SENSITISATION TO TRAIL-INDUCED APOPTOSIS BY TARGETED INHIBITION OF KINASES

A DISSERTATION
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I. Abstract

The tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in cancer cell lines but not in normal cells. This property of TRAIL led to its development as a novel cancer drug. However, most primary tumour cells are TRAIL-resistant, yet, they can be sensitised by combining TRAIL with other cancer drugs. Kinase inhibitors have emerged as a new class of cancer drugs with high therapeutic potential and cancer cell specificity. The aim of this thesis was to determine the mechanism of TRAIL apoptosis sensitisation by inhibition of certain kinases that are specifically and aberrantly activated in cancer cells. When studying the TRAIL-induced phosphorylation of Bid it was discovered in this thesis that this phosphorylation was independent of ATM which has previously been described to phosphorylate Bid at this specific site. Remarkably, the ATM inhibitor KU-55933 used in this context was able to further sensitise HeLa cells to TRAIL-induced apoptosis and could break TRAIL resistance of the colon carcinoma cell line DLD1. As the combination of TRAIL and KU-55933 might represent a promising treatment option for cancer therapy this study focused on investigating the molecular mechanism that leads to TRAIL sensitisation by KU-55933. Surprisingly, TRAIL sensitisation by KU-55933 was independent of specific inhibition of ATM and, instead, achieved by inhibition of the phosphoinositide 3-kinase (PI3K) p110α isoform. Aberrant activation of PI3K α is a frequent tumour-specific alteration in various types of cancer including breast and colon carcinoma. It could be demonstrated that TRAIL apoptosis sensitisation of TRAIL-resistant DLD1 colon carcinoma cells by KU-55933 or PIK75, a specific inhibitor for p110α, required concomitant down-regulation of the cellular FLICE-inhibitory protein (cFLIP) and the X-linked Inhibitor of Apoptosis Protein (XIAP). Whilst suppression of cFLIP enhanced caspase-8 activation at the TRAIL death-inducing signalling complex (DISC), resulting in first cleavage of caspase-3, loss of XIAP enabled further cleavage and full activation of caspase-3. These results suggest that the combination of TRAIL or other TRAIL receptor agonists with inhibitors of PI3Kα may be an effective new strategy in cancer treatment capable of overcoming therapy resistance.

II. Declaration

I, Kerstin Papenfuss, declare that this PhD Thesis is my own work and has not been submitted in any form for another degree at any university or other institute of tertiary education. Information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is given in the bibliography.

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Kerstin Papenfuss

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VI. Table of contents

1. Introduction

“One aspect of the cell lineage particularly caught my attention: in addition to the 959 cells generated during worm development and found in the adult, another 131 cells are generated but are not present in the adult. These cells are absent because they undergo programmed cell death” - Horvitz: Nobel Prize lecture "Worms, Life and Death," 2002. This simple observation about the nematode *Caenorhabditis elegans* by Robert Horvitz in the 1970’s opened up a new area of research- programmed cell death, which was later coined apoptosis by Kerr, Wiley and Currie (Kerr et al., 1972). Over the past decades research identified apoptosis as an important regulatory process in development. It has evolved to facilitate tissue remodelling and homeostasis and to remove unwanted and potentially dangerous cells from an organism (Los, Wesselborg et al. 1999; Vaux and Korsmeyer 1999). Tumour cells are characterised by their ability to avoid the normal regulatory mechanisms of cell growth, division and death. Classical chemotherapy aims to kill tumour cells by causing DNA damage-induced apoptosis. However, as many tumour cells possess mutations in intracellular apoptosis-sensing molecules like p53, they are not capable of inducing apoptosis on their own and are therefore resistant to chemotherapy. With the discovery of the death receptors the opportunity arose to directly trigger apoptosis from the outside of tumour cells, thereby circumventing chemotherapeutic resistance. Death receptors belong to the tumour necrosis factor (TNF) receptor superfamily, with TNF-Receptor-1, CD95 and TNF-related apoptosis-inducing ligand (TRAIL)-Receptor (R) 1 and -R2 being the most prominent members. Unfortunately early hopes to use TNF or CD95 as anti-tumour therapeutics had to be abandoned due to profound toxicity (Creagan et al., 1988; Creaven et al., 1987; Galle et al., 1995; Ogasawara et al., 1993). In contrast to this TRAIL has been shown to selectively kill tumour cells, while sparing normal tissue. This attribute makes TRAIL an attractive drug candidate for cancer therapy (Walczak et al., 1999). Although most primary tumour cells turned out to be primarily TRAIL-resistant, recent studies evidence that a variety of cancers can be sensitised to TRAIL-induced apoptosis upon pre-treatment with chemotherapeutic agents or irradiation, while normal cells remain TRAIL-resistant.

1.1. Forms of cell death

Cell death is part of the counterbalance to cell division and determines the overall growth rate of a tissue. There are three main forms of cell death, namely necrosis, autophagy and apoptosis. These three processes can be distinguished on the basis of the morphological changes that occur.

Necrosis occurs after exposure to high concentrations of detergents, oxidants, ionophores or high intensities of pathologic insult (Nicotera et al., 1999). Necrosis is characterised by clumps of cells in a tissue that act together. Cells swell; cytoplasmatic granules disintegrate rapidly while they give up any metabolic activity. The DNA and cellular constituents start to disintegrate in a random, uncontrolled fashion. Subsequently, cells burst, organelles get destructed and leak out of the cell. The host tissue reacts by inducing an inflammatory reaction that leads to damage of the surrounding tissue (reviewed in Potten, 2004).

A second process referred to as **autophagic cell death** has also been proposed to be a form of programmed cell death. It is defined as a catabolic process which involves the degradation of a cell's own components through the lysosomal machinery resulting in the total destruction of the cell. During autophagy, long-lived proteins or whole organelles are sequestered into double membrane vesicle referred to as autophagosomes. Autophagy-related genes (atg) are required for the formation of these autophagosomes which fuse with lysosomes where the contents are enzymatically digested. However, in cells with intact apoptotic machinery, it is unclear whether autophagy indeed acts as direct death execution pathway. Autophagic cell death has mainly been observed in cells in which the apoptotic machinery was dysfunctional or blocked, e.g. caspase-blockage by the use of the caspase-inhibitor zVAD-fmk. Under these conditions, autophagic cell death is hallmarked by emerging autophagic vacuoles and the early degradation of organelles. Generally autophagy is responsible for the degradation of long-lived proteins and is the only known pathway which degrades whole organelles (Klionsky and Emr, 2000). It is important to bear in mind that under conditions of nutrient deprivation, autophagy is rather thought to act as a survival mechanism.

In contrast to necrosis, **apoptosis** is a programmed, genetically controlled, active ATP-dependent process. It is possible to observe cell shrinkage of a single cell and breaking of cell-to-cell contacts with neighbouring cells. Cells become round and smaller, so that cytoplasmic internal membranes, ribosomes, mitochondria and other organelles are more concentrated in

the cytoplasm. However, the organelles remain intact and retain their metabolic activity. The condensed chromatin generates a crescent shaped area which follows the contour of the nuclear membrane. The DNA is cut between the nucleosomes thereby creating fragments of multiples of 180 bp in length. These fragments form the characteristic “DNA ladder” of apoptotic cells that can be observed in an agarose gel (Cohen and Duke, 1984). The nucleus then fragments into pieces and likewise the cell splits into smaller pieces; this is a process referred to as blebbing. Blebbing results in the formation of apoptotic bodies which have an intact membrane, thus preventing the leakage of cellular contents into the extracellular space. Another feature of apoptotic cells is the exposure of phosphatidylserine (PS) on the outer plasma membrane which then serves as an important "eat me" signal. Subsequently, these cells are phagocytosed by neighbouring cells and macrophages (Fadok et al., 1992). The apoptotic body is then digested within the phagosome without induction of an inflammatory response.

Physiologically, apoptosis plays a major role in the removal of cells during developmental and differentiation processes, in homoeostasis of tissues and in the immune system. It is also very important in the removal of senescent cells and cells with damaging potential (Yin, 2003). Apoptosis can be induced from inside the cell using the **intrinsic pathway** or from outside the cell, via the **extrinsic pathway** involving the activation of death receptors. The intrinsic pathway is triggered under different stress conditions, e.g. DNA damage, and leads to the release of apoptogenic proteins from the mitochondria. This pathway is also referred to as “Bcl-2 controlled pathway” as it is activated and controlled by members of the Bcl-2 protein family (reviewed in Youle and Strasser, 2008). This protein family is introduced in chapter 1.2.2. The extrinsic pathway is activated when death receptors belonging to the Tumour Necrosis factor (TNF)-Receptor superfamily are oligomerised by their cognate ligands. After binding of the ligand, the death-inducing signalling complex (DISC) is formed which is essential for subsequent signal transduction by intracellular proteins and induction of apoptosis. The main players in this respect are cysteine-dependent, aspartate-specific proteases (caspases) (see chapter 1.2.1) which cleave a variety of cellular substrates, initiating the morphological changes attributed to apoptosis.

1.2. Executioners of apoptosis

1.2.1. Caspases

Based on his initial observation that 131 cells of the 1090 somatic cells were eliminated during development in *C. ele*gans, Robert Horvitz established the importance of caspases in apoptosis (Ellis et al., 1991). In a mutagenesis screening he found that the *ced-3* gene was required for programmed cell death. The protein encoded by the *ced-3* gene was a cysteine protease with similar properties to the mammalian interleukin-1-beta converting enzyme (ICE) (now known as caspase 1) which at the time was the only known caspase (Yuan et al., 1993). Up to now 14 different homologues have been found in humans. They were termed caspases as in cysteine-aspartate specific proteases. Upon apoptosis induction a caspase cascade is initiated that leads to cleavage of a variety of cellular substrates, contributing to the destruction of the cell and ultimately leading to cell death. Caspases are synthesised as inactive pro-enzymes (zymogens). Structurally, caspases are organised into a pro-domain region, a large subunit and a small subunit. Upon activation, the large and small subunits are released from the pro-enzyme by cleaving an Asp-X bond between the pro-domain and the large subunit. Similarly, the large and small subunits are separated via as second cleavage between the two domains. Active caspases are generally heterotetrameric, comprising two large and two small sub-units. An example of the activation of caspase-3 is depicted in figure 1.

