**: 3097-3110.

Haj-Ahmad, Y. and F. L. Graham (1986). "Characterization of an adenovirus type 5 mutant carrying embedded inverted terminal repeats." Virology **153**: 22-34.

Harada, T., N. N. Miura, Y. Adachi, M. Nakajima, T. Yadomae and N. Ohno (2002). "IFN-gamma induction by SCG, 1,3-beta-D-glucan from Sparassis crispa, in DBA/2 mice in vitro." J Interferon Cytokine Res **22**: 1227-1239.

Hayflick, L. and P. S. Moorhead (1961). "The serial cultivation of human diploid cell strains." Exp Cell Res **25**: 585-621.

Hetschko, H., V. Voss, S. Horn, V. Seifert, J. H. Prehn and D. Kogel (2008). "Pharmacological inhibition of Bcl-2 family members reactivates TRAIL-induced apoptosis in malignant glioma." J Neurooncol **86**: 265-272.

Hinz, S., A. Trauzold, L. Boenicke, C. Sandberg, S. Beckmann, E. Bayer, H. Walczak, H. Kalthoff and H. Ungefroren (2000). "Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis." Oncogene **19**: 5477-5486.

Hirata, T., M. Nakamoto, M. Nakamura, N. Kinjo, A. Hokama, F. Kinjo and J. Fujita (2007). "Low prevalence of human T cell lymphotropic virus type 1 infection in patients with gastric cancer." J Gastroenterol Hepatol **22**: 2238-2241.

Holman, D. H., D. Wang, K. Raviprakash, N. U. Raja, M. Luo, J. Zhang, K. R. Porter and J. Y. Dong (2007). "Two complex, adenovirus-based vaccines that together induce immune responses to all four dengue virus serotypes." Clin Vaccine Immunol **14**: 182-189.

Huang, Y., Y. C. Park, R. L. Rich, D. Segal, D. G. Myszka and H. Wu (2001). "Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain." Cell **104**: 781-790.

Iacomino, G., M. C. Medici and G. L. Russo (2008). "Valproic acid sensitizes K562 erythroleukemia cells to TRAIL/Apo2L-induced apoptosis." Anticancer Res **28**: 855-864.

Ibrahim, S. M., J. Ringel, C. Schmidt, B. Ringel, P. Muller, D. Koczan, H. J. Thiesen and M. Lohr (2001). "Pancreatic adenocarcinoma cell lines show variable susceptibility to TRAIL-mediated cell death." Pancreas **23**: 72-79.

Inoue, S., M. MacFarlane, N. Harper, L. M. Wheat, M. J. Dyer and G. M. Cohen (2004). "Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies." Cell Death Differ **11 Suppl 2**: S193-206.

Insinga, A., S. Minucci and P. G. Pelicci (2005). "Mechanisms of selective anticancer action of histone deacetylase inhibitors." Cell Cycle **4**: 741-743.

Insinga, A., S. Monestiroli, S. Ronzoni, V. Gelmetti, F. Marchesi, A. Viale, L. Altucci, C. Nervi, S. Minucci and P. G. Pelicci (2005). "Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway." Nat Med **11**: 71-76.

Ishdorj, G., B. A. Graham, X. Hu, J. Chen, J. B. Johnston, X. Fang and S. B. Gibson (2008). "Lysophosphatidic acid protects cancer cells from histone deacetylase (HDAC) inhibitor-induced apoptosis through activation of HDAC." J Biol Chem **283**: 16818-16829.

Jacobson, M. D. (1997). "Programmed cell death: a missing link is found." Trends Cell Biol **7**: 467-469.

Janardhanan, R., N. L. Banik and S. K. Ray (2008). "N-(4-Hydroxyphenyl)retinamide induced differentiation with repression of telomerase and cell cycle to increase interferon-gamma sensitivity for apoptosis in human glioblastoma cells." Cancer Lett **261**: 26-36.

Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu and M. J. Thun (2009). "Cancer statistics, 2009." CA Cancer J Clin **59**: 225-249.

Jiang, X. and X. Wang (2004). "Cytochrome C-mediated apoptosis." Annu Rev Biochem **73**: 87-106.

Kafri, T., D. Morgan, T. Krahl, N. Sarvetnick, L. Sherman and I. Verma (1998). "Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy." Proc Natl Acad Sci U S A **95**: 11377-11382.

Karikari, C. A., I. Roy, E. Tryggestad, G. Feldmann, C. Pinilla, K. Welsh, J. C. Reed, E. P. Armour, J. Wong, J. Herman, D. Rakheja and A. Maitra (2007). "Targeting the apoptotic

machinery in pancreatic cancers using small-molecule antagonists of the X-linked inhibitor of apoptosis protein." Mol Cancer Ther **6**: 957-966.

Khanbolooki S., S.T. Nawrocki, T. Arumugam, R. Andtbacka, M.S. Pino, R. Kurzrock, C. D. Logsdon, J. L. Abbruzzese, D. J. McConkey. “Nuclear factor-kappaB maintains TRAIL resistance in human pancreatic cancer cells.” Mol Cancer Ther. **9:**2251-60.

