Characterization of TRAIL-mediated apoptotic pathways in the lung cancer cell line NCI-H460 with focus on caspase-3 and XIAP

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Erklärung

I hereby declare that I performed the present thesis independently without further help or other materials than stated.

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List of abbreviations

˚C	degree Celsius
Apaf-1	apoptosis protease activating factor-1
APS	ammonium persulfate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma extra large
BH	Bcl-2 homology domain
Bid	BH3 interacting domain death agonist
BIR	baculovirus IAP repeat
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
cFLIP	cellular FLICE inhibitory protein
cm	centimeter
CO_2	carbon dioxide
CRD	cysteine-rich domain
$Db_{\alpha EGFR}$-scTRAIL	EGFR targeting diabody-single chain TRAIL
DD	death domain
DED	death effector domain
DIABLO	direct IAP-binding protein with low isoelectric point
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e.g.	*exempli gratia* (example given)
EGFR	epidermal growth factor receptor
et al.	*et alii* (and others)
FACS	fluorescence activated cell sorting
FADD	Fas associated protein with death domain
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
g	grams
gt	goat
h	hours
HRP	horseradish peroxidase
i.e.	id est (that is)
IAP	inhibitor of apoptosis protein
IgG	immunoglobulin G
iz	isoleucine zipper
Kb	kilo basepairs
kDa	kilo Dalton
m	milli
M	molar
mA	milli Ampère
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinases
MDM2	mouse double minute 2 homolog
min	minutes
ml	milliliters
MOMP	mitochondrial outer membrane permeabilization
MW	molecular weight
n	nano (10^{-9})
NFκB	nuclear factor 'kappa-light-chain enhancer' of activated B cells
NSCLC	non-small cell lung cancer
OD	optical density
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
PARP	poly ADP ribose polymerase
PBA	PBS with BSA and azide
PBS	phosphate buffered saline
PBST	PBS with Tween-20
PCR	polymerase chain reaction
PCD	programmed cell death
PFA	paraformaldehyde

PLAD	pre-ligand binding assembly domain
PMSF	phenylmethylsulphonyl fluoride
RANKL	receptor activator of nuclear factor κB ligand
rpm	revolutions per minute
RT	room temperature
s	seconds
SCLC	small cell lung cancer
SDS	sodium dodecyl sulfate
siRNA	short-interfering RNA
Smac	Second mitochondrial-derived activator of caspase
tBid	truncated Bid
TEMED	N, N, N', N'-tetramethylethyl diamine
TMRM	tetramethylrodamine methyl ester
TNF	tumor-necrosis factor
TNFR	TNF receptor
TRADD	TNF receptor type-1 associated protein with death domain
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR1	TRAIL receptor 1 (death receptor 4)
TRAILR2	TRAIL receptor 2 (death receptor 5)
TRAILR3	TRAIL receptor 3 (decoy receptor 1)
TRAILR4	TRAIL receptor 4 (decoy receptor 2)
Tris	Tris- (hydroxymethyl) -aminomethan
U	unit
V	volt
v/v	volume/volume
w/v	weight/volume
wt	wild type
XIAP	X-chromosome-linked IAP (inhibitor of apoptosis protein)
$\Delta\Psi_m$	mitochondrial membrane potential

Summary

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily of cytokines, was discovered in mid-90s. Due to its unique capability of selectively killing tumor cells without affecting normal tissues, it gained much attention as a potential tumor therapeutic. Intensive research has been done during the last two decades to utilize the natural cytokine and derived molecules thereof as effective death inducer for cancer therapy. Unfortunately a big hurdle is the primary or acquired resistance of many tumor cells to TRAIL. Since TRAIL can activate both the extrinsic as well as the intrinsic pathway of apoptosis, blocking the intrinsic mitochondrial amplification loop in so-called type II cells can confer apoptosis resistance to TRAIL.

The human non-small cell lung cancer cell line NCI-H460 is characterized as a chemoresistant cell line due to a dysfunctional mitochondrial apoptotic pathway. This thesis aimed at the investigation of the underlying molecular mechanisms that regulate the intrinsic apoptotic pathway of NCI-H460 in response to TRAIL. Therefore a NCI-H460 cell line, stably overexpressing Bcl-2, was established. The resulting 2-3 fold increase in Bcl-2 was capable to switch the TRAIL sensitive NCI-H460 cell line into a highly TRAIL resistant phenotype, demonstrating that the mitochondria are indispensable for NCI-H460 cells to execute apoptosis. Further, overexpression of Bcl-2 preserved the outer mitochondrial membrane integrity after TRAIL application and fully inhibited cytochrome c and Smac release into the cytosol. Consequently, this resulted in the inhibition of caspase-8, -3 and -9 activation and full processing. In particular, the final cleavage step of the executioner caspase-3 fragment p19 to p17 was effectively blocked by Bcl-2 overexpression. In addition, Bcl-2 overexpression delayed activation of the initiator caspase-8. While caspase-8 is generally believed to be activated both upstream and downstream of the mitochondrial pathway, inhibition of its activation in cells with blocked mitochondria points to the importance of downstream activation of caspase-8 in NCI-H460 cells *via* a positive feedback loop initiated by activated caspase-3.

Since the inhibitor of apoptosis protein family member XIAP is accepted as a central regulator of apoptosis by directly inhibiting caspases, another focus of the present work was to elucidate its role in TRAIL-mediated apoptosis in NCI-H460. Overexpression of full length XIAP or its BIR domains showed limited protective effects in TRAIL-induced apoptosis. In contrast, targeting

XIAP *via* a Smac mimetic or by siRNA-mediated downregulation of its protein expression rendered Bcl-2 overexpressing cells sensitive to TRAIL. Importantly, XIAP knockdown in combination with TRAIL promoted the autocleavage of caspase-3 to generate the catalytically active p17 fragment, whose production was otherwise blocked in Bcl-2 overexpressing NCI-H460 cells. Thus our data strongly suggest that XIAP-mediated inhibition of caspase-3 is required to protect NCI-H460 cells from TRAIL-induced apoptosis. Taken together we hypothesize that the endogenous cytosolic XIAP levels allow efficient inhibition of caspase-3 thereby playing a crucial role in TRAIL resistance of NCI-H460/Bcl-2 cells. These results are in accordance with a model from literature proposing that reduced XIAP expression can bypass the mitochondria-dependent (type II-like) activation of caspases, thereby phenotypically converting a type II cell to type I. It can be concluded that in NCI-H460 cells especially the expression level of XIAP and its inhibition of activated caspase-3 as well as the counteracting XIAP degradation by activated caspase-3 control the apoptotic program and hence the final cellular fate.

Zusammenfassung

Das Zytokin Tumornekrosefaktor-verwandter Apoptose-induzierender Ligand (tumor necrosis factor-related apoptosis-inducing ligand, TRAIL) ist ein Mitglied der TNF Superfamilie und wurde Mitte der 90er Jahre entdeckt. Wegen seiner besonderen Fähigkeit selektiv Tumorzellen zu töten, dabei aber normalen Zellen nicht zu schaden, hat es viel Beachtung als ein potentielles Tumortherapeutikum erlangt. Tatsächlich wurden während der letzten 20 Jahre intensive Forschungen durchgeführt mit dem Ziel das natürliche Zytokin bzw. davon abgeleitete Moleküle als effektive Auslöser von Zelltod in der Krebstherapie einzusetzen. Allerdings stellte sich heraus, dass viele Tumore resistent gegen TRAIL sind oder während einer Behandlung resistent werden. Da TRAIL fähig ist sowohl den extrinsischen als auch den intrinsischen Weg der Apoptose zu aktivieren, kann eine Blockade der intrinsischen mitochondrialen Verstärkunsschleife in sogenannten Typ II Zellen eine Resistenz gegen TRAIL verursachen.

Die humane nicht-kleinzellige Lungenkrebszelllinie NCI-H460 wird auf Grund ihres dysfunktionalen mitochondrialen Signalweges als chemoresistent bezeichnet. In dieser Arbeit sollten nun die dem intrinsischen apoptotischen Signalweg zugrundeliegenden molekularen Regulationsmechanismen in NCI-H460 nach TRAIL Stimulation untersucht werden. Hierfür wurde eine stabil Bcl-2 überexprimierende NCI-H460 Zelllinie generiert. Tatsächlich konnte hiermit gezeigt werden, dass die mitochondriale Verstärkungsschleife notwendig ist, um TRAIL vermittelten Zelltod in NCI-H460 auszulösen, da die erreichte 2-3 fache Erhöhung der Bcl-2 Menge ausreichte, die an sich TRAIL empfindlichen NCI-H460 Zellen in einen TRAIL resistenten Phänotyp umzuwandeln. Desweiteren schützte die Überexpression von BCl-2 die Integrität der äußere mitochondriale Membran nach TRAIL Applikation und verhinderte vollständig die Ausschüttung von cytochrom c und Smac/Diablo in das Zytoplasma, was in Folge zu einer Inhibierung der Caspase-8,-3-und-9 Aktivierung bzw. deren kompletten Prozessierung führte. Insbesondere wurde der finale Schritt in der Prozessierung von Caspase 3 (p19 → p17) durch die Überexprimierung von Bcl-2 verhindert und zusätzlich die Aktivierung von Caspase-8 verzögert. Bisher wurde allgemein angenommen, dass Caspase-8 im Signalweg vor aber auch dem Mitochondrium nachgeschaltet aktiviert werden kann. Die Inhibierung der Aktivierung von Caspase-8 in Bcl-2 überexprimierenden und damit in der mitochondrialen Verstärkungsschleife blockierten NCI-H460 Zellen deutet allerdings daraufhin, dass in unserem zellulären Modell vor allem die

dem Mitochondrium nachgeschaltete Aktivierung von Caspase-8 durch einen positive Rückkopplungsschleife über Caspase-3 wichtig ist.

Da XIAP, ein Mitglied der Familie der Apoptose hemmenden IAP-Proteine, durch seine Fähigkeit zur direkten Inhibierung von Caspasen als zentraler Regulator der Apoptose angesehen wird, wurde im Rahmen dieser Arbeit auch dessen Rolle in NCI-H460 Zellen untersucht. Überexpression von vollständigem XIAP, genauso wie der der einzelnen BIR2 bzw. BIR3 Domäne zeigte kaum Auswirkungen in Bezug auf TRAIL induzierte Apoptose. Im Gegensatz dazu führte die Behandlung mit dem Smac-Mimetikum SM83 genauso wie ein siRNA-vermittelter knockdown des Moleküls zu einer Sensitivierung der Bcl-2 überexprimierenden Zellen gegenüber TRAIL. Desweiteren bewirkte der *knockdown* von XIAP in Kombination mit TRAIL Stimulation eine proteolytische Aktivierung von Caspase-3 bis hin zum katalytisch aktiven p17 Fragment, dessen Produktion in Bcl-2 überexprimierenden Zellen zuvor blockert war. Unsere Daten deuten somit stark daraufhin, dass die Inhibierung von Caspase-3 durch XIAP notwendig ist um TRAIL induzierte Apoptose in NCI-H460 Zellen zu verhindern. Zusammenfassend gesagt vertreten wir die Hypothese, dass erhöhte Mengen von XIAP im Zytoplasma und deren effiziente Hemmung von Caspase-3 eine vorherrschende Rolle in der TRAIL Resistenz von NCI-H460/Bcl-2 Zellen spielt. Diese Ergebnisse stehen im Einklang mit einem Modell aus der Literatur welches vorschlägt, dass verminderte XIAP Expression die Mitochondrien abhängige (Typ II) Aktivierung von Caspasen umgehen kann. Daraus kann geschlossen werden, dass in NCI-H460 Zellen vor allem die Expression von XIAP in Zusammenhang mit der daraus folgenden Inhibierung von aktivierter Caspase-3, genauso wie der entgegenwirkende Abbau von XIAP durch aktivierte Caspase-3 das apoptotische Programm und daher auch das Schicksal der Zellen kontrollieren.

1. Introduction

1.1. Lung cancer

Cancer is a wide group of diseases characterized by abnormal cell growth with the possibility to spread gradually to other parts of the body. In 2012 it caused 8.2 million deaths, which is approximately 15% of all human deaths (World Health Organization). More than 100 types of cancer are known, the most frequent tumor in males are lung, prostate, colorectal and stomach cancer while in females, the most common types are breast, colorectal, lung and cervical cancer (World cancer report 2014, WHO).

Worldwide, lung cancer is the most common cause of cancer-related death in men and among women has the third highest incidence, and was responsible for 1.56 million deaths in 2012 (World Health Organization). It is characterized by the uncontrolled cell growth in tissues of the lung. Lung carcinomas are categorized by the size and appearance of malignant cells into two types: non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). NSCLC are the most frequent form accounting for 85-90% of total lung cancers (American Cancer Society). NSCLC can be further divided into different forms depending upon the site of origin. Squamous or epidermoid carcinoma begins in squamous cells, cancer that may begin in large cells is called large cell carcinoma while adenocarcinoma is the name for cancer which initiates in the cells that line the alveoli and produce for example mucus. There are also some other types which are not very common like pleomorphic, carcinoid tumor and salivary gland carcinoma. Development of targeted therapy plays a huge role in the treatment of many cancers but its role is limited in treating lung cancer due to acquired drug resistance, possibly caused by specific mutations in certain genes like epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling pathways. Identification of these mutations has helped a lot in developing molecular targeted therapy to improve the survival of patients with metastatic disease.

1.2. Apoptosis

The word ‘‘apoptosis’’ comes from the Greek words apo and ptosis, which mean falling off of the leaves of trees in autumn, and was first introduced by John Kerr in 1972 referring to the morphological feature of ‘‘apoptotic bodies’’ formation from a cell (Kerr et al., 1972). Apoptosis is

a tightly regulated form of controlled cellular self-destruction also called the programmed cell death. A range of morphological changes has been discovered that occur during apoptosis. Cell shrinkage and chromatin condensation are the most characteristic features of early apoptosis (Kerr et al., 1972). Sub cellular changes like chromatin condensation can be better identified by electron microscopy. Massive plasma membrane blebbing occurs and cell fragments are separated into apoptotic bodies by a process called “budding”. These bodies are composed of cytoplasm with firmly packed organelles enclosed within an intact plasma membrane. Consequently these apoptotic bodies are engulfed and phagocytosed by macrophages, parenchymal or neoplastic cells. Another feature of apoptosis is the appearance of the phospholipid phosphatidylserine in the outer leaflet of the plasma membrane. Phosphatidylserine is usually found in the inner leaflet of the plasma membrane. During the apoptotic process however, this phospholipid flips to the outer leaflet, thus becoming a signal for macrophages to dispose of the cell. Importantly, apoptosis causes no inflammation because 1) cellular components of the apoptotic cells are not released in the surrounding tissue; 2) they are quickly engulfed and digested by surrounding cells hence preventing secondary necrosis; 3) macrophages that phagocytosed these bodies, do not release any inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003).

Apoptotic processes are of widespread biological significance, being involved in development, differentiation, proliferation, homeostasis, regulation and function of the immune system and in the removal of defective and therefore harmful cells. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions. Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative disorders, acquired immune deficiency syndrome (AIDS) and ischemic diseases are caused or enhanced by excessive apoptosis.

At the center of apoptosis is a cascade of proteases, the caspases, which finally cleave key components within cells, thus leading to the inevitable destruction of the cell. The term caspases is derived from cysteine-dependent aspartate-specific proteases. In the cell, caspases are synthesized as inactive zymogens, so called procaspases. Upon maturation, the procaspases are proteolytically processed resulting in a small and a large subunit. A heterotetramer consisting of two small and two large subunits then forms an active caspase. Caspases can be divided into a group of initiator caspases including caspases-2, -8, -9, -10, and into a group of executioner (effector) caspases including caspasese-3, -6 and -7.

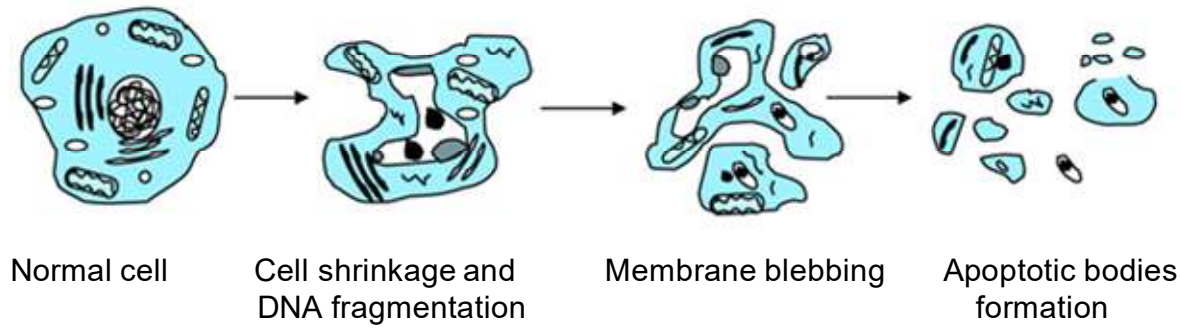

Figure 1: Morphological changes in the cell during apoptosis

During the early process of apoptosis the cell will shrink and chromatin condensation will occur, which is the most characteristic feature of apoptosis. Later on, the cell detaches from the surrounding tissues and extensive plasma membrane blebbing occurs followed by formation of apoptotic bodies. These apoptotic bodies consist of cytoplasm with tightly packed organelles within an intact plasma membrane. Neighboring cells, including macrophages, parenchymal or neoplastic cells, rapidly phagocytose these apoptotic bodies (modified from Padanilam, 2003).

In contrast to programmed cell death, necrosis is another major form of cell death which is considered to be uncontrolled and toxic. Nature of the cell death stimuli, tissue type and developmental stage of the tissue are the factors which are in part responsible for whether a cell will die by apoptosis or necrosis (reviewed in Nikoletopoulou et al., 2013). Most important morphological changes observed in necrosis are cell swelling, cytoplasmic vacuole formation, swollen and ruptured mitochondria, disrupted organelle membranes and ultimately disruption of the cell membrane (Kerr et al., 1972; Majno and Joris, 1995; Trump et al., 1997). Cytoplasmic contents are released into the surrounding tissues due to the loss of cell membrane integrity, sending chemotactic signals to the inflammatory cells.

1.2.1. Extrinsic and intrinsic pathways of apoptosis

Two main signaling pathways have been delineated to initiate the apoptotic suicide program in mammalian cells, the intrinsic and extrinsic pathways (Ashkenazi, 2002). The extrinsic pathway is induced by the activation of transmembrane receptors of the so called “death receptor” subgroup within the TNF receptor family which transmit apoptotic signals after ligation with their specific ligands. Death receptors share common cysteine-rich extracellular ligand-binding domains, typical for all TNF receptor family members, but also a cytoplasmic region comprising of a so called death domain of approximately 80 amino acids (Ashkenazi and Dixit, 1998). Activated death receptors recruit intracellular adopter molecules such as tumor necrosis factor

receptor type 1-associated protein with death domain (TRADD) and Fas-associated protein with death domain (FADD), *via* death domain interaction. A death-inducing signaling complex (DISC) is formed by the interactions of the death effector domains (DED), which are present in both FADD and pro-caspase-8. Cellular FLICE inhibitory protein (c-FLIP), a natural antiapoptotic protein, can interfere with the interaction between FADD and caspase-8 by connecting to their DEDs thereby preventing caspase-8 activation (Tschopp et al., 1998). The precise molecular mechanism is still in discussion how procaspase-8 is activated within the DISC, but it is proposed that the low intrinsic protease activity of the procaspase comes into operation by high concentration and close proximity of procaspase-8, resulting subsequently in autoproteolysis and activation of caspase-8 (Padanilam, 2003). Once activated, caspase-8 can in turn cleave and activate downstream caspases to further activate the caspase cascade. Activated effector caspases finally cleave numerous important cellular substrates to trigger the cellular apoptotic events like DNA cleavage and membrane blebbing. In death receptor-mediated apoptosis two distinct cell types have been described, called type I and type II. In type I cells, caspase-8 is directly and strongly activated within the DISC, and activated caspase-8 then directly triggers the activation of caspase-3 and -7 without a need for the involvement of the intrinsic pathway. On the other hand, in so called type II cells, the mitochondrial pathway of apoptosis is required for amplification of the apoptotic signal (Scaffidi et al., 1998).

As illustrated in Figure 2, in type II cells, caspase-8 activated at the DISC cleaves and activates the proapoptotic Bcl-2 family member Bid. Truncated Bid (tBid) then through Bak/Bax activation triggers the mitochondria to release cytochrome c and additional proapoptotic factors like Smac. Cytosolic cytochrome c then initiates apoptosome formation that triggers the activation of the initiator caspase-9 (Acehan et al., 2002). Activated caspase-9 subsequently activates caspase-3 and -7 resulting in cell death. On the other hand Smac promotes apoptosis by binding to inhibitor of apoptosis proteins.

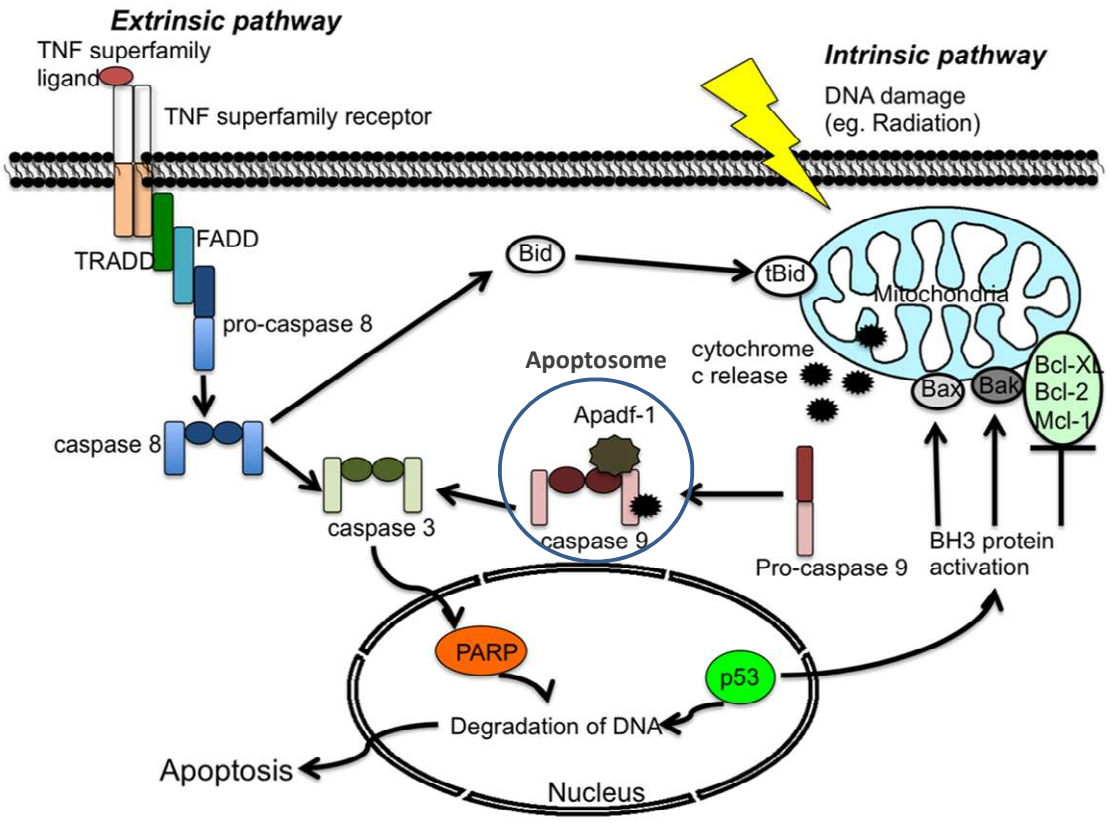

Figure 2: Overview of the extrinsic and intrinsic apoptosis pathways

The extrinsic pathway is triggered by the binding of the TNF family ligand (TRAIL, FasL etc.) to their respective death receptors, followed by the activation of caspase-8 within the DISC. Activated caspase-8 moves to the cytoplasm where it can directly activate effector caspases like caspase-3. Alternatively, active caspase-8 can activate the mitochondrial permeability transition and the intrinsic pathway through the cleavage of Bid into its active form truncated Bid (tBid). tBid translocates from the cytosol to mitochondria, where it induces polymerization of Bak/Bax resulting in mitochondrial depolarization and the release of intermembrane space proapoptotic proteins cytochrome c and Smac into the cytosol. Cytochrome c forms a complex with apoptosis protease activating factor 1, (Apaf-1) and procaspase-9, called the apoptosome while Smac promotes apoptosis by antagonizing inhibitor of apoptosis proteins (modified from Yuan et al., 2012).

The intrinsic apoptotic pathway is activated in response to signals resulting from DNA damage, loss of cell survival factors, or other types of severe cellular stress. The key event in this pathway is the permeabilization of the mitochondrial outer membrane (MOMP), integrity of mitochondrial membrane is mainly controlled by Bcl-2 family members. This large protein family consists of both antiapoptotic and proapoptotic members which either induce or inhibit MOMP (Tsujimoto, 2003). Changes in mitochondrial integrity are primarily initiated by Bak and Bax. It has been suggested that BH3-only proteins Bid or Bad release Bak and Bax from antiapoptotic

antagonists, to become freely available in the cytosol or in mitochondria enabling them to induce apoptosis by competing with the binding partners of Bak and Bax (Cory et al., 2003). Mitochondrial outer membrane permeabilization results in the release of soluble intermembrane proteins into the cytosol. These proapoptotic proteins activate caspase proteases and trigger apoptosis. The intrinsic pathway hinges on the balance of activity between pro and antiapoptotic signals of the Bcl-2 family (Cory and Adams, 2002).

1.2.2. Cross talk between intrinsic and extrinsic pathway

Previously it was considered that execution of cell death by death-receptor and mitochondrial signals takes two separate pathways, but actually these pathways are connected by caspase-8-mediated activation of the BH3-only protein Bid. Even though only a small amount of caspase-8 is activated in type II cells, it is able to provoke the cleavage of Bid; truncated form of Bid (tBid) can migrate from cytosol to mitochondria and causes mitochondrial outer membrane permeabilization and release of proapoptotic proteins from mitochondria, via Bak/Bax activation (Tang et al., 2000).

The topic of cross-talk in different signaling pathways is very common now, existence of cross-talk between death receptor-mediated and mitochondrial pathway is admitted widely (Roy and Nicholson, 2000). Accordingly it is believed that the intrinsic apoptotic pathway is capable to integrate a large number of pro- and antiapoptotic signals finally leading to a clear decision of the cells to either survive or to die.

1.2.3. Apoptosis and cancer

Apoptosis is a major natural process to keep the balance between cell proliferation and death by removing unhealthy and mutated cells. Inhibition of the apoptotic pathway is believed to be crucial in development of cancer, dysregulation of apoptosis is thought as one of the six major hallmarks of cancer (Hanahan and Weinberg, 2000). The established model to explain how cancer therapies work is that anticancer agents prompt the cell to undergo apoptosis, therefore a general resistance to apoptosis will result in resistance to therapy (Brown and Attardi, 2005). Accordingly, the role of defects in the apoptotic machinery that cause therapeutic drug resistance is intensively discussed. However, it is widely accepted that drugs induce cytotoxicity in transformed cells mainly by triggering the apoptotic pathway (Johnstone et al., 2002). Tumor cells which have modifications in proteins engaged in apoptosis frequently resist to

chemotherapy and it is hard to treat them with therapeutic drugs that mainly work by provoking apoptosis, nevertheless targeting proteins e.g. death receptors, Bcl-2 and XIAP that regulate apoptosis, could be a promising strategy in drug development to treat resistant cells.