Active caspases are able to activate other members of the caspase family which subsequently results in the proteolysis of various cellular proteins. Caspases are highly specific proteases that cleave their substrates after specific tetrapeptide-motifs (P4-P3-P2-P1). P1 is always an aspartate residue. The residue P4 is the most critical in determining the substrate specificity of the individual caspase, for example DEVD (Asp-Glu-Val-Asp), is the tetrapeptide that is recognised by caspase 3 (Villa et al., 1997).

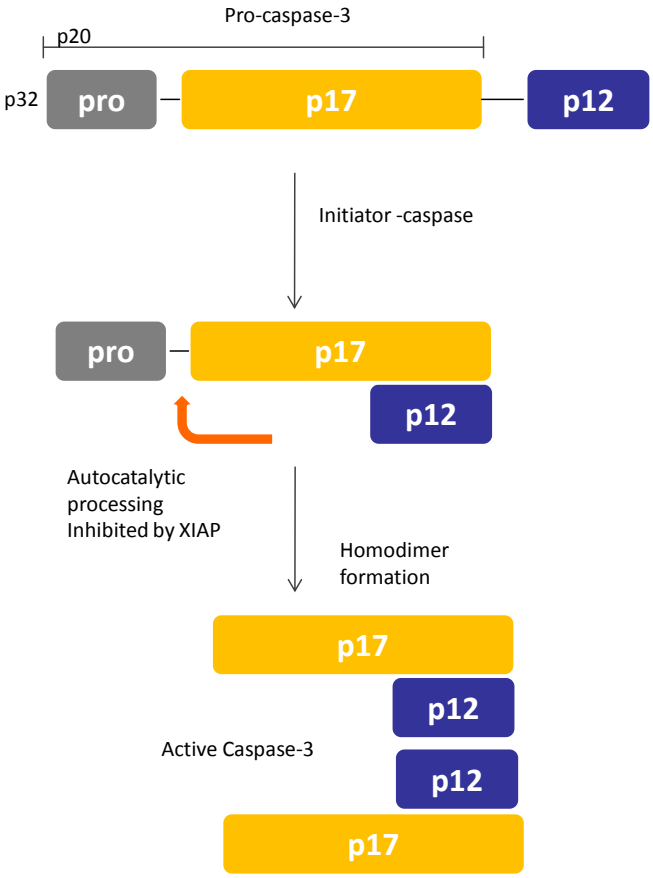


figure 1. Activation of caspase-3.

Cleavage at the amino acid position aspartate 175 by initiator caspases leads to autocatalytic processing of caspase-3. The prodomain of caspase-3 is cleaved off at aspartate 28. X-linked inhibitor of apoptosis protein (XIAP) can block this autoactivation. p32, full length caspase-3; p20, large subunit inclusive prodomain; p17, large subunit; p12, small subunit.

The caspase gene family can be grouped into two major sub-families, namely inflammatory caspases (caspases 1, 4, 5, 12L), whose primary role seems to be in cytokine processing, and apoptotic caspases (caspases 2, 3, 6, 7, 8, 9, 10) (figure 2). Apoptotic caspases can be further subdivided into initiator and effector caspases. Initiator caspases possess long pro-domains which either contains a caspase recruitment domain (CARD) (caspases 2 and 9), or a death effector domain (DED) (caspases 8 and 10). These pro-domains enable the caspases to interact with other proteins that regulate their activation. Activation of initiator caspases occurs at multiprotein complexes including the DISC (caspases 8 and 10) (Walczak and Haas, 2008), the apoptosome (caspase-9) (Riedl and Salvesen, 2007), the inflammasome (caspase-1) (Martinon and Tschopp, 2007) and the piddosome (caspase-2) (Tinel and Tschopp, 2004). Several hypotheses concerning the activation of initiator caspases exist. In the "induced-

proximity model", recruitment of initiator caspases to the receptor complex by the Fas-Associated protein with Death Domain (FADD) leads to clustering of initiator caspase zymogens resulting in self-activation of the caspases via a cross-proteolysis mechanism (Salvesen and Dixit, 1999). In contrast to this the "proximity-induced dimerisation" model states that the formation of dimers is the driving force behind activation of initiator caspases. The adaptor protein complexes serve to promote dimerisation by increasing the local concentration of initiator caspases (Shi, 2004). Dimerisation of the procaspases is crucial for initiator caspase activation, even though the processing of the caspases stabilises the active dimers. The most recent model by Chao *et al.* is the "induced conformation model" (Chao et al., 2005). In this model the conformation change of the active site of the initiator caspase which is attained through direct interaction with the adaptor protein complex is a prerequisite for the activation. Most of the studies concerning initiator caspase activation have been focused on the activation of caspase-9 at the apoptosome. However, the same molecular concepts might also apply for the activation of other initiator caspases at their respective activation platforms.

In contrast to initiator caspases, effector caspases have shorter pro-domains and do not show CARD motifs. Therefore, they can only be activated by other caspases. During apoptosis, they are cleaved by initiator caspases and can then autoactivate themselves by autocatalytic cleavage of their pro-domain. The autocatalytic activation step can be inhibited by inhibitor of apoptosis proteins (IAPs). The major task of effector caspases is amplifying the caspase cascade (Slee et al., 1999).

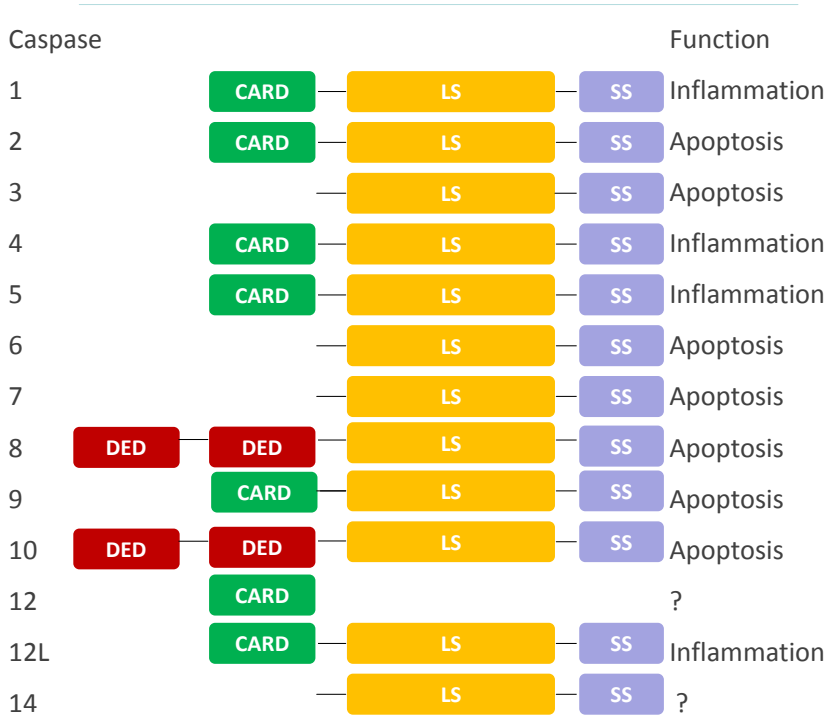


figure 2. The caspase family.

All caspases have a similar domain structure comprising a pro-peptide followed by a large (LS) and a small subunit (SS). The pro-peptide can vary in length and, can be used to recruit the enzyme to activation platforms as in the case of initiator caspases. Two distinct, but structurally related, pro-peptides have been identified; the death effector domain (DED) and the caspase recruitment domain (CARD), which typically facilitate interaction with proteins that contain the same motifs. Not all mammalian caspases participate in apoptosis. For example, caspase-1, caspase-4, caspase-5 and caspase-12 are involved in the processingand regulation of inflammatory cytokine and are activated during innate immune responses. Adapted from Taylor et al.(Taylor et al., 2008).

Activated caspases are able to cleave hundreds of proteins (Nicholson, 1999). Among them are proteins which are involved in all important cellular processes, for instance cell cycle and replication, DNA damage and repair, transcription and translation, and signal transduction as well as cytoskeletal and structural proteins. Caspase activity can have two different effects: destruction of protein and activation of proteins that are important for the process of programmed cell death.

1.2.2. **Bcl-2 family proteins**

Members of the B-cell lymphoma (Bcl-2) family are important regulators of the initiation of apoptosis in mammals. They can be grouped into anti-apoptotic members, that inhibit the initiation of the death program, and pro-apoptotic members that sense death signals within the cell. Bcl-2 is a proto-oncogene and homolog to ced-9 found in *C.elegans* (Hengartner and Horvitz, 1994). A key feature of Bcl-2 family proteins is that they share sequence homology in four domains, namely: Bcl-2 homology (BH) 1, BH2, BH3 and BH4. However, not all members possess all domains. They can be subdivided according to their domain structure and function (figure 3). Proteins that posses all BH domains are classified as anti-apoptotic and are required for death repression, e.g. Bcl-2, Bcl-XL, Bcl-W, Mcl-1, Bcl-B and A1. In contrast, pro-apoptotic molecules comprise only the domains BH1-BH3 (Bax, Bak and Bok). A third divergent class of BH3-only proteins (Bad, Bik, Bid, Hrk, Bim, Bmf, Noxa and Puma) has a conserved BH3 domain that can bind and regulate anti-apoptotic BCL-2 proteins to promote apoptosis (reviewed in Youle and Strasser, 2008). Simplified, one can say that the ratio of pro-apoptotic and anti-apoptotic molecules determines the fate of the cell. An excess of anti-apoptotic molecules keeps the cell alive while an excess of pro-apoptotic molecules induces apoptosis. The pro-apoptotic family members Bax and Bak are essential for the induction of the mitochondrial outer membrane permeabilisation (MOMP) and the subsequent release apoptogenic molecules such as cytochrome *c* and SMAC/DIABLO which leads to caspase activation. Anti-apoptotic family members, such as Bcl-2 and Bcl-XL, counteract Bax and Bak. Although it is commonly thought that Bax and Bak form pores in the mitochondrial membrane, the biochemical nature of these pores and how anti-apoptotic Bcl-2 family proteins might regulate them ist still a controversial issue in the field of apoptosis (Chipuk et al., 2006).

BH3-only proteins are pro-apoptotic and function as initial sensors of apoptotic signals that emanate from various cellular processes. There are two models concerning the activation of Bax and Bak by BH3-only proteins. One model suggests that BH3-only proteins (specifically Bim, tBid and Puma) directly activate Bax and Bak (Youle, 2007). However, recent evidence indicates that BH3-only proteins de-repress Bax and Bak by direct binding and inhibition of Bcl-2 and other anti-apoptotic family (Willis et al., 2007).