Kerr, J. F. (1972). "Shrinkage necrosis of adrenal cortical cells." J Pathol **107**: 217-219.

Kerr, J. F. (2002). "History of the events leading to the formulation of the apoptosis concept." Toxicology **181-182**: 471-474.

Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon and R. H. Plasterk (2001). "Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans." Genes Dev **15**: 2654-2659.

Khanbolooki, S., S. T. Nawrocki, T. Arumugam, R. Andtbacka, M. S. Pino, R. Kurzrock, C. D. Logsdon, J. L. Abbruzzese and D. J. McConkey (2006). "Nuclear factor-kappaB maintains TRAIL resistance in human pancreatic cancer cells." Mol Cancer Ther **5**: 2251-2260.

Kim, J. Y., E. H. Kim, S. S. Park, J. H. Lim, T. K. Kwon and K. S. Choi (2008). "Quercetin sensitizes human hepatoma cells to TRAIL-induced apoptosis via Sp1-mediated DR5 up-regulation and proteasome-mediated c-FLIPS down-regulation." J Cell Biochem **105**: 1386-1398.

Kim, K., M. J. Fisher, S. Q. Xu and W. S. el-Deiry (2000). "Molecular determinants of response to TRAIL in killing of normal and cancer cells." Clin Cancer Res **6**: 335-346.

Kim, S., J. B. Lee, G. K. Lee and J. Chang (2009). "Vaccination with recombinant adenoviruses and dendritic cells expressing prostate-specific antigens is effective in eliciting CTL and suppresses tumor growth in the experimental prostate cancer." Prostate **69**: 938-948.

Kim, Y. H., J. W. Park, J. Y. Lee and T. K. Kwon (2004). "Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells." Carcinogenesis **25**: 1813-1820.

Kisielow, M., S. Kleiner, M. Nagasawa, A. Faisal and Y. Nagamine (2002). "Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA." Biochem J **363**: 1-5.

Kominsky, S. L., P. S. Subramaniam, H. M. Johnson and B. A. Torres (2000). "Inhibitory effects of IFN-gamma and acyclovir on the glioblastoma cell cycle." J Interferon Cytokine Res **20**: 463-469.

Krammer, P. H. (2000). "CD95's deadly mission in the immune system." Nature **407**: 789-795.

Kuida, K., T. S. Zheng, S. Na, C. Kuan, D. Yang, H. Karasuyama, P. Rakic and R. A. Flavell (1996). "Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice." Nature **384**: 368-372.

Kuraoka, K., S. Matsumura, Y. Sanada, K. Nakachi, K. Imai, H. Eguchi, K. Matsusaki, N. Oue, H. Nakayama and W. Yasui (2005). "A single nucleotide polymorphism in the extracellular domain of TRAIL receptor DR4 at nucleotide 626 in gastric cancer patients in Japan." Oncol Rep **14**: 465-470.

Langaas, V., S. Shahzidi, J. I. Johnsen, B. Smedsrod and B. Sveinbjornsson (2001). "Interferon-gamma modulates TRAIL-mediated apoptosis in human colon carcinoma cells." Anticancer Res **21**: 3733-3738.

Larbouret, C., B. Robert, I. Teulon, D. Azria and A. Pelegrin (2007). "[Combined anti-EGFR and anti-HER2 monoclonal antibodies: preclinical efficacy in the treatment of pancreatic cancer]." Med Sci (Paris) **23**: 800-803.

Lavrik, I., A. Golks and P. H. Krammer (2005). "Death receptor signaling." J Cell Sci **118**: 265-267.

Lee, J. Y., H. J. Jung, I. S. Song, M. S. Williams, C. Choi, S. G. Rhee, J. Kim and S. W. Kang (2009). "Protective role of cytosolic 2-cys peroxiredoxin in the TNF-alpha-induced apoptotic death of human cancer cells." Free Radic Biol Med **47**: 1162-1171.

Lemiale, F., W. P. Kong, L. M. Akyurek, X. Ling, Y. Huang, B. K. Chakrabarti, M. Eckhaus and G. J. Nabel (2003). "Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system." J Virol **77**: 10078-10087.

Li, M., H. Yang, H. Chai, W. E. Fisher, X. Wang, F. C. Brunicardi, Q. Yao and C. Chen (2004). "Pancreatic carcinoma cells express neuropilins and vascular endothelial growth factor, but not vascular endothelial growth factor receptors." Cancer **101**: 2341-2350.

Libikova, H., J. Pogady, V. Wiedermann and S. Breier (1975). "Search for herpetic antibodies in the cerebrospinal fluid in senile dementia and mental retardation." Acta Virol **19**: 493-495.

Lippens, S., M. Kockx, M. Knaapen, L. Mortier, R. Polakowska, A. Verheyen, M. Garmyn, A. Zwijsen, P. Formstecher, D. Huylebroeck, P. Vandenabeele and W. Declercq (2000). "Epidermal differentiation does not involve the pro-apoptotic executioner caspases, but is associated with caspase-14 induction and processing." Cell Death Differ **7**: 1218-1224.