Disorder in the intrinsic pathway of apoptosis is very frequent in tumor cells, and p53 as a sentinel of cellular stress, is an important regulator of this pathway (Lowe and Lin, 2000). Upon activation p53 plays its role in regulating genes that are involved in either cell cycle arrest, e.g. p21 and p27, or the intrinsic pathway of apoptosis, e.g. Bcl-2 family proteins (Mahalingam et al., 2009). The importance of the p53 pathway in cancer development is emphasized by the fact that in nearly half of all tumors investigated so far p53 was mutated and/or dysregulated. Moreover, deletion of p53 in transgenic mouse models has been shown to accelerate tumor development (Attardi, 2005). MDM2 is an important negative regulator of p53, which binds to the tumor suppressor protein thus interfering with its activity to induce apoptosis in response to cellular stress like DNA damage. Therefore, approaches intended to prevent for example interaction between p53 and its inhibitor MDM2 might be interesting for cancer treatment (Lane and Lain, 2002). Another group of proteins that play a central role in regulating programmed cell death (PCD) are Bcl-2 proteins. Antiapoptotic Bcl-2 proteins are overexpressed in a wide range of tumors, including lung, multiple myeloma, prostate, ovarian, cervical, pancreatic, breast and colorectal cancers, and this overexpression of antiapoptotic members of the family has been shown to be involved in resistance to different cytotoxic stimuli (Ohmori et al., 1993; Minn et al., 1995). Consequently many tumor cells can be sensitized to apoptosis by targeting Bcl-2 proteins (Baell and Huang, 2002). Synthetic peptides have been designed that mimic the action of BH3-only proapoptotic proteins and they are in different phases in clinical trials. An additional downstream group of inhibitor proteins, which play an important role in regulating apoptosis by directly binding caspases, are the IAP molecules. A large number of studies have shown that high expression levels of IAPs can induce apoptotic resistance in tumor cells (Yang et al., 2003; Wrzesien-Kus et al., 2004; Schimmer et al., 2006). XIAP, one particular member of the IAP family, gained much attention due to its ability to directly bind and inhibit initiator and effector caspases. It has been shown that drug-induced apoptosis is interrupted by inhibitor of apoptosis proteins (Deveraux and Reed, 1999). Since chemoresistance of tumor cells has been often associated with dysregulated expression of IAPs these molecules have been considered to represent potential candidates for new targeted tumor therapy approaches (Notarbartolo et al., 2004; Schimmer et al., 2004).

1.3. TNF-related apoptosis inducing ligand (TRAIL)

TNF-related apoptosis-inducing ligand (TRAIL; Apo2L; CD253; TNFSF10) is a type II transmembrane protein of about 34 kDa, which has been discovered by two groups in 1995/1996 (Wiley et al., 1995; Pitti et al., 1996). TRAIL is a member of the tumor necrosis factor (TNF) superfamily of cytokines and like most members of this family it can be cleaved at the cell surface by metalloproteases to form a soluble molecule. TRAIL is constitutively expressed in several cells and tissues, however, few normal cell populations (hepatocytes) are sensitive to this ligand, whereas multiple malignant cell types are highly sensitive (Pitti et al., 1996; Ashkenazi et al., 1999). Preclinical data in mice and non-human primates have shown that TRAIL can suppress tumor growth without systemic toxicity (Ashkenazi et al., 1999; Walczak et al., 1999). Thus, TRAIL has promising potential as therapeutic agent for human cancer.

TRAIL forms homotrimers and can bind to five distinct receptors: TRAILR1 (death receptor 4, DR4), TRAILR2 (death receptor 5, DR5), TRAILR3 (decoy receptor 1, DcR1), TRAILR4 (decoy receptor 2, DcR2) and osteoprotegerin (OPG) (see Figure 3). OPG is a soluble receptor, originally identified as a receptor which binds to ligand for receptor activator of nuclear factor κB (RANKL) and prevents bone resorption by inhibiting the reaction between RANKL and RANK. Later on, it was shown that OPG can also bind TRAIL, however with low affinity (Kelley and Ashkenazi, 2004). The role of OPG under physiological conditions is still unclear, because compared to other TRAIL receptors the affinity of OPG for TRAIL is relatively weak (Truneh et al., 2000).

TRAIL receptors have been divided into two groups; death and decoy receptors, based on the differences in their intracellular domains, though the extracellular domains of all receptors have high homology. TRAILR1 and TRAILR2 are death domain (DD) containing type I transmembrane proteins which mediate apoptosis. In contrast, TRAILR3 lacks an intracellular DD while TRAILR4 retains a cytoplasmic region but has a truncated death domain. Accordingly, neither TRAILR3 nor TRAILR4 is capable to mediate apoptosis upon ligand binding. High expression of TRAILR4 was found to inhibit apoptosis, studies have also shown that overexpression of TRAILR4 can also activate NFκB (Degli-Esposti et al., 1997; Lalaoui et al., 2011). It has been therefore suggested that these receptors may act as regulatory or decoy receptors.

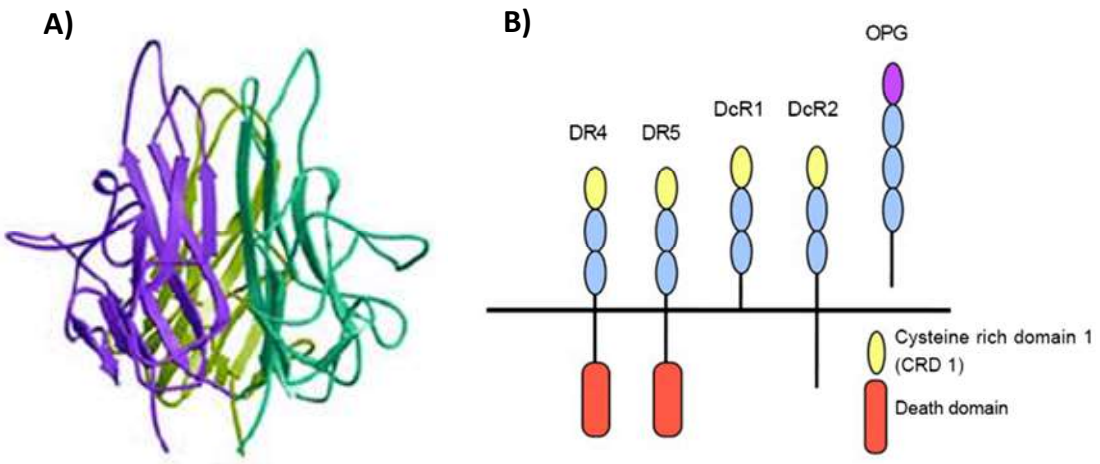

Figure 3: TRAIL and its receptors

A) Side view of ribbon structure of TRAIL trimer depicts β-strands, different colors represents each subunit. **B)** Schematic presentation of five TRAIL receptors. Colored ovals represent the extracellular cysteine-rich domains. TRAILR1 and TRAILR2 possess a death domain (red box) in their cytoplasmic tail. TRAILR3 and TRAILR4 lack death domains and are thus unable to induce apoptotic signaling; hence they can act as decoy receptors. Osteoprotegerin (OPG) is a soluble decoy receptor (modified from Kimberley and Screaton, 2004).

TNFR superfamily members were initially thought to exist as monomers that trimerize on binding of their cognate trimeric ligands. However, substantial evidence now indicates that at least certain TNFRs are preassembled into oligomers before ligation (Chan, 2000). A pre-ligand binding assembly domain (PLAD), located in the first, membrane distal cysteine-rich domain (CRD) of the receptor, mediates the association between monomers. High molecular weight ligand/receptor complexes which are shown to be necessary for signal induction by TNF and FasL are assumed to be formed by not only ligand-receptor interaction but also by receptor-receptor interactions mediated by the PLAD (Krippner-Heidenreich et al., 2002; Branschädel et al., 2010; Lewis et al., 2012). Formation of large receptor-receptor clusters have been also recently identified and suggested to play an important role in signal initiation by TRAIL (Wagner et al., 2007; Valley et al., 2012). However, up to now no precise molecular mechanisms are known about PLAD-mediated TRAIL receptor-receptor interactions. Some studies hypothesized that the formation of homo- and heteromeric receptor complexes is strictly ligand dependent (Merino et al., 2006). On contrary, Clancy and colleagues demonstrated that TRAIL independent receptor-receptor interactions between TRAILR4 and TRAILR2 inhibit TRAIL-induced apoptosis. Furthermore they also showed that the PLAD of TRAILR4 is required for its interactions with TRAILR2 (Clancy et al., 2005). Later on, ligand independent homo- and

heteromeric receptor complexes formation was confirmed by other studies (Lee et al., 2005; Neumann et al., 2014).

Ligation of TRAILR1 or/and TRAILR2 complexes by trimeric TRAIL promotes further clustering of the receptors into high molecular weight complexes and drives formation of the death-inducing signaling complex (Wagner et al., 2007). DISC is composed of oligomerized receptors, the adaptor molecule FADD, the initiator proteases caspase-8 and/or caspase-10, and the cellular FLICE-inhibitory protein (c-FLIP). FADD consists of two protein interaction domains: a death domain (DD) and a death effector domain (DED). FADD binds to the receptor through interactions between DDs and to procaspase-8/-10 through DED. Recruitment of procaspase-8/-10 through FADD leads to its auto-cleavage and activation. Cellular FLIP (c-FLIP) is also capable of binding FADD. Short c-FLIP isoforms, c-FLIPS and c-FLIPR, block DR-induced apoptosis by inhibiting procaspase-8 activation at the DISC (Krueger et al., 2001). The role of the long isoform c-FLIPL at the DISC is still a matter of controversy (Yu and Shi, 2008). Active caspase-8 and -10, in turn, activate effector caspases such as caspase-3 causing the cell to undergo apoptosis (Ashkenazi and Dixit, 1998; Schulze-Osthoff et al., 1998; Suliman et al., 2001).

Besides apoptosis signaling TRAIL has been shown to also induce survival pathways e.g. *via* NFκB activation. NFκB has been reported to induce the expression of cFLIP, Bcl-xL and XIAP, which have been shown to protect cells from death (Dolcet et al., 2005). TRAIL-mediated NFκB activation is significantly attenuated and delayed as compared to that of TNF, and requires a high concentration of the ligand. Thus, NFκB induction by TRAIL may be a secondary, indirect event (Sheridan et al., 1997).

1.3.1. Physiological role of TRAIL

Despite of the fact that TRAIL has been known widely as a potential anti-tumor agent, its role in normal physiological condition is still under debate. The physiological role of TRAIL was mainly investigated by studies which used TRAIL knockout mouse models. TRAIL-deficient mice develop normally without any apparent defects in growth and fertility (Sedger et al., 2002), so it could be concluded that it has no essential function in development. However, defects in the immune system were identified in TRAIL knockout mice indicating that it plays a curial role in regulation of the immune system (Diehl et al., 2004), e.g. in homeostasis of memory T-cells (Janssen et al., 2005). Additionally, TRAIL plays an essential role as negative regulator of

autoimmunity, since increased proliferation of auto reactive lymphocytes was observed after blocking TRAIL in mice (Song et al., 2000).

An important role of TRAIL is contributing to the host immune system in preventing tumor development and metastasis. A number of studies using different tumor cell lines showed that TRAIL can induce apoptosis and suppress tumor growth of carcinomas starting to develop in different tissues including colon, lung, kidney, skin, breast, bladder and pancreas (Ashkenazi et al., 1999; Walczak et al., 1999). In TRAIL$^{-/-}$ mice rapid progression of renal cell carcinoma was observed as compared to wild type control mice and tumors showed enhanced metastasis to the liver. IFNɣ-induced increased expression of TRAIL was observed in natural killer (NK) cells, playing an important role as anti-metastatic cells (Smyth et al., 2001). Another study suggested that TRAIL displays strong proangiogenic properties (Secchiero et al., 2004).

1.3.2. TRAIL in tumor therapy

The current view of developing successful anticancer drugs is that it should selectively kill tumor cells with high potency without harming normal cells (Hall and Cleveland, 2007). TRAIL has proven to be a promising tumor therapeutic since it has been shown to induce apoptosis in many tumor cells which were resistant to chemo- and radiotherapy while the majority of normal cells remained unaffected (Ashkenazi et al., 1999; Walczak et al., 1999). Initially this incident of selective tumor cell killing was thought to be due to a stronger expression of death receptors (TRAILR1 and TRAILR2) on tumor cells, but later on it has been shown that numerous intra- and extracellular factors play a role in protecting normal cells from TRAIL-induced cytotoxicity (MacFarlane, 2003).

A number of modified forms of recombinant TRAIL have been generated with tags like polyhistidine; FLAG or isoleucine zipper (iz). In addition the non-tagged wild-type form of TRAIL has been optimized for the efficiency and selectivity in various tumor models. Some tagged versions of TRAIL appeared to kill tumor cells with higher efficacy but could also show to initiate some toxicity towards human hepatocytes (Lawrence et al., 2001). However, additional work disclosed the fact that only aggregated (His or FLAG tagged) forms of TRAIL are toxic to normal hepatocytes (Koschny et al., 2007), whereas wild type and non-tagged trimerized versions of TRAIL did not show significant cytotoxicity to human hepatocytes and keratinocytes. Monoclonal antibodies against the death receptors (TRAILR1/R2) have also been designed to overcome the resistance mediated by antagonistic receptors. This approach has been proved to be very

effective as these monoclonal antibodies have extended half-lives (days) as compared to recombinant human TRAIL (rhTRAIL) and are efficient inducer of apoptosis (Wiezorek et al., 2010). Recently, apomab, an agonistic antibody which selectively binds and activates TRAILR2 (DR5) was used to treat lung and colon cancer and was shown to trigger a strong tumor cell death induced by clustering of DR5 and activation of caspase-8 (Adams et al., 2008). Other examples of TRAILR2 antibodies are lexatumumab and tigatozumab while mapatumumab is a TRAILR1 agonistic monoclonal antibody.

A major hurdle in developing TRAIL-based therapies is the primary or acquired resistance to TRAIL, as it has been reported that around 50% of all tumor cell lines are unresponsive to TRAIL treatment (Walczak et al., 1999). Likely reasons for this resistance could be either overexpression of antiapoptotic proteins like Bcl-2 or XIAP (Fulda et al., 2002a; Cummins et al., 2004) or defects in death receptor signaling pathways mediated by e.g. epigenetic silencing or mutations in TRAIL receptors (Fisher et al., 2001; Horak et al., 2005). Sensitivity to TRAIL can be restored by using this molecule in combination with conventional chemotherapy or targeted agents. The mechanisms behind the enhancement of apoptosis after combination therapy are different and depend on the drug combined with TRAIL. In various studies, treating a broad range of tumor types with TRAIL in combination with chemotherapeutic drugs like gemcitabine, oxaliplatin and cisplatin or radiotherapy resulted in synergistically sensitizing the tumors to co-treatment. With the advancement in knowledge of TRAIL signaling, administration of sub toxic concentrations of agents against antiapoptotic molecules in combination with TRAIL showed promising results. A few examples include Bcl-2 inhibitors, XIAP inhibitors and Smac mimetics. Prosurvival signaling induced by TRAIL *via* NFκB can be prevented by using proteasome inhibitors like bortezomib. Bortezomib inhibits the degradation of IκB and subsequently prevents NFκB activation. However, this is not the sole function of bortezomib, as it enhances TRAIL-induced cytotoxicity for example in lung cancer cells by mediating an increased TRAILR2 expression, a downregulation of the antiapoptotic protein cFLIP and an enhancement of expression of proapoptotic members of Bcl-2 family (BH3-only) members (Voortman et al., 2007). With the identification of the point(s) of disturbances in the apoptosis signaling pathway in individual cancer type it would more convenient to decide which combination therapy will be more effective in that specific case (Hellwig and Rehm, 2012).

1.4. Bcl-2 family members as regulators of apoptosis

The Bcl-2 family is an important group of proteins which are crucially involved in the regulation of apoptosis. Up to now 25 members of this family have been recognized (Adams and Cory, 1998). As indicated by its name, the gene was first discovered in human B-cell lymphoma, where it was overexpressed due to dysregulation of bcl-2 gene expression at the transcriptional level. Bcl-2 family members were initially characterized by their role in the mitochondrial apoptosis pathway by regulating the integrity of the outer mitochondrial membrane (Green and Evan, 2002). On the basis of their function Bcl-2 and all other family members are categorized as either antiapoptotic or proapoptotic Figure 4. Most cells express a number of both subgroups and the overall balance between pro- and antiapoptotic members finally determines the decision whether to survive or to die. All Bcl-2 family proteins at least share one common functional domain called Bcl-2 homology domain (BH), which is important for the interactions between the family proteins, as heterodimer formation is essential for their regulatory functions.

Antiapoptotic members of the family contain all four defined bcl-2 homology domains (BH1-BH4). The most important members of this group are Bcl-2, Bcl-xL, Bcl-w and Mcl-1; they can directly inhibit the proapoptotic counterparts of the family and maintain the integrity of the outer mitochondrial membrane. The proapoptotic class of the family is subdivided into two groups on the basis of sequence homology within the BH domains i.e. effector proteins and BH3-only proteins.

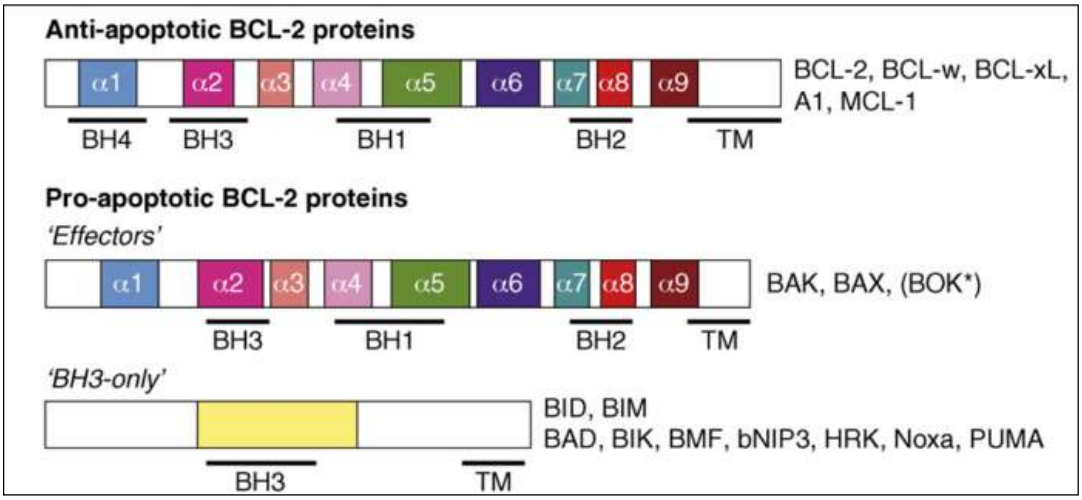

Figure 4: Schematic presentation of Bcl-2 family members

On the basis of Bcl-2 homology domain (BH) composition, members of this family are categorized into three groups. The antiapoptotic members contain four BH domains (BH1-BH4), this group includes Bcl-2, Bcl-xL, Bcl-w and Mcl-1. The proapoptotic members are subdivided into two groups, proapoptotic multi domains (Bax and Bak) which contain BH1-3 domains and BH3-only proteins contain only one conserved domain, the BH3. Mostly, BH3-only members are sub grouped into activators (Bid and Bim) and sensitizers (Bad, Bik, Noxa and PUMA) (taken from Chipuk and Green, 2008).

The Bcl-2 protein gained much attention after realizing that this proto-oncogene can prevent program cell death (PCD) and the inhibition of PCD is a central step in tumorigenesis. Although the complex interaction pattern of the Bcl-2 family is not yet fully understood, its role in mitochondrial membrane integrity was the center of attention for scientist in recent years. As already mentioned, a crucial event in the intrinsic pathway is the permeabilization of mitochondrial outer membrane followed by release of cytochrome c and other proteins into the cytosol. Translocation of Bax from the cytosol to mitochondria is thought to be responsible for the release of cytochrome c (Wolter et al., 1997) by the formation of large multimers which results in pore formation in the mitochondrial outer membrane (Antonsson et al., 1997). Bax and Bak proapoptotic proteins of the Bcl-2 family are extremely important for apoptosis, as integrity of mitochondrial outer membrane was not affected after stimulation with various death inducing agents like ultraviolet (UV), staurosporine, DNA damage etc, in cells with Bak/Bax double knockdown (Wei et al., 2001). Two opposing models exist for Bak/Bax activation, one of them is called direct model, and according to this activator proteins of BH3-only group (Bim, Bid etc.) can directly bind to Bak and Bax, causing conformational changes and activation. Studies have confirmed this interaction using different techniques (Lovell et al., 2008). According to the indirect model, antiapoptotic proteins (Bcl-2, Bcl-xl etc.) prevent the activation of Bak/Bax by binding to them; BH3-only proteins set Bak/Bax free from the inhibition by binding to antiapoptotic proteins (Willis et al., 2007). Activated Bak and Bax oligomers then form pores and permeabilize the outer mitochondrial membrane, followed by release of various proapoptotic proteins (Wang, 2001).

In various tumors, the antiapoptotic proteins of the Bcl-2 family are overexpressed thereby causing apoptosis inhibition and drug resistance. It is generally believed that tumor cells can sustain their survival by Bcl-2 overexpression. Inhibition of Bcl-2 and other antiapoptotic proteins of the family e.g. Bcl-xL and Mcl-1 sensitize tumor cells to chemotherapy, so combining traditional therapy with Bcl-2 inhibitors showed promising success in treating resistant tumors (Kang and Reynold, 2009).

1.5. Inhibitor of apoptosis protein (IAP) family

The first inhibitor of apoptosis (IAP) gene and its product was discovered in baculovirus where it efficiently protected against death of infected cells (Crook et al., 1993). Later on, homologous genes were identified in a number of other organisms ranging from yeast to humans (Deveraux and Reed, 1999). Till now, eight members of the family have been identified in humans: NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/ML-IAP (BIRC7) and IAP-like protein 2 (BIRC8) (Vucic and Fairbrother, 2007). IAP family proteins are characterized by a domain of ~70 amino acids termed as baculoviral IAP repeat (BIR) which are thought to be important for their antiapoptotic activity (Crook et al., 1993). Some members of the group also contain a c-terminal RING finger domain (Borden, 2000). XIAP, c-IAP1 and c-IAP2 contain three BIR domains and a RING domain. The RING finger domain has been shown to have E3 ubiquitin ligase activity (Yang et al., 2000).

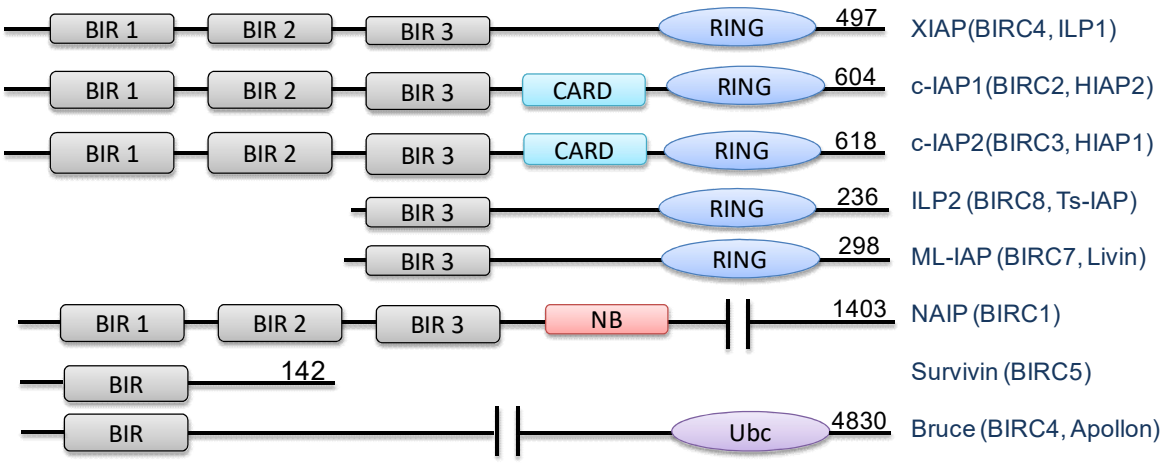

Figure 5: Inhibitor of apoptosis protein (IAP) family

Eight human IAP family members have been identified so far, characterized by the presence of at least one BIR domain. In addition to the BIR domain many IAP molecules also contain an E3 ligase, RING finger motif. On the basis of homology of BIR domains and the presence of RING domains the IAP family is divided into three classes. XIAP, c-IAP1, c-IAP2, ILP2 and ML-IAP are classified as Class I, they contain one RING finger domain in addition to BIR domain. Class II comprises of NAIP, the first discovered member of the family, consists of three BIR domains but no RING domain. Survivin and Bruce are included in class III, having no RING domain. Survivin has one BIR domain while Bruce possesses an ubiquitin conjugating (Ubc) domain in addition to one BIR domain.

The IAP family is considered to comprise the most important negative regulators of apoptosis since these molecules can control both the intrinsic and the extrinsic pathway (Deveraux and Reed, 1999). Although additional cell processes are also known to be regulated by this family,

the most extensively recognized mechanism by which IAPs inhibit apoptosis is caspase inhibition. Alterations in the expression level of IAP proteins have been observed in chemoresistant tumor cells (Hunter et al., 2007).

XIAP, the best characterized IAP member so far, is generally recognized as the most potent endogenous caspase inhibitor (Eckelman et al., 2006). It can inhibit the effector caspase-3 and -7 with its linker-BIR2 domain and caspase-9 through its BIR3 domain as illustrated in Figure 6. Caspase inhibition is thought to be indispensable for the antiapoptotic effects of XIAP (Bratton et al., 2002). Smac, a proapoptotic protein which translocates from mitochondria into the cytoplasm during apoptosis can antagonize XIAP action releasing caspases from XIAP-mediated inhibition. Although the role of XIAP as a direct caspase inhibitor is widely accepted, recently it was found that XIAP possesses an E3 ligase activity and can therefore cause ubiquitination of important proteins in the cell death signaling pathway by the proteasome (Galban and Duckett, 2010). Recently in an *in vitro* study from Gillissen and colleges it was suggested that HCT-116 colon cancer cells can be sensitized to TRAIL by inhibiting XIAP-mediated ubiquitination of active caspase-3 (Gillissen et al., 2013). Besides its regulatory function in caspase inhibition and ubiquitination activity, XIAP has also been identified to have role in NFκB and JNK1 signal pathways by binding to the TAB-1 adapter protein through its BIR1 domain. Although this molecular mechanism is still under investigation and not yet very well defined it has been proposed that these pathways are crucial for the prosurvival activities of XIAP (Hofer-Warbinek et al., 2000).