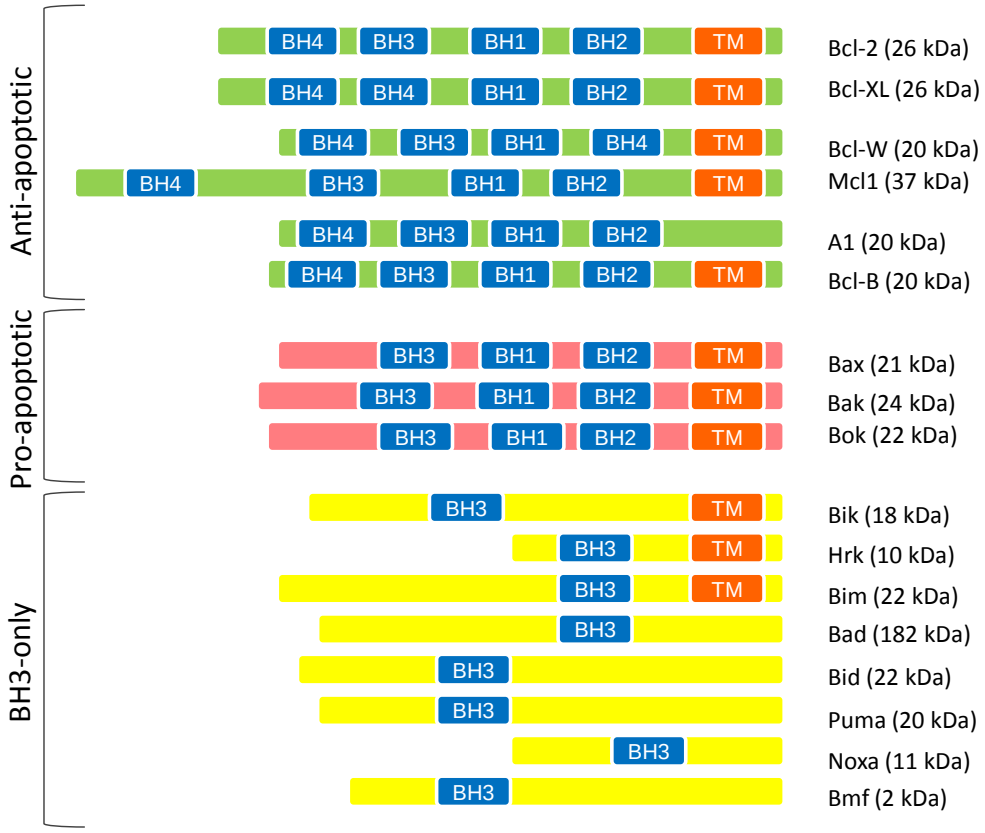


figure 3. The Bcl-2 family.

Bcl-2-family proteins have a crucial role in the regulation of apoptosis through their ability to control mitochondrial cytochrome c release. The Bcl-2 family comprises three subfamilies that contain between one and four Bcl-2 homology (BH) domains. The anti-apoptotic subfamily members contain four BH domains. Most members of this subfamily are typically associated with membranes and therefore also contain transmembrane domains (TM). The pro-apoptotic subfamily lacks BH4 domains and promotes apoptosis by forming pores in mitochondrial outer membranes. The BH3-only subfamily is a structurally diverse and only displays homology within the small BH3 motif. Adapted from Taylor et al. (Taylor et al., 2008).

BH3-interacting domain death agonist (Bid)

Bid was indentified and cloned based on its ability to interact with the Bcl-2 family members Bax and Bcl-2 via its BH3 domain in 1995 (Yang et al., 1995). It has a special role among the BH3-only proteins as it acts a molecular linker between the extrinsic and the intrinsic apoptotic pathway. It becomes activated by cleavage by caspase-8 after death-receptor engagement (Li et al., 1998). Bid in its uncleaved form is already able to kill cells as shown in an overexpression system with a Bid mutant lacking the caspase-8 cleavage site (Sarig et al., 2003). Additionally, endogenous full length Bid translocates to the mitochondria in anoikis

(Valentijn and Gilmore, 2004). Anoikis is a form of apoptosis induced by detachment of cells from the extra-cellular matrix. The truncated form of Bid (tBid) is much more powerful in terms of apoptosis induction. The cleavage of Bid into tBid by caspase-8 leads to its translocation to the mitochondria where it facilitates effective activation of Bax and Bak. There are different hypotheses on how tBid leads to the induction of MOMP. A recent study suggests that voltage-dependent anion channel (VDAC) 2 is responsible for the recruitment of Bax to the mitochondrial membrane which is crucial for tBid-induced MOMP (Roy et al., 2009). In contrast this other studies show that tBid rather binds to the Bcl-XL which is bound to the mitochondrial membrane (Garcia-Saez et al., 2009), potentially displacing it from Bak or Bax. A third hypothesis is that tBid interacts with the mitochondrial lipid cardiolipin and directly induces pore formation (Petit et al., 2009).

Besides caspase-8, caspase-10 is recruited to the DISC and is also able to process Bid. It does not only create the p15 fragment usually referred to as tBid, but it can also create a shorter p13 fragment of tBid by cleaving at the residue D75 (Fischer et al., 2006). However, the role of this p13 fragment is not understood yet. Bid cannot only be cleaved by caspases but also by other proteases, e.g. cathepsins, calpains, and Granzyme B (Barry et al., 2000; Chen et al., 2001; Cirman et al., 2004). Therefore, it can be considered as a sentinel for death signals mediated by proteases. figure 4 shows an overview of the structure of human Bid, its protease cleavage sites and other post-translational modifications. Proteolytic cleavage is not the only post-translational modification regulating the function of Bid. Zha *et al.* (2000) reported that the amino-terminus of tBid becomes N-myristoylated after having been cleaved by caspase-8. The myristoylated form of tBid is considered to be 350 time more potent than the unmodified version. In contrast, a negative regulation of the pro-apoptotic activity of Bid is conferred by phosphorylation. Casein kinase (CK) I and II have been shown to phosphorylate murine Bid at residues T59, S61 and S64 which interferes with the cleavage of Bid by caspase-8 (Desagher et al., 2001). Of note is that so far this has only been shown in the murine system and that residue S61 is not conserved in humans. Therefore, the physiological relevance of this mechanism in the human system cannot be assessed.

For a long time, it has been assumed that Bid only possesses a killing function. However, recent studies provide evidence that it also has a proliferative effect (Bai et al., 2005) and that it acts as a sensor for DNA damage and introduces cell cycle arrest (Kamer et al., 2005; Zinkel et al., 2005). It has been observed that Bid-deficient mouse embryonic fibroblasts

(MEFs) enter into the cell cycle in a delayed fashion when mitogenically stimulated compared to wild type MEFs (Bai et al., 2005) but the mechanism behind this is still unclear. The participation of Bid in cell cycle regulation was discovered when Bid-deficient MEFs were exposed to DNA damage. Normally, a proliferating cell arrests in S-phase when exposed to DNA damage allowing for repair of damaged DNA before the cells proceed in the cell cycle. Interestingly, Zinkel *et al.* (Zinkel et al., 2005) showed that Bid-deficient MEFs failed to accumulate in S-phase, suggesting a role of Bid in the S-G_2 cell cycle checkpoint. In this context, phosphorylation of Bid at residue S78 in the human and at residues S61 and S78 in the murine form of Bid by Ataxia telangiectasia mutated (ATM), a kinase that becomes activated upon DNA damage, seems to be essential for S-phase arrest. Using a non-phosphorylatable Bid mutant, Zinkel *et al.* showed that this mutant was not able to restore the S-phase arrest when introduced into Bid-deficient MEFs. In addition, this non-phosphorylatable Bid mutant rendered cells more susceptible to etoposide-induced apoptosis (Kamer et al., 2005). The ability of Bid to induce S-phase arrest when DNA damage occurs also suggests that Bid could be a key player in tumourigenesis. Bid deficient-MEFs suffer from genomic instability followed by leukemogenesis probably due to the accumulation of DNA failures (Zinkel et al., 2005).

However, the function of Bid with respect to DNA damage is disputed. Kaufmann *et al.* (Kaufmann et al., 2007) created a different strain of Bid-deficient mice on the C57BL/6 background. These mice did not show the phenotype described by Zinkel *et al.* (Zinkel et al., 2005) and no implication of Bid in DNA damage- and stress-induced apoptosis could be detected, rendering Bid dispensable for these processes. These contradictory results are most likely due to subtle changes in the experimental conditions (Zinkel et al., 2007).

Another study touching up on this issue claims a dual function for Bid. It could be shown in hepatocellular carcinoma cells that Bid sensitises cells to apoptosis when treated with high concentrations of etoposide, which cause irreparable DNA damage. In contrast, when cells were treated with low doses of etoposide that only cause repairable damage, Bid induced S-phase arrest (Song et al., 2008a). These findings were further supported in a recent study in which low doses of the carcinogen anti-(±)-5-methylchrysene-1,2-diol-3,4-epoxide (5-MCDE) induced increased apoptosis in $Bid^{-/-}$ MEFs and reconstitution of Bid expression in $Bid^{-/-}$ cells could inhibit the increased apoptosis (Luo et al., ,2010). However, the phosphorylation status of Bid was not investigated in these studies.

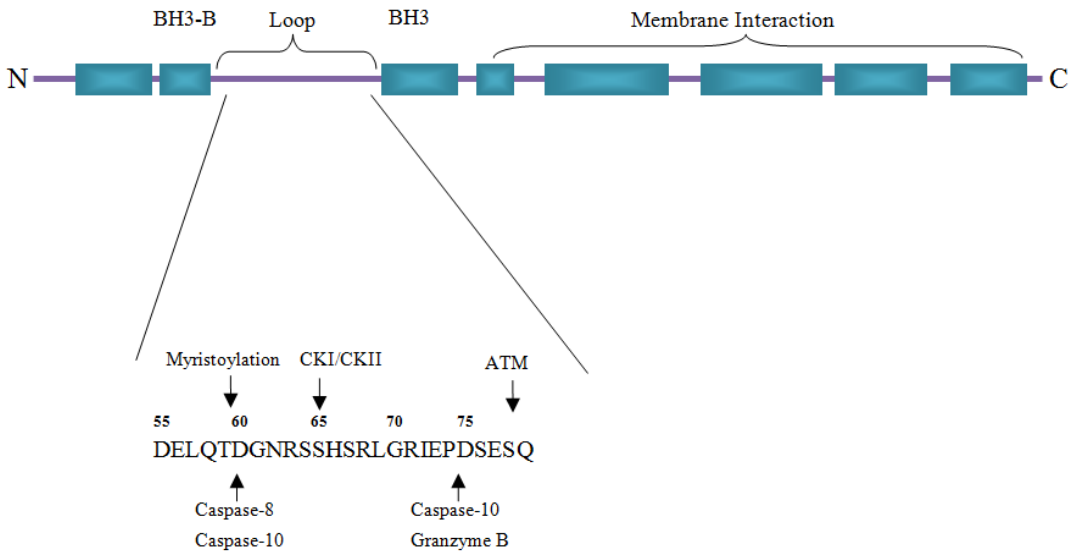


figure 4. Schematic overview of the human Bid structure and its posttranslational modifications.