Lissat, A., T. Vraetz, M. Tsokos, R. Klein, M. Braun, N. Koutelia, P. Fisch, M. E. Romero, L. Long, P. Noellke, C. L. Mackall, C. M. Niemeyer and U. Kontny (2007). "Interferon-

gamma sensitizes resistant Ewing's sarcoma cells to tumor necrosis factor apoptosis-inducing ligand-induced apoptosis by up-regulation of caspase-8 without altering chemosensitivity." Am J Pathol **170**: 1917-1930.

Liu, X., H. Zou, C. Slaughter and X. Wang (1997). "DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis." Cell **89**: 175-184.

Lockshin, R. A. and C. M. Williams (1965). "Programmed Cell Death--I. Cytology of Degeneration in the Intersegmental Muscles of the Pernyi Silkmoth." J Insect Physiol **11**: 123-133.

Louvet, C., R. Labianca, P. Hammel, G. Lledo, M. G. Zampino, T. Andre, A. Zaniboni, M. Ducreux, E. Aitini, J. Taieb, R. Faroux, C. Lepere and A. de Gramont (2005). "Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial." J Clin Oncol **23**: 3509-3516.

Ma, J. J., B. L. Chen and X. Y. Xin (2009). "XIAP gene downregulation by small interfering RNA inhibits proliferation, induces apoptosis, and reverses the cisplatin resistance of ovarian carcinoma." Eur J Obstet Gynecol Reprod Biol **146**: 222-226.

MacFarlane, M., S. Inoue, S. L. Kohlhaas, A. Majid, N. Harper, D. B. Kennedy, M. J. Dyer and G. M. Cohen (2005). "Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1." Cell Death Differ **12**: 773-782.

Mackenzie, R. P. and A. D. McCollum (2009). "Novel agents for the treatment of adenocarcinoma of the pancreas." Expert Rev Anticancer Ther **9**: 1473-1485.

Makhov, P., K. Golovine, R. G. Uzzo, J. Rothman, P. L. Crispen, T. Shaw, B. J. Scoll and V. M. Kolenko (2008). "Zinc chelation induces rapid depletion of the X-linked inhibitor of apoptosis and sensitizes prostate cancer cells to TRAIL-mediated apoptosis." Cell Death Differ **15**: 1745-1751.

Marzo, I., C. Brenner, N. Zamzami, S. A. Susin, G. Beutner, D. Brdiczka, R. Remy, Z. H. Xie, J. C. Reed and G. Kroemer (1998). "The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins." J Exp Med **187**: 1261-1271.

Meister, N., T. Shalaby, A. O. von Bueren, P. Rivera, R. Patti, C. Oehler, M. Pruschy and M. A. Grotzer (2007). "Interferon-gamma mediated up-regulation of caspase-8 sensitizes medulloblastoma cells to radio- and chemotherapy." Eur J Cancer **43**: 1833-1841.

Micheau, O., M. Thome, P. Schneider, N. Holler, J. Tschopp, D. W. Nicholson, C. Briand and M. G. Grutter (2002). "The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex." J Biol Chem **277**: 45162-45171.

Micheau, O. and J. Tschopp (2003). "Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes." Cell **114**: 181-190.

Mikolajczyk, J., K. M. Boatright, H. R. Stennicke, T. Nazif, J. Potempa, M. Bogyo and G. S. Salvesen (2003). "Sequential autolytic processing activates the zymogen of Arg-gingipain." J Biol Chem **278**: 10458-10464.

Miyagawa, T., T. Ohtsuki, T. Koyano, T. Kowithayakorn and M. Ishibashi (2009). "Cardenolide glycosides of Thevetia peruviana and triterpenoid saponins of Sapindus emarginatus as TRAIL resistance-overcoming compounds." J Nat Prod **72**: 1507-1511.

Mohr, A., C. Buneker, R. P. Gough and R. M. Zwacka (2008). "MnSOD protects colorectal cancer cells from TRAIL-induced apoptosis by inhibition of Smac/DIABLO release." Oncogene **27**: 763-774.

Mohr, A., G. Henderson, L. Dudus, I. Herr, T. Kuerschner, K. M. Debatin, H. Weiher, K. J. Fisher and R. M. Zwacka (2004). "AAV-encoded expression of TRAIL in experimental human colorectal cancer leads to tumor regression." Gene Ther **11**: 534-543.

Mohr, A., M. Lyons, L. Deedigan, T. Harte, G. Shaw, L. Howard, F. Barry, T. O'Brien and R. Zwacka (2008). "Mesenchymal stem cells expressing TRAIL lead to tumour growth inhibition in an experimental lung cancer model." J Cell Mol Med **12**: 2628-2643.

Mohr, A., M. Lyons, W. Mohr and R. M. Zwacka (2007). "[Gene therapy strategies for colorectal cancer]." Dtsch Med Wochenschr **132**: 567-570.

Mohr, A. and R. M. Zwacka (2007). "In situ trapping of initiator caspases reveals intermediate surprises." Cell Biol Int **31**: 526-530.

Mohr, A., R. M. Zwacka, K. M. Debatin and K. Stahnke (2004). "A novel method for the combined flow cytometric analysis of cell cycle and cytochrome c release." Cell Death Differ **11**: 1153-1154.