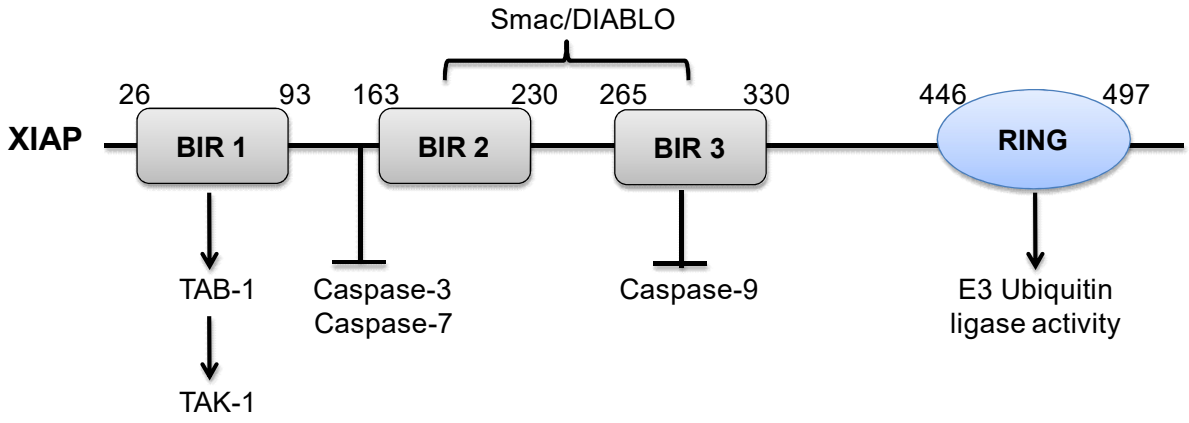

Figure 6: Schematic presentation of XIAP structure and interactions

XIAP contains three BIR domains on its N-terminal part and a RING finger motif on its C-terminus. The linker region proximal to BIR2 binds to caspase-3 and -7 and inhibits the catalytic sites of these enzymes, while the BIR3 region has been shown to bind and inhibit caspase-9 processing. BIR1 domain is involved

in signal transduction pathways e.g. JNK1 and NFκB activation by binding to TAB-1, which in turn recruits TAK-1 to the receptor complex (Yamaguchi et al., 1999). Smac/DIABLO can bind to both BIR2 and BIR3 domain thus competing with caspases and releases them from XIAP inhibition.

As XIAP and other members of the family are highly expressed in various tumors and this overexpression is linked to tumorigenesis, accelerated tumor growth and resistance to conventional therapies, they are thought to represent potential targets for therapeutic development (Fulda and Vucic, 2012). Antisense oligonucleotides targeting IAPs and small molecules that mimic the action of endogenous IAP inhibitor Smac are two current approaches to antagonize IAPs in different clinical trials for tumor treatment. Downregulation of XIAP levels using antisense oligonucleotides has been shown to sensitize tumor cells *in vitro* as well as in *in vivo* mouse models to chemotherapy (Bilim et al., 2003; Hu et al., 2003). Importantly, Smac peptides strongly enhanced cytotoxic effects in tumor cells in different mouse models when used in combination with TRAIL (Fulda et al., 2002b).

1.6. Aim of the thesis

Dysregulation of the mitochondrial signaling pathway of apoptosis represents a major problem in tumor therapy. The objective of the presented work was therefore to investigate role of the intrinsic (mitochondrial) pathway in the cell line NCI-H460 upon induction of apoptosis by TRAIL. For this, a transfectant stably overexpressing FLAG-tagged Bcl-2 was established to block triggering of the mitochondrial pathway following TRAIL stimulation. Effects of this overexpression were then investigated in terms of caspase activation and processing as well as the mitochondrial integrity regarding the membrane potential and the release of various proapoptotic molecules into the cytosol. Furthermore, it was of interest to gain insight into the controversially discussed positive feedback mechanism leading from activated effector caspases to initiator procaspases. Importantly, XIAP has been recently proposed in literature to represent a major determinant of the apoptotic phenotype deciding whether the cell dies as a type I or a type II cell. Therefore, the second main objective of this thesis was to explore the functional role of XIAP in TRAIL-induced apoptosis in NCI-H460 cells. Both, overexpression and downregulation/inhibition of XIAP should be achieved to gain detailed insight into the regulation processes mediated by this molecule. Furthermore it was of special interest to characterize the cells with blocked mitochondrial pathway, i.e. the Bcl-2 overexpressors, when in addition XIAP was downregulated/inhibited. These simultaneous changes in expression of two central

regulatory proteins were expected to shed light on the mutual relationship between the crucial players controlling TRAIL sensitivity as well as the regulation of the type of apoptotic cell death.

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2. Materials and Methods

2.1. Materials

2.1.1. Instruments

Table 1: List of instruments

Equipment	Company
Agfa Curix60 (Film developing machine)	Agfa HealthCare GmbH, Cologne, Germany
Balances	Kern ALJ120-4, Kern & Sohn GmbH, Balingen Frommern, Germany and Precision Standard, OHAUS Co., Parsippany, New Jersey, USA
Centrifuges	Centrifuge 5415R and Centrifuge 5810R, Eppendorf, Hamburg, Germany
CO_2 Incubator	Zapf, Sarstedt, Germany
Cryo 1 °C Freezing Containers	Thermo Fisher Scientific, Ulm, Germany
Electrophoresis chambers	Phase, Lübeck, Germany
Fluorescence microscope	Cell Observer® SD, Carl Zeiss Micro Imaging GmbH, Jena, Germany
LI-COR Odyssey	LI-COR Biotechnology GmbH, Bad Homburg, Germany
MACSQuant® Flow cytometer	MACS Miltenyi Biotec, Bergisch Gladbach, Germany
NanoDrop® ND-1000 (Spectrophotometer)	peQLab, Erlangen, Germany
Nucleofector™	Lonza, Basel, Switzerland
Olympus CKX41 (microscope)	Olympus Deutschland GmbH, Hamburg, Germany
pH meter	pH 530; Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Pipettes	Eppendorf Research Family, Eppendorf AG, Hamburg, Germany
Power supplies	Electrophoresis Power Supply EPS 301 Amersham Pharmacia Biotech; GE and EPS 601, Healthcare Europe, Germany
Semi-dry blotting chamber	Phase, Lübeck, Germany
Sonopuls HD 200 (sonicator)	Bandelin, Berlin, Germany

Tecan Infinite 200 Reader	Tecan, Männedorf, Switzerland
Thermomixer	Eppendorf AG, Hamburg, Germany
Vortexer	VF2; Janke & Kunkel, IKA-Werke GmbH & Co. KG, Staufen, Germany
Water bath	Julabo GmbH, Seelbach, Germany

2.1.2. Consumables

Table 2: List of consumables

Consumables	Company
96-well E-plates	Roche Diagnostics, Mannheim
Blotting membrane	Protran BA85; Whatman, Schleicher & Schüll, Dassel, Germany
Blotting paper, 3 mm	Whatman, Schleicher & Schüll, Dassel, Germany
Cell culture flasks and plates	CELLSTAR; Greiner bio-one, Frickenhausen, Germany
Counting chamber	Neubauer counting chamber, Marienfeld, Lauda-Königshofen, Germany
Cover slips 18×18 mm	Menzel-Gläser, Braunschweig, Germany
Cryo vials 1ml	CELLSTAR; Greiner bio-one, Frickenhausen, Germany
Falcon tubes (15 ml and 50 ml)	Greiner, Frickenhausen, Germany
Flow cytometry tubes	Polystyrene tubes, round bottom; Greiner bio-one, Frickenhausen, Germany
iBlot Gel Transfer Stacks Nitrocellulose, Regular	Life technologies, Carlsbad, CA, USA
Reaction tubes (1.5 and 2 ml)	Eppendorf AG, Hamburg, Germany
X-ray film	CEA, Strangnas, Sweden

2.1.3. Cell lines, cell culture media and supplements

Table 3: List of cell lines

Cell line	Description
NCI-H460	Human lung cancer cells, obtained from ATCC, Manassas, USA

NCI-H460/Bcl-2	Lung carcinoma cell line stably expressing FLAG-Bcl-2, Institute of Cell Biology and Immunology, University of Stuttgart
BT474	Nancy Hynes ,Friedrich Miescher Institute, Basel, Switzerland
Colo 205	Colon cancer line, obtained from Banca Biologica e Cell Factory, Genova, Italy
HCT-116	Colon cancer line, obtained from Banca Biologica e Cell Factory, Genova, Italy
MDA-MB 231	CLS, Heidelberg, Germany

Table 4: List of media and other cell culture reagents

Media and other cell culture reagents	Description
DMEM	Life Technologies, Gibco, Karlsruhe, Germany
Eosine solution	6mM eosine, 90% (v/v)PBS, 10% (v/v) FCS, 0.02% (w/v) NaN3
Fetal calf serum (FCS)	PAN Biotech GmbH, Aidenbach, Germany
Freezing medium	90% (v/v) FCS, 10% (v/v) DMSO
OptiMEM®	Life Technologies, Gibco, Karlsruhe, Germany
RPMI 1640 + L-glutamine	Life Technologies, Gibco, Karlsruhe, Germany
RPMI 1640 + L-glutamine, phenol red-free	Life Technologies, Gibco, Karlsruhe, Germany
Trypsin/EDTA 10X	Life Technologies, Gibco, Karlsruhe, Germany

2.1.4. Chemicals, solvents and reagents

Table 5: List of chemicals, solvents and reagents

Chemicals	Company
Acrylamide, Rotiphorese Gel 30	Carl Roth GmbH & Co, Karlsruhe, Germany
Agarose	Carl Roth GmbH & Co, Karlsruhe, Germany
Ammonium persulfate (APS)	Carl Roth GmbH & Co, Karlsruhe, Germany
ß-Mercaptoethanol	Sigma-Aldrich, Taufkirchen, Germany
Blocking reagent	Roche Diagnostics, Basel, Switzerland

Bovine Serum Albumin (BSA)	Sigma-Aldrich, Taufkirchen, Germany
Bradford reagent	Bio-Rad Protein Assay, Bio-Rad Laboratories GmbH, München
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Invitrogen, Carlsbad, CA, USA
Complete Protease Inhibitor Cocktail	Roche Diagnostics, Mannheim, Germany
Crystal violet	Carl Roth GmbH & Co., Karlsruhe, Germany
DharmaFECT 3 (transfection reagent)	Dharmacon, GE healthcare, UK
Dimethylsulfoxide (DMSO)	Carl Roth GmbH & Co., Karlsruhe, Germany
Dithiothreitol (DTT)	Carl Roth GmbH & Co, Karlsruhe
DAPI (4',6-diamidino-2-phenylindole)	Sigma, Steinheim, Germany
Eosine	Carl Roth GmbH & Co., Karlsruhe, Germany
Ethanol	Carl Roth GmbH & Co., Karlsruhe, Germany
Ethidiumbromide (EtBr)	Carl Roth GmbH & Co., Karlsruhe, Germany
Fluoromount G	Southern Biotech, Birmingham, USA
HCl (37% v/v)	Carl Roth GmbH & Co., Karlsruhe, Germany
Isopropanol	Carl Roth GmbH & Co., Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co., Karlsruhe, Germany
Milk powder skim	Carl Roth GmbH & Co., Karlsruhe, Germany
MitoTracker® Mitochondrion-Selective Probes	Molecular Probes, invitrogen detection technologies, Germany
NP-40 (Nonidet P40)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
PageRuler prestained protein ladder	Fermentas GmbH, St. Leon-Roth, Germany
Paraformaldehyde (PFA)	Carl Roth GmbH & Co., Karlsruhe, Germany
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Propidium iodide (PI)	Invitrogen, Karlsruhe, Germany
Protein G Sepharose	GE Healthcare Europe GmbH, Freiburg, Germany

Puromycin	Applichem GmbH, Darmstadt, Germany
Sodiumdodecylsulfate (SDS)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co., Karlsruhe, Germany
N,N,N',N'-Tetramethyl-ethylene-diamine (TEMED)	Carl Roth GmbH & Co., Karlsruhe, Germany
Tetramethylrodamine methyl ester (TMRM)	Invitrogen, Carlsbad, CA, USA
Tris-hydroxymethyl-aminomethane (Tris)	Carl Roth GmbH & Co., Karlsruhe, Germany
Triton X-100	Carl Roth GmbH & Co., Karlsruhe, Germany
Tween-20	Carl Roth GmbH & Co., Karlsruhe, Germany
Zeocin	Life Technologies, Invitrogen, Karlsruhe, Germany

2.1.5. Buffers and solutions

Table 6: List of buffers with recipes

Buffers	Recipe
10% APS solution	10% (w/v) APS in H_2O
4X SDS sample buffer	40mM Tris/HCl, pH 8.0 0.4mM EDTA 140mM SDS 4.4M glycerine 0.4% (w/v) bromphenol blue
Blotting buffer	200mM glycine 25mM Tris base 20% (v/v),methanol
Caspase-activity Buffer	10mM HEPES, pH 7.4 220mM Mannitol 68mM Sucrose 2mM NaCl 2.5mM KH_2PO_4 0.5mM EGTA 2mM $MgCl_2$ 5mM Pyruvat
Caspase lyses buffer	100mM Tris-HCl 200mM NaCl 1% NP-40 pH 7.4
Crystal violet solution	0.5% (w/v) crystal violet 20% (v/v) methanol

Lysis buffer (western blot)	20mM Tris-HCl 150mM NaCl 1mM Na_2 EDTA 1mM EGTA, 1% (v/v) Triton X-100 2.5mM sodium pyrophosphate 1mM β glycerophosphate 1mM NaF, pH 7.5
PBA	PBS + 0.05% (w/v) BSA 0.02% (w/v) NaN_3
Phosphate Buffered Saline (PBS)	2.67mM KCl 1.47mM KH_2PO_4 137.93mM NaCl 8.06mM Na_2HPO_4
PBS-Tween	0.05% (v/v) Tween 20 in PBS
Propidium iodide (PI) staining solution	PI (50 µg/µl) RNase (20 pg/µl) in PBS
SDS-PAGE running buffer	50mM Tris-HCl 380mM glycine 4mM SDS pH 8.3
SDS-PAGE stacking gel buffer	5% (v/v) acrylamide 130mM Tris-HCl, pH 6.8 0.1% (v/v) SDS 0.1% APS 0.1% TEMED in H_2O
SDS-PAGE separating gel buffer	12% or 15% (v/v) acrylamide 375mM Tris, pH 8.8 0.1% (v/v) SDS 0.1% APS 0.1% TEMED in H_2O
Tris-Acetat-EDTA (TAE) buffer	40mM Tris-acetate 1mM EDTA, pH 8.3

2.1.6. Oligonucleotides (primers and siRNAs)

Table 7: List of primers and siRNAs

Primers and siRNAs	Sequence 5'-3'
XIAP-BIR2-forward	GCCTCGAGGCAGAGATCATTTTGCC
XIAP-BIR2-reverse	GCGAATTCTTAAATATTAAGATTCCG
XIAP-BIR3-forward	GCCTCGAGCAAATTCAACAAATCTT
XIAP-BIR2-reverse	GCGAATTCTTACTCAAGTGAATGAGT

siXIAP	GGAUAUACUCAGUUAACAAtt
siSmac	UCUCUUUACCGACAAUAUAtt
siCaspase-9	CGGUGAAAGGGAUUUAUAAtt

2.1.7. Antibodies

Table 8: List of primary antibodies

Antibody	Species	Company
anti-actin C-11	goat pAb	Santa Cruz Biotechnology, California, USA
anti-Bcl-2	rabbit pAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-caspase-3 (8G10)	rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-caspase-8 (1C12)	mouse mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-cleaved caspase-3 (Asp175)	rabbit pAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-cleaved caspase-8 (Asp 391)	rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-caspase-9	mouse mAb	BD Pharmingen, Heidelberg, Germany
anti-caspase-9	rabbit pAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-cleaved PARP (Asp214)	mouse mAb	BD Pharmingen, Heidelberg , Germany
anti-COX IV (3E11)	rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-cytochrome c	mouse mAb	BD Pharmingen, Heidelberg , Germany
anti-FLAG M2	rabbit pAb	Cell signaling Technology Inc., Danvers, MA, USA
anti-Smac/Diablo	mouse mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-c-IAP1 (D5G9)	rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti.c-IAP2 (58C7)	rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-survivin (71G4B7)	rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-GFP	mouse mAb	Roche Diagnostics, Mannheim, Germany

anti-TRAILR1	mouse mAb	R&D Systems, Wiesbaden, Germany
anti-TRAILR2	mouse mAb	R&D Systems, Wiesbaden, Germany
anti-TRAILR3	mouse mAb	R&D Systems, Wiesbaden, Germany
anti-TRAILR4	mouse mAb	R&D Systems, Wiesbaden, Germany
anti-α-tubulin	mouse mAb	Sigma-Aldrich, Taufkirchen, Germany
anti-XIAP (3B6)	rabbit mAb	Cell Signaling Technology Inc., Danvers, MA, USA
anti-XIAP	mouse mAb	BD Pharmingen, Heidelberg , Germany
IgG_1 isotype control	mouse mAb	BD Pharmingen, Heidelberg , Germany
IgG_{2b} isotype control	mouse mAb	BD Pharmingen, Heidelberg , Germany

Table 9: List of secondary antibodies

Conjugated secondary Antibody	Company
anti-goat IgG HRP-conjugated	Santa Cruz Biotechnology, California, USA
anti-mouse IgG + IgM (H+L) FITC-labeled	Dianova, Hamburg, Germany
anti-mouse IgG +IgM (H+L) HRP- conjugated	Dianova, Hamburg, Germany
anti-mouse IgG (H+L) IRDye 800 CW	LI-COR Biotechnology GmbH, Bad Homburg, Germany
anti-mouse IgG + IgM (H+L) phycoerythrin (PE)-labeled	Dianova, Hamburg, Germany
anti-rabbit IgG (H+L) HRP-conjugated	Dianova, Hamburg, Germany

2.1.8. Substrates, inhibitors and ligands

Table 10: List of substrates, inhibitors and ligands

Substrate, ligands and inhibitors	Company
ABT-737 sc-207242	Santa Cruz Biotechnology, California, USA
Caspase-3 substrate Ac-DMQD-AMC	Enzo Life Sciences, Switzerland
Caspase-8 substrate Ac-IEPD-AMC	Enzo Life Sciences, Switzerland

Caspase-9 substrate Ac-LEHD-AMC	Enzo Life Sciences, Switzerland
Caspase-9 inhibitor (z-LEHD-fmk)	R&D Systems, Wiesbaden, Germany
Diabody-scTRAIL ($Db_{\alpha EGFR}$-scTRAIL)	DbαEGFR-scTRAIL (Db-scTRAIL), kindly provided by Dr. Martin Siegemund, Institute of Cell Biology and Immunology, University of Stuttgart, Germany
Pan caspase inhibitor (zVAD-fmk)	R&D Systems, Wiesbaden, Germany

2.1.9. Software

Table 11: List of software

Software	
Clone Manager 7.04	Scientific & Educational Software, Cary, North Carolina, USA
GraphPad Prism 4.03	GraphPad, La Jolla, California, USA
MACSQuantify	MACS Miltenyi Biotec, Bergisch Gladbach, Germany
ImageJ 1.46r	Wayne Rasband, National Institutes of Health, USA
Odyssey Infrared Imaging System 2.1.12	Free Software Foundation Inc. (Boston, USA)
Tecan i-control 1.5.14	Tecan, Mannedorf, Switzerland

Table 12: List of kits

Kits	**Company**
AmaxaTM Cell Line NucleofectorTM Kit T	LONZA, Basel, Switzerland
Annexin V-FITC apoptosis detection kit	Sigma-Aldrich, Taufkirchen, Germany
Mitochondria isolation kit for Mammalian cells	Thermo Scientific, Rockford IL, USA
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific, Rockford IL, USA

2.2. Methods

2.2.1. Maintenance of cell lines

Human non-small cell lung cancer cell line NCI-H460 was obtained from ATCC, Manassas, USA. Cells were cultured in RPMI-1640 medium supplemented with L-glutamine and 5% Fetal Calf Serum (FCS) and kept in an incubator at 37°C with 5% CO_2 and 96% relative humidity. Transfectants of NCI-H460 cells stably overexpressing Bc-2 (NCI-H460/Bcl-2) were supplemented by medium; containing 3µg/ml puromycin. Cells were not grown over 80% confluency. To split cells, medium was removed and cells were washed with 5-6ml PBS, 2ml 1x Trypsin-EDTA was used to detach cells from the bottom of the flask. 8ml of medium was added to stop the activity of Trypsin and cells were pelleted at 1000 rpm for 5min. The cell pellet was resuspended in fresh medium containing FCS and transferred to 75 cm^2 flasks. For all experiments cells from exponentially growing cultures were used. Cell cultures were checked regularly for myco plasma to ensure the absence of any contamination in the used cultures. Before seeding the cells for an experiment, cells were counted using Neubauer counting chamber after staining them with eosin. Eosin can only penetrate into the wall of dead cells, so all eosin negative cells were counted and cell number per ml was calculated by the formula.

2.2.1.1. Freezing and thawing of cells

To freeze the cells, they were trypsinized and counted as described earlier, after centrifugation the cell pellet was dissolve in cold freezing medium (FCS+10% DMSO), and transferred to cryovials. DMSO was used as a cryoprotectant which prevents crystallization of water that will cause cells to lyse during freezing and thawing. The cells were slowly cooled to -80°C in cryoboxes filled with isopropanol, and then transferred to the liquid nitrogen for long-term storage at -196°C. To take new culture of frozen cells, cells were thawed at 37˚C and quickly transferred to a falcon containing pre-warmed culture medium and centrifuged to remove DMSO. Cell pellet was dissolved in growth medium and transferred to cell culture flask.

2.2.2. Cytotoxicity assay

Sensitivity of NCI-H460 cell line to death stimuli (Db-scTRAIL) was investigated with cytotoxicity assay. 15000 cells per well were plated in 96-well plate (flat bottom) the day before stimulation. Next day cells were stimulated with serial dilution of Db-scTRAIL starting with 1nM. Cells were incubated at 37°C for 24h. After the completion of stimulation time, media was removed and cells were washed with PBS. Crystal violet solution (50µl per well) was added and adherent cells were stained for 15min at room temperature. The plate was washed gently to remove the residual dye completely and dried overnight. 50µl methanol per well was added and after 10min of shaking, the absorbance was measured in a microplate reader at λ=550nm (Infinite M200, Tecan). All the values were normalized to the values from unstimulated wells.

2.2.3. Caspase activity assay

To investigate the activity of caspase-3, -8 and -9 in Db-scTRAIL stimulated cells, synthetic fluorogenic caspase substrates, namely Ac-DMQD-AMC (caspase-3), Ac-IEPD-AMC (caspase-8) and Ac-LEHD-AMC (caspase-9) were used. Briefly, 300,000 cells from both cell lines (NCI-H460 and NCI-H460/Bcl-2) were seeded in 6-well plates and incubated overnight at 37°C, next day stimulated with Db-scTRAIL for the indicated times points and one well was left unstimulated as a control. After the stimulation cells were scraped and the whole medium containing floating and attached cells was transferred to micro reaction tubes and centrifuge for 5min at 1500 rpm. Cell pellet was then washed with PBS. 30µl caspase lysis buffer with freshly added protease inhibitors was used to resuspend the pellets and incubated on ice for 15-20min. Cells were centrifuged for 10min at 13000 rpm at 4°C and the supernatant was transferred to new labeled reaction tubes. Protein concentration was determined using Bradford assay. 4µg of total protein was used to measure caspase-3 activity, while 32µg were used for caspase-8 and 20µg for caspase-9. The experiment was conducted in a non-transparent 96-well plate. Cell lysates were transferred to the wells and mixed with 125µl caspase activity buffer containing the appropriate caspase substrate. The change in fluorescence intensity was detected in a microplate reader every 2min over 2h at 37˚C. The activity was calculated from the slopes of the resulting lines and normalized to the highest value.

2.2.4. Cell lysis, SDS-PAGE and immunoblotting

2.2.4.1. Cell lysis and protein quantification

Cells were stimulated, harvested and pelleted by centrifugation, washed with PBS and resuspended in lysis buffer (described earlier in materials) with freshly added protease inhibitor. After 10-15min incubation on ice, samples were centrifuged at 13000 rpm at 4˚C for 10min. Supernatants were transferred to new labeled Eppendorf tubes and either stored at -20˚C or preceded further with protein measurement. Protein concentration was determined by Bradford assay. Bovine albumin serum (BSA) was used to make the standard curve of absorbance versus microgram of protein. Protein concentrations of unknown samples were determined by comparing to the standard curve.

2.2.4.2. Subcellular fractionation

To investigate the translocation of cytochrome c and Smac, cytosolic and mitochondrial extracts were prepared using digitonin based subcellular fractionation technique. Briefly, cells were seeded in 6-well plates at a density of 5×10^5 per well in culture medium and allowed to proliferate for 24h before treatment. After stimulation with 0.5nM Db-scTRAIL for indicated time points, cells were collected and lysed in a permeabilization buffer containing 200µg/ml digitonin for 5min. Plasma membrane permeabilization was checked by trypan blue staining and the lysate was centrifuged at 2000 rpm. Supernatant which is the cytoplasmic fraction was collected in the new labeled Eppendorf tubes and the pellet was resuspended in lysis buffer containing 1% Triton-X100 and incubated on ice for 15min, centrifuged at high speed (13000 rpm) for 10min and supernatants were transferred to new reaction tubes and stored at -20˚C. Protein concentrations of both extracts were measured *via* Bradford assay using BSA as a standard. Western blot analysis with cytosolic and mitochondrial fractions was performed as described below, using mouse monoclonal anti cytochrome c antibody (BD Pharmingen) and mouse anti Smac antibody (Cell Signaling technology) at a 1:1000 dilution.

2.2.4.3. SDS-PAGE

Before loading the gel, 50µg of cell lysates were mixed with protein sample buffer (2x) and ß-mercaptoethanol (5% (v/v)) and denatured by boiling for 5min at 95°C. As a standard, a

marker (CPPM, ColorPlus Prestained Protein Marker) which was composed of different proteins of variable molecular weights, was run with the samples to estimate the sizes of applied proteins. Proteins were separated on 12.5% and 15% polyacrylamide gels for 90min and at a constant voltage of 150 V.

2.2.4.4. Immunoblotting

Separated proteins were transferred onto nitrocellulose membrane using a semi dry blotting chamber for 90min at 10V and 80-90mA current. Whatman paper and nitrocellulose membrane was equilibrated with transfer buffer 10min prior the transfer started. After transfer the membrane was blocked for 1h at room temperature with blocking reagent (Roche, 1x) or 10% (w/v) non-fat milk powder dissolved in PBST (PBS + 0.05% Tween-20). Membrane was washed 3 times with PBST after blocking and was incubated with antibody of interest overnight on shaker at 4°C. Next day, membrane was washed 3 times with PBST. Subsequently, the blots were incubated with secondary antibody diluted in PBST at room temperature for one hour on a shaker. Membrane was washed again and developed using ECL substrate SuperSignal West Pico (Thermo Fisher Scientific, Waltham USA) according to the manufacturer protocol. X-ray films were exposed to the membrane for varying time periods and the films were developed with Agfa Curix 60 X-ray developing machine. 0.2% NaOH was used to strip the membranes, after 5 min incubation in 0.2% NaOH, membranes were washed with PBST and incubated with fresh antibody dilution after 1h blocking with Roche blocking reagent.

2.2.4.5. Quantification of western blots

Kinetics of procaspase-3, -8 and -9 after Db-scTRAIL stimulation for increasing time points were quantified using ImageJ. The intensities were normalized to the intensities of loading controls used.