Full length Bid consists of 195 amino acids structured in eight α-helices depicted in blue. One of these α-helices constitutes the BH3 domain which is responsible for the interaction with other Bcl-2 family members. α -helices 6 and 7 are hydrophobic and are buried inside the full length protein. They are potentially responsible for membrane interaction once the protein is cleaved into tBid. Especially the loop region of Bid is subjected to posttranslational modifications, it becomes cleaved by different proteases (Caspases 8 and 10 and Granzyme B) at two different sites (D60 and D75), myristoylated and phosphorylated by CKII and ATM (S78).

1.3. Apoptosis-induction by TRAIL

1.3.1. The TRAIL/TRAIL-receptor system

TRAIL is expressed as a type II transmembrane protein consisting of 281 amino acids in human. It consists of a short intracellular N-terminus and a long extracellular receptor binding domain. Similar to TNF or CD95L, TRAIL can be cleaved off the membrane to form a soluble trimer which is stabilised by cysteine residues that are coordinated by a zinc ion (Hymowitz et al., 1999). Noteworthy, unlike CD95L and TNF, which are cleaved off by metalloproteases, soluble TRAIL is generated by the action of cysteine proteases (Mariani and Krammer, 1998). It is assumed that membrane bound TRAIL has greater cytotoxic potential than the soluble form as has recently been shown for CD95L (LA et al., 2009).

TRAIL was identified in a screen based on sequence homology with CD95L (Pitti et al., 1996; Wiley et al., 1995). However, instead of binding to CD95, TRAIL has been shown to bind to five different receptors in humans TRAIL-R1 (DR4, TNFRSF10A), TRAIL-R2 (DR5, TNFRSF10B, Killer, TRICK2), TRAIL-R3 (DcR1, TRID), TRAIL-R4 (DcR2) and Osteoprotegerin (OPG), which form a rather complex receptor system unique within the TNF-R superfamily (figure 5). All five receptors share the typical cysteine rich domain (CRD) structure, but only TRAIL-R1 and TRAIL-R2 are capable of transmitting the apoptotic signal to the cell's inside because they are the only classical death receptors containing the intracellular death domain (DD) (Pan et al., 1997a; Pan et al., 1997b; Screaton et al., 1997; Sheridan et al., 1997; Walczak et al., 1997; Wu et al., 1997). Both receptors are characterised by the presence of two cysteine rich repeats (CRRs) in their extracellular parts facilitating TRAIL binding. It is still not completely understood why two apoptosis-inducing TRAIL receptors are expressed in humans though only one receptor is sufficient to induce apoptosis in a variety of tumour cell lines following TRAIL application (Sprick et al., 2002). Thus, there has to be a differential function of TRAIL-R1 and TRAIL-R2, respectively, which remains to be elucidated.

Although TRAIL-R3 (Degli-Esposti et al., 1997b) and TRAIL-R4 (Degli-Esposti et al., 1997a) are highly homologous in their extracellular domains to their apoptosis-inducing counterparts, they are unable to induce apoptosis due to a complete or partial lack of the DD, respectively. TRAIL-R3 and -R4 are generally referred to as decoy-receptors. However, a decoy-function has so far only been demonstrated in an overexpression system, whereas evidence in a more physiological setting is still missing. Merino *et al.* (Merino et al., 2006) showed for the first time that the two receptors might use different mechanism to inhibit TRAIL-induced apoptosis. On the one hand, TRAIL-R3 titrates TRAIL within lipid rafts, therefore blocking TRAIL-induced cell death by competition. On the other hand, a TRAIL-dependent interaction of TRAIL-R4 with TRAIL-R2 might result in impaired formation of a death receptor-signalling complex, accompanied by reduced levels of caspase-8 activation, the main executor of apoptosis (Merino et al., 2006). However, as these studies were not performed under physiological expression levels, more studies are required to demonstrate that the role of TRAIL-R3 and TRAIL-R4 is more "regulatory" than "decoy". Accordingly, although all TRAIL-receptors are widely expressed within normal as well as malignant cell types, the expression of TRAIL-R3 and TRAIL-R4 does not correlate with the sensitivity of a

given cell towards TRAIL-induced apoptosis. Thus, the mechanism of TRAIL-restricted apoptosis of tumour cells remains elusive.

OPG is the fifth, rather low-affinity receptor for TRAIL (Emery et al., 1998; Truneh et al., 2000), whose function is linked to bone metabolism. Upon binding to Receptor Activator of NF-κB ligand (RANKL), another member of the TNF-superfamily, OPG competitively inhibits the RANKL-RANK interaction, thereby suppressing osteoclast formation. Surprisingly, not only TRAIL, but also its receptors are widely spread through human tissues, including spleen, thymus, peripheral blood lymphocytes, prostate, testis, ovary, uterus and multiple tissues along the gastrointestinal tract as has been shown on mRNA level (Walczak et al., 1997; Wiley et al., 1995). Thus, in contrast to the CD95 system, which is controlled by tight expression of CD95L, the control point for TRAIL-induced apoptosis does not seem to refer to the transcriptional level, but rather the level of surface expression. However, it remains to be elucidated how TRAIL-R surface expression is indeed regulated on protein level.

The murine TRAIL-R system differs profoundly from the human TRAIL-R system. In mice there is only one apoptosis-inducing receptor, referred to as TRAIL-R (mDR5, murine killer-MK). It cannot be regarded as an ortholog of one of the human TRAIL-Rs as it exhibits similar sequence homology to both human TRAIL-Rs (76% and 79% sequence identity for TRAIL-R1 and TRAIL-R2, respectively) (Wu et al., 1999). The other murine receptor, mDcR1 and the splice variants mDcR2L and mDcR2S, have not been studied yet, besides their identification in a clustered locus (Schneider et al., 2003). These receptors are only distantly related to human receptors because they possess a different CRD structure. Potentially they exert similar functions as human TRAIL-R3 and TRAIL-R4.

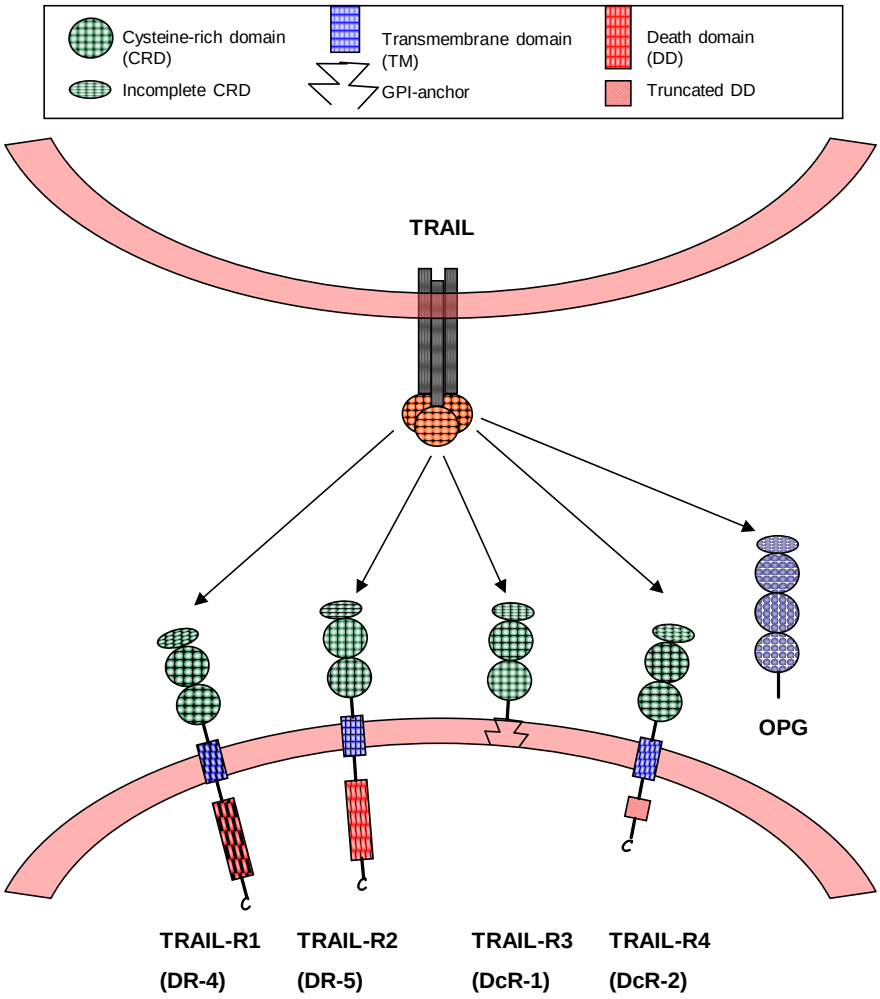


figure 5. The TRAIL/TRAIL-R system in humans.

Trimerised TRAIL can bind to five different receptors. Of them, only TRAIL-R1 and TRAIL-R2 can induce apoptosis because they contain a DD. TRAIL-R3 and TRAIL-R4 cannot induce apoptosis as they lack the DD or have a truncated DD, respectively. The soluble receptor OPG can also bind to TRAIL but with rather low affinity (Cordier et al., 2009).

1.3.2. **TRAIL-receptor signalling**

Apoptosis is a tightly controlled process regulated by a complex signal machinery with a variety of check-points at several levels of signalling (figure 6). Binding of membrane-bound or soluble TRAIL to its two death-inducing receptors TRAIL-R1 and TRAIL-R2 induces receptor oligomerisation, thereby bringing the intracellular DDs of the receptors into close proximity. Protein crystallography experiments suggested TRAIL to bind as a trimer to pre-assembled receptor complexes that are connected via their pre-ligand binding-assembly domain (PLAD), which themselves are not yet capable of transmitting a death signal (Chan et

al., 2000). However, once TRAIL is bound to this pre-assembled receptor complex, juxtaposition of the DDs creates a structure referred to as death-inducing signalling complex (DISC), to which a variety of adaptor and signalling molecules is recruited. Among them is FADD, which binds via its DD to the DD of the TRAIL-receptor. Subsequently, pro-caspase-8 and -10 are recruited to the DISC upon interaction of the death-effector domain (DED) of FADD with the DED of these caspases. While caspase-8 is essential in the induction of apoptosis, the role of caspase-10 in this process remains controversial. Sprick *et al.* demonstrated that although caspase-10 is recruited to the DISC by FADD, it is not required for apoptosis induction and unable to functionally substitute for caspase-8 (Sprick et al., 2002). Thus, one might suggest that caspase-10 possesses alternative functions in apoptosis induction, for instance in diversifying the apoptotic signal.