Mohr, A., R. M. Zwacka, G. Jarmy, C. Buneker, H. Schrezenmeier, K. Dohner, C. Beltinger, M. Wiesneth, K. M. Debatin and K. Stahnke (2005). "Caspase-8L expression protects CD34+ hematopoietic progenitor cells and leukemic cells from CD95-mediated apoptosis." Oncogene **24**: 2421-2429.

Mori, T., R. Doi, A. Kida, K. Nagai, K. Kami, D. Ito, E. Toyoda, Y. Kawaguchi and S. Uemoto (2007). "Effect of the XIAP inhibitor Embelin on TRAIL-induced apoptosis of pancreatic cancer cells." J Surg Res **142**: 281-286.

Murray, T. V., J. M. McMahon, B. A. Howley, A. Stanley, T. Ritter, A. Mohr, R. Zwacka and H. O. Fearnhead (2008). "A non-apoptotic role for caspase-9 in muscle differentiation." J Cell Sci **121**: 3786-3793.

Mymryk, J. S. and S. T. Bayley (1994). "Multiple pathways for activation of E2A expression in human KB cells by the 243R E1A protein of adenovirus type 5." Virus Res **33**: 89-97.

Natoni, F., L. Diolordi, C. Santoni and M. S. Gilardini Montani (2005). "Sodium butyrate sensitises human pancreatic cancer cells to both the intrinsic and the extrinsic apoptotic pathways." Biochim Biophys Acta **1745**: 318-329.

Ndozangue-Touriguine, O., M. Sebbagh, D. Merino, O. Micheau, J. Bertoglio and J. Breard (2008). "A mitochondrial block and expression of XIAP lead to resistance to TRAIL-induced apoptosis during progression to metastasis of a colon carcinoma." Oncogene **27**: 6012-6022.

Neoptolemos, J. P., D. D. Stocken, H. Friess, C. Bassi, J. A. Dunn, H. Hickey, H. Beger, L. Fernandez-Cruz, C. Dervenis, F. Lacaine, M. Falconi, P. Pederzoli, A. Pap, D. Spooner, D. J. Kerr and M. W. Buchler (2004). "A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer." N Engl J Med **350**: 1200-1210.

Neuhaus, J., F. Pfeiffer, H. Wolburg, L. C. Horn and W. Dorschner (2005). "Alterations in connexin expression in the bladder of patients with urge symptoms." BJU Int **96**: 670-676.

Nicholson, D. W. (1999). "Caspase structure, proteolytic substrates, and function during apoptotic cell death." Cell Death Differ **6**: 1028-1042.

Nicoletti, I., G. Migliorati, M. C. Pagliacci, F. Grignani and C. Riccardi (1991). "A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry." J Immunol Methods **139**: 271-279.

Oikonomou, E., V. Kosmidou, A. Katseli, K. Kothonidis, D. Mourtzoukou, G. Kontogeorgos, L. Andera, G. Zografos and A. Pintzas (2009). "TRAIL receptor upregulation and the implication of KRAS/BRAF mutations in human colon cancer tumors." Int J Cancer **125**: 2127-2135.

Osada, T., X. Y. Yang, Z. C. Hartman, O. Glass, B. L. Hodges, D. Niedzwiecki, M. A. Morse, H. K. Lyerly, A. Amalfitano and T. M. Clay (2009). "Optimization of vaccine responses with an E1, E2b and E3-deleted Ad5 vector circumvents pre-existing anti-vector immunity." Cancer Gene Ther **16**: 673-682.

Palacios, C., R. Yerbes and A. Lopez-Rivas (2006). "Flavopiridol induces cellular FLICE-inhibitory protein degradation by the proteasome and promotes TRAIL-induced early signaling and apoptosis in breast tumor cells." Cancer Res **66**: 8858-8869.

Paul, C. P., P. D. Good, I. Winer and D. R. Engelke (2002). "Effective expression of small interfering RNA in human cells." Nat Biotechnol **20**: 505-508.

Peter, M. E. (2004). "The flip side of FLIP." Biochem J **382**: e1-3.

Peter, M. E. and P. H. Krammer (2003). "The CD95(APO-1/Fas) DISC and beyond." Cell Death Differ **10**: 26-35.

Pignolo, R. J., M. O. Rotenberg and V. J. Cristofalo (1994). "Alterations in contact and density-dependent arrest state in senescent WI-38 cells." In Vitro Cell Dev Biol Anim **30A**: 471-476.

Pollok, S., C. Bauerschmidt, J. Sanger, H. P. Nasheuer and F. Grosse (2007). "Human Cdc45 is a proliferation-associated antigen." Febs J **274**: 3669-3684.

Pompeiano, M., A. J. Blaschke, R. A. Flavell, A. Srinivasan and J. Chun (2000). "Decreased apoptosis in proliferative and postmitotic regions of the Caspase 3-deficient embryonic central nervous system." J Comp Neurol **423**: 1-12.

Potthoff, A., S. Schwannecke, G. Nabi, D. Hoffmann, T. Grunwald, O. Wildner, N. H. Brockmeyer, K. Uberla and M. Tenbusch (2009). "Immunogenicity and efficacy of intradermal tattoo immunization with adenoviral vector vaccines." Vaccine **27**: 2768-2774.