2.2.5. Flow cytometry

2.2.5.1. Measurement of cell surface expression of TRAIL receptors

Flow cytometry was performed to investigate the expression of TRAIL receptors on the surface of NCI-H460 and NCI-H460/Bcl-2 cells. Briefly, 3×10^5 cells for each sample were harvested by trypsin and centrifuged at 1500 rpm for 5min; the pellet was resuspended in 100µl cold PBA and transferred to 96 well plates. 3µg/ml primary antibody or isotype-matched nonbinding antibodies to control the nonspecific binding, was added to the cells. After 1h incubation on ice cells were centrifuged, pellet was washed again with PBA and resuspended in PBA containing FITC conjugated goat-anti-mouse secondary antibody at the dilution of 1:100. After 1h of incubation on ice and protected from light, cells were washed with PBA resuspended in 400µl PBA, transferred to FACS tubes and analyzed by the MACSQuant® flow cytometer. 10^4 events were analyzed for each sample and the same instrument settings were used for all experiments.

2.2.5.2. Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Tetramethylrhodamine methylester (TMRM) was used to monitor the mitochondrial membrane potential. TMRM is a fluorescent cationic dye which accumulated in the negatively charged mitochondria in healthy cells, forming aggregates which give a specific fluorescence. Upon apoptosis mitochondrial outer membrane permeabilizes and this cationic dye diffuses into the cytoplasm thus leads to a decrease in fluorescence intensity, which can be detected via flow cytometer. For monitoring changes in mitochondrial membrane potential, cells were stimulated with 0.5nM Db-scTRAIL for the indicated time points, 30min prior to stop stimulation cells were loaded with 60nM tetramethylrhodamine methylester (TMRM) in culture medium. After incubation time has completed, cells were harvested using trypsin and the cell pellet was washed once with PBS followed by resuspending the pellet in PBA for measuring the TMRM intensity using MACSQuant® flow cytometer

2.2.5.3. Intracellular staining

Cells were harvested, transferred into reaction tubes, centrifuged at 100 rpm for 5min and fixed with 4% PFA at room temperature for 15min. After one washing step with PBA, cells

were permeabilized with 1x permeabilization buffer from BD Pharmingen for 10min at RT. After centrifugation cells were dissolve in 100µl PBA and transferred to v-bottom 96-well plate, and blocked for 30min. Primary antibody was diluted in blocking buffer (PBA + 10% FCS), and the cells were incubated with primary antibodies for 60min on ice followed by two washing steps with blocking buffer. Cells were incubated with PE-labeled secondary antibody which was also diluted in blocking buffer at 1:100. After 1h incubation with secondary antibody in dark, washed twice and resuspended in 400µl PBA and transferred to FACS tubes and analyzed *via* flow cytometer.

2.2.5.4. PI/Annexin V staining or apoptosis assay

An important feature of apoptosis is the appearance of the phospholipid phosphatidylserine in the outer leaflet of the plasma membrane. Phosphatidylserine is usually found in the inner leaflet of the plasma membrane. During the apoptotic process however, this phospholipid flips to the outer leaflet, which can be detected via annexin V staining. Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit II (BD Pharmingen) was used according to the manufacturer's instructions. In brief, 48h after siRNA XIAP and non-targeting siRNA transfection, NCI-H460/Bcl-2 cells were treated with Db-scTRAIL up to 4h. Both floating and attached cells were harvested and washed twice with PBS. After centrifugation, cells were stained by suspending in 100µl binding buffer containing propidium iodide and annexin V-FITC for 15-20min at room temperature. Total 10^4 events were considered for each sample and fluorescence intensities were measured using flow cytometer. Data were analyzed using MACSQuant software.

2.2.6. Immunofluorescence

Cells were seeded on coverslips and kept in incubator at 37˚C overnight. Following day, 100nM mitoTracker was added to the medium for 30min to stain the mitochondria. Then, the media was removed, cells were washed with 1x PBS and fixed with 4% ice-cold paraformaldehyde (PFA) for 20min at 4˚C. Cells were washed with 1x PBS, permeabilized with 0.2% Triton X-100 in 1x PBS for 15min at room temperature and washed once with PBS. After 1h incubation in blocking solution (RPMI supplemented with 10% FCS), primary antibody was added (anti-Flag-M2, dilution 1:1000 in RPMI + 10%FCS) for 1h. Cells were washed two times with PBS and one time with 0.1% Triton X-100 in PBS. After staining with

secondary antibody (anti-mouse-IgG-AlexaFluor 488, 1:500 in RPMI + 10% FCS),cells were washed using 0.1% Triton X-100 in PBS and the coverslips mounted on microscope slides using Fluromount G containing DAPI. Slides were kept in dark until analysis using cell observer microscope (Carl Zeiss Micro Imaging AG, Jena).

2.2.7. Time-lapse fluorescence microscopy

To investigate cell death and other effects of mitochondrial outer membrane potential on the cell cycle, fluorescent microscope (Cell Observer) was used to make videos over 30h of cells loaded with TMRM. Briefly, 40.000 cells per compartment were seeded in a 35mm glass bottom dish and incubated overnight. The next day medium was replaced by RPMI1640 + L-Glutamine, 5% FCS without phenol red containing 60nM tetramethylrhodamine methylester (TMRM) and incubated for 30min at 37˚C. Cells were washed twice and either treated with Db-scTRAIL or left untreated. The dish was placed in the incubation chamber (37°C, 5% CO_2) of the Cell Observer. The cells were observed at 20X magnification, bright field and fluorescent images were taken in a 15min time interval.

2.2.8. Cell transfection

2.2.8.1. Nucleofection

For transient expression of full length XIAP and its BIR domains, cells were nucleofected using Amaxa NucleofectorTM Kit T from Lonza according to manufacturer's instruction. Briefly, 1-2 million cells per reaction were resuspended in 100µl T-buffer (supplemented with the kit) and 2µg plasmid DNA (pEGFP–C1, pEGFP-XIAP, pEGFP-BIR2, and pEGFP-BIR3) was added. Cells were nucleofected with the program T20 using Nucleofector II (Amaxa biosystems). Rapidly after transfection, growth medium was added to the cells and were seeded in a 6-well plate. Transfection efficiency was checked after 24h by GFP positive cells under the microscope and afterwards, cells were harvested and analyzed for protein level of XIAP and BIR domains by immunoblotting using anti-GFP antibody.

2.2.8.2. Short interfering RNAs (siRNA) transfection

For knockdown experiments, cells were transiently transfected with short interfering RNAs (siRNAs) at a final concentration of 8nM using DharmaFECT according to the manufacturer's instructions. In brief, for a 6-well format, in one reaction tube 8µl of siRNAs (final conc. 8nM) were diluted in 192µl Opti-MEM serum free medium. In the second reaction tube 5µl of DharmaFECT 3 was diluted in 195µl of Opti-MEM, contents of both tubes were mixed and incubated for 20min at room temperature. Meanwhile 1600µl fresh growth medium was added to the cell; 400µl of transfection mix (siRNA+DharmaFECT 3+ Opti-MEM) was added on the top and incubated for 48h at 37˚C. 24h later transfection medium was changed and cells were supplemented with fresh growth medium. Protein expression levels were confirmed *via* western blot analysis. For transfection in other tissue culture plates, volumes were calculated according to the surface area of the plate.

2.2.9. Molecular biology

2.2.9.1. Cloning of BIR domains

Plasmid pEGFPC1-XIAP encoding full length XIAP, GFP (pEGFP-C1) tag and kanamycin as a selection marker was used as template plasmid. The gene sequences encoding the BIR2 and BIR3 domain of XIAP were amplified using appropriate primers (Table 7). The amplified fragments were run on Agarose gel electrophoresis to check the size of the amplified cDNAs, and the sequences were verified by DNA sequencing. The PCR mix and program used is stated in Table 13 and Table 14.

For analysis and purification of cDNAs (PCR products), samples were mixed with 6× DNA sample buffer and separated on agarose gels containing 1% agarose and 1µg/ml ethidiumbromide in TAE buffer. Gels were run at 80–100 V for 60–90 min. DNA bands were visualized under UV light. Bands of expected sizes were excised and isolated from the gel slice using Nucleo Spin Extract II kit according to the manufacturer's protocol.

The RCR reaction mix was prepared as follow.

Table 13: Reagents for PCR mix

Reagent	Volume
Forward primer (10pmol/µl)	2µl
Reverse primer (10pmol/µl)	2µl
DNA template (10ng/µl)	3µl
DNA polymerase (1U/µl)	1µl
dNTPs (2mM each)	5µl
10X PCR buffer	5µl
$MgSO_4$ (25mM)	2.5µl
ddH_20	30µl

The following program was used for amplification of BIR2 and BIR3 forward and reverse oligonucleotides

Table 14: program for the thermal cycler

Step	Temperature	Time	Cycles
Denaturation	95°C	1min	1
Annealing	60°C	30sec	30
Elongation	72°C	1min	
Final elongation	72°C	10min	1

2.2.9.2. Transformation and plasmid isolation from bacteria

Competent NEB cells were transformed with digested PCR product; Briefly NEB cells were thawed on ice and incubated with 5µl digested and ligated PCR products on ice for 30min followed by heat shock at 42˚C for 20sec and placed again on ice for 5min, 500µl prewarmed SOC medium was added and the transformation mix was incubated at 37˚C for 1h for recovery. The mixture was centrifuged and supernatant was discard leaving 100µl which was spread on LB-agar plates containing appropriate antibiotics (kanamycin). Single colonies of transformed bacteria were picked and used to inoculate 2ml LB-medium containing kanamycin which was then incubated overnight at 37˚C on a shaker. The next day bacteria were collected

by centrifugation and DNA was isolated using Nucleo Spin Plasmid Kit according to the manufacturer's protocol. To produce large amounts of DNA, picked bacterial colony was inoculated into 100ml of LB medium, and incubated overnight at 37˚C with shaking. Next day cells were collected by centrifugation and plasmid DNA was isolated using NucleoBond Xtra Midi kit according to manufacturer's instructions. The DNA pellet was washed with 70% ethanol, dried and resuspended in 2µl ddH_20. Concentration of the DNA was determined by UV-absorption while successful plasmid construction was verified by DNA sequencing (GATC Biotech AG, Konstanz, Germany).

3. Results

3.1. Characterization of a cell line stably overexpressing Bcl-2

Transfectants of NCI-H460 cells stably overexpressing Bcl-2 protein at different levels had already been established at the institute. One of these transfectants after dilution cloning was selected and used for all subsequent experiments after confirming proper expression of Bcl-2. Prior to starting any experiments, the stably Bcl-2 expressing NCI-H460 cell line (NCI-H460/Bcl-2) was characterized in context of the Bcl-2 level as compared to the parental cell line. Immunoblot analysis showed the wild type Bcl-2 with a molecular weight (MW) of about 26 kDa was expressed both in the control NCI-H460 cell line as well as in NCI-H460/Bcl-2 (Figure 7A). The latter cells in addition showed expression of the transfected protein possessing a higher MW of about 27 kDa due to the FLAG tag, but migrating always on SDS gel somewhat faster than the wild type protein. As a next step to precisely determine the overexpression level of Bcl-2 in the transfectant, immunoblot assays were done using the same amounts of protein (5, 10, 20, 40µg of cell lysates) in a first set of experiments (Figure 7C). In a second set of experiments identical numbers of cells were used from both cell lines and endogenous and overexpressed Bcl-2 was detected *via* western blotting (Figure 7D). Intensities of the bands were quantified via ImageJ software. In the third set of experiments, intracellular staining of Bcl-2 was done after fixing and permeabilizing the cells (Figure 7E). Flow cytometric analysis revealed approximately 2.4 fold increased expression of Bcl-2 in NCI-H460/Bcl-2 cells. Together with the other experiments similar relative overexpression levels of Bcl-2 of 2.2, 2.1 and 2.4 fold, respectively, were obtained, revealing an average level of 2.2 fold. Together, in NCI-H460/Bcl-2 cells the total Bcl-2 molecule amount was 2-3 fold enhanced compared to wild type cells, i.e. comparably moderately overexpressed as referred to the amount of the endogenous protein. In addition, no indication was found that Bcl-2 overexpression would affect the level of the endogenous protein.

Bcl-2 family proteins are among the critical regulators of apoptosis, they can be classified as either proapoptotic or antiapoptotic. One major site of action of these important regulators of apoptosis is at the outer mitochondrial membrane (OMM). Therefore colocalization of the transfected Bcl-2 protein with mitochondria was analyzed by confocal microscopy (Figure 7B). As expected, indirect immunofluorescence showed prominent staining of the FLAG tag

of overexpressed Bcl-2 protein in NCI-H460/Bcl-2 cells in colocalization with the mitochondrial staining, but not with nuclei.

While the antiapoptotic role of Bcl-2 is widely accepted, it has also been suggested that cell proliferation is being affected by this protein. Indeed, overexpression of Bcl-2 in numerous mammalian cell lines resulted in a reduction of cellular proliferation (Borner, 1996). Another study investigating the underlying mechanism suggested that Bcl-2 might affect the progression of cell cycle by prolonging cell division at the G_1 to S transition (Mazel et al., 1996). Based on these data the cell cycle length of Bcl-2 overexpressing NCI-H460 cells was compared with the parental cell line using live cell imaging. Cell cycle lengths of approximately 100 cells were calculated, revealing that the average cell cycle length of NCI-H460/Bcl-2 cells was 16.9h (data not shown) which was nearly identical with the cell cycle length of 16h of NCI-H460 wild type (data from Aline Lindner, bachelor thesis, 2012). So it can be concluded that at least in our cellular system obtained moderate overexpression of Bcl-2 does not significantly interfere with the cell cycle progression.

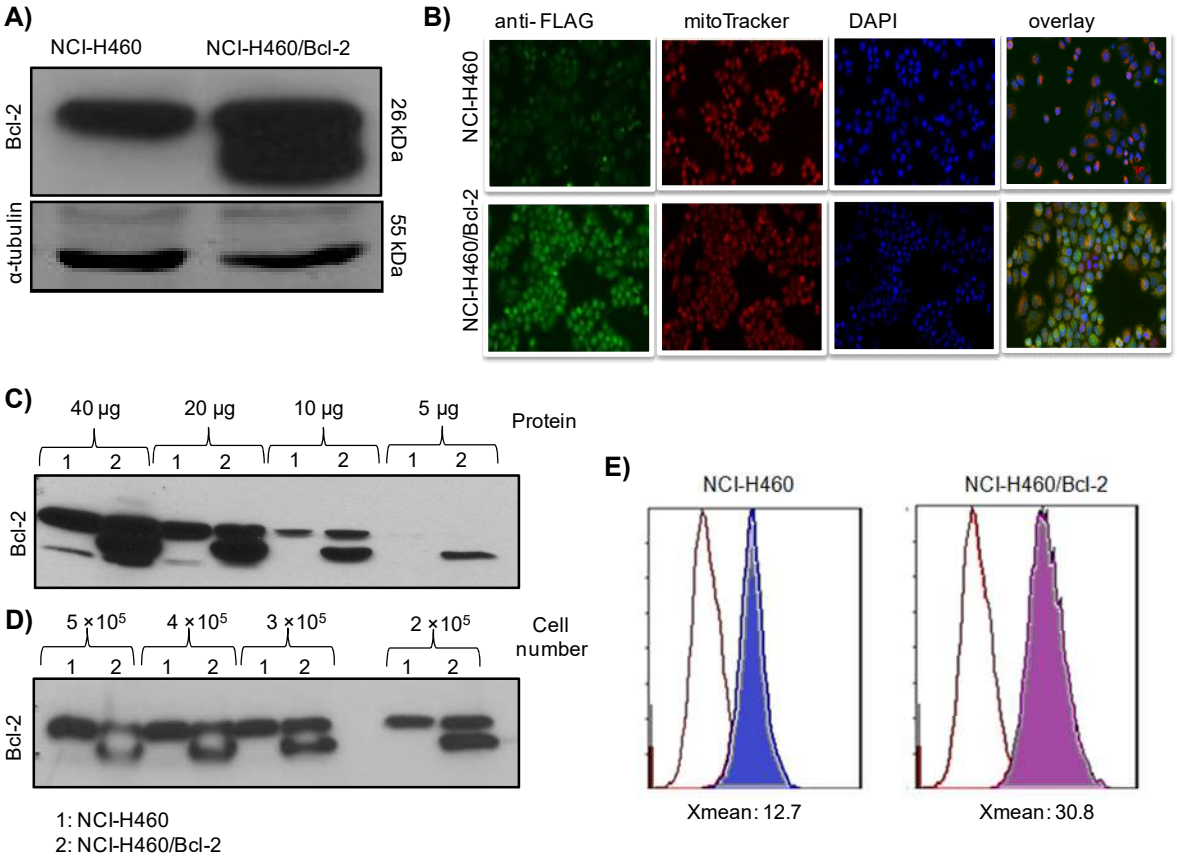

Figure 7: Characterization of Bcl-2 overexpressing cell line

A) To verify the overexpression of Bcl-2, equal amounts of whole cell lysates (50µg) from NCI-H460 wild type and NCI-H460/Bcl-2 were analyzed by immunoblotting using anti-Bcl-2 antibody followed by anti-rabbit-HRP-conjugated secondary antibody. Equal loading was confirmed by re-probing the membrane with α-tubulin specific antibody. **B)** NCI-H460 cells and NCI-H460 cells stably transfected with FLAG tagged-Bcl-2 were fixed and immunostained with α-FLAG M2 primary antibody followed by Alexa Fluor® 488-conjugated anti-mouse secondary antibody (first panel). Mitochondria were stained with MitoTracker (second panel) while DAPI was used to stain cell nuclei (third panel). The images were overlaid in the last panel. DAPI: 4', 6-diamidino-2-phenylindol. Data shown is one of two independent experiments showing identical results. **C)** and **D)** To quantify the Bcl-2 expression level equal amounts of protein or lysates from equal number of cells were loaded on gels and were analyzed *via* immunoblotting using anti-Bcl-2 antibody. Intensities of the bands were quantified using ImageJ software. **E)** Cells were fixed with 4% PFA, permeabilized with permeabilization buffer (BD Pharmingen) and stained with anti-Bcl-2 primary antibody and PE-labeled secondary antibody. Cells were analyzed by flow cytometry. Data shown is one out of three independent experiments with comparable results.

3.1.1. Expression of major proteins involved in the apoptotic pathway are not affected by Bcl-2 overexpression

To exclude the possibility that overexpression of Bcl-2 influences the cell surface expression of TRAIL receptors (TRAILR1, TRAILR2, TRAILR3 and TRAILR4) and other key proteins of the apoptotic pathway induced by TRAIL, a next set of experiments was performed. First, the cell surface expression of all four TRAIL receptors in NCI-H460 and NCI-H460/Bcl-2 cells was analyzed by flow cytometric analyses and the endogenous levels of apoptotic key proteins *via* western blot analysis were compared. These data revealed that NCI-H460 and its Bcl-2 overexpressing counterpart showed strong and comparable expression of TRAILR1 and TRAILR2, which was significantly higher as those of TRAILR3 and TRAILR4 on the cell surface (Figure 8A). As shown further in Figure 8 overexpression of Bcl-2 has no significant impact on the surface expression of TRAIL receptors (Figure 8A) or the level of major proteins of the apoptotic signaling pathway like caspase-8, caspase-3 and XIAP (Figure 8B), since both cell lines showed similar endogenous levels of these proteins. Thus, any differences in TRAIL sensitivity are unlikely to be based on differences in the level of these proteins.

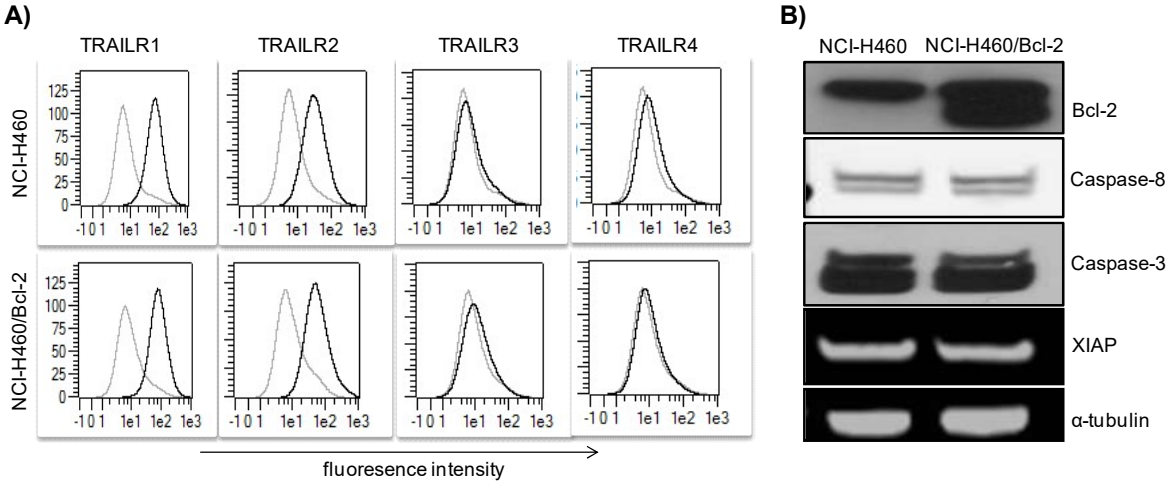

Figure 8: Bcl-2 overexpression does not affect the expression of major proteins of the TRAIL-induced apoptotic pathway

A) Surface expression of the four human TRAIL receptors was analyzed by flow cytometry in wild type and Bcl-2 overexpressing NCI-H460 cells. Briefly, equal numbers of cells were used for all samples, cells were stained with mouse monoclonal antibodies specific for human TRAILR1, TRAILR2, TRAILR3 or TRAILR4, followed by incubation with anti-mouse IgG FITC-conjugated antibody (black

lines/peaks). IgG_1 Isotype control was used for TRAILR1, TRAILR3 and TRAILR4 while IgG_{2B} was used for TRAILR2 (grey lines/peaks). Fluorescence intensity is plotted against cell counts. Data shown is representative of three independent experiments. **B)** Equal amounts of protein (50µg) from whole cell lysates were separated on 12% acrylamide gel and subjected to immunoblotting using antibodies against full length XIAP and capase-8, caspase-3. α-tubulin was used as loading control. One representative experiment out of three is shown.

3.2. Effect of Bcl-2 overexpression on TRAIL-induced apoptosis

3.2.1. Bcl-2 overexpressing cells are inhibited in TRAIL-induced cell death

To investigate the effect of Bcl-2 overexpression on TRAIL-induced cell death, susceptibilities of NCI-H460 and NCI-H460/Bcl-2 cells to Db-scTRAIL (TRAIL) were compared. In this work Db-scTRAIL was used because of its higher bioactivity as compared to wild type TRAIL and because this type of an EGF receptor (EGFR)-targeted molecule represents an interesting molecule for clinical development. The enhanced bioactivity of Db-scTRAIL can be caused by any combination of three different molecular mechanisms, a) the higher valency of this molecule (six *versus* three TRAIL monomers in a single molecule), b) a membrane TRAIL-like enhanced activity caused by the antibody residue binding to EGFR and c) by affecting EGFR signaling *via* the antibody part. However, as we used NCI-H460 cells in this study, the third mechanism of action can be excluded because these cells carry a KRAS mutation. Accordingly, in NCI-H460 cells Db-scTRAIL acts simply as a TRAIL version with enhanced bioactivity without affecting EGFR signaling.

Cytotoxicity assays were performed with increasing concentrations of Db-scTRAIL for 24h. As shown in Figure 9A overexpression of Bcl-2 almost completely protected the NCI-H460 cells from TRAIL-induced apoptosis in a dose- and time-dependent manner whereas the parental cell line was susceptible to Db-scTRAIL as about 90% of the cells underwent apoptosis as compared to only about 10% cell death in NCI-H460/Bcl-2 cells at a Db-scTRAIL concentration of 1nM. Figure 9B shows the cytotoxic effects as a function of time after addition of 1nM of Db-scTRAIL. In a next step to confirm the results about the effect of Bcl-2 in protecting NCI-H460/Bcl-2 cells from TRAIL-induce apoptosis, an inhibitor of Bcl-2, Bcl-xl and Bcl-w, known as ABT-737 was used (Oltersdorf et al., 2005). At low concentrations (2.5µM) of ABT-737 that were not toxic to the cells as such (data not shown and see Figure 9C, far most left data point), this molecule was able to synergistically enhance the cytotoxicity of Db-scTRAIL in NCI-H460/Bcl-2 cells (Figure 9C). Co-incubation

with increasing concentrations of Db-scTRAIL in the presence of ABT-737 resulted in an increase of dead cells up to approximately 90% at highest concentration of Db-scTRAIL used (1nM).

To verify that the induction of cell death in Bcl-2 overexpressing cells following treatment with ABT-737 in combination with Db-scTRAIL was caused by apoptosis, the broad range pan caspase inhibitor zVAD-fmk (zVAD) was used. Co-treatment with zVAD-fmk very efficiently protected the cells from ABT-737/Db-scTRAIL-induced cell death, hence confirming that ABT-737 enhances TRAIL-induced apoptosis in NCI-H460/Bcl-2 cells in a caspase-dependent manner (Figure 9D). In summary, an increase in Bcl-2 expression significantly protected NCI-H460 from TRAIL-induced apoptosis. Further, Bcl-2 inhibition using the small molecule inhibitor ABT-737 can reverse this resistance resulting again in a strong TRAIL-mediated activation of the caspase cascade.