Assembly of the DISC creates a structure allowing for auto-catalytic cleavage of caspases, thereby producing active caspase-8 and -10. To promote the apoptotic process following pro-caspase-8 (and -10) cleavage, pro-caspase-3 is activated in a two-step mechanism. Initially, active caspase-8 separates the large from the small subunit. However, to become fully activated, caspase-3 has to remove its pro-domain during an autocatalytic maturation step. Once activated, it cleaves a variety of cellular proteins, including Poly(ADP-ribose) polymerase (PARP), lamins and cytokeratins. Furthermore, it inactivates ICAD, the inhibitor of Caspase Activated DNase (CAD). Thus, CAD is no longer restrained by ICAD, but able to enter the nucleus and to fragment the DNA, thereby producing the "DNA ladder" characteristic for apoptotic cells.

TRAIL-receptor cross-linking is also able to activate the BH3-only protein Bid, which is cleaved by receptor-activated caspase-8 (and -10) into truncated Bid (tBid). tBid then translocates to the mitochondria to induce the release of pro-apoptotic factors via Bax and Bak. Thus, Bid forms a bridge connecting the extracellular and intracellular pathways. Due to increased permeability of the outer membrane and a breach in mitochondrial integrity, cytochrome *c* and other pro-apoptotic molecules are released. Together with Apaf-1 and pro-caspase-9, cytochrome *c* forms a structure referred to as apoptosome (Baliga and Kumar, 2003). Like caspase-8, apoptosome-activated caspase-9 is also able to activate pro-caspase-3. Once caspase-3 is activated, it will not only cleave its target proteins, but also new pro-caspase-9 molecules that in turn further activate pro-caspase-3. This positive feedback loop ensures apoptosis to be inevitably carried out.

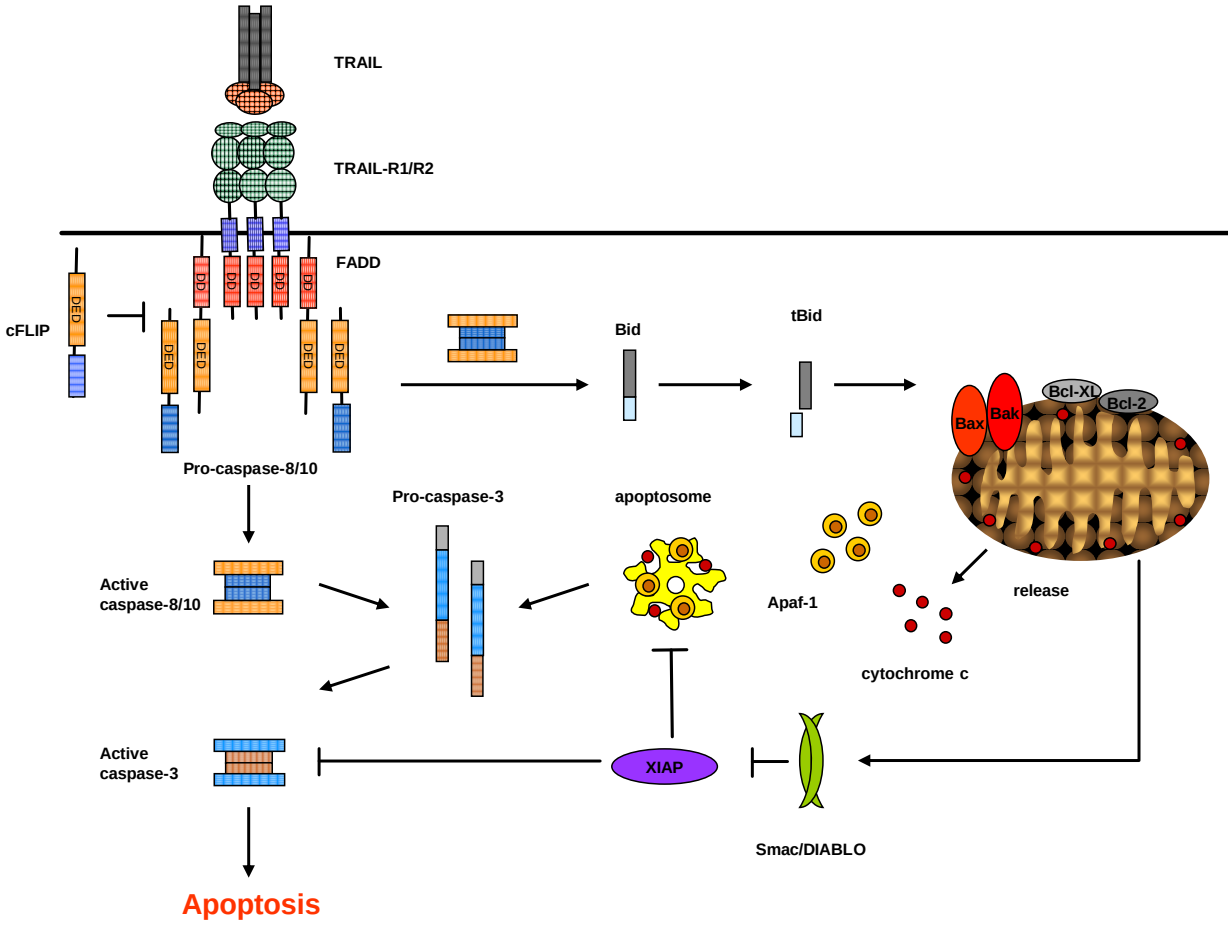


figure 6. The TRAIL-apoptosis pathway.

The TRAIL signal is initiated by binding of the ligand to the respective receptor, TRAIL-R1 and /or TRAIL-R2. The receptors trimerise and recruit several intracellular adaptor molecules like FADD, cFLIP and procaspases 8 and 10, which are autocatalytically activated and from the DISC. Active caspases 8 and 10 either directly cleave effector caspase-3 or involve the mitochondrial apoptosis paythway via processing of Bid into tBid. tBid activates Bax/Bak, which are usually blocked by Bcl-2, Bcl-XL or Mcl-1, to release cytochrome c and SMAC/DIABLO from the mitochondrial intermembrane space. Cytochrome c binds Apaf-1 and forms the apoptosome together with caspase-9 which in turn activates caspase-3. SMAC/DIABLO further facilitates apoptosis by binding to XIAP that usually blocks caspase-3 maturation (Cordier et al., 2009).

Depending on the need of the intrinsic apoptotic pathway to undergo death receptor induced apoptosis, cells can be classified as type I and type II cells, respectively. Type I cells are characterised by low expression of XIAP, the inhibitor of caspase-3, which allows for the direct activation of caspase-3 activation by caspase-8. In contrast, XIAP levels in type II cells are high. Thus, these cells additionally require the mitochondrial amplification loop to efficiently activate effector caspases to undergo apoptosis (Jost et al., 2009).

The apoptotic signal is regulated at several stages. Due to the presence of two DEDs, the cellular FLICE-like inhibitory protein (cFLIP) competes with caspase-8 for the binding to FADD (Krueger et al., 2001). Displacement of caspase-8 from the DISC prevents the initiation of a caspase cascade responsible for apoptosis transmission. The amount of cFLIP within a cell inversely correlates with the amount of caspases that are activated at the DISC and therefore with the decision whether apoptosis is induced. Three splice variants of cFLIP are reported referred to as $cFLIP_L$, $cFLIP_s$ and $cFLIP_R$ (Golks et al., 2005). Comprising two DEDs and an additional C-terminal caspase domain, $cFLIP_L$ closely resembles caspase-8 in its overall structural organisation. However, due to the lack of a critical cysteine residue within the active centre, $cFLIP_L$ does not possess proteolytic activity. Whereas $cFLIP_S$ and $cFLIP_R$ already inhibit the recruitment of pro-caspase-8 to the DISC, $cFLIP_L$ rather interferes with the full maturation of DISC-recruited pro-caspase-8. However interestingly, a heterodimer of caspase-8 and $cFLIP_L$ has been shown to possess stronger caspase-8 activity than a homodimer of solely caspase-8 in a cell free system (Micheau et al., 2002). Also possessing a pro-apoptotic activity in this context, the role of $cFLIP_L$ might be more complex than initially assumed.

Other proteins inhibiting the apoptotic process are the cellular inhibitor of apoptosis proteins (IAPs) (Salvesen and Duckett, 2002). As already mentioned, XIAP is the most prominent member that is known to prevent the activation of caspase-3 and -9 by direct interaction (Riedl et al., 2001). XIAP blocks the removal of the inhibitory pro-domain of caspase-3, therefore inhibiting its complete maturation. Alternatively, XIAP is also able to catalyse its ubiquitination, therefore leading to its proteasomal degradation (Vaux and Silke, 2005). Other members of the IAP family are cIAP1, cIAP2 and survivin. However their role in TRAIL-induced apoptosis is not completely understood. The activity of IAPs themselves is in turn controlled by another set of proteins that antagonise their function. Once released from the mitochondrial inter-membrane space, the pro-apoptotic SMAC/DIABLO protein interacts and sequesters XIAP, thereby removing it from caspase-3 and -9. Caspase-3 can then be auto-catalytically cleaved, therefore allowing apoptosis to proceed.

Taken together, apoptosis is a complex, highly regulated process that is influenced by a variety of pro- as well as anti-apoptotic proteins. While caspases are the main executors of apoptosis, intracellular factors like anti-apoptotic Bcl-2 family members, cFLIP and IAPs are able to reduce the sensitivity of a given cell towards apoptosis. It is therefore not surprising

that many tumour cells overexpress these inhibitory molecules or down-regulate pro-apoptotic Bcl-2 proteins. In many cancers, the balance of anti- and pro-apoptotic effectors is shifted in favour of the former, indicating that cells continue to replicate in spite of being damaged.