Raff, M. C. (1992). "Social controls on cell survival and cell death." Nature **356**: 397-400.

Ray, D., Y. Terao, D. Nimbalkar, L. H. Chu, M. Donzelli, T. Tsutsui, X. Zou, A. K. Ghosh, J. Varga, G. F. Draetta and H. Kiyokawa (2005). "Transforming growth factor beta facilitates beta-TrCP-mediated degradation of Cdc25A in a Smad3-dependent manner." Mol Cell Biol **25**: 3338-3347.

Ray, D., Y. Terao, D. Nimbalkar, L. H. Chu, M. Donzelli, T. Tsutsui, X. Zou, A. K. Ghosh, J. Varga, G. F. Draetta and H. Kiyokawa (2005). "Transforming growth factor beta facilitates beta-TrCP-mediated degradation of Cdc25A in a Smad3-dependent manner." Mol Cell Biol **25**: 3338-3347.

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz and G. Ruvkun (2000). "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans." Nature **403**: 901-906.

Renatus, M., H. R. Stennicke, F. L. Scott, R. C. Liddington and G. S. Salvesen (2001). "Dimer formation drives the activation of the cell death protease caspase 9." Proc Natl Acad Sci U S A **98**: 14250-14255.

Ricci, M. S., Z. Jin, M. Dews, D. Yu, A. Thomas-Tikhonenko, D. T. Dicker and W. S. El-Deiry (2004). "Direct repression of FLIP expression by c-myc is a major determinant of TRAIL sensitivity." Mol Cell Biol **24**: 8541-8555.

Riedl, S. J., W. Li, Y. Chao, R. Schwarzenbacher and Y. Shi (2005). "Structure of the apoptotic protease-activating factor 1 bound to ADP." Nature **434**: 926-933.

Riedl, S. J. and Y. Shi (2004). "Molecular mechanisms of caspase regulation during apoptosis." Nat Rev Mol Cell Biol **5**: 897-907.

Rubinson, D. A., C. P. Dillon, A. V. Kwiatkowski, C. Sievers, L. Yang, J. Kopinja, D. L. Rooney, M. Zhang, M. M. Ihrig, M. T. McManus, F. B. Gertler, M. L. Scott and L. Van Parijs (2003). "A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference." Nat Genet **33**: 401-406.

Ruiz-Ruiz, C., C. Munoz-Pinedo and A. Lopez-Rivas (2000). "Interferon-gamma treatment elevates caspase-8 expression and sensitizes human breast tumor cells to a death receptor-induced mitochondria-operated apoptotic program." Cancer Res **60**: 5673-5680.

Saelens, X., N. Festjens, L. Vande Walle, M. van Gurp, G. van Loo and P. Vandenabeele (2004). "Toxic proteins released from mitochondria in cell death." Oncogene **23**: 2861-2874.

Salvesen, G. S. and V. M. Dixit (1999). "Caspase activation: the induced-proximity model." Proc Natl Acad Sci U S A **96**: 10964-10967.

Saraste, A. and K. Pulkki (2000). "Morphologic and biochemical hallmarks of apoptosis." Cardiovasc Res **45**: 528-537.

Sasagawa, T., M. Hlaing and T. Akaike (2000). "Synergistic induction of apoptosis in murine hepatoma Hepa1-6 cells by IFN-gamma and TNF-alpha." Biochem Biophys Res Commun **272**: 674-680.

Sasajima, J., S. Tanno, K. Koizumi, Y. Nakano, A. Habiro, A. Chiba, T. Fujii, Y. Sugiyama, K. Nakamura, T. Nishikawa, Y. Mizukami, T. Okumura and Y. Kohgo (2009). "[Gemcitabine in combination with S-1 or UFT in patients with advanced pancreatic cancer]." Gan To Kagaku Ryoho **36**: 1657-1661.

Schiedner G., S. Hertel and S. Kochanek (2000) “Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production.” Hum Gene Ther **11**:2105-2116.

Shabram, P. W., D. D. Giroux, A. M. Goudreau, R. J. Gregory, M. T. Horn, B. G. Huyghe, X. Liu, M. H. Nunnally, B. J. Sugarman and S. Sutjipto (1997). "Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles." Hum Gene Ther **8**: 453-465.

Sharma, A., X. Li, D. S. Bangari and S. K. Mittal (2009). "Adenovirus receptors and their implications in gene delivery." Virus Res **143**: 184-194.

Shayakhmetov, D. M., T. Papayannopoulou, G. Stamatoyannopoulos and A. Lieber (2000). "Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector." J Virol **74**: 2567-2583.

Shenoy, K., Y. Wu and S. Pervaiz (2009). "LY303511 enhances TRAIL sensitivity of SHEP-1 neuroblastoma cells via hydrogen peroxide-mediated mitogen-activated protein kinase activation and up-regulation of death receptors." Cancer Res **69**: 1941-1950.

Shi, Y. (2002). "Mechanisms of caspase activation and inhibition during apoptosis." Mol Cell **9**: 459-470.