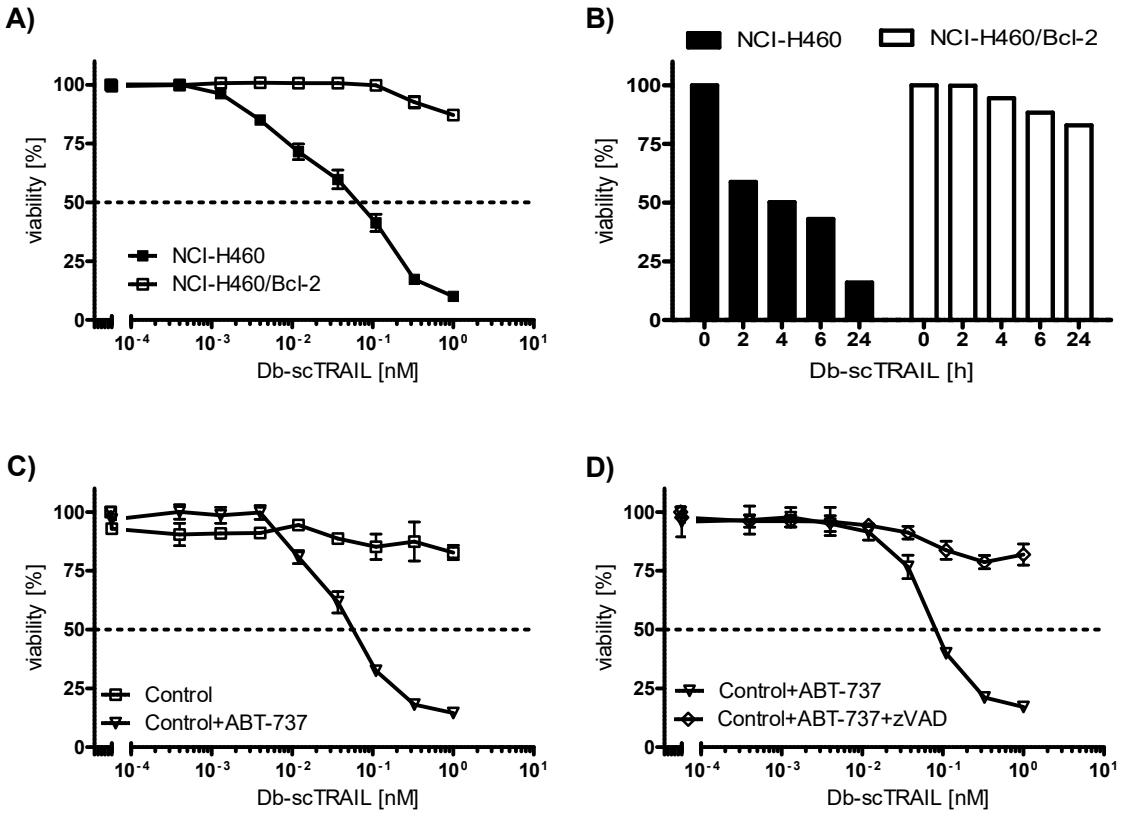

Figure 9: Overexpression of Bcl-2 effectively suppresses TRAIL-induced apoptosis

A) For cytotoxicity assays cells were seeded in 96-well tissue culture plates at densities of 1×10^4 cells per well. Next day cells were treated with increasing concentrations of Db-scTRAIL for 24h, viable

cells were stained with crystal violet. Dye was dissolved in methanol and the absorbance was measured at 550nm. All the values were normalized to values from unstimulated cells. Values shown are mean ± SD calculated from triplicates. Data shown is representative of three independent experiments. **B)** Cells were seeded in 96-well plate the day prior to stimulation, next day they were treated for indicated time points with 1nM Db-scTRAIL. After the completion of stimulation time, attached cells were stained with crystal violet and air dried overnight. Stained cells were dissolved in methanol and the absorbance was measured by Tecan reader. Values were normalized to unstimulated wells. **C)** and **D)** NCI-H460/Bcl-2 cells were used in these experiments; they were seeded in 96-well plates. Prior to stimulation with Db-scTRAIL the cells were preincubated with 2.5μM ABT-737 alone (C, open triangle) or in combination with 20μM caspase inhibitor zVAD-fmk (D, open diamond) for 1h followed by Db-scTRAIL treatment. After 24h incubation with increasing concentrations of Db-scTRAIL (highest 1nM) at 37˚C, viable cells were stained with crystal violet, dissolved in methanol and the absorbance was measured at 550nm. All the values were normalized to values from unstimulated cells. Values shown are mean ± SD calculated from triplicates. Data shown is representative of three independent experiments.

3.2.2. Bcl-2 overexpression impairs TRAIL-induced activation of caspases

To gain insight into the molecular mechanism of apoptosis inhibition by Bcl-2, we first explored whether overexpression of Bcl-2 has an effect on TRAIL-induced activation of caspases. Cells were either left untreated or were treated with Db-scTRAIL for indicated time intervals up to 8h. Specific fluorogenic substrates were used to analyze the enzymatic activities of the caspases-8, -3 and -9. Cleavage of these substrates resulted in an increase of fluorescence correlating with the activity of the caspases. It is evident from the results that relative activities of caspase-8, -3 and -9 were significantly higher in NCI-H460 wild type cells as compared to the Bcl-2 overexpressing cells (Figure 10B). Following Db-scTRAIL treatment of NCI-H460 resulted in an increase in caspase-3 like activity in a time-dependent manner. Maximum increase in the activity was observed after 6h for all three caspases following Db-scTRAIL treatment in wild type cells, after which it started to decrease. There was a slight increase in caspase-8 activity with stimulation time in NCI-H460/Bcl-2, but the maximal relative caspase-8 activity reached was only about 10% of maximal activity (6h) determined in parental cell line (Figure 10B). In consistence with caspase-8 activity, caspase-3 activity in Db-scTRAIL stimulated Bcl-2 overexpressing cells was also very low and reached only about 30% of maximal activity observed in wild type NCI-H460 cells. Also, relative caspase-9 activity was much lower in NCI-H460/Bcl-2 cells. Overall these findings demonstrated that Bcl-2 overexpression was found to strongly reduce the enzymatic activity of initiator and effector caspases which were investigated here.

In the next step, cleavage kinetics of caspase-8, -3 and -9 were determined to study the sequence of apoptotic events at the biochemical level in more detail after Db-scTRAIL treatment. As it is well known that caspase-8 is the key caspase which is essential to initiate the caspase cascade *via* death receptors, the cleavage pattern between wild type and Bcl-2 overexpressing cells were compared. Procaspase-8 is present as a 53/55 kDa protein, upon Db-scTRAIL stimulation, first cleavage product of caspase-8, p41/43, appeared with identical intensities and kinetics in both cell lines (Figure 10A). Although, the first cleavage products could be detected after 2h of Db-scTRAIL treatment; the enzymatically full active p18 fragment was detected after 4h of stimulation in NCI-H460 wild type cells only. In fact, generation of the catalytically active p18 fragment appeared to be largely blocked in NCI-H460/Bcl-2 cells. As illustrated in Figure 10A, efficient cleavage of the pro form of caspase-3 into its small fragments was noticed in NCI-H460 wild type cells, first cleavage product p19 appeared within 3h of treatment, which is due to cleavage between large and small subunit of caspase-3 mediated by caspase-8 (Ferreira et al., 2012). After 4h of stimulation p19 fragment was auto processed to the fully enzymatically active p17 fragment and increased further in concentration until 6h after stimulation. Interestingly, the first cleavage product p19 in NCI-H460/Bcl-2 appeared after 3h, which was therefore comparable to the parental line. However, auto processing from p19 to p17 appeared to be exclusively blocked in NCI-H460/Bcl-2 cells (Figure 10A).

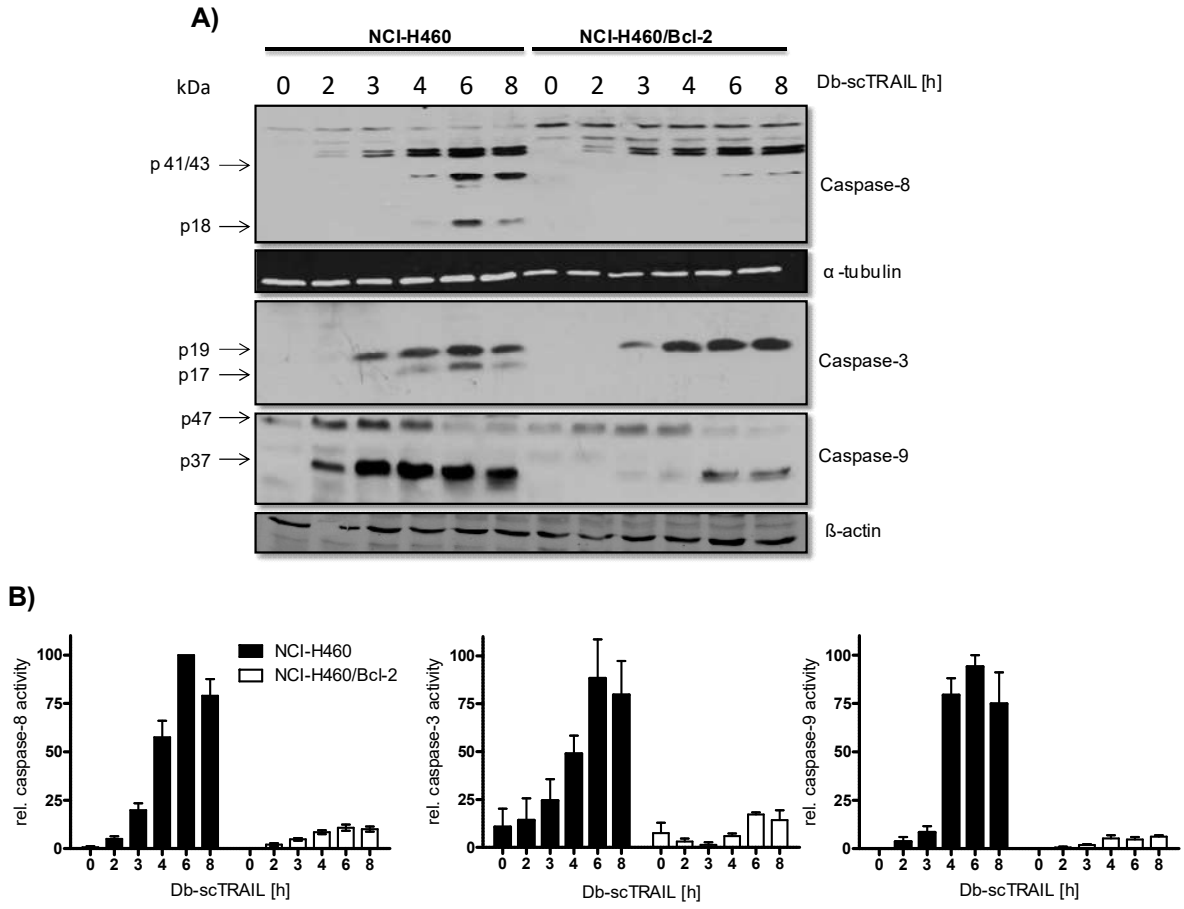

Figure 10: Overexpression of Bcl-2 impairs caspase activation

A) Cells were seeded in 6-well plates one day prior to stimulation. Next day stimulated with 1nM Db-scTRAIL up to 8h, one well was left untreated as a control. After stimulation cells were lysed on ice in cold lysis buffer. Equal amounts (50µg) of whole cell lysates from each sample were subjected to immunoblot analysis using cleaved caspase-8, cleaved caspase-3 and caspase-9 specific antibodies followed by HRP-conjugated secondary antibody. Membranes were reprobed with anti α-tubulin and actin antibodies to confirm equal loading. **B)** Cells were stimulated with Db-scTRAIL (1nM) for the indicated time periods. Whole protein lysates were incubated with fluorogenic caspases-3 substrate (Ac-DMQD-AMC), caspase-8 substrate (Ac-IEPD-AMC) and caspase-9 substrate (Ac-LEHD-AMC). The increasing fluorescence was measured every 2 minutes for 2 hours at λ=460 nm. To determine the relative increase in proteolytic activity, untreated control cells were included in the experiment. Data points are mean values ± SD calculated from 3 independent experiments, normalized to the highest value of each experiment.

As early as 2h after Db-scTRAIL stimulation in NCI-H460 cells, declining levels of procaspase-9 and the corresponding generation of processed caspase-9 (p37) were observed, as expected this processing of caspase-9 was also delayed in NCI-H460/Bcl-2 cells as it took 6h until the first cleavage form of caspase-9 could be observed in Bcl-2

overexpressing cells (Figure 10A). Taken together these results indicate that stimulation of NCI-H460 cells with Db-scTRAIL up to 8h results in quite significant and comparable initial cleavage of initiator and effector caspases in both cell lines but the further auto cleavage processes to generate enzymatically fully active fragments of caspase-8, -3 and -9 are largely inhibited in Bcl-2 overexpressing cells by downstream inhibitory mechanisms. So resistance to Db-scTRAIL in NCI-H460/Bcl-2 cells could be, at least in part, due to inhibition of caspases activation.

3.2.3. Comparable and significant initial cleavage of caspases in wild type and cells overexpressing Bcl-2

As distinct activation and cleavage pattern of caspases had been observed in the experiments described above, we were interested to compare the decrease in proenzyme levels after Db-scTRAIL stimulation in NCI-H460 and its counterpart overexpressing Bcl-2 in more detail. Western blot analysis was used to quantify the decline in protein expression levels of the pro forms of caspase-8, -3 and -9 after Db-scTRAIL treatments in NCI-H460 and NCI-H460/Bcl-2 cells. Decline of procaspase band intensities were quantified by ImageJ software and normalized to α-tubulin serving as a loading control. Values from untreated cells were taken as 100%. In general, quantification data clearly showed a comparable decline in proenzyme levels in a time dependent manner after treatment with 0.5nM Db-scTRAIL in parental and Bcl-2 overexpressing NCI-H460 cell lines (Figure 11).

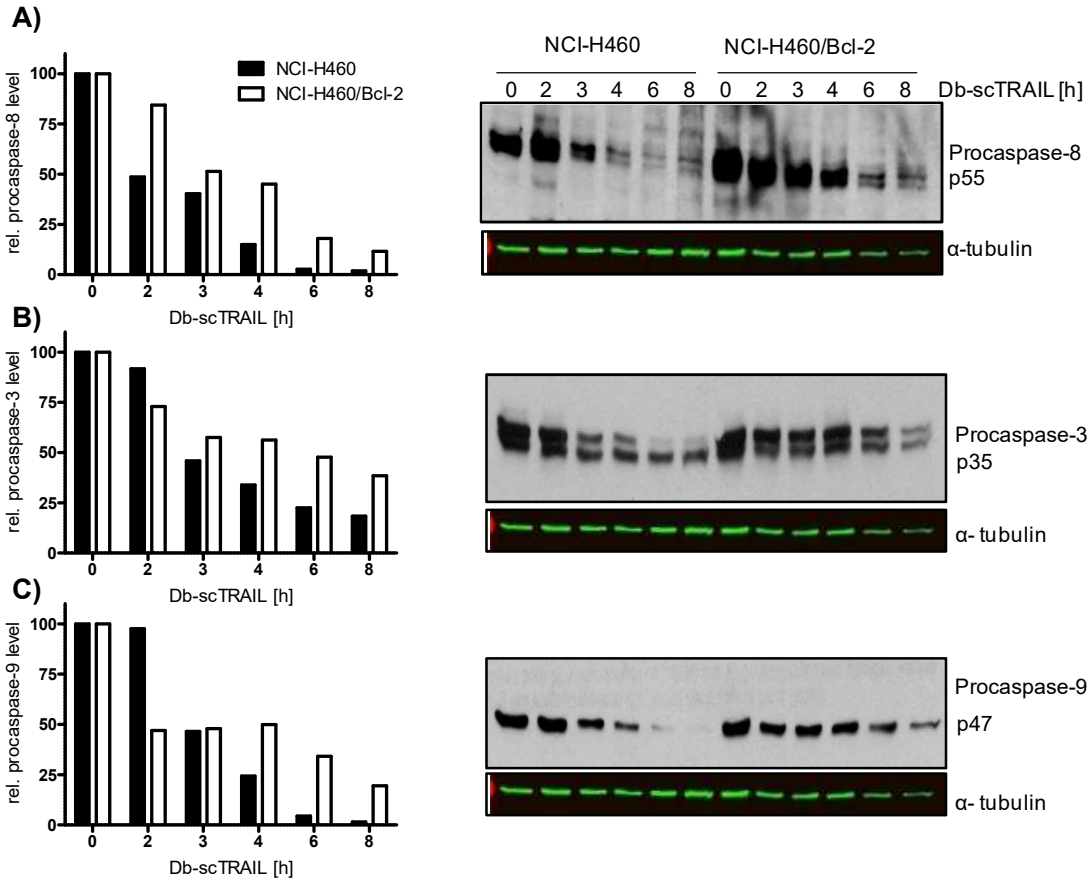

Figure 11: Comparable initial cleavage of caspases in wild type and Bcl-2 overexpressing cells

A) Cells were treated with 0.5nM Db-scTRAIL up to 8h; whole cell lysates were subjected to immunoblot analysis using antibodies specific to detect mature (pro) forms of caspases, i.e. caspase-8 **(A)**, caspase-3 **(B)** and caspase-9 **(C)**. Values on the graph represent the quantification data. Values are normalized to α-tubulin loading and untreated cells were taken as 100%.

3.3. Bcl-2 overexpression blocks mitochondrial perturbations after TRAIL treatment

3.3.1. Differential regulation of mitochondrial membrane potential after TRAIL stimulation

Permeabilization of the outer membrane of mitochondria plays a key role in the intrinsic pathway of apoptosis induction. In fact, it has been shown that depolarization of the mitochondrial membrane potential and the subsequent release of cytochrome c and Smac is induced by the opening of the mitochondrial permeability transition pore. In the literature conflicting data exist about the mitochondrial membrane potential ($\Delta\psi_m$) suggesting that loss $\Delta\psi_m$ may or may not occur early in the apoptotic pathway depending on the cell system. To check whether dissipation of the mitochondrial membrane potential is an early event in our cellular systems, and to further gain insight how overexpression of Bcl-2 might affect this process, the fluorescent dye TMRM (tetramethyl-rhodamine-methylester) was used to monitor changes in the mitochondrial membrane potential. Reduction in the TMRM fluorescence, indicating loss in mitochondrial potential, was measured by flow cytometry after Db-scTRAIL treatment. As shown in Figure 12A and B there was a time-dependent decrease in mitochondrial membrane potential in NCI-H460 cells as a drop in $\Delta\psi_m$ was observed between 2 and 4h of stimulation. Approximately 85% of all cells had lost their membrane potential after 8h of Db-scTRAIL treatment. By comparison, cells overexpressing Bcl-2 showed only a minute loss of $\Delta\psi_m$ induced by Db-scTRAIL within the first 4h of stimulation, rather a steady but limited loss of TMRM fluorescence was observed within 8h of stimulation.

3.3.2. Bcl-2 overexpression inhibits cytochrome c and Smac release from mitochondria after TRAIL treatment

Permeabilization of the mitochondrial outer membrane has been correlated with the release of apoptogenic proteins from the intermembrane space into the cytosol. Bcl-2 family members control the permeability of the mitochondrial outer membrane to release various proapoptotic proteins (Joza et al., 2002). To further confirm that Bcl-2-mediated protection against Db-scTRAIL is due to blocked mitochondrial depolarization, we investigated the release of two important proapoptotic molecules from mitochondria, cytochrome c and Smac.

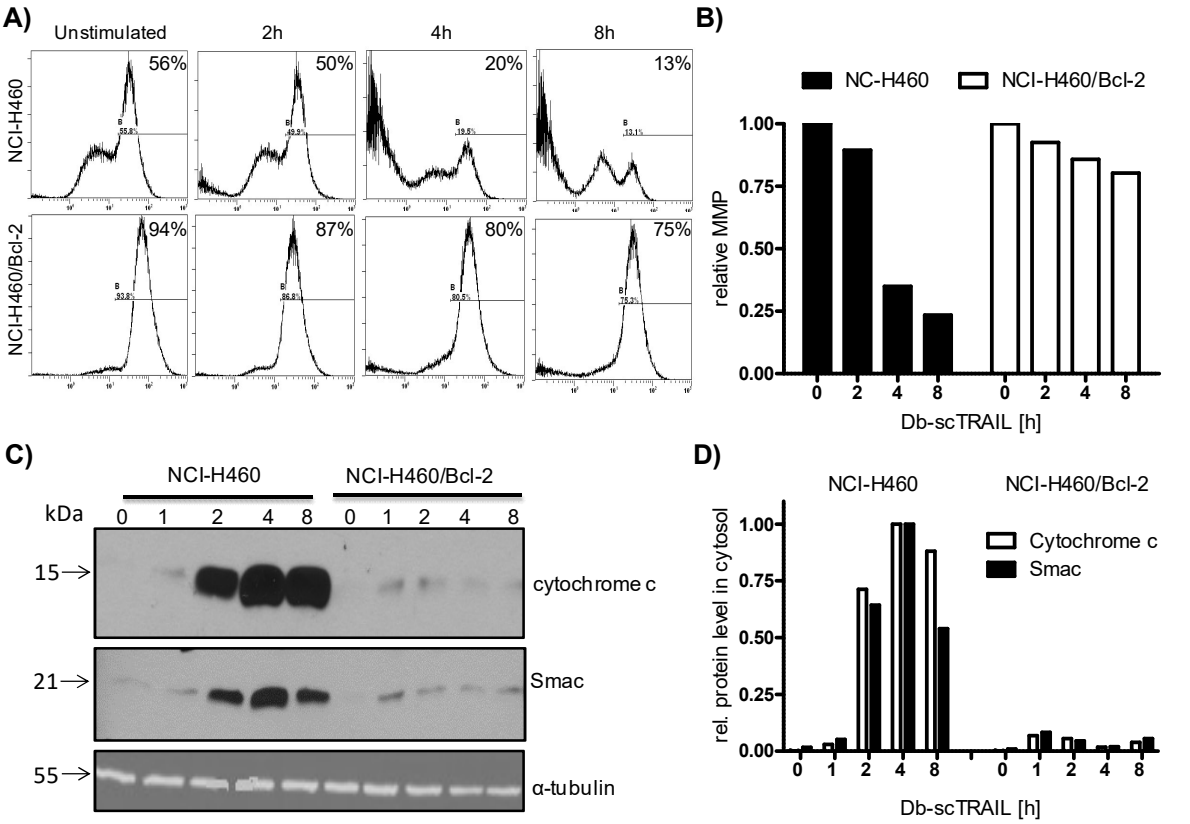

Figure 12: Overexpression of Bcl-2 blocks TRAIL-induced permeabilization of the mitochondrial outer membrane and release of proapoptotic proteins into the cytosol

A) For flow cytometry analysis both cell lines were left untreated or treated with 0.5nM Db-scTRAIL for the indicated time periods. In the last 30min of stimulation cells were loaded with 60nM TMRM. Cells were washed with PBS, resuspended in PBA and the fluorescence intensity was measured immediately by flow cytometry. **B)** Quantification of the results from flow cytometry normalized to values from unstimulated cells. Data shown is representative of three independent experiments. **C)** To determine the release of cytochrome c and Smac, cells were left untreated or treated with Db-scTRAIL (0.5nM) for the indicated time periods up to 8h. Cytosolic extracts from the cells harvested were subjected to western blotting with cytochrome c and Smac specific antibodies. Equal loading was confirmed by reprobing the membrane with α-tubulin antibody. **D)** Quantification of the results from western blot experiment normalized to maximum release observed (set to 1.0). Data shown is representative of three independent experiments.

Cytosolic cytochrome c promotes the formation of a multiprotein complex, the apoptosome, leading to activation of procaspase-9 and subsequent activation of effector caspases like caspase-3 and -7. On the other hand release of Smac into the cytosol neutralizes XIAP-mediated inhibition of caspases.

As illustrated in Figure 12C increased levels of cytochrome c could be detected in cytosolic fractions from NCI-H460 cells already after 2h of stimulation with Db-scTRAIL. However, this release was almost completely blocked in Bcl-2 overexpressing NCI-H460 cells. Even after 8h of Db-scTRAIL stimulation, there were no or only minimal detectable amounts of cytochrome c found in cytosolic fractions of NCI-H460/Bcl-2 cells. The kinetics of Smac release into the cytosol in NCI-H460 was comparable with those of cytochrome c release (Figure 12C). Again, after two hours of Db-scTRAIL stimulation significant amounts of Smac had been translocated into cytosol, although the absolute amount released appeared less than those of cytochrome c assuming comparable protein amounts in the cell and that both antibodies are of comparable quality.

As cytochrome c is involved in activation of procaspase-9, our data adequately fit to the caspase-9 activation data shown in Figure 10 where we could observe generation of the p37 fragment also as early as 2h after Db-scTRAIL stimulation. Together, our data suggest that the only 2-3 fold increase in Bcl-2 protein is capable to completely preserve mitochondrial integrity after Db-scTRAIL stimulation, thereby preventing the drop of the mitochondrial outer membrane potential and subsequently release of cytochrome c and Smac (and likely other proapoptotic factors) from mitochondria.

3.4. Overexpression of XIAP or its sub domains hardly affects TRAIL sensitivity

The second important aim of the present study was to evaluate the role of XIAP in TRAIL-induced apoptotic signaling pathways in the non-small cancer cell line NCI-H460. High expression levels of XIAP have been observed in many types of tumors and this elevated expression is thought to contribute to therapy resistance (Hunter et al., 2007). First we overexpressed full length XIAP and its BIR domains, as mutational studies on XIAP have revealed that its BIR2 domain is indispensable to inhibit caspase-3 and -7 while BIR3 is important for caspase-9 inhibition (Eckelman et al., 2006).

In order to examine the effect of each BIR domain of XIAP in TRAIL-induced apoptosis, after transfecting the cells with full length XIAP or BIR domains (BIR2 and BIR3) susceptibility of NCI-H460 to Db-scTRAIL was compared to untreated controls. Transfection efficiency was determined 24h after transfection *via* western blot analysis and also microscopically because

a plasmid coding for a GFP fusion protein had been used (Figure 13A). Expression of BIR3 domain was more efficient than that of BIR2 or full length XIAP as determined by western blotting (Figure 13B). Unexpectedly, cytotoxicity assays revealed only a very limited protection of NCI-H460 against Db-scTRAIL in cells overexpressing BIR2 and BIR3, whereas in XIAP transfected cells hardly any effect could be observed (Figure 13C).

Based on these results we concluded that the amounts of XIAP and the BIR domains overexpressed were not sufficient to protect the cells from Db-scTRAIL. As NCI-H460 cells possess 10 times more procaspase-3 molecules as compared to XIAP (Nadine Pollak, unpublished data), we assumed that there still remain sufficient amounts of free caspase-3 which can proceed with substrate cleavage and apoptosis induction despite increased amount of XIAP.

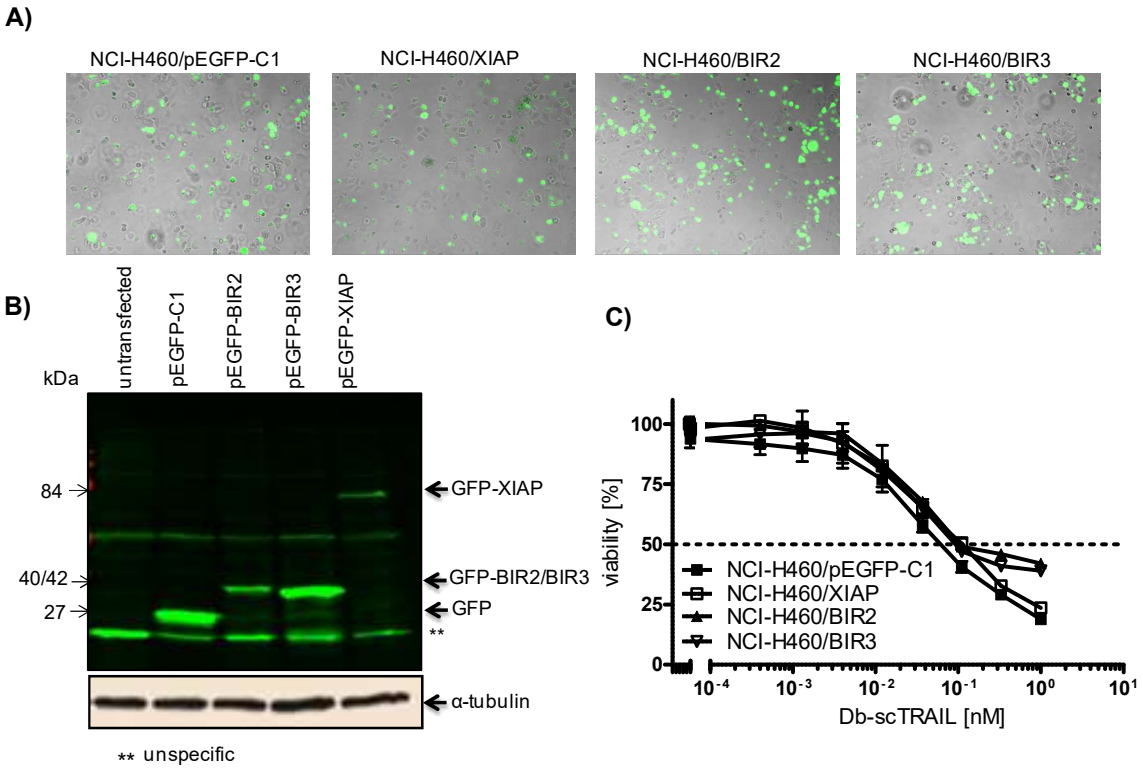

Figure 13: Overexpression of XIAP or its sub domains (BIR domains) and the effects on TRAIL-induced apoptosis in NCI-H460 cells

A) Cells were transiently transfected with empty vector (pEGFP-C1) as a control or vectors encoding full length XIAP, BIR2 and BIR3 domains. 24h later the transfection efficiency was checked by visualizing GFP signal under inverted microscope (EVOS) **B)** Whole cell lysates (50µg) from each transfection described in (A) was separated on a 12% acrylamide gel and overexpression was

confirmed by immunoblot analysis using anti-GFP antibody. **C)** To investigate the effect of overexpression on TRAIL-induced apoptosis, transfected cells from (A) were seeded in a 96-well plate and one day after transfection cells were stimulated with increasing concentrations of Db-scTRAIL for 24h. Viable cells were stained with crystal violet, dissolved in methanol and the absorbance was determined at 55nm. Cells transfected with pEGFP-C1 were used as control. Values shown are mean ± SD calculated from triplicates. All the values from stimulated cells were normalized to unstimulated cells. One representative experiment out of three is shown.