Although the molecules so far detected in the TRAIL-receptor DISC are similar to those of the CD95 DISC, the biological outcome of the action of both molecules is extremely diverse. While systemic CD95 stimulation also kills normal cells including hepatocytes, TRAIL specifically eliminates malignantly transformed cells without damaging healthy tissue. Therefore, it is highly likely that the receptor composition of these two systems has to differ in some way. Thus, new studies are required to explicitly compare both receptor complexes following stimulation in order to detect novel factors that are only present in one of the two systems and might therefore explain for the difference in functional outcome.

1.4. The physiological role of TRAIL

Over the last decade several TRAIL$^{-/-}$ and TRAIL-R$^{-/-}$ mice have been developed by different groups. The first TRAIL-deficient mice were developed by two groups in parallel in 2002 (Cretney et al., 2002; Sedger et al., 2002). In both studies mice were viable, fertile and had no obvious phenotype except for an enlarged thymus. Therefore, a role for TRAIL in development could be excluded. The same holds true for TRAIL-R$^{-/-}$ mice. To date three different groups have generated TRAIL-R deficient mice which showed enhanced innate immune responses (Diehl et al., 2004), defects in radiation-induced apoptosis (Finnberg et al., 2005) and increased susceptibility to lymph node metastasis (Grosse-Wilde et al., 2008).

By now TRAIL$^{-/-}$ mice are commercially available and many studies investigating their phenotype have been carried out. Many of them found that TRAIL plays a role in innate and adaptive immune responses and in infectious and autoimmune diseases, which might not be surprising as TRAIL and TRAIL-Rs are expressed on a variety of immune cells. In close relation to this TRAIL was also found to have an immune surveillance function against tumours and metastases. Furthermore, triggered by the debate about liver toxicity of certain TRAIL-preparations, the role of TRAIL in liver disease has been studied.

Noteworthy, TRAIL has also been shown to bind to OPG, an osteoblast-secreted decoy receptor that functions as a negative regulator of bone resorption (Emery et al., 1998). As

TRAIL and TRAIL-R knockout mice do not show a bone phenotype, the physiological importance of the TRAIL-OPG interaction is still elusive.

1.4.1. TRAIL in the immune system

Stimulation-induced expression of TRAIL was found on the surface of cells from the innate as well as the adaptive immune system. TRAIL has been shown to be up-regulated upon antigen–receptor engagement, stimulation of Toll-like receptors and exposure to interferons (INFs). For example TRAIL up-regulation on monoctyes and macrophages is triggered by Lipopolysaccharides (LPS), INF-β and INF-γ, which has also been shown to be responsible for the up-regulation of TRAIL on the surface of dendritic cells and natural killer cells (Ehrlich et al., 2003; Halaas et al., 2000).

TRAIL in innate immune cells

TRAIL has an important effector function in NK cell-induced killing of target cells. This has initially been shown *in vitro*, where NK-mediated killing of tumour cells could only be ablated when TRAIL was neutralised in combination with CD95L and perforin (Kayagaki et al., 1999c). Later on this finding could also be confirmed in an *in vivo* setting. Takeda *et al*. (Takeda et al., 2001) used a metastasis model to show that reduction of the tumour burden was greatly dependent on INF-induced up-regulation of TRAIL. Although induction via INF-γ is a prerequisite for TRAIL expression on NK cells in adult mice, a small subpopulation exists that constitutively expresses TRAIL. This subpopulation has been shown to mainly consist of immature NK cells. These cells are most likely a remainder form earlier stages in life as high TRAIL expression can be found in fetal and neonatal mice, due to an autocrine production of INF-γ (Takeda et al., 2005).

Similarly, a subset of dendritic cells (DCs) which produces INF-γ has been identified which is hallmarked by high TRAIL expression levels (Chan et al., 2006; Taieb et al., 2006).

TRAIL in infectious diseases

One decade ago, it has been discovered that TRAIL plays a role in viral infections. It was noticed that virus infected cells were rendered more TRAIL-sensitive, e.g. usually TRAIL-resistant fibroblasts were sensitised to TRAIL after infection with human cytomegalovirus (Sedger et al., 1999). Accordingly, Sato *et al*. (Sato et al., 2001) showed two years later that the blockage of NK cell derived TRAIL increased viral titers ultimately leading to an earlier

death of encephalomyocarditis virus infected mice. One study using TRAIL-R knock-out mice investigated the response of TRAIL to different pathogens, not only viruses (Diehl et al., 2004). Absence of TRAIL-R only influenced the response to virus infection (murine cytomegalovirus) but not to other pathogens. Surprisingly, TRAIL-receptor deficient mice were more resistant to virus infection than the wild type mice. As TRAIL seems to be necessary for the clearance of virus infected cells, this finding seems counterintuitive. Interestingly, murine cytomegalovirus infection led to increased serum levels of Interleukin (IL)-12 and IFN-γ in TRAIL-R -/- mice, possibly produced by DCs, macrophages and NK cells. Thus, TRAIL-R might be a negative regulator of innate immune responses by influencing antigen presenting cells (Diehl et al., 2004).

TRAIL in T cells

Similar to NK cells, TRAIL is absent on naïve T cells but can be induced by different stimuli, e.g. anti-CD3 (Jeremias et al., 1998), type I INFs (Kayagaki et al., 1999a), LPS, phytohemagglutinin (PHA) and IL-2 (Ehrlich et al., 2003). TRAIL contributes to the cytotoxic activity of T lymphocytes as shown for $CD4^+$ cells (Kayagaki et al., 1999b) and $CD8^+$ T cells, which can kill virus infected cells via TRAIL (Mirandola et al., 2004). In this context, TRAIL-R deficient mice also had more severe influenza infections due to a decreased $CD8^+$ T-cell mediated killing (Brincks et al., 2008).

Additionally, the TRAIL/TRAIL-R system may also play a role in the homeostasis of a particular subset of $CD8^+$ T cells. "Helpless" $CD8^+$ T cells are primed in the absence of $CD4^+$ T cells and are unable to undergo a second round of clonal expansion upon restimulation with their cognate antigen (Shedlock et al., 2003). As TRAIL deficient "helpless" $CD8^+$ T cells can still expand a second time, this effect was thought to be mediated via TRAIL. Thus, the absence of $CD4^+$ T cells results in short-lived antigen-specific $CD8^+$ T cells and defective secondary $CD8^+$ T cell responses because of TRAIL-mediated apoptosis (Janssen et al., 2005). By now, IL-15 has been identified as a mediator of $CD4^+$ help for $CD8^+$ T cell longevity and avoidance of TRAIL-mediated apoptosis by down-regulating pro-apoptotic Bax and increasing anti-apoptotic Bcl-XL in $CD8^+$ T cells (Oh et al., 2008). A third study went one step further and showed that the induction of tolerance by apoptotic cells was mediated by $CD8^+$ suppressor T cells with a "helpless phenotype"(Griffith et al., 2007b). Hence, animals deficient in TRAIL were resistant to tolerance induction by apoptotic cells.

However, the role of TRAIL in the homeostasis of helpless $CD8^+$ is heavily contested. Two independent studies could not reproduce the results and claim that helpless $CD8^+$ T cells still proliferate, but in a delayed fashion (Badovinac et al., 2006; Sacks and Bevan, 2008).

Another role for TRAIL in T cells might be regulating Th1 versus Th2 cell responses. Th1 cells have been shown to up-regulate CD95L upon stimulation with anti-CD3 *in vitro*, whereas Th2 cells rather seem to up-regulate TRAIL. These Th2 cells also are more TRAIL-resistant than their Th1 counterparts (Roberts et al., 2003; Zhang et al., 2003). The cause for this might be an up-regulation of cFLIP also induced by the treatment with anti-CD3. In a model for allergic airway disease $TRAIL^{-/-}$ mice showed an ameliorated disease outcome. TRAIL deficiency led to decreased homing of Th2 cells to the airways and as a result, to decreased release of Th2 cytokines, which in turn induce allergy (Weckmann et al., 2007). This could be caused by TRAIL-mediated apoptosis induction of Th1 cells leading to a stronger Th2 response. Therefore, blocking TRAIL in the airway of asthma patients might be a treatment approach for asthma.

1.4.2. **TRAIL in liver disease**

As already mentioned earlier, immature NK cells express TRAIL in the liver. TRAIL receptors become up-regulated in various liver diseases, among them Hepatitis B virus, Hepatitis C virus or cirrhosis conditions that are hallmarked by increased apoptosis and chronic inflammation. This might contribute to the liver damage caused by TRAIL which has been observed *in vivo* in different hepatitis models (reviewed in Herr et al., 2007).

The first indication that TRAIL plays a role in hepatitis was a result of the studies by Zheng *et al.* (Zheng et al., 2004). In their study they were able to show that TRAIL deficient mice were resistant to Concanavalin A- induced and *Listeria cytogenes*- induced hepatitis.

These findings were corroborated by another study, which addressed CD95L-induced hepatitis (Corazza et al., 2006). In this model hepatitis was induced using the CD95-antibody Jo-2. Wild type mice died within hours after administration of the antibody due to hepatocyte death and liver failure. Although it was widely believed that this death was mainly dependent on TNF, this study now showed that TRAIL might contribute to Jo-2-induced death of hepatocytes. For some TRAIL-deficient mice, death was only delayed by 1–2 hours, but 43 %

of TRAIL-deficient mice survived over 24 hours. These data suggest that TRAIL facilitates CD95L-induced liver damage and thereby enhances CD95L-induced lethality.

In a bile duct ligated mouse model, which mimics cholestasis (the retention of bile fluid in the liver) and is achieved by a surgical block of the bile duct, hepatocytes have been shown to become TRAIL-sensitive to endogenous TRAIL present on NK cells (Kahraman et al., 2008).

Furthermore, using a model a viral hepatitis that more resembles physiological conditions, it was found that adenoviral application of TRAIL induced hepatitis, however, only when cells have been infected with adenovirus before (Mundt et al., 2003). In a second study the same group investigated patient samples and found that TRAIL was up-regulated in Hepatitis C patients. The expression of TRAIL in virally infected livers induced hepatic steatosis (the deposition of fatty acids in the liver) and apoptosis (Mundt et al., 2005). Furthermore, liver slices of HCV-infected organs and from livers suffering from steatosis were shown to be killed by different preparations of TRAIL.