Shimizu, S., Y. Eguchi, W. Kamiike, Y. Funahashi, A. Mignon, V. Lacronique, H. Matsuda and Y. Tsujimoto (1998). "Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux." Proc Natl Acad Sci U S A **95**: 1455-1459.

Shin, E. C., J. M. Ahn, C. H. Kim, Y. Choi, Y. S. Ahn, H. Kim, S. J. Kim and J. H. Park (2001). "IFN-gamma induces cell death in human hepatoma cells through a TRAIL/death receptor-mediated apoptotic pathway." Int J Cancer **93**: 262-268.

Shrader, M., M. S. Pino, L. Lashinger, M. Bar-Eli, L. Adam, C. P. Dinney and D. J. McConkey (2007). "Gefitinib reverses TRAIL resistance in human bladder cancer cell lines via inhibition of AKT-mediated X-linked inhibitor of apoptosis protein expression." Cancer Res **67**: 1430-1435.

Siegel, R. M., J. K. Frederiksen, D. A. Zacharias, F. K. Chan, M. Johnson, D. Lynch, R. Y. Tsien and M. J. Lenardo (2000). "Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations." Science **288**: 2354-2357.

Siegelin, M. D., D. E. Reuss, A. Habel, C. Herold-Mende and A. von Deimling (2008). "The flavonoid kaempferol sensitizes human glioma cells to TRAIL-mediated apoptosis by proteasomal degradation of survivin." Mol Cancer Ther **7**: 3566-3574.

Song, J. H., A. Bellail, M. C. Tse, V. W. Yong and C. Hao (2006). "Human astrocytes are resistant to Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis." J Neurosci **26**: 3299-3308.

Song, J. H., D. K. Song, B. Pyrzynska, K. C. Petruk, E. G. Van Meir and C. Hao (2003). "TRAIL triggers apoptosis in human malignant glioma cells through extrinsic and intrinsic pathways." Brain Pathol **13**: 539-553.

Song, J. J., J. Y. An, Y. T. Kwon and Y. J. Lee (2007). "Evidence for two modes of development of acquired tumor necrosis factor-related apoptosis-inducing ligand resistance. Involvement of Bcl-xL." J Biol Chem **282**: 319-328.

Sorensen, M. R., P. J. Holst, H. Pircher, J. P. Christensen and A. R. Thomsen (2009). "Vaccination with an adenoviral vector encoding the tumor antigen directly linked to invariant chain induces potent CD4(+) T-cell-independent CD8(+) T-cell-mediated tumor control." Eur J Immunol **39**: 2725-2736.

Spee, B., M. D. Jonkers, B. Arends, G. R. Rutteman, J. Rothuizen and L. C. Penning (2006). "Specific down-regulation of XIAP with RNA interference enhances the sensitivity of canine tumor cell-lines to TRAIL and doxorubicin." Mol Cancer **5**: 34.

Srinivasula, S. M., A. Saleh, M. Ahmad, T. Fernandes-Alnemri and E. S. Alnemri (2001). "Isolation and assay of caspases." Methods Cell Biol **66**: 1-27.

Stennicke, H. R., Q. L. Deveraux, E. W. Humke, J. C. Reed, V. M. Dixit and G. S. Salvesen (1999). "Caspase-9 can be activated without proteolytic processing." J Biol Chem **274**: 8359-8362.

Sui, G., C. Soohoo, B. Affar el, F. Gay, Y. Shi, W. C. Forrester and Y. Shi (2002). "A DNA vector-based RNAi technology to suppress gene expression in mammalian cells." Proc Natl Acad Sci U S A **99**: 5515-5520.

Sussman, R. T., M. S. Ricci, L. S. Hart, S. Y. Sun and W. S. El-Deiry (2007). "Chemotherapy-resistant side-population of colon cancer cells has a higher sensitivity to TRAIL than the non-SP, a higher expression of c-Myc and TRAIL-receptor DR4." Cancer Biol Ther **6**: 1490-1495.

Takahashi, R., Q. Deveraux, I. Tamm, K. Welsh, N. Assa-Munt, G. S. Salvesen and J. C. Reed (1998). "A single BIR domain of XIAP sufficient for inhibiting caspases." J Biol Chem **273**: 7787-7790.

Tamer, R, L. Voti, L. E. Fleming, J. MacKinnon , D. Thompson , M. Blake , J. A. Bean , L.C. Richardson (2003). “A feasibility study of the evaluation of the Florida breast cancer early detection program using the statewide cancer registry.” Breast Cancer Res Treat. 3:187-94.

Teitz, T., T. Wei, M. B. Valentine, E. F. Vanin, J. Grenet, V. A. Valentine, F. G. Behm, A. T. Look, J. M. Lahti and V. J. Kidd (2000). "Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN." Nat Med **6**: 529-535.

Thiagalingam S. , K. H. Cheng, H.J. Lee, N. Mineva, A. Thiagalingam, J.F. Ponte (2003). “Histone deacetylases: unique players in shaping the epigenetic histone code.” Ann N Y Acad Sci. 983:84-100.

Thompson, C. B. (1995). "Apoptosis in the pathogenesis and treatment of disease." Science **267**: 1456-1462.