3.5. XIAP inactivation restores TRAIL sensitivity in Bcl-2-transfected NCI-H460 cells

3.5.1. Inhibition of XIAP or XIAP knockdown restore TRAIL-induced cytotoxicity in NCI-H460 cells overexpressing Bcl-2

XIAP, a potent endogenous inhibitor of apoptosis, is known to act by directly binding to caspase-3, -7 and -9. To validate the role of XIAP in the protection of Bcl-2 overexpressing cells against TRAIL-induced cell death we used two different approaches. In a first approach Smac mimetic SM83 was used, a compound known to mimic the function of Smac by binding to XIAP, but also shown to inhibit c-IAP1 and c-IAP2. Cotreatment of NCI-H460/Bcl-2 cells with Db-scTRAIL and a sub toxic concentration (1µM) of SM83 strongly restored apoptosis induction in Bcl-2 overexpressing cells (Figure 14B). In contrast, addition of SM83 to untransfected control cells revealed only little enhancement of apoptosis induction in comparison to Db-scTRAIL only (Figure 14A).

In a different approach NCI-H460 and NCI-H460/Bcl-2 cells were transfected with XIAP specific siRNAs. Figure 14E demonstrates that the protein expression level of XIAP could indeed be significantly reduced in both cell lines. Cytotoxicity assays demonstrated that NCI-H460 cells overexpressing Bcl-2 were significantly sensitized to Db-scTRAIL-induced cell death by treatment with siRNA against XIAP, as approximately 90-100% cell death could be achieved at higher Db-scTRAIL concentrations (Figure 14D). In contrast, no significant enhancement in apoptosis was observed in NCI-H460 control cells after XIAP knockdown, as these showed a high TRAIL sensitivity anyway (Figure 14). Preincubation of NCI-H460/Bcl-2 cells deficient in XIAP with the broad caspase inhibitor zVAD-fmk (zVAD) completely protected these cells, demonstrating that TRAIL-mediated cell death in cells with downregulated XIAP but overexpressed Bcl-2 is in fact caspase-dependent (Figure 14F).

It has been shown previously in some studies that downregulation of XIAP can be compensated for by upregulation of other IAP family members like c-IAP1 and c-IAP2 (Harlin et al., 2001). However, we could not observe any significant changes in the protein levels of c-IAP1 or survivin after siRNA-mediated knockdown of XIAP, whereas c-IAP2 was not detectable (data not shown). It is therefore likely that the observed changes in TRAIL-mediated sensitivity caused by XIAP downregulation are in fact specific for XAIP and are not caused by secondary, indirect effects.

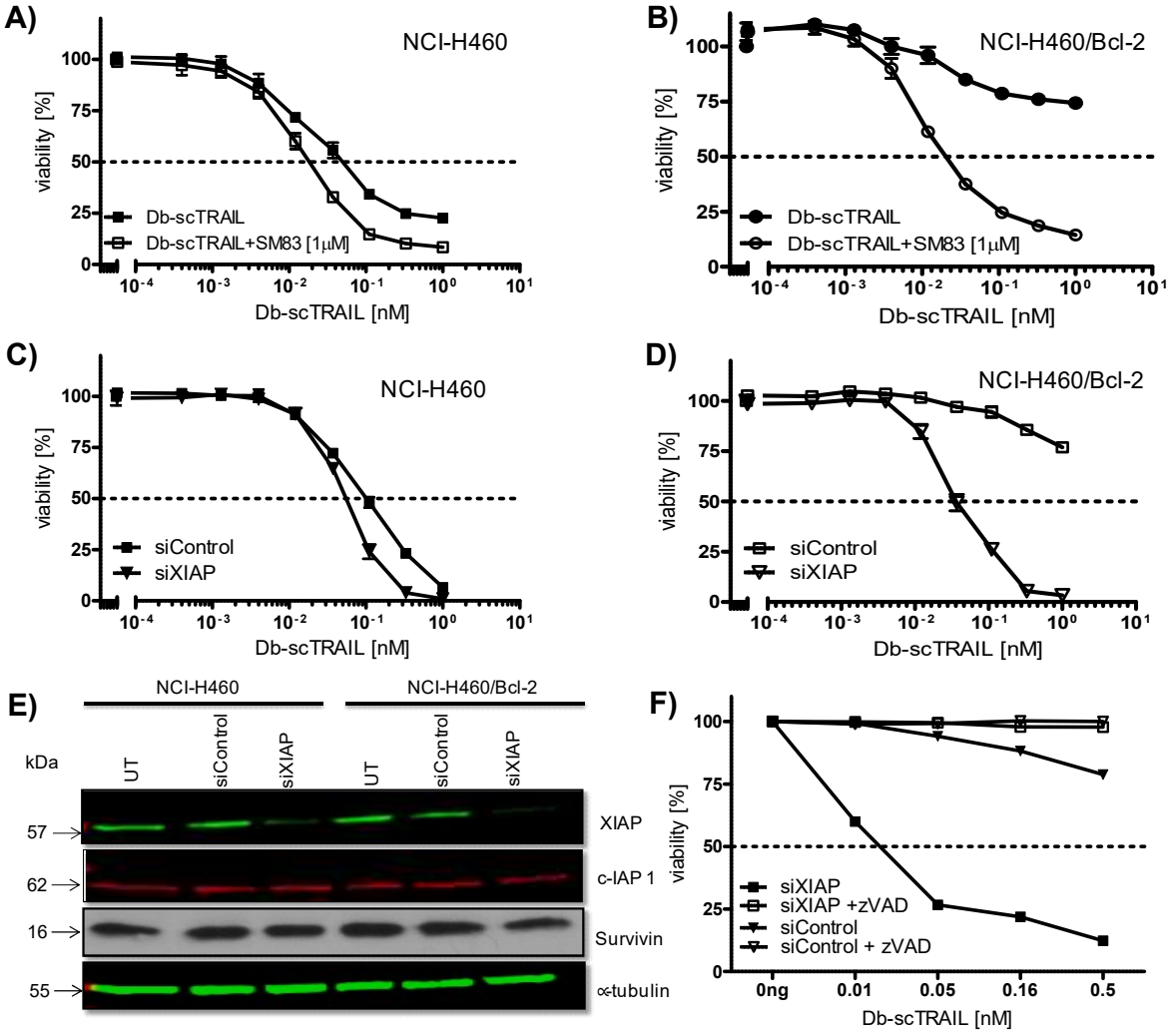

Figure 14: XIAP inhibition or downregulation sensitize Bcl-2 overexpressing cells to TRAIL-induced apoptosis

A) and **B)** NCI-H460 wild type and NCI-H460/Bcl-2 cells were seeded in 96-well plates. Prior to stimulation with Db-scTRAIL the cells were preincubated with 1µM Smac mimetic SM83 followed by

stimulation with increasing concentrations of Db-scTRAIL for 24h. Viable cells were stained with crystal violet, dissolved in methanol and the absorbance was determined at 550nm. Values shown are mean ± SD calculated from triplicates. All the values from stimulated cells were normalized to unstimulated cells. One representative experiment out of three is shown. **C)** and **D)** NCI-H460 wild type and NCI-H460/Bcl-2 cells were seeded in 96-well plates, adherent cells were then transiently transfected with 8nM of either control (filled and open square) or XIAP (filled and open triangle) specific siRNA. 48h post transfection cells were treated with increasing concentrations of Db-scTRAIL. After 24h incubation at 37˚C, viable cells were stained with crystal violet. All the values were normalized to those from unstimulated cells. The experiment was performed in triplicates and the data shown are representative of three independent experiments. **E)** Cells were seeded one day prior to transfection in a 6-well plate. Next day when confluency was approximately 50-60%, cells were left untreated (UT) or were transiently transfected with non-targeting (siControl, 8nM) or siRNA specific for XIAP (siXIAP, 8nM). Protein expression was determined 48h post transfection from whole cell lysates *via* immunoblot assay using an XIAP specific antibody. Protein expression of other closely related proteins (c-IAP1 and survivin) was also investigated using the respective antibodies. In order to confirm equal loading, the membrane was reprobed with α-tubulin specific antibody. **F)** NCI-H460/Bcl-2 cells were transiently transfected with siRNA (either control or XIAP-specific, 8nM) and 48h post transfection the cells were either preincubated with the caspase inhibitor zVAD-fmk (20μM) (open square and triangle) or only culture medium (filled square and triangle) for one hour before being stimulated with different concentrations of Db-scTRAIL for 24h. Cell viability was then determined by crystal violet staining. Data shown is from two independent experiments with similar results.

3.5.2. Enhancement of caspase processing after XIAP knockdown in Bcl-2 overexpressing cells

To gain insight into the mechanism involved in the sensitization of Bcl-2 overexpressing to Db-scTRAIL after XIAP knockdown, we first analyzed the processing of initiator and effector caspases in control and XIAP knockdown cells. Approximately 70% reduction in XIAP protein level was observed after siRNA transfection for 48h (Figure 15A). As shown in Figure 10 that full activation of initiator and effector caspases is largely inhibited in Bcl-2 overexpressing cells. Notably, western blot analyses after Db-scTRAIL treatment of XIAP deficient NCI-H460/Bcl-2 cells confirmed a pronounced enhancement in proteolytic processing of caspase-8, -3 and -9 (Figure 15B). As shown in Figure 15B in control-transfected cells incomplete processing of caspase-3 to its intermediate cleavage product p19 was observed. In contrast, caspase-3 was largely processed to its fully active fragments p17 in XIAP knockdown cells. In line with caspase-3 processing, a dramatic increase in the appearance of the enzymatically fully active p18 product of caspase-8 was observed in NCI-H460/Bcl-2 cells downregulated in XIAP as compared to control-transfected cells. Moreover, an enhancement in caspase-9 processing was also observed (Figure 15B). This again points to the

involvement of mitochondria in enhancement of cell death after Db-scTRAIL treatment in XIAP deficient Bcl-2 overexpressing cells.

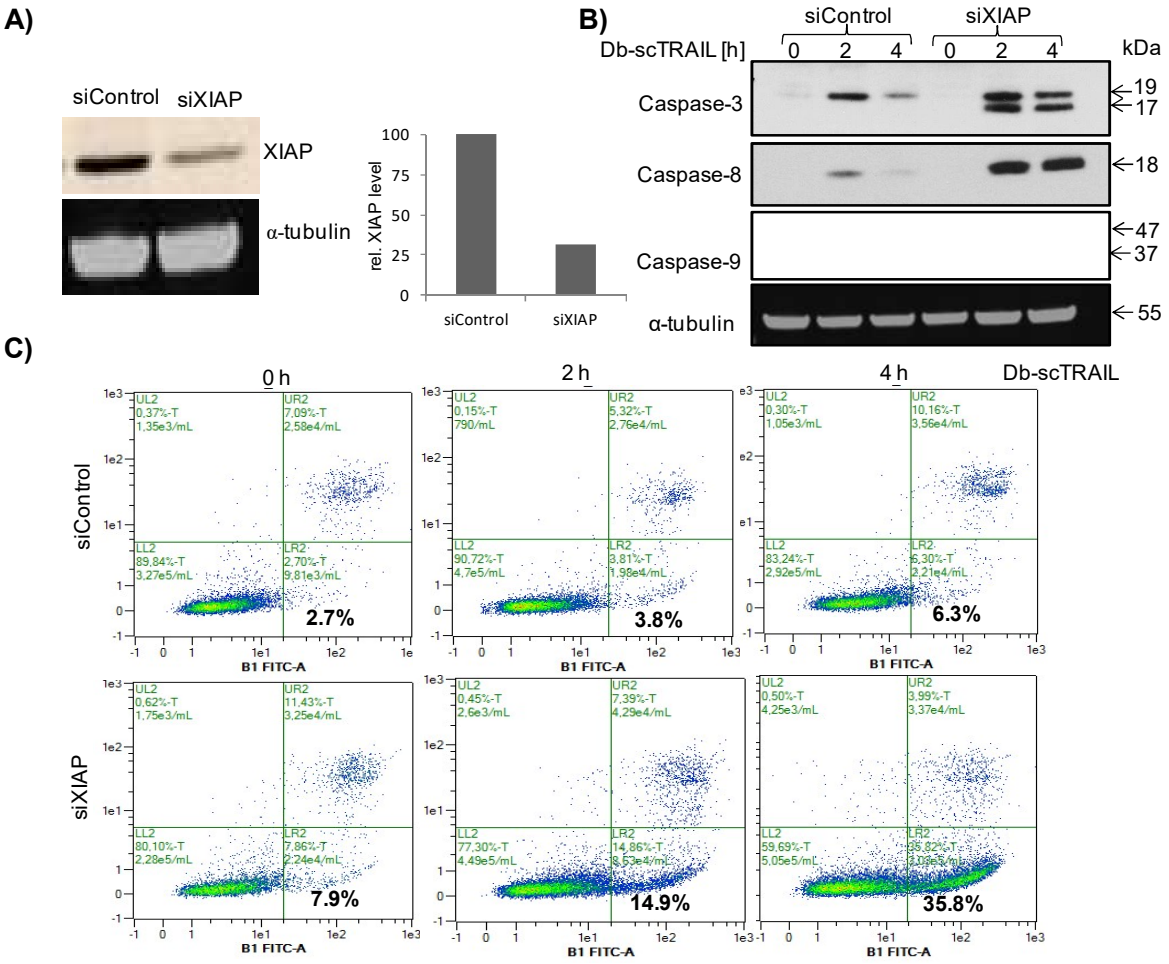

Figure 15: XIAP knockdown enhances caspase processing in Bcl-2 overexpressing cells after TRAIL stimulation

A) The expression of XIAP was determined 48h after transient transfection by immunoblot assay using an antibody specific for full length XIAP. Quantification of the resulting knockdown was performed using ImageJ software, which revealed 70% reduction in the expression of XIAP in siXIAP transfected cells as compared to siControl transfected cells. **B)** To determine the processing of caspases, NCI-H460/Bcl-2 cells were transiently transfected with either non-targeting siRNA (siControl) or XIAP specific siRNA (siXIAP). 48h post transfection cells were treated with 1nM Db-scTRAIL for 2 and 4h, one well was left unstimulated. Identical amounts of whole cell lysates (50µg) were loaded on SDS gel to separate the proteins. Proteins were transferred to nitrocellulose membranes, and detected using specific antibodies against cleaved caspase-3, cleaved caspase-8 and caspase-9. α-tubulin was used to verify equal loading. One experiment out of two with very similar results is shown here. **C)** To confirm apoptosis induction after Db-scTRAIL treatment in XIAP knockdown cells, Bcl-2 overexpressing NCI-H460 cells had been transiently transfected with control or siRNA specific for

XIAP (8nM) for 72h. Cells were then treated with 1nM Db-scTRAIL up to 4h and the extent of apoptosis was quantified by annexin V/PI staining followed by flow cytometric analyses. Quadrants are characterized as live (left lower), early apoptosis (right lower), late apoptosis (right upper) and necrotic cells (left upper).

Results from cytofluorometric analyses of annexin V/PI staining of the XIAP knockdown cells revealed 35% of annexin V positive cells after 4h of stimulation with Db-scTRAIL as compared to only 6% cell death in control transfected cells (Figure 15C). These data further demonstrate that the enhanced cell death in Bcl-2 overexpressing cells after downregulation of XIAP is based on apoptosis induction.

3.6. Caspase-3 activation remains unaffected after caspase-9 silencing

In context of the type I/II cell death model proposed initially for the Fas system and later on for TRAIL (Scaffidi et al., 1998; Suliman et al., 2001), our model cell line NCI-H460 dies after Db-scTRAIL treatment as a so-called type II cell, as blockage of mitochondria protects cells from apoptosis. Interestingly, the data so far established demonstrate that XIAP knockdown completely resensitizes Bcl-2 overexpressing cells to TRAIL-induced apoptosis. Assuming that the mitochondria are still protected by Bcl-2 in these cells one might therefore argue that in NCI-H460 cells the interaction between XIAP and Smac might be the central regulatory mechanism after mitochondrial depolarization. More simply said, the release of Smac from mitochondria to neutralize XIAP seems to be the more crucial event in sensitizing cells to Db-scTRAIL.

The role of caspase-9 in apoptosis induction in type II cells is still not very clear, since differential contribution has been described in various TRAIL sensitive tumor cell lines including colon (HCT-116, SW480) and lung (NCI-H460) (Ozoren et al., 2000). Based on these considerations in the next step we wanted to address whether caspase-9 deficiency affects the TRAIL sensitivity in NCI-H460 cells. siRNA specific for caspase-9 was used to downregulate its protein level but also the small inhibitor molecule z-LEHD-fmk was applied which irreversibly binds to caspase-9. The HCT-116 colon cancer cell line was included in these studies as a control, because from literature it is known that z-LEHD-fmk completely protects HCT116 from TRAIL-induced apoptosis (Ozoren et al., 2000). As shown in Figure 16A pretreatment with the caspase-9 inhibitor resulted in no significant protection against

TRAIL-induced apoptosis in NCI-H460 cells while HCT-116 cells were completely protected (data not shown). In contrast, the broad caspase inhibitor zVAD-fmk was very effective in protecting both cell lines against TRAIL-induced apoptosis as well as inhibiting caspase-9 processing (Figure 16B). To further analyze the underlying molecular mechanisms we investigated the cleavage of caspase-9 in cells treated with Db-scTRAIL alone or in combination with the zVAD-fmk or z-LEHD-fmk. In HCT-116 cells the cleavage of procaspase-9 into its active fragments was completely blocked when using z-LEHD-fmk (Figure 16B). On the other hand processing of caspase-9 was hardly affected and cleavage into the p37/35 fragments could be observed even after cotreatment of NCI-H460 cells with the caspase-9 inhibitor z-LEHD-fmk and Db-scTRAIL.

As all these caspase inhibitors are not highly selective, in a next set of experiments a siRNA-based approach was used to downregulate caspase-9. As shown in Figure 16C caspase-9 protein level was significantly reduced 48h post transfection. Both short-term (4h; Figure 16E) and long-term (24h; Figure 16D) apoptosis assays were performed after caspase-9 knockdown to investigate the effect of caspase-9 silencing on TRAIL-induced apoptosis. Our results clearly demonstrate that despite a strong downregulation of caspase-9 NCI-H460 cells remained fully sensitive to Db-scTRAIL, as no significant differences in dose (Figure 16D) and time dependent (Figure 16E) cell death was observed.

Effector caspase-3 can be activated by both caspase-8 and caspase-9 *via* a positive feedback loop. Therefore we investigated whether downregulation of caspase-9 does have some effects on caspase-3 processing. As suggested already from the results of the cytotoxicity assays caspase-3 processing remained unaffected after caspase-9 knockdown (Figure 16F). In fact, caspase-3 was similarly cleaved into its p19/17 fragments in the presence or absence of caspase-9. Based on these results it might be concluded that the contribution of caspase-9 for caspase-3 activation is only of minor importance in NCI-H460 cells whereas direct activation of caspase-3 by caspase-8 should play an important role.

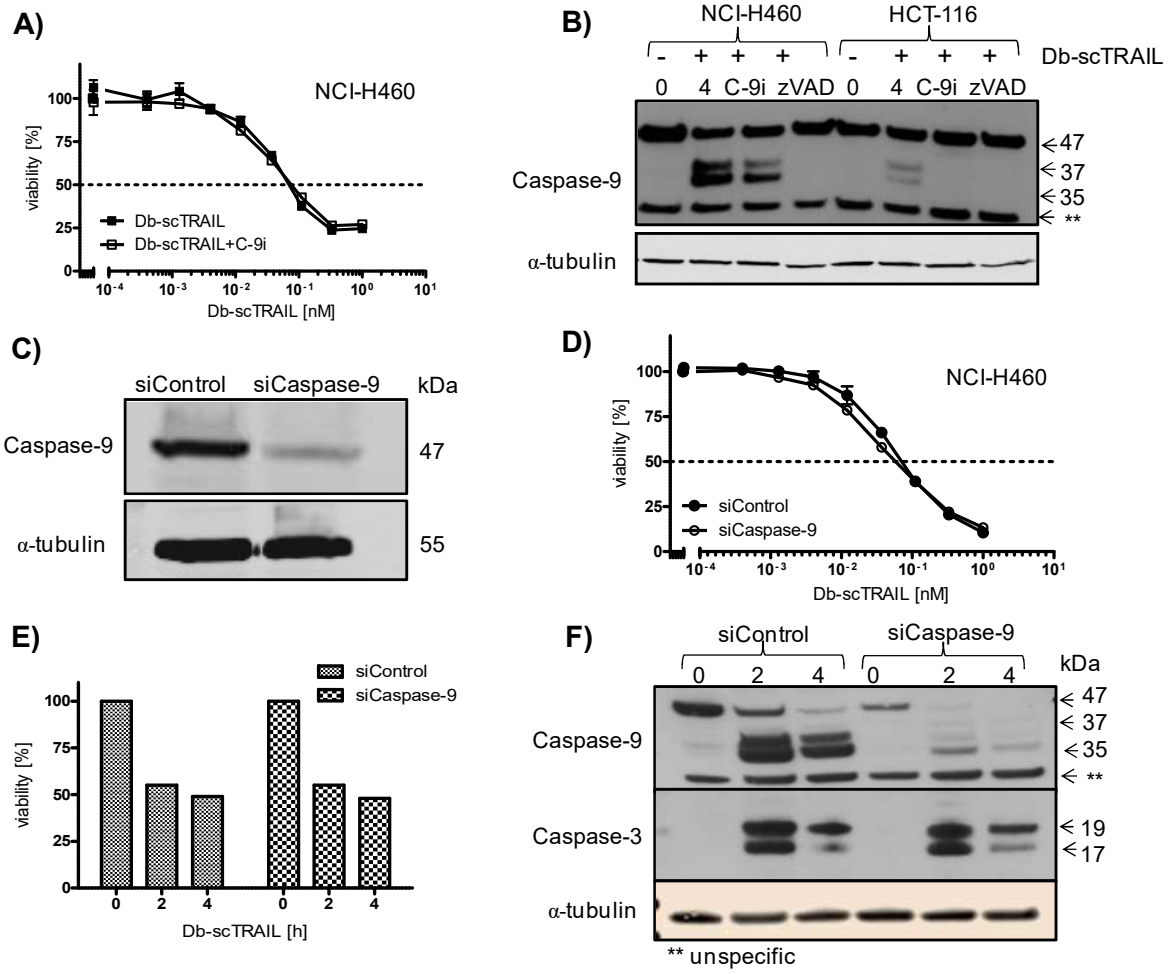

Figure 16: Caspase-9 plays a minor role in TRAIL-induced apoptosis in NCI-H460 cells

A) Cells were cultured in 96-well plates the day prior to stimulation, next day cells were preincubated with 50µM caspase-9 inhibitor (z-LEHD-fmk) for one hour, followed by stimulation with different concentrations of Db-scTRAIL for 24h. Viable cells were stained with crystal violet and absorbance was measured at 550nm after dissolving the dye in methanol. Data represent one out of two independent experiments. **B)** NCI-H460 and HCT-116 (used as control) cells were seeded in 6-well plates, preincubated with 50µM caspase-9 inhibitor (C-9i) or 20µM broad range caspase inhibitor zVAD-fmk (zVAD) for one hour, followed by Db-scTRAIL stimulation for 4h. Whole cell lysates were used to check caspase-9 processing by western blotting using anti-caspase-9 antibody. **C)** NCI-H460 cells were transfected with 8nM siRNAs (siControl and siCaspase-9). Transfection efficiency was confirmed by western blotting after 48h. **D)** NCI-H460 were transfected with either non-targeting (siControl) or caspase-9 (siCaspase-9) siRNAs in a 96-well plate; 48h post transfection cells were stimulated with increasing concentrations of Db-scTRAIL for further 24h. Viability was measured by staining adherent cells with crystal violet, and measuring the absorbance of the dye at 550nm. **E)** and **F)** Cells were transiently transfected with non-targeting siRNAs and siRNA against caspase-9 for 48h, followed by 2 and 4h treatment with 1nM Db-scTRAIL, one well was left untreated. Viability of cells was determined by crystal violet staining (E). 30µg whole cell lysate from each sample were subjected

to western blot analysis using specific antibodies directed against caspase-9 and caspase-3. α-tubulin was used to confirm the equal loading (F).

3.7. Downregulation of Smac is not sufficient to protect NCI-H460 cells from TRAIL-induced apoptosis

Disruption of mitochondria results in the release of proapoptotic molecules like Smac, Omi and others, in addition to cytochrome c. Protection of the mitochondrial pathway in NCI-H460 cells almost completely prevented Db-scTRAIL-mediated apoptosis. However, sensitivity to TRAIL could be recovered by XIAP knockdown, and in addition the cell permeable Smac-mimetic SM83 significantly enhanced cell death in NCI-H460/Bcl-2 cells after Db-scTRAIL treatment. From these data it can be assumed that Smac release from mitochondria to antagonize XIAP is an important step of Db-scTRAIL-induced apoptosis in our model cell line NCI-H460.

In the next step we therefore evaluated in more detail the role of endogenous Smac. Again, this molecule was transiently downregulated by siRNA. Several studies have investigated the expression of Smac and its role in cancer progression and treatment in lung cancer cells, but outcome with contradictory results. Sekimura and co-workers demonstrated that Smac expression was significantly reduced in lung cancer cells as compared to normal lung tissues, Additionally they correlated the low expression of Smac with poor prognosis of patients (Sekimura et al., 2004), whereas two other studies found higher expression of Smac mRNA in different lung cancer types in comparison to normal cells. Moreover observed higher expression of Smac was linked to cancer progression (Krepela et al., 2006; Yang et al., 2008). Based on these conflicting results we were interested to study the effect of Smac downregulation on Db-scTRAIL-induced apoptosis in NCI-H460.