The concept of TRAIL being a mediator of liver disease raises concerns about the clinical application of TRAIL-receptor targeting drugs. Profound liver toxicity in mice has hampered the use of CD95L in the clinics (Ogasawara et al., 1993). So far TRAIL has been administered safely in mice and non-human primates and is also well tolerated in clinical trials (discussed in detail in section 1.6.3). However, there is a debate about toxicity of TRAIL for hepatocytes which might greatly depend on the TRAIL-preparation and on the models system used. Taking all of this into account, in case TRAIL-induced liver toxicity turns out to be a clinically relevant issue, patients should be investigated for liver diseases prior to treatment with TRAIL and might have to be excluded when they present liver diseases.

1.4.3. **TRAIL in autoimmunity**

Autoimmune diseases develop as a result of inappropriate immune responses to self-antigens. Although TRAIL-R$^{-/-}$ and TRAIL$^{-/-}$ mice do not develop spontaneous autoimmune diseases, the induction of autoimmunity showed strong effects in TRAIL$^{-/-}$ mice in different autoimmune models: collagen-induced arthritis (Song et al., 2000), diabetes I (Lamhamedi-Cherradi et al., 2003), experimental autoimmune encephalomyelitis (EAE) (Cretney et al., 2005) and experimental autoimmune thyroiditis (EAT) (Wang et al., 2005). For example, BL6

wild type mice were resistant to collagen-induced arthritis, whereas TRAIL$^{-/-}$ mice readily developed the disease.

At first the role of TRAIL in autoimmunity was attributed to a function in thymic negative selection (Lamhamedi-Cherradi et al., 2003). It was proposed that TRAIL-R $^{-/-}$ mice failed to delete self-reactive T-cells. However, it is now widely accepted that the TRAIL-TRAIL-R system does not have a function in central tolerance. TRAIL is not expressed on dendritic and epithelial cells in the thymus, which are the major mediators of negative selection in the thymus (Tanaka et al., 1993). Furthermore, Cretney *et al.* (Cretney et al., 2005) failed to identify a role for TRAIL in an acute model of peptide antigen-specific negative selection using a T cell receptor (TCR) transgenic system as well as antibody-mediated TCR/CD3 ligation *in vitro* and *in vivo.* These results combined with the fact that aged TRAIL$^{-/-}$ mice showed no signs of autoimmunity, strongly indicate that intrathymic negative selection occurs normally in the absence of TRAIL-signalling. Therefore, the mechanism, how TRAIL influences autoimmunity, has yet to be determined.

1.4.4. **TRAIL in tumourigenesis**

After the discovery that TRAIL efficiently kills tumour cells, many groups set out to investigate the influence of TRAIL on tumourigenesis, using TRAIL- and TRAIL-R deficient mice or TRAIL-blocking antibodies. Although many studies have been conducted to date, they did not yield a conclusive picture after all.

The first indication that TRAIL may suppress tumour growth already showed in the initial TRAIL knockout study by Sedger *et al.* (1999), which demonstrated that a syngenic tumour transplant of a B cell lymphoma line grew much faster in the absence of endogenous TRAIL. In line with this, tumour growth of other syngenic tumour cell lines, e.g. the mammary carcinoma cell line 4T1 or the renal cell line Renca, was elevated in TRAIL$^{-/-}$ mice or after the treatment with TRAIL-blocking antibodies (Cretney et al., 2002; Takeda et al., 2001). Noteworthy, metastasis formation of Renca cells was affected in TRAIL$^{-/-}$ mice. These mice showed enhanced formation of metastasis in the liver but not in the lung, which might be due to the constitutive expression of TRAIL on NK cells in the liver mentioned earlier. Taken together, endogenous TRAIL was repeatedly shown to have an effect on tumour growth and metastasis formation in transplanted tumour models.

In contrast to this, the situation in autochthonous tumour models is not very clear. TRAIL$^{-/-}$ and TRAIL-R$^{-/-}$ mice do not develop spontaneous tumours at an early age. Only one study detected an increased formation of lymphoma in TRAIL$^{-/-}$ mice at a later stage in life (300-500 days after birth) (Zerafa et al., 2005). Especially, disseminated cancers like lymphoma, which reflect the situation of injected cell lines, seem to be affected by the loss of TRAIL. For instance, Eμ-myc induced formation of lymphoma was increased in TRAIL-R mice. (Finnberg et al., 2005). Similarly, loss of TRAIL promoted lymphoma formation induced by p53 heterozygocity.

In solid tumours models the role of TRAIL remains unclear. Zerafa *et al.* (2005) detected an increased incidence of sarcoma in p53$^{+/-}$ mice. Furthermore, in a chemically induced tumour model using MCA (methylcholanthrene) TRAIL$^{-/-}$ mice suffered from enhanced formation of fibrosarcoma (Cretney et al., 2002). In contrast to this, Finnberg *et al*. (2005) failed to observe a significant difference in diethylnitrosamine (DEN)-induced hepatocarcinogenesis. In all other studies conducted in epithelial tumour models TRAIL consistently did not play a role in the development of primary tumours. No difference was found between wild type mice and TRAIL-R or TRAIL deficient mice concerning the formation of intestinal tumours (Yue et al., 2005) or Her2/neu driven mammary carcinoma, respectively (Zerafa et al., 2005). Furthermore, in the chemically induced DMBA/TPA model, which mimics multiple steps in skin tumourgenesis, no significant increases in papilloma or carcinoma could be detected (Grosse-Wilde et al., 2008). Instead this study for the first time described TRAIL as a specific suppressor of lymph node metastasis in an autochthonous model in which primary tumour formation was not influenced by the absence of TRAIL. It is still elusive whether this specific metastasis suppressor function of TRAIL-R is confined to metastases in lymphoid organs, and which type(s) of cells are responsible for the TRAIL-mediated effect. If the tumour suppressor function of TRAIL also applies to other types of metastasis, this function of TRAIL could be exploited therapeutically in anti-metastatic therapies.

1.5. Sensitivity and resistance to TRAIL-induced apoptosis

In contrast to systemic treatment with CD95L or TNF, TRAIL selectively induces apoptosis in about 50% of tumour cell lines while leaving normal cells unharmed (Ashkenazi et al., 1999; Walczak et al., 1999). This key discovery opened up the possibility to use TRAIL as anti-cancer drug. However, recent studies revealed that most primary tumour cells are TRAIL resistant in the first place. Yet, many of these primary cancer cells can be sensitised to

TRAIL-induced apoptosis by combinational treatment with chemotherapeutics or irradiation. To be able to treat cancer efficiently it is hence crucial to understand the mechanisms underlying TRAIL-resistance.

1.5.1. **Resistance mechanisms**

Looking at TRAIL–R signalling, resistance to TRAIL can occur at different levels of the signalling cascade: at the level of TRAIL-Receptors, at the DISC level, at the mitochondria, at the level of caspase-3 activation or at any other step in the pathway that is required for TRAIL-induced apoptosis.

First of all the expression of the apoptosis-inducing receptors themselves can be down-regulated (Horak et al., 2005b). Accordingly, TRAIL-R1 expression was reported very low in some cells, e.g. in ovarian cancer which coincides with TRAIL-resistance. Low expression was caused by hypermethylation of the TRAIL-R1 promoter. Hence, resistance could be overcome by treatment with demethylating agent that restored TRAIL-R1 expression (Horak et al., 2005a). Noteworthy, a down-regulation of one of the apoptosis-inducing TRAIL-Rs must not necessarily result in resistance, e.g. in systems in which TRAIL apoptosis signal is mainly transmitted via the other TRAIL-R as has been reported for the ovarian cancer cell line A2780 (Saulle et al., 2007). In addition to the regulation of the two death-inducing TRAIL-Rs, TRAIL-R3 and TRAIL-R4 can also be regulated. As mentioned in section 1.3.1 their decoy function is still a matter of debate. TRAIL-R3 has be shown to be over-expressed in many TRAIL-resistant primary tumours from the gastrointestinal tract (Sheikh et al., 1999), however, others studies report the opposite, a down-regulation of TRAIL-R3 in aggressive prostate cancer (Hornstein et al., 2008). Furthermore, recently several studies have shown that elevated expression of OPG, the soluble TRAIL-receptor, can account for TRAIL-resistance by interacting with TRAIL and preventing it to bind to TRAIL-R1 and R2 (De Toni et al., 2008; Patino-Garcia et al., 2009; Rachner et al., 2009).

One step further down in the TRAIL-signalling cascade, resistance conferred at the DISC levels seems to be regulated by cFLIP and caspase-8 levels. For instance in highly malignant human neuroblastoma or neural stem and progenitor cells, resistance to TRAIL was reported to correlate with silenced caspase-8 expression (Hopkins-Donaldson et al., 2000; Ricci-Vitiani et al., 2004). Elevated cFLIP could be observed in 40% of human ovarian carcinoma samples. In many cases a knockdown of cFLIP is sufficient to restore TRAIL sensitivity.

Furthermore, high levels of PED/PEA-15, an anti-apoptotic factor recruited to the DISC which is predominantly expressed in the central nervous system, particularly in astrocytes, were shown to confer TRAIL-resistance in neural stem and progenitor cells (Ricci-Vitiani et al., 2004).

On the mitochondrial level over-expression of anti-apoptotic Bcl-2 family members like Bcl-2 and Bcl-XL and Mcl-1 blocks the disruption of the mitochondria and has often been shown to confer TRAIL-resistance (Barnhart et al., 2003; Fulda et al., 2002; Taniai et al., 2004).

As already mentioned in section 1.3.2, IAPs inhibit the activation of caspases. Consequently, over-expression of XIAP induces TRAIL-resistance (Makhov et al., 2008) . In this scenario, SMAC/DIABLO released from the mitochondria is not able to antagonize XIAP and facilitate activation of caspases (Micheau and Merino, 2004).

Taken together, resistance to TRAIL can occur at every step of TRAIL apoptosis pathway and some tumour cells use a combination of different resistance mechanisms to evade TRAIL-induced apoptosis (Vogler et al., 2008). To devise treatments that overcome multiple mechanisms of resistance will be crucial for the success of TRAIL-based therapy in the future.

1.5.2. **Non-apoptotic signalling of TRAIL**

Intriguingly, TRAIL does not only induce apoptosis, but triggers proliferation, migration and invasion of tumour cells that are resistant to TRAIL-induced apoptosis. Already in the initial characterisation of TRAIL-R it was discovered that TRAIL can induce activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), a major pro-inflammatory transcription factor (Wajant, 2004). This activation of NF-κB was initially thought to only negatively regulate TRAIL-signalling. Later on it was shown that TRAIL-induced tumour cell migration and invasion of apoptosis resistant cholangiocarcinoma cells was dependent on the activation of NF-κB by TRAIL (Ishimura et al., 2006). Furthermore, TRAIL-induced survival and proliferation in TRAIL-resistant Jurkat cells was dependent on the presence of the receptor interacting protein-1 (RIP1) (Ehrhardt et al., 2003). RIP1 is well described in its role in TNF-signalling. There, it facilitates the activation of NF-κB by activating the inhibitor of κB kinase (IKK) complex. RIP1 has been reported to be recruited to the TRAIL DISC and might be the link between the TRAIL DISC and NF-κB activation. RIP1-deficient fibroblasts

show no IKK activation upon TRAIL stimulation (Lin et al., 2000). Non-apoptotic signalling of TRAIL has been proposed to be mediated by a secondary intracellular signalling complex, which consists of the DISC components FADD and caspase-8 but also RIP1, TNF-receptor associated factor-2 (TRAF2) and IKKγ. This complex can not only trigger activation of NF-κB but also c-Jun N-terminal kinase (JNK) and p38 (Varfolomeev et al., 2005).

But also other survival pathways seem to be triggered by TRAIL, namely the extracellular regulated kinase (ERK) pathway and the phosphoinositide 3-kinases (PI3K) pathway. TRAIL-resistant glioma cells proliferate after TRAIL treatment which correlates with increased ERK phosphorylation (Vilimanovich and Bumbasirevic, 2008). The authors of this study claimed that this ERK activation is dependent on cFLIP, as a knockdown of cFLIP reduced ERK phosphorylation. However it might also be possible that a knockdown of cFLIP tips the scale towards apoptosis induction and in turn there is less non-apoptotic signalling going on in the cell. Interestingly, only very low amounts of TRAIL (100 pg/ml) are sufficient to trigger TRAIL-induced ERK phosphorylation in human vascular smooth muscle cells which also induced migration and proliferation (Secchiero et al., 2004). This concentration is comparable to soluble TRAIL in the human plasma and was also sufficient to promote migration of human bone marrow multipotent stromal cells which could be blocked by pre-treatment with pharmacological inhibitors of the ERK1/2 pathway (Secchiero et al., 2008). In parallel to ERK activation TRAIL caused survival and proliferation of primary human vascular endothelial cells by activating AKT, also referred to as Protein kinase B (PKB). Conversely, treatment of TRAIL-resistant glioma cells with migration inhibitors not only stopped migration but also sensitised these to TRAIL-induced apoptosis, which correlated with a loss of phosphorylation of AKT (Joy et al., 2003). These finding suggest that a crosstalk between TRAIL-signalling and signalling usually responsible for survival and migration exists.

The significance of non-apoptotic signalling of TRAIL has also been confirmed *in vivo*. In model of liver metastasis using orthotopically transplanted human pancreatic ductal adenocarcinoma cells Trauzold *et al.* (Trauzold et al., 2006) observed a dramatic increase in metastatic spread following TRAIL treatment. The fact that the TRAIL signal can be rerouted from apoptosis into pro-survival or migration signalling might also explain why tumours do not lose expression of TRAIL-Rs to evade apoptosis.

Taken together, these findings suggest that TRAIL can induce a diverse range of effects besides inducing apoptosis. One has to bear in mind that these functions of TRAIL under certain conditions might alter the outcome of TRAIL-based anti-cancer therapies.

1.5.3. **Sensitisation to TRAIL-induced apoptosis**

As already mentioned, TRAIL as a single agent is not able to induce apoptosis in most primary tumour cells. Fortunately, encouraging results have been obtained showing that the additional use of other anti-cancer drugs sensitises tumour cells to the effects of TRAIL. The synergistic effect of cytotoxic agents and TRAIL is believed to be mainly due to changes on the transcriptional levels of proteins important for the TRAIL pathway (Cretney et al., 2006; Wajant et al., 2002). Many studies suggest that changes on the receptor level, e.g. up-regulation of TRAIL-R1 and TRAIL-R2, are already sufficient for the observed sensitising effect. Though up-regulation may correlate with the sensitising effect observed, it is not necessarily the cause, e.g. combinatorial treatment with 5-FU or bortezomib leads to an up-regulation of TRAIL-R1 and -R2 (Koschny et al., 2007a). However, this change on the receptor level was not the only factor contributing to the sensitisation. Sensitising agents rather generally shift the threshold of tumour cells for apoptosis. They do so by down-regulating anti-apoptotic molecules like IAPs, cFLIP, Bcl-2, Bcl-XL and Mcl-1 and by up-regulating pro-apoptotic molecules including death receptors, caspase-8, FADD, Bak or Bax (Held and Schulze-Osthoff, 2001; Kelley and Ashkenazi, 2004; Mitsiades et al., 2002).

For a safe use of a combinatorial therapy, it is important that preferentially tumour cells become sensitised to TRAIL-induced apoptosis while normal cells remain resistant. So far, only very high doses of the frequently applied chemotherapeutic cisplatin or the proteasome inhibitor bortezomib were shown to induce toxicity in primary human hepatocytes at day 4 of *in vitro* culture (Ganten et al., 2005). However, the concentration of bortezomib was about 40 times higher than actually needed for TRAIL-sensitisation of tumour cells. Thus, combinational treatment of TRAIL and in combination with another drug might open up a therapeutic window for treatment of tumour patients without severe toxicity. Noteworthy, data obtained with different proteasome inhibitor show that each combination has to be assessed carefully, even though the sensitisers belong to the same class of cytotoxic agents. In this respect, normal primary human keratinocytes were sensitised to TRAIL even with low concentrations of the proteasome inhibitor MG-115 (Leverkus et al., 2003).

Apart from classical chemotherapeutic drugs like actinomycin D (Zisman et al., 2001), cisplatin and carboplatin (Mizutani et al., 2001) and irradiation (Maduro et al., 2008) new classes of anti-cancer drugs have been successfully applied as a means to sensitise to TRAIL. Among them are: proteasome inhibitors, Histone deacetylase inhibitors (HDACi), SMAC mimetics, BH3 mimetics and kinase inhibitors.

Proteasome inhibitor like bortezomib or MG-115 already mentioned above have a direct anti-cancer effect and have been found to sensitise a wide range of tumour cells to TRAIL (reviewed in Sayers and Murphy, 2006). Treatment of tumour cells with bortezomib results in multiple biological effects including inhibition of the cell cycle, inhibition of NF-κB activation, changes in cell adherence and increased apoptosis. They have also been shown to sensitise cells to TRAIL-induced apoptosis by shifting the ratio of cFLIP, caspase-8 and FADD at the TRAIL-DISC leading to an increased DISC formation and apoptotic signal transduction independently of NF-κB (Ganten et al., 2005). Furthermore, proteasome inhibition has also been shown to reduce XIAP levels in keratinocytes (Leverkus et al., 2003).

Another class of sensitising agents are histone deacetylase inhibitors. They have been reported to lead to enhanced FADD recruitment to the DISC (Inoue et al., 2009) and to increase expression of TRAIL-Rs and other pro-apoptotic molecules (Caspase-8, Bax, Bak) whilst down-regulating anti-apoptotic factor (cFLIP, XIAP, Survivin) (Guo et al., 2004). However, the mechanism behind HDACi-dependent sensitisation to TRAIL-induced apoptosis is still unclear. The combination of TRAIL and HDACi efficiently induces apoptosis in hepatoma cell lines (Schuchmann et al., 2006), primary AML and CCL cells (Inoue et al., 2004; MacFarlane et al., 2005; Nebbioso et al., 2005), while primary human hepatocytes, normal peripheral mononuclear blood cells and myeloid progenitors remain unharmed.

SMAC mimetics and BH3 mimetics are the results of rational drug design. As the name implies SMAC mimetics have been designed to mimic the structure of SMAC and inhibit the action of IAPs. SMAC mimetics have *in vitro* and *in vivo* anti-tumour activity whilst remaining non-toxic for untransformed cells (Wu et al., 2007). Smac mimetics potently synergise with TRAIL to kill tumour cell lines (Dai et al., 2009; Li et al., 2004) and have already been successfully applied to sensitise primary ovarian carcinoma cells to TRAIL (Petrucci et al., 2007). BH3 mimetics are small molecules that mimic BH3-only proteins by binding to and inhibiting pro-survival members of the Bcl-2 family. The best charactersed

BH3 mimetic is ABT-737, generated by Abbott Laboratories through a combination of NMR-based screening, parallel synthesis and structure-based design (Oltersdorf et al., 2005). ABT-737 synergised with TRAIL in several cancer types, including those expressing high levels of Mcl-1 i*n vitro* and *in vivo* (Mason et al., 2008; Song et al., 2008b; Tagscherer et al., 2008).

Taken together, these pre-clinical data point towards a great potential of combinational treatment in cancer therapy. However, there is still a need for systematic research to understand the principles under which conditions transformed cells but not normal cells become sensitised to TRAIL to further refine TRAIL-based cancer therapeutic approaches.

Sensitisation by kinase inhibition

Kinases are the main mediators of survival signalling and transmit growth and survival signals into the cells. Deregulated survival signalling, e.g. through activating mutations of kinases has been shown to drive tumourigenesis. Over the last decade, kinase inhibitors have emerged as novel class of targeted cancer therapeutics with more than 10,000 patent applications in the US alone since 2001 (Akritopoulou-Zanze and Hajduk, 2009). They have revolutionised the treatment of a particular group of diseases, e.g. chronic myeloid leukaemia or gastrointestinal stromal tumours where kinase inhibitors have achieved multi-year increases in survival (Druker et al., 2001; Heinrich et al., 2003). These diseases are driven by a singly oncogenic kinase. In contrast to this kinase inhibitors have been least effective in cancers with high mortality rates, such as prostate cancer, lung cancer, colorectal cancer and pancreatic cancer. Identifications of markers for patients that are likely to respond to the given kinase inhibitor will be crucial to improve the results of kinase inhibitor based therapy, as has already been shown for KRAS mutations in advanced colorectal cancer (Karapetis et al., 2008).

To date, three different strategies exist to design kinase inhibitors which are selective for a certain kinase (reviewed in Fedorov et al., 2010). The most prevalently used approach for the development of selective inhibitors is by targeting the ATP binding site of the kinase in question. The ATP binding site is situated in the deep cleft between the two catalytic domains and can be targeted by low molecular weight inhibitors. The ATP-bindings site is rigid and also well conserved within the kinase family adding to the difficulty to design selective ATP-competitive inhibitors. However, using the ever growing toolkit of chemical design strategies, very potent and selective ATP-competitive kinase inhibitors have