Tong, H. X., C. W. Lu, J. H. Zhang and J. H. Zhang (2008). "Enhanced effect of IFNgamma on the induced apoptosis of neuroblastoma cells by cytotoxic drugs." Pediatr Hematol Oncol **25**: 549-558.

Tucker, S. N., D. W. Tingley and C. D. Scallan (2008). "Oral adenoviral-based vaccines: historical perspective and future opportunity." Expert Rev Vaccines **7**: 25-31.

Tuve, S., Y. Liu, K. Tragoolpua, J. D. Jacobs, R. C. Yumul, Z. Y. Li, R. Strauss, K. E. Hellstrom, M. L. Disis, S. Roffler and A. Lieber (2009). "In situ adenovirus vaccination engages T effector cells against cancer." Vaccine **27**: 4225-4239.

Twiddy, D., D. G. Brown, C. Adrain, R. Jukes, S. J. Martin, G. M. Cohen, M. MacFarlane and K. Cain (2004). "Pro-apoptotic proteins released from the mitochondria regulate the protein composition and caspase-processing activity of the native Apaf-1/caspase-9 apoptosome complex." J Biol Chem **279**: 19665-19682.

van Gurp, M., N. Festjens, G. van Loo, X. Saelens and P. Vandenabeele (2003). "Mitochondrial intermembrane proteins in cell death." Biochem Biophys Res Commun **304**: 487-497.

van Loo, G., X. Saelens, F. Matthijssens, P. Schotte, R. Beyaert, W. Declercq and P. Vandenabeele (2002). "Caspases are not localized in mitochondria during life or death." Cell Death Differ **9**: 1207-1211.

Varfolomeev, E. E., M. Schuchmann, V. Luria, N. Chiannilkulchai, J. S. Beckmann, I. L. Mett, D. Rebrikov, V. M. Brodianski, O. C. Kemper, O. Kollet, T. Lapidot, D. Soffer, T. Sobe, K. B. Avraham, T. Goncharov, H. Holtmann, P. Lonai and D. Wallach (1998). "Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally." Immunity **9**: 267-276.

Vaux, D. L. (2002). "Apoptosis timeline." Cell Death Differ **9**: 349-354.

Vaux, D. L., S. Cory and J. M. Adams (1988). "Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells." Nature **335**: 440-442.

Vaux, D. L. and S. J. Korsmeyer (1999). "Cell death in development." Cell **96**: 245-254.

Vaux, D. L. and J. Silke (2003). "HtrA2/Omi, a sheep in wolf's clothing." Cell **115**: 251-253.

Vogler, M., K. Durr, M. Jovanovic, K. M. Debatin and S. Fulda (2007). "Regulation of TRAIL-induced apoptosis by XIAP in pancreatic carcinoma cells." Oncogene **26**: 248-257.

Vogler, M., H. Walczak, D. Stadel, T. L. Haas, F. Genze, M. Jovanovic, U. Bhanot, C. Hasel, P. Moller, J. E. Gschwend, T. Simmet, K. M. Debatin and S. Fulda (2009). "Small molecule XIAP inhibitors enhance TRAIL-induced apoptosis and antitumor activity in preclinical models of pancreatic carcinoma." Cancer Res **69**: 2425-2434.

Vogler, M., H. Walczak, D. Stadel, T. L. Haas, F. Genze, M. Jovanovic, J. E. Gschwend, T. Simmet, K. M. Debatin and S. Fulda (2008). "Targeting XIAP bypasses Bcl-2-mediated resistance to TRAIL and cooperates with TRAIL to suppress pancreatic cancer growth in vitro and in vivo." Cancer Res **68**: 7956-7965.

Wang, L. D., S. T. Shi, Q. Zhou, S. Goldstein, J. Y. Hong, P. Shao, S. L. Qiu and C. S. Yang (1994). "Changes in p53 and cyclin D1 protein levels and cell proliferation in different stages of human esophageal and gastric-cardia carcinogenesis." Int J Cancer **59**: 514-519.

Wang, P., J. Zhang, A. Bellail, W. Jiang, J. Hugh, N. M. Kneteman and C. Hao (2007). "Inhibition of RIP and c-FLIP enhances TRAIL-induced apoptosis in pancreatic cancer cells." Cell Signal **19**: 2237-2246.

White-Gilbertson, S. J., L. Kasman, J. McKillop, T. Tirodkar, P. Lu and C. Voelkel-Johnson (2009). "Oxidative stress sensitizes bladder cancer cells to TRAIL mediated apoptosis by down-regulating anti-apoptotic proteins." J Urol **182**: 1178-1185.

Wolf, B. B. and D. R. Green (1999). "Suicidal tendencies: apoptotic cell death by caspase family proteinases." J Biol Chem **274**: 20049-20052.

Wolpin, B. M., A. F. Hezel, T. Abrams, L. S. Blaszkowsky, J. A. Meyerhardt, J. A. Chan, P. C. Enzinger, B. Allen, J. W. Clark, D. P. Ryan and C. S. Fuchs (2009). "Oral mTOR inhibitor everolimus in patients with gemcitabine-refractory metastatic pancreatic cancer." J Clin Oncol **27**: 193-198.