The expression level of Smac was significantly, but not very strongly reduced, about 50% reduction was observed 48h after transfection as illustrated in Figure 17A. Treatment with Db-scTRAIL, however, had no significant effect on apoptosis induction in comparison to control transfectants (Figure 17B). In fact, the cell death pattern was comparable at both low and higher doses of Db-scTRAIL. XIAP NCI-H460 cells treated with siXIAP were included in this experiment as an additional control and as expected these cells showed enhanced Db-scTRAIL sensitivity particularly at low concentrations (Figure 17B). However, different activation kinetics of caspase-3 could be observed after Smac knockdown as compared with

cells transfected with control siRNA after Db-scTRAIL stimulation (Figure 17C). Following Smac knockdown the p17 fragment of caspase-3 was hardly detectable at all time points investigated after Db-scTRAIL treatment. In addition the p19 fragment disappeared in a time-dependent manner. In contrast, in cells transfected with siXIAP, p17 and p19 could be observed as strong bands at all time points. These data indicate that downregulation of Smac in fact had significant effects on Db-scTRAIL-induced apoptosis induction.

In a next attempt we used the proteasome inhibitors MG132 and bortezomib to inhibit the degradation of the active fragments of caspase-3. This was based on the assumption that Smac deficiency results in elevated levels of XIAP which are subsequently responsible for ubiquitination and degradation of active caspase-3. The results presented in Figure 17D in fact demonstrate that both protease inhibitors caused an elevation of the protein levels of fragments, p17 and p19.

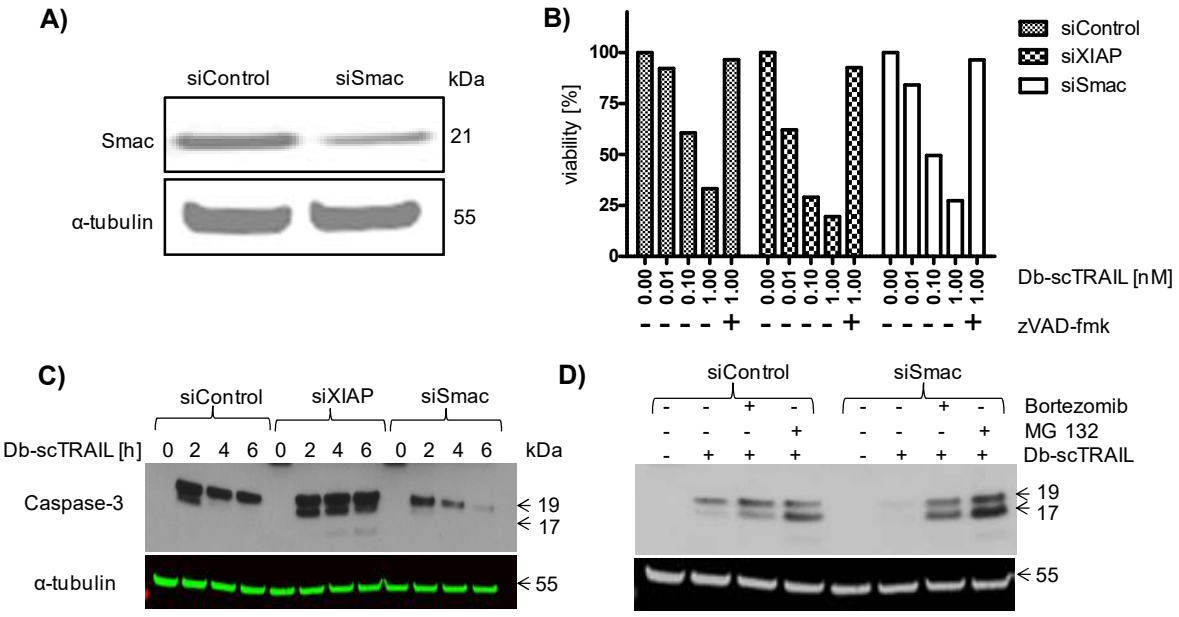

Figure 17: Smac knockdown results in enhanced caspase-3 degradation

A) NCI-H460 cells were transfected with 8nM siRNAs against Smac (siSmac) or non-targeting (siControl). Expression levels of Smac were determined 48h after transfection *via* western blotting using Smac specific antibody. Equal loading was confirmed by detection of α-tubulin. **B)** NCI-H460 cells were transfected with siRNAs specific for XIAP or Smac, non-targeting siRNA was included as control. 48h post transfection, cells were stimulated with Db-scTRAIL (0.01-1nM), in the last wells caspase inhibitor (zVAD-fmk) was added at a final concentration of 20µM. At the end of the stimulation time (24h) surviving cells were stained with crystal violet, dried over night, dissolve in 50µl/well methanol and the absorbance were measured by micro plate reader at 550nm. All the values from

stimulated cells were normalized to unstimulated cells. One representative experiment out of three is shown. **C)** 48h post transfection with XIAP, Smac and non-targeting siRNAs, cells were treated with Db-scTRAIL (1nM) up to 6h. Equal amounts of whole cell lysates (30µg) were separated on a 12% acrylamide gel and analyzed by immunoblotting using cleaved caspase-3 antibody. **D)** NCI-H460 cells were transfected with control and Smac siRNAs. 48h after transfection, cells were preincubated with MG132 (25µM) or Bortezomib (1µM) followed by stimulation with 1nM Db-scTRAIL for 4h. Immunoblotting was performed to detect caspase-3 processing using cleaved caspase-3 antibody. α-tubulin was used to confirm the equal loading.

3.8. NCI-H460 cells represent a heterogeneous population with regard to mitochondrial potential

While investigating the mitochondrial membrane potential ($\Delta\psi_m$) an interesting observation was the heterogeneity among NCI-H460 cells, as remarkable subpopulations existed having differences in their mitochondrial membrane potential (Figure 12A). These results raised the question whether this heterogeneity among the cell population with regard to mitochondrial potential is a specific feature of NCI-H460 cells only or whether this might also exist in other tumor cell lines. Therefore, together with NCI-H460 cells five different tumor cell lines, including colon (Colo 205, HCT-116) and breast cancer cell lines (BT 474, MDA-MB 231) were investigated for their mitochondrial potential using TMRM. The results presented in Figure 18A demonstrate that no subpopulations could be observed in all other tumor cell lines investigated. These results indicate that some particular changes or even defects in the mitochondrial integrity occur in the NCI-H460 cell population, which can be overcome by Bcl-2 overexpression as no heterogeneity was observed in the $\Delta\psi_m$ of the NCI-H460/Bcl-2 cell population (Figure 12A).

To assure the above mentioned observation and to gain further insight into this heterogeneous cell population among NCI-H460 cells, the cells were loaded with TMRM and sorted on the base of differences in TMRM intensities. After sorting of two distinct cell populations, having high and low TMRM intensities (Figure 18B), these were cultured for one more week followed by reanalysis of the TMRM intensities. It was evident from the results that the cells having high mitochondrial membrane potential were stable as we still observed a relatively homogenous population with regard to the TMRM intensity, i.e. the mitochondrial potential. On the other hand those cells having low TMRM intensities showed after 7 days of cell culture a comparably broad distribution of TMRM intensities, indicating a quite heterogeneous mitochondrial membrane potential. This experiment was repeated two times,

revealing comparable results each time. Further studies are needed to explain and investigate the role of this difference in mitochondrial potential and the factors responsible for such heterogeneity within a population.

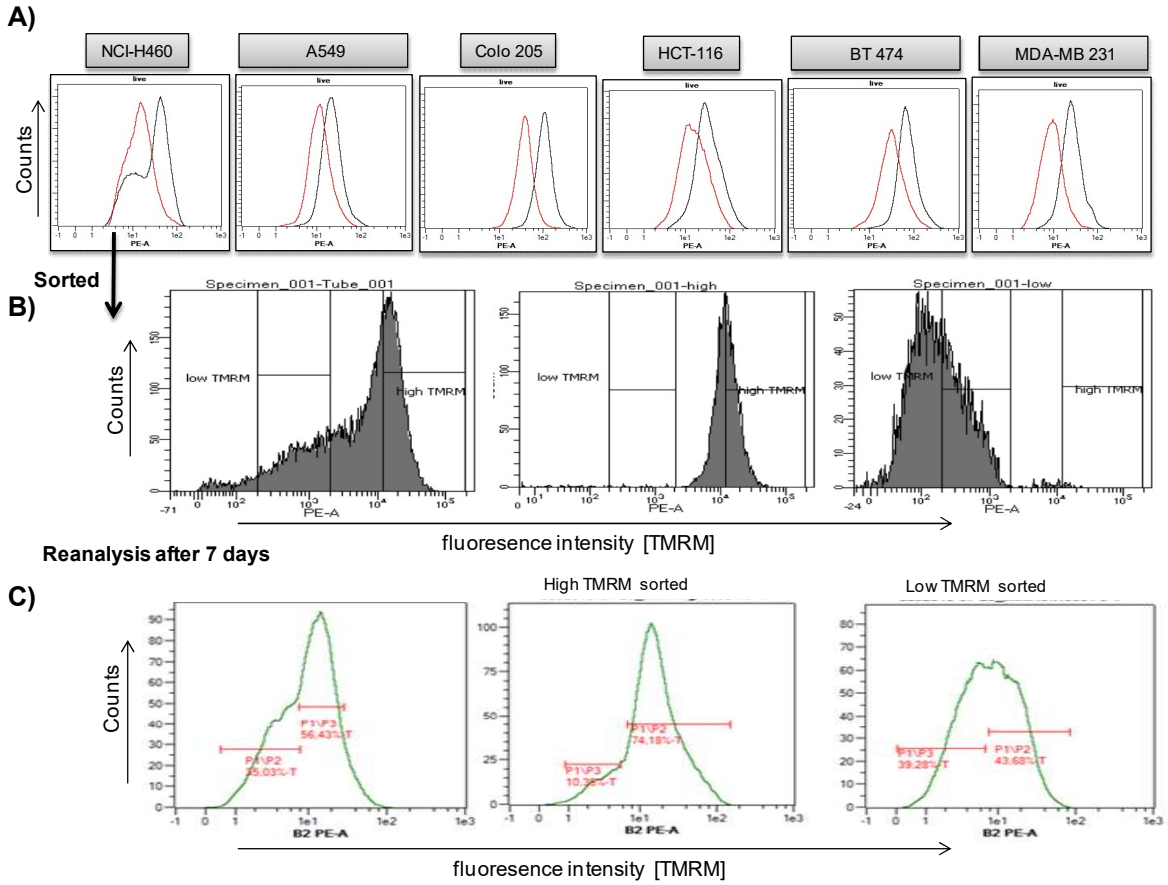

Figure 18: Sorting of NCI-H460 with regard to differences in mitochondrial potential

A) NCI-H460 cells were loaded with either 60nM TMRM alone (black histograms) or in combination with 80µM CCCP (red histograms), which was used a control for a breakdown of the mitochondrial potential for 30min at 37˚C. Cells were gently trypsinized, washed once with PBS, resuspended in PBA and immediately analyzed by flow cytometry CCCP: carbonyl cyanide *m*-chlorophenylhydrazone. **B)** NCI-H460 cells were incubated with 60nM TMRM for 30min at 37˚C, gently trypsinized and resuspended in PBS containing 10% FCS, filtered (70µm) and separated according to the TMRM intensity using a FACSAria III (left: cells before sort; middle: sorted population with high TMRM; right: sorted cells with low TMRM intensity). **C)** Sorted cells were cultured in media containing 10% FCS for further 7 days, and then reanalyzed after TMRM loading to investigate the stability of the sorted cells with regard to TMRM intensities.

3.8.1. TRAIL susceptibility is comparable in NCI-H460 sub-populations with different mitochondrial membrane potential

While investigating the effect of Db-scTRAIL on mitochondrial membrane potential ($\Delta\psi_m$) *via* flow cytometry analysis, existence of a subpopulation with relatively low TMRM fluorescence has been observed in untreated NCI-H460 (Figure 19A). To further evaluate the observed heterogeneity within the population with regard to $\Delta\psi_m$, cells were analyzed using live cell imaging. For this cells were loaded with TMRM and imaged by fluorescence microscopy. The background-corrected cellular TMRM fluorescence intensity was calculated for 100 cells. As shown in Figure 19B, a NCI-H460 cell population can be separated into two subpopulations having a different TMRM fluorescence intensity, hence distinct mitochondrial membrane potential. The results confirmed the flow cytometry data that already in unstimulated cells there exists a remarkable subpopulation (approx. 30-40%) with relatively low mitochondrial membrane potential.

Surely, one would now be interested to know the effect of these variations in mitochondrial membrane potential on apoptosis induced by TRAIL. To explore this, after loading NCI-H460 cells with TMRM, they were stimulated with Db-scTRAIL. The cellular fate was followed by live cell imaging. Keeping in mind the fact that NCI-H460 cells are of type II which are highly dependent on mitochondrial pathway for apoptosis induction, faster apoptosis execution was expected for cells with low TMRM fluorescence intensity. Surprisingly, there was no difference in Db-scTRAIL susceptibility between the subpopulations and apoptosis was induced in more than 90% of the cells between two to four hours post treatment (Figure 19C).

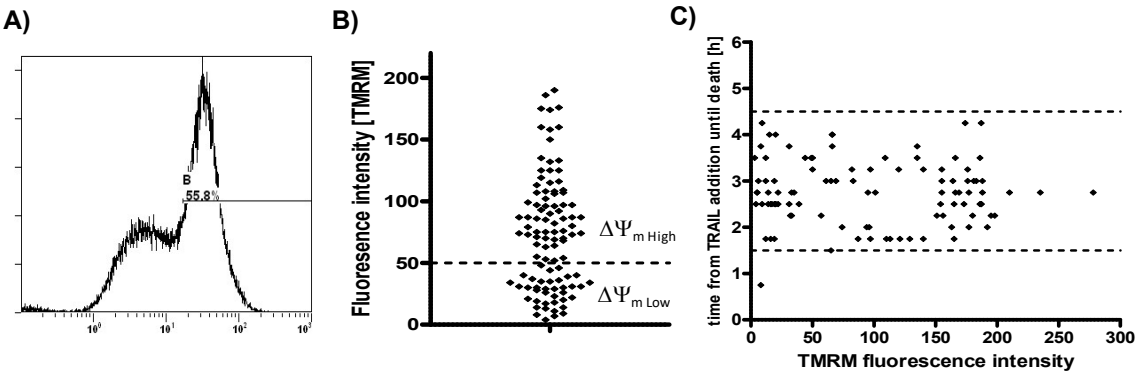

Figure 19: Equal susceptibility of two cellular sub-populations with different $\Delta\psi_m$ to TRAIL

A) NCI-H460 cells were incubated with 60nM TMRM for 30min. Cells were washed with PBS, resuspended in PBA and the fluorescence intensity was measured immediately by flow cytometry. **B)** NCI-H460 cultivated on 35mm glass-bottom dishes were loaded with 60nM TMRM for 30min at 37°C and images were taken using a fluorescence microscope. TMRM fluorescence and corresponding cell size were analyzed for 100 randomly selected cells. Cellular TMRM fluorescence intensity shown is background corrected and presented in a scatter dot plot. **C)** NCI-H460 cells were loaded with 60nM TMRM for 30min and 0.1nM TRAIL was added onto the cells. Images were taken in 15min intervals using a fluorescence microscope. Time from TRAIL addition to death is shown at the single cell level and is plotted in a scatter dot plot as a function of TMRM fluorescence intensity.

4. Discussion

Lung cancer is the leading cause of cancer related death worldwide. Although tremendous improvements have been made in chemo- and radiotherapy the survival rate is still very low. Selection of the treatment strongly depends on the type and stage of the tumor; lung tumors diagnosed in early stages are typically removed by surgery followed by chemotherapy. The established chemotherapeutics which are used to treat the advanced stages of non-small cell lung cancer (NSCLC) include cisplatin, paclitaxel and gemcitabine. However, intrinsic or acquired resistance to chemotherapy is a big hurdle for effective treatment. Therefore, it is needed to explore particular mechanism of resistance to improve the efficiency of cancer therapeutics.

The current focus of cancer research is to develop tumor-specific therapeutics which efficiently target tumor cells without harming normal cells, and TRAIL is supposed to fit these criteria. Unlike other known ligands of this cytokine family, such as TNF and FasL, it has been shown that TRAIL can selectively induce apoptosis in transformed cells leaving normal cells unharmed. Although TRAIL has been demonstrated to induce apoptosis in many tumor cell lines, the fact that nearly half of all tumor cell lines show resistance to TRAIL presents the major hurdle in developing TRAIL based therapies (Walczak et al., 1999). TRAIL resistance can be caused at multiple points within the signaling pathway, starting at the cell membrane level with alterations in the amount of death and/or decoy receptors and followed by subsequent signaling proteins, e.g. like overexpression of antiapoptotic Bcl-2 proteins and inhibitor of apoptosis (IAP) proteins (Zhang and Fang, 2005).

Defects in the mitochondrial pathway of apoptosis contribute to resistance against therapy in many tumors including lung cancer. TRAIL has also been shown to induce apoptosis *via* the intrinsic pathway in some cellular systems, i.e. the so-called type II cells. In order to improve the therapeutic value of TRAIL it is necessary to elucidate the molecular mechanisms behind resistance development. Here we used the non-small cell lung carcinoma cell line NCI-H460, which has been characterized as a type II cell because of its dependency on the mitochondrial pathway for apoptosis induction by death receptors. A NCI-H460 cell variant stably overexpressing the Bcl-2 protein has been established to investigate the effects of blocked mitochondria on different downstream events in comparison to parental cells in TRAIL signaling.

4.1. Regulation of TRAIL-induced apoptosis upstream of mitochondria

4.1.1. The role of Bcl-2 proteins

Overexpression of antiapoptotic proteins of the Bcl-2 family like Bcl-2 and Bcl-xL can inhibit apoptosis in various cancer cells. Since aberrant expression of these antagonistic molecules has been observed in various tumor types, they are suggested to be tightly linked to the resistance shown by these tumor cells against multiple therapeutic agents (Hinz et al., 2000; Fulda et al., 2002a). Notably abnormal expression of the Bcl-2 oncoprotein was reported in nearly 15-25% non-small cell lung tumor cell lines (Pezzella et al., 1993; Apolinario et al., 1997).

In this study a Bcl-2 overexpressing lung cancer cell line has been generated to explore the contribution of the intrinsic (mitochondrial) pathway in TRAIL-induced apoptosis. As one can assume changes at the molecular level during the long cloning and antibiotic selection processes, the expression levels of various key proteins were compared in parental and transfected cells in order to avoid any such possibility. The first checkpoint for TRAIL resistance is located at the membrane level and might be caused by alterations in the expression of agonistic and antagonistic TRAIL receptors. As shown in Figure 8 NCI-H460 wild type and Bcl-2 overexpressing cells express equal and quite high levels of TRAILR1 and TRAILR2 while TRAILR3 and TRAILR4 are found in very low amounts. It is therefore very likely that TRAIL sensitivity in NCI-H460 cells and the Bcl-2 overexpressing cells is hardly affected by the so-called decoy receptors i.e.TRAILR3 and TRAILR4, even if one takes into account that these can be very powerful as they can act in a dominant negative manner by formation of heteromeric non-signaling receptor complexes (Neumann et al., 2014). Whether there exist any functional differences between TRAILR1 and TRAILR2 signaling is still much undefined. In addition to the TRAIL receptor, other key proteins like caspase-8, caspase-3 and XIAP were also considered (Figure 8B). Our results show that the expression levels of all these key proteins investigated were comparable between the two cell lines as no significant differences could be observed.

The balance between pro- and antiapoptotic members of the Bcl-2 family at the outer membrane of the mitochondria is very critical for the cell fate. Even though NCI-H460 cells

expressed high amounts of Bcl-2 protein as compared to other tumor cell lines investigated in our laboratory (data not shown), this cell line is sensitive to TRAIL. Nevertheless the level of the antiapoptotic Bcl-2 family members is of high importance, because a clone with only a 2-3 fold increase in Bcl-2 expression levels in comparison to wild type NCI-H460 showed a strong protection of the cells against TRAIL-induced apoptosis. Hence, only moderate variation in Bcl-2 protein level dramatically change the cell's response to TRAIL (Figure 9) resulting in a near to full TRAIL unresponsiveness, because the high concentration of the improved molecule Db-scTRAIL of 1nM used here showed no significant cytotoxic effects. In order to further demonstrate that the resistance shown by the NCI-H460/Bcl-2 cell line was selectively caused by Bcl-2 overexpression, cells were pretreated with ABT-737, an inhibitor of Bcl-2 family proteins (Bcl-2, Bcl-xl and Bcl-w) (Oltersdorf et al., 2005). ABT-737 treated NCI-H460/Bcl-2 cells showed a comparable sensitivity towards Db-scTRAIL as observed in wild type NCI-H460 cells, suggesting that blocked mitochondrial pathway by highly expressed Bcl-2 is the only defect within the TRAIL pathway in NCI-H460/Bcl-2 cells.

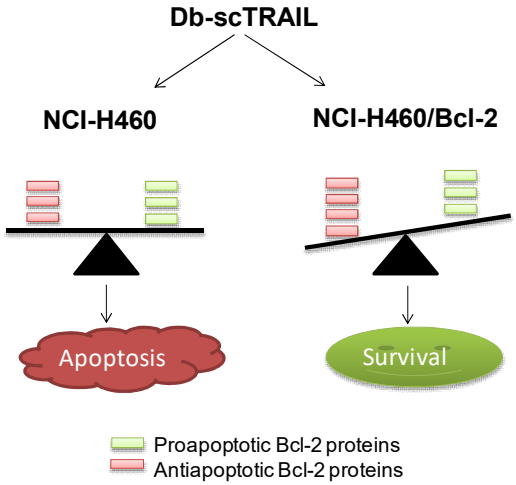

Figure 20: Schematic presentation of balance between Bcl-2 family proteins

A (mis)balance between pro and antiapoptotic proteins of the Bcl-2 family is responsible for the cell fate after death stimulus. NCI-H460 cells are sensitive to Db-scTRAIL; however a 2-3 fold increase in the antiapoptotic Bcl-2 protein made them resistant to TRAIL.

Enzymatic assays using fluorogenic caspase-3, -8 and -9 substrates were used to determine the proteolytic activity of the respective caspases. The results shown in Figure 10B clearly demonstrate that Bcl-2 overexpression inhibited the enzymatic activity of not only effector (caspase-3 and -9) but also initiator caspases (caspase-8) due to blocked mitochondria. As

caspase-8 is activated directly within the DISC upstream of the mitochondria this enzyme might be assumed to be activated equally in both NCI-H460 wild type and Bcl-2 overexpressing cells. Interestingly, however, western blot analyses of the cleavage pattern showed that indeed the cleavage of precursor caspase-8 (p53/55) to its p41/p43 fragments was identical in both cell lines whereas further processing was inhibited in NCI-H460/Bcl-2 cells, and the enzymatically fully active p18 cleavage product was therefore not detectable even after 8h of Db-scTRAIL stimulation. These results are comparable with data from other reports suggesting that effector caspase-3 can amplify the apoptotic signaling by cleaving caspase-8 *via* a positive feedback loop, hence caspase-8 can be activated both upstream and downstream of mitochondria (Slee et al., 2001; Fulda et al., 2002a). Already early mathematical models have been designed to understand this crosstalk and to predict a feedback amplification loop between activated caspase-3 and residual caspase-8 (Eissing et al., 2004). But also results from the group of Ferreira can be considered where they claimed that in NCI-H460 cells caspase-8 activation is mitochondria-dependent by a yet to be defined mechanism, hence overexpression of Bcl-2 can result in blocking caspase-8 activation in chemotherapy-induced apoptosis pathway (Ferreira et al., 2000). In any case the results obtained here suggest that a positive feedback loop mediated by caspase-3 and acting on caspase-8 mainly affects further processing steps of p41/p43, whereas these latter products are formed directly within the receptor signaling complex and are thus unaffected by the mitochondrial pathway.

Significant differences in the activation pattern of caspase-3 were also detected (see Figure 10). Even though caspase-8-mediated initial cleavage of caspase-3 between the large and small subunits to generate the p19 fragment was comparable in both cell lines since it appeared similarly within 2 to 3h of Db-scTRAIL treatment, the autocleavage step to generate the fully active p17 fragment was blocked in Bcl-2 overexpressing cells. One likely explanation for this result argues that the high level of XIAP protein in the cytosol effectively inhibits caspase-3 maturation in NCI-H460/Bcl-2 cells, because the overexpression of Bcl-2 in these cells blocks the release of Smac, the endogenous antagonist of XIAP, from the mitochondria. In wild type cells, however, Smac is released into the cytosol upon mitochondrial depolarization and subsequently neutralizes XIAP, thereby allowing effective further maturation of caspase-3.

The protective role of Bcl-2 has been convincingly demonstrated, as overexpression of Bcl-2 in certain cell types so called type II cells resulted in complete protection from Fas-induced apoptosis (Scaffidi et al., 1998). However its role in TRAIL system is somehow contradictory as some initial studies using Jurkat and CEM T-cells, which are classified as type II in Fas system, demonstrated that overexpression of Bcl-2 did not protect them from TRAIL-induced apoptosis (Keogh et al., 2000; Walczak et al., 2000; Rudner et al., 2004). Additionally Rudner et al. suggested that the protecting role of Bcl-2 is limited to low TRAIL concentrations. However numerous studies, while working with various solid tumor cell lines, strongly suggested that Bcl-2 overexpression can protect cells of type II from TRAIL-induced apoptosis, mainly by inhibiting the release of proapoptotic proteins from mitochondria, hence blocking the amplification of caspase activation *via* the intrinsic pathways (Munshi et al., 2001; Fulda et al., 2002a; Sinicrope et al., 2004). One possible reason for this discrepancy in the results could be that the inhibitory effect of Bcl-2 might be cell-type dependent and most of the initial studies used lymphoma cells. Consistent with these reports the results from this study also confirmed that overexpression of Bcl-2 in NCI-H460 cells render them resistant to TRAIL primarily by impairing caspase processing *via* conserving mitochondrial outer membrane integrity.

Role of the Bcl-2 protein in resistance against apoptotic stimuli can be dependent on the type of cell culture as it has been shown that the lung cancer cell lines A459 and H1299 are sensitive to TRAIL and bortezomib treatment in monolayer culture, whereas they acquire resistance when grown as 3D cell spheroid (Yang et al., 2009). Additionally these authors could show that Bcl-2 was highly upregulated in spheroids and furthermore this acquired resistance could be reversed by inhibition of Bcl-2 with ABT-737, which is in line with our results (Yang et al., 2009). To study solid tumors like lung cancer, three-dimensional spheroid cell culture models may represent a better approach to uncover the mechanism behind multicellular resistance by tumors.

4.2. Regulation of TRAIL-induced apoptosis at the mitochondria

During apoptosis mitochondria undergo various changes; these alterations include mitochondrial outer membrane permeabilization (MOMP), collapse of mitochondrial potential ($\Delta\Psi m$) and release of various proteins from the intermembrane space into the cytosol. Bcl-2 family proteins are important regulators of mitochondrial function during apoptosis. Activated caspase-8 cleaves the proapoptotic Bcl-2 family member protein Bid, which in turn results in Bak/Bax activation and subsequently pore formation in the outer mitochondrial membrane (OMM). Once the mitochondrial outer membrane is permeabilized, the cell will certainly undergo apoptosis, hence MOMP is considered as “point of no return” in this process. How the Bcl-2 protein maintains the mitochondrial integrity thereby controlling the release of mitochondrial components is still not very clear, but generally two models have been proposed for Bak/Bax activation and its control by antiapoptotic Bcl-2 member, called the direct and the indirect model. In the direct model it has been suggested that activator BH3 proteins (Bid, Bim, and Puma etc) directly interact with Bax/Bak resulting in their activation while antiapoptotic proteins can prevent this interaction by binding to BH3 activator proteins (Lovell et al., 2008). According to the indirect model, antiapoptotic proteins (Bcl-2, Bcl-xl etc.) prevent the activation of Bak/Bax by binding to these; BH3-only proteins set Bak/Bax free from the inhibition by binding to antiapoptotic proteins (Willis et al., 2007).