Yao, H. S., Q. Wang, W. J. Wang and Z. Q. Hu (2008). "Intraoperative allogeneic red blood cell transfusion in ampullary cancer outcome after curative pancreatoduodenectomy: a clinical study and meta-analysis." World J Surg **32**: 2038-2046.

Yao, J., Q. Y. Ma, L. C. Wang, M. Zhang and S. G. Shen (2007). "[Construction of the recombinant adenovirus mediated shRNA to silence PTN in pancreatic carcinoma and the effect of DRGn on neurite in vitro]." Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi **23**: 797-800.

Yasui, H., M. Adachi, H. Hamada and K. Imai (2005). "Adenovirus-mediated gene transfer of caspase-8 sensitizes human adenocarcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand." Int J Oncol **26**: 537-544.

Yeh, C. C., Y. T. Deng, D. Y. Sha, M. Hsiao and M. Y. Kuo (2009). "Suberoylanilide hydroxamic acid sensitizes human oral cancer cells to TRAIL-induced apoptosis through increase DR5 expression." Mol Cancer Ther **8**: 2718-2725.

Yodkeeree, S., B. Sung, P. Limtrakul and B. B. Aggarwal (2009). "Zerumbone enhances TRAIL-induced apoptosis through the induction of death receptors in human colon cancer cells: Evidence for an essential role of reactive oxygen species." Cancer Res **69**: 6581-6589.

Yoo, C., J. Y. Hwang, J. E. Kim, T. W. Kim, J. S. Lee, D. H. Park, S. S. Lee, D. W. Seo, S. K. Lee, M. H. Kim, D. J. Han, S. C. Kim and J. L. Lee (2009). "A randomised phase II study of modified FOLFIRI.3 vs modified FOLFOX as second-line therapy in patients with gemcitabine-refractory advanced pancreatic cancer." Br J Cancer **101**: 1658-1663.

Yoshida, T., M. Horinaka, M. Takara, M. Tsuchihashi, N. Mukai, M. Wakada and T. Sakai (2008). "Combination of isoliquiritigenin and tumor necrosis factor-related apoptosis-inducing ligand induces apoptosis in colon cancer HT29 cells." Environ Health Prev Med **13**: 281-287.

Young, J. I. and J. R. Smith (2001). "DNA methyltransferase inhibition in normal human fibroblasts induces a p21-dependent cell cycle withdrawal." J Biol Chem **276**: 19610-19616.

Yu, J. Y., S. L. DeRuiter and D. L. Turner (2002). "RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells." Proc Natl Acad Sci U S A **99**: 6047-6052.

Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis and H. R. Horvitz (1993). "The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme." Cell **75**: 641-652.

Yuan, J. and B. A. Yankner (2000). "Apoptosis in the nervous system." Nature **407**: 802-809.

Zabner, J., P. Freimuth, A. Puga, A. Fabrega and M. J. Welsh (1997). "Lack of high affinity fiber receptor activity explains the resistance of ciliated airway epithelia to adenovirus infection." J Clin Invest **100**: 1144-1149.

Zamore, P. D. (2002). "Ancient pathways programmed by small RNAs." Science **296**: 1265-1269.

Zangemeister-Wittke, U. (2003). "Antisense to apoptosis inhibitors facilitates chemotherapy and TRAIL-induced death signaling." Ann N Y Acad Sci **1002**: 90-94.

Zhang, L. and B. Fang (2005). "Mechanisms of resistance to TRAIL-induced apoptosis in cancer." Cancer Gene Ther **12**: 228-237.

Zhang, L., H. Zhu, F. Teraishi, J. J. Davis, W. Guo, Z. Fan and B. Fang (2005). "Accelerated degradation of caspase-8 protein correlates with TRAIL resistance in a DLD1 human colon cancer cell line." Neoplasia **7**: 594-602.

Zhao, P., Y. H. Zhu, J. X. Wu, R. Y. Liu, X. Y. Zhu, X. Xiao, H. L. Li, B. J. Huang, F. J. Xie, J. M. Chen, M. L. Ke and W. Huang (2007). "Adenovirus-mediated delivery of human IFNgamma gene inhibits prostate cancer growth." Life Sci **81**: 695-701.

Zhu, H., Y. Zhu, J. Hu, W. Hu, Y. Liao, J. Zhang, D. Wang, X. Huang, B. Fang and C. He (2007). "Adenovirus-mediated small hairpin RNA targeting Bcl-XL as therapy for colon cancer." Int J Cancer **121**: 1366-1372.

Zhu, H. B., X. F. Huang, J. Z. Hu, W. Zhou, W. Chen, L. L. Chen and C. He (2008). "[Overcoming acquired resistance to tumor necrosis factor-related apoptosis-inducing ligand by Bcl-XL small interfering RNA in human colon cancer]." Zhonghua Zhong Liu Za Zhi **30**: 245-249.

Zong, H., B. Yin, J. Chen, B. Ma, D. Cai and X. He (2009). "Over-expression of c-FLIP confers the resistance to TRAIL-induced apoptosis on gallbladder carcinoma." Tohoku J Exp Med **217**: 203-208.

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