Here we used the cationic fluorescent dye TMRM to monitor changes in the mitochondrial membrane potential following Db-scTRAIL stimulation. Treating NCI-H460 wild type cells with Db-scTRAIL causes a drop in $\Delta\Psi m$ in a time-dependent manner; in contrast Bcl-2 overexpressing cells show a rather minute loss of $\Delta\Psi m$ (see Figure 12), pointing at the ability of antiapoptotic Bcl-2 proteins to preserve the mitochondrial integrity. Since Bax/Bak-mediated pore formation results in the release of a range of proteins from mitochondrial intermembrane space to the cytosol, next we compared the release pattern of cytochrome c and Smac between wild type and Bcl-2 overexpressing NCI-H460 cells. The results shown in Figure 12 further confirmed the necessity of mitochondria in execution of apoptosis in our model cell line, as dramatically increased levels of cytochrome c were observed in the cytosol after Db-scTRAIL treatment, suggesting that TRAIL-induced intrinsic and extrinsic pathways of apoptosis had been activated in NCI-H460 cells. This is in contrast to the report from Keogh et al. where the authors suggested that Bcl-2 overexpression in CEM cells (type II cells) fails to inhibit cytochrome c release from mitochondria and a subsequent apoptosis

induction by TRAIL (Keogh et al., 2000). It is clear, at least in our cellular system, that the release of cytochrome c and Smac can be abolished by overexpression of Bcl-2 in NCI-H460.

While monitoring changes in the mitochondrial membrane potential we noticed the presence of subpopulations having distinct $\Delta\Psi_m$ in untreated NCI-H460 wild type cells. As in NCI-H460/Bcl-2 cells no heterogeneity regarding mitochondrial membrane potential has been observed, it can be assumed that some alterations or defects in mitochondrial integrity may exist in NCI-H460 wild type which can be overcome by Bcl-2 overexpression (Figure 12A). Up till now very limited literature is available regarding heterogeneity within a tumor cell population and its effect on therapy resistance, here we tried to address the question whether significant differences in the mitochondrial potential within NCI-H460 cell population are correlated to TRAIL susceptibility. To this the cellular fate of NCI-H460 cells after Db-scTRAIL stimulation was followed by live cell imaging. The results demonstrated that despite of differences in $\Delta\Psi_m$ more than 90% of the cells died between 2 to 4h after Db-scTRAIL stimulation, hence no correlation between the intrinsic mitochondrial potential and TRAIL sensitivity was found.

It has been suggested in the literature that differences in the intrinsic $\Delta\Psi_m$ within colon and breast carcinoma cell population are linked to tumor phenotypes, cells with high $\Delta\Psi_m$ were suggested to be involved in tumor progression and development (Heerdt et al., 2005). However, further studies are necessary to explain and investigate the role of these differences in mitochondrial potential and the factors involved in these differences within a cell population.

4.3. Regulation of TRAIL-induced apoptosis downstream of mitochondria

4.3.1. The role of cytochrome c, caspase-9 and Smac

It is well established that so-called type II cells depend on the mitochondrial pathway of apoptosis for the activation of effector caspases. These results have been originally described in the Fas system but later on also in other death receptor signaling pathways including TRAIL (Scaffidi et al., 1998; Suliman et al., 2001). The precise molecular

mechanism, however, by which mitochondria contribute to the final activation of executioner caspases, is still not fully understood. Originally caspase-9 was considered to represent an apical caspase in non receptor-mediated apoptosis as its curial role was confirmed in various tumor cell lines following treatment with chemotherapeutic agents (Kuwahara et al., 2000; Liu et al., 2002; Wu and Ding, 2002). With the discovery and definition of mitochondria-independent (type I) and mitochondria-dependent (type II) cell types in Fas-mediated apoptosis, caspase-9 was demonstrated to play a central role in death receptor-mediated apoptotic signaling at least in some cellular systems (Samraj et al., 2006).

In an attempt to characterize the importance of caspase-9 within the TRAIL-induced apoptotic pathway in our model cell line NCI-H460 in more detail, the endogenous enzyme has been inhibited using a small molecule inhibitor but was also downregulated at the RNA level using caspase-9 specific siRNAs. It is evident from Figure 16A and D that neither caspase-9 inhibition nor its silencing significantly protected NCI-H460 from Db-scTRAIL-induced death even at very moderate ligand concentrations used (e.g. at a concentration of 1nM). We are convinced that these negative results are valid because colon cancer cells HCT-116 had been included in the first set of experiments as a positive control, as they had been shown in literature to be protected from TRAIL-mediated cell death by the caspase-9 inhibitor z-LEHD-fmk (Ozoren et al., 2000). As expected, caspase-9 inhibition significantly protected HCT-116 cells (data not shown) while NCI-H460 retained full sensitivity to Db-scTRAIL (Figure 16). Western blot analysis corroborate these data as we see processing of caspase-9 into its p37/35 fragments in NCI-H460 cells after cotreatment with Db-scTRAIL and z-LEHD-fmk, but not with the broad caspase inhibitor zVAD-fmk (Figure 16B). Since caspase-9 activation is generally believed to enhance caspase-3 processing, next we investigated the effect of caspase-9 deficiency on caspase-3 cleavage. Interestingly no differences in caspase-3 processing were observed in control transfected and caspase-9 deficient cells (Figure 16F). Caspase-3 was processed to p19/17 active fragment within 2h of Db-scTRAIL treatment in caspase-9 knockdown cells indicating that full processing of caspase-3 to generate the catalytically active fragment does not depend on caspase-9 activity in NCI-H460 cells. It has been earlier suggested in a model of Fas-induced cell death that the requirement of caspase-9 activity for caspase-3 activation depends at least on the cellular system used (Hakeem et al., 1998).

Together, these data strongly suggest only a minor role of caspase-9 in TRAIL-induced apoptosis in NCI-H460 cells. Accordingly we propose a caspase-9-independent and caspase-3-dependent pathway after ligation of Db-scTRAIL to its death receptors. Using different assay systems to trigger the intrinsic pathway of apoptosis Ferreira and colleges have generated lavish amount of data about the functional role of caspase-9 in NCI-H460 cells. They confirmed that apoptosis induced by chemotherapeutic drugs like cisplatin, topotecan, and gemcitabine do not require the activation of caspase-9 and proposed a model where caspase-8 becomes activated in a mitochondria-dependent manner but independent of caspase-9 (Ferreira et al., 2000). In line with these results, we here show that caspase-9 is not an essential player in extrinsically induced apoptosis by TRAIL death receptors. Clearly these results might be cell specific as for example in a recent study using caspase-9 deficient Jurkat cells it was shown that deficiency of caspase-9 not only renders these cells resistant to Fas stimulation but also to TRAIL-mediated apoptosis. Hence, cytochrome c-mediated apoptosome formation and subsequent caspase-9 activation is crucial in this particular Jurkat model (Samraj et al., 2006). Later on another study confirmed these results using the same cell system and these authors demonstrated that Apaf-1 deficient Jurkat cells lacking caspase-9 activation were resistant to etoposide treatment but were on the same time susceptible to death receptor-mediated apoptosis, hence a stimulus-dependent varying role of caspase-9 was suggested (Shawgo et al., 2009). It is therefore likely that the functional role of caspase-9 activation for the development of the apoptotic phenotype depends on both the type of stimulus as well as the cellular context (Hakem et al., 1998). In future studies it might be helpful to add Jurkat cells as a control because their dependency on caspase-9 activation is well established.

A number of proapoptotic proteins are released from mitochondria following mitochondrial outer membrane permeability including Smac, cytochrome c, apoptosis inducing factor (AIF) and HtrA2/Omi. In our cellular model overexpression of Bcl-2 completely protected the cells from Db-scTRAIL-induced apoptosis strongly suggesting that at least one of these factors is important to amplify the apoptotic pathway. Different studies using various cell lines and stimuli revealed conflicting results regarding the role of proteins released from mitochondria. A number of studies suggested that Smac release is a key event and represents thus a major determinant of cellular responsiveness to various death inducing agents, as compared to the release of cytochrome c and subsequent caspase-9 activation (Deng et al., 2002; Sun et al., 2002). In most of these studies Smac was overexpressed or a Smac mimetic was

used, i.e. a small peptide that can bind to IAPs, to investigate the role of this mitochondrial protein in apoptosis induced by various antitumor drugs (e.g. paclitaxel, doxorubicin, cisplatin, and etoposide) but also death ligands (Srinivasula et al., 2000; Arnt et al., 2002; Fulda et al., 2002b). Particularly overexpression of Smac was efficient to sensitize lung cancer cell lines A459 and 95D to cisplatin-induced cell death. Although these results underscore the strong apoptotic potential of Smac, the question remains open asking for the role of the endogenous levels of Smac. In our studies we therefore decided to downregulate endogenous Smac to get insight into its functional role in the TRAIL-mediated apoptotic pathway in type II lung tumor cells. Sekimura and co-workers showed that Smac expression was significantly reduced in lung tumor cells as compare to normal lung tissue (Sekimura et al., 2004), whereas two other studies found higher expression of Smac mRNA in different lung tumor types as compared to normal cells (Krepela et al., 2006; Yang et al., 2008).

As illustrated in Figure 17B apoptosis induction with various Db-scTRAIL concentrations was comparable in cells transfected with either Smac or control siRNAs, and no significant protection could be observed after Smac silencing in NCI-H460 wild type cells. These results are in line with some studies demonstrating that Smac deficiency had no effect on apoptosis induced by numerous stimuli (Okada et al., 2002; Bartling et al., 2004), although Smac has been suggested as one of the important factor in promoting apoptosis following various death stimuli (Zhang et al., 2001; Deng et al., 2002; Rudy et al., 2008). As in our work the siRNA-mediated downregulation was significant but residual protein was clearly detectable, one could argue that the protein amounts of Smac being detectable 48h after transfection are still sufficient to effectively antagonize IAP proteins. Another possibility would be that downregulation of Smac is compensated for by upregulation of other proapoptotic proteins like Omi/HtrA2 (Bartling et al., 2004).

Although Smac knockdown alone was incapable of protecting NCI-H460 cells even at moderate TRAIL concentrations, a distinct capase-3 activation pattern could be observed in control and Smac siRNA treated cells (Figure 17C). In general, cells with decreased Smac level showed lower amounts of cleaved caspase-3, the p17 fragment of caspase-3 was not detectable even after 6h treatment with Db-scTRAIL and the p19 fragment also disappeared in a time-dependent manner. Activated caspase-3 fragments p19/p17 were detected in higher levels in the presence of proteasome inhibitors, indicating XIAP-mediated caspase-3 ubiquitination and degradation. Together, our data suggest some regulatory role of Smac as

a promoter of TRAIL-induced apoptosis, but do not argue for a central role of it. To get further insight into this regulatory network one should investigate the protein levels of IAPs and particularly XIAP in control and Smac siRNA transfected cells. These data should be valuable, as the group of Okada observed no differences in the protein levels of XIAP, c-IAP1, c-IAP2 and survivin in primary and Smac knockout mouse embryonic fibroblasts (Okada et al., 2002).

4.3.2. The role of XIAP

IAP family members have been reported to play roles in various cellular processes in parallel like regulation of cell division and control of intracellular signal pathways in part by their E3 ligase activity causing protein degradation (Altieri, 2003; Liston et al., 2003). However, the most well distinguished function of IAP proteins is their ability to counteract apoptosis induction, especially by direct inhibition of caspases (Deveraux and Reed, 1999). Aberrant expression of antiapoptotic molecules like Bcl-2 and XIAP is believed to be an important mechanism to induce TRAIL resistance in a variety of tumor cell lines (Fulda and Debatin, 2004). Expression of IAP protein family members, particularly XIAP, has been reported to be significantly elevated in prostate, pancreatic, breast, colon and renal carcinomas (Krajewska et al., 2003; Yang et al., 2003; Mizutani et al., 2007).

To investigate the role of XIAP in our cellular system we transiently overexpressed full length XIAP or its subdomains to evaluate the resulting protective effects on TRAIL-induced apoptosis. XIAP overexpression has been shown to protect lung tumor cells from apoptosis induced by multiple death receptor agonists (Holcik et al., 2000) in contrast, our results shown in Figure 13C demonstrate that overexpression of XIAP or individual domains resulted in only very limited protection against Db-scTRAIL-mediated cell death. The possible reasons for these small effects include at first low transfection efficiency (Figure 13), i.e. a high background of untransfected cells. Even in transfected cells, if NCI-H460 cells possess high amounts of endogenous XIAP protein, it can be assumed that this level is sufficient for its inhibitory role while further increase would not additionally protect from TRAIL-induced apoptosis. Interestingly, in NCI-H460 cells the endogenous caspase-3 level is almost 10 fold higher on a molecular basis as compared to XIAP (Nadine Pollak, unpublished results), and activated caspase-3 is known to cleave and degrade this inhibitor (Hornle et al., 2011). At a first glance, from these data one could expect that strong XIAP overexpression must be

protective which has been in fact shown in several cellular models in literature (Deveraux and Reed, 1999; Wilkinson et al., 2004; Cheng et al., 2010). On the other hand it has been also shown elevated levels of XIAP alone are not be sufficient to make HeLa cells resistant to various drugs, rather the equilibrium between XIAP and its antagonists is the crucial factor (Seeger et al., 2010).

The obviously sufficiently high level of XIAP in NCI-H460 cells suggests that targeting of XIAP and its inhibition or downregulation should sensitize cells to TRAIL. Targeting of IAP proteins is an attractive approach for the development of antitumor drugs. Intensive research has been performed in the last years to develop small molecule inhibitors like the Smac mimetics which mimic the action of the endogenous antagonist of several IAP proteins, Smac. Strong synergism between TRAIL and Smac mimetics has been observed in various tumor cells (Fulda et al., 2002b). Here two different approaches have been used to inhibit XIAP activity, in the first method a small molecule Smac mimetic was used at a sub toxic concentration. Using the Smac mimetic SM83 in combination with Db-scTRAIL in wild type NCI-H460 cells revealed only a very limited additional sensitization of the cells. Especially at lower Db-scTRAIL concentrations there were no differences detectable in the cytotoxic effects with and without SM83 (Figure 14A). At high TRAIL concentrations the maximum cytotoxic effects were somewhat enhanced by this molecule, indicating that SM83 did not enhance the TRAIL sensitivity of the cells *per se*, rather than allowing to induce apoptosis in those cells which were otherwise TRAIL resistant if the cytokine was given alone.

Interestingly, the Smac mimetic SM83 was in fact capable to sensitize Bcl-2 overexpressing cells which were otherwise TRAIL resistant. These results show that this molecule can be highly effective in the proper cellular context in an additive or more likely synergistic manner together with TRAIL. It is known that antagonizing IAPs is not the only mechanism by which Smac mimetics sensitize cells to apoptotic stimuli; rather multiple factors like NFκB inhibition and activation of TNFα signaling have been demonstrated to be additional mechanisms (Probst et al., 2010; Fulda and Vucic, 2012). The molecular mechanism how this Smac mimetic sensitized Bcl-2 overexpressing cells to TRAIL was not investigated further; in future it would be interesting to investigate effect of Smac mimetic in combination with TRAIL, not only on XIAP but also on c-IAP1/2 and survivin inhibition and degradation.

Since Smac mimetics can also engage other signaling pathways by binding to c-IAP1 and c-IAP2, in order to better validate the role of XIAP in NCI-H460, cells were transfected with XIAP specific siRNAs to downregulate the expression of XIAP. An efficient downregulation of XIAP was achieved with no visible effects on the expression of other members of the family like c-IAP1 and survivin. As expected, targeting of XIAP with siRNA followed by Db-scTRAIL treatment resulted in prompt induction of apoptosis. As evident from the Figure 14C and D enhancement of cell death was much more pronounced in Bcl-2 overexpressing cells. Ndozangue-Touriguine and colleagues defined two blocked points during the TRAIL-induced apoptosis in type II colon tumor cell lines, one at the level of mitochondria and the other in the cytosol at the level of XIAP. Our results are in line with their results showing that downregulation of XIAP is sufficient to sensitize TRAIL resistant SW620 tumor cells despite of their block at the mitochondria, by a defect in Bax translocation (Ndozangue-Touriguine et al., 2008).

While investigating the mechanism of sensitization to Db-scTRAIL in NCI-H460/Bcl-2 cells it has been observed that XIAP knockdown resulted in the enhancement of caspase-3, caspase-8 and caspase-9 processing into their active forms. The increased level of caspase-8 p18 fragment is possibly caused by a positive feedback loop leading from fully active caspase-3 fragment (p17) to the caspase-8 intermediate product as suggested by Ferreira et al. that targeting of XIAP would result in enhanced caspase-3 activity followed by an enhanced cleavage of caspase-8 p41/43 intermediate into p18 fragment (Ferreira et al., 2012).

The role of caspase-3 has been investigated intensively and it has been concluded that this protease contributes to cell death critically, although not in all cellular systems, dependent on cell type, tissue and death stimulus. In fact a wide range of studies using cells from caspase-3 defective mice revealed no major changes in the apoptotic phenotype following a broad range of death stimuli (Woo et al., 1998). Different evidences come from the human MCF7 breast cancer cell line, as in these cells no DNA fragmentation and apoptotic bodies' formation was observed following a variety of apoptotic stimuli, as these cells lack caspase-3 due to a genetic mutation.

As illustrated in Figure 10A compared to Db-scTRAIL treated parental cells the autocleavage of caspase-3 in their Bcl-2 overexpressing counterparts was fully blocked. The hypothesis

that the second cleavage step of caspase-3 is controlled by cytochrome c/caspase-9 appears unlikely as we cannot see any significant effect of caspase-9 silencing on caspase-3 activation. However, most likely the processed form of caspase-3 might be inhibited by IAPs, specifically XIAP, as no interaction between c-IAP1, c-IAP2 and caspase-3 has been found (Sun et al., 2002). The most striking difference in XIAP deficient Bcl-2 overexpressing and vector transfected cells after Db-scTRAIL treatment was caspase-3 processing to its fully active p17 fragment (Figure 15B). Therefore our data strongly suggest that XIAP-mediated inhibition of caspase-3 is required to protect cells from TRAIL-induced apoptosis. These findings are in contrast to some early studies which have been shown that caspase-3 inhibition is not the central step for the protective effects of XIAP, as XIAP inhibits cell death prior to activation of the effector caspases like caspase-3 (Silke et al., 2002). Later on, Wilkinson and coworkers suggested that the apoptosis regulating properties of XIAP depend on the nature of the death stimulus. These authors showed that caspase-3 inhibition by XIAP is not essential for protection against etoposide which targets the intrinsic pathway, whereas it was indispensable to protect cells from death receptor-mediated apoptosis (Wilkinson et al., 2004). Taking into account that caspase-3 can be activated by both caspase-8 and caspase-9, it has yet to be clarified whether enhancement in caspase-3 processing has to be attributed to activated caspase-9 after XIAP knockdown or whether it is independent from mitochondrial activation in terms of a type I signaling pathway. In our cellular system we have clearly demonstrated that targeting of XIAP alone can be sufficient to allow TRAIL-induced apoptosis in otherwise resistant cells because of the overexpression of Bcl-2. TRAIL resistant NCI-H460/Bcl-2 cells can be sensitize to TRAIL by XIAP knockdown which results in the full activation of effector caspase-3 along with enhancement of other caspase activation too.

Two possible explanations for XIAP knockdown-mediated enhancement of Db-scTRAIL-induced apoptosis in Bcl-2 overexpressing cells can be proposed. Either the cells are forced to die *via* the type I cell death pathway because of pronounced caspase-3 cleavage and a positive feedback leading to further activation of caspase-8 (Gillissen et al., 2013). Alternatively the cells still die *via* a type II death pathway possibly using a positive feedback leading from activated caspase-3 to cleave Bid or Bcl-2 thereby disturbing the balance between pro- and antiapoptotic Bcl-2 proteins, to finally cause mitochondrial outer membrane permeabilization and a release of Smac and cytochrome c into the cytoplasm (Kirsch et al., 1999; Slee et al., 2000; Chawla-Sarkar et al., 2004; Fakler et al., 2009). This second

possibility is quite interesting and convincing because it is widely accepted that antiapoptotic molecules like Bcl-2, Bcl-xL and Akt are substrates for caspases and their inhibitory functions can be inactivated by cleavage. Moreover, their cleavage products then can play a proapoptotic rather than antiapoptotic role (Cheng et al., 1997; Clem et al., 1998; Grandgirard et al., 1998). Furthermore MCF7 cells lacking endogenous caspase-3 were unable to cleave Bcl-2 following staurosporine treatment, notably transfection of the cells with caspase-3 resulted in Bcl-2 cleavage and cytochrome c release, then amplifying the caspase cascade (Kirsch et al., 1999).

These findings indicate that part of the XIAP knockdown-mediated enhancement of apoptosis in NCI-H460/Bcl-2 cells is provoked by promoting caspase-3 processing to the p17 fragment. So we can argue that XIAP-mediated inhibition of caspase-3 is one important factor causing resistance to Db-scTRAIL-induced apoptosis in Bcl-2 overexpressing cells. In conclusion, overexpression of Bcl-2 results in TRAIL resistance in NCI-H460 cells, however, additional XIAP knockdown resensitizes cells to TRAIL treatment by an increased activation of the effector caspase-3. Further studies are required to evaluate additional possible mechanisms behind the enhancement of cell death after XIAP knockdown in NCI-H460 cells overexpressing Bcl-2 cells, nevertheless XIAP is likely to represent a promising candidate for targeted therapy as a wide range of tumors express elevated levels of Bcl-2 and XIAP simultaneously and XIAP knockdown can also overcome Bcl-2-mediated resistance in our cell system. Our results therefore strongly support XIAP targeting approaches for cancer therapy.

Figure 21 illustrates the conclusive outlook of the presented work. How XIAP expression regulates the TRAIL signaling pathway in NCI-H460 is quite clear now, using three cellular models we assume a predominant role of XIAP. Although in NCI-H460 (wild type) cells the endogenous level of XIAP appears quite high it does not reach a threshold necessary for effective apoptosis inhibition and hence the cells are sensitive to TRAIL. On the other hand blocking of the mitochondrial pathway (NCI-H460/Bcl-2) make these cells resistant to TRAIL, based on the endogenous XIAP levels, most likely due to absence of its endogenous inhibitors, like Smac. In XIAP deficient/downregulated NCI-H460/Bcl-2 cells increased caspase-3 activity is found although we suppose no Smac or cytochrome c. The major reason might be the positive feedback leading from caspase-3 to caspase-8, thereby resensitizing the cells to TRAIL. This interpretation of our data is in accordance with the

results of Jost et al., who propose that downregulation of XIAP changes the phenotype of a type II cell to that of a type I cell (Jost et al., 2009). However, their interpretation is not in accordance with the original definition of a type I cell, where it was assumed that caspase-8 activation within the DISC is sufficient for sufficient effector caspase activation, which holds not true in our cellular model.

Nevertheless there are still important questions yet to be answered. First of all, although blocking of the mitochondrial amplification loop by enhanced Bcl-2 levels very efficiently induced TRAIL resistance in NCI-H460 cells, the individual knockdown of either Smac or caspase-9 was not very effective. Based on this observation one might assume two possibilities: Either simultaneous release and action of both of these proapoptotic molecules is necessary for efficient apoptosis induction or additional contribution(s) by the mitochondria may exist. Furthermore, as we observed heterogeneity in NCI-H460 cell population regarding the mitochondrial potential, it would be interesting to explore the consequences of these subpopulations with remarkably distinct mitochondrial potential on overall response to TRAIL treatment.

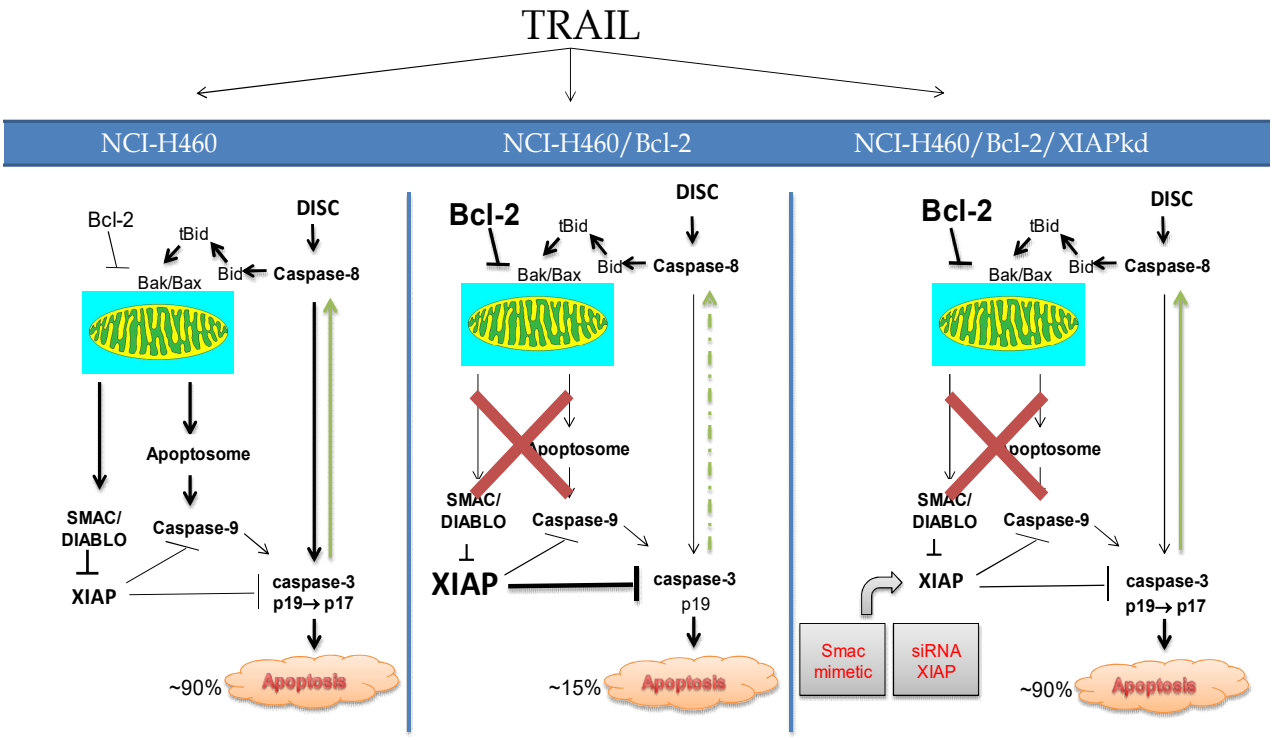

Figure 21: Schematic presentation of XIAP-mediated differential regulation of signaling pathways

Three different cellular models have been investigated regarding differential involvement of the mitochondrial pathway and the cytosolic XIAP level. Overexpression of Bcl-2 protected NCI-H460 from TRAIL-induced apoptosis whereas an additional XIAP knockdown restores TRAIL sensitivity in Bcl-2 overexpressing cells at least in part by enhancing caspase-3 activity. Black arrows $\rightarrow$ represent the reactions during the signaling while $\perp$ is used to represent the inhibitory mechanisms/reactions. Thickness of $\rightarrow$ and $\perp$ indicates the strength of the reaction. Green arrows represent the positive feedback

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Poster presentation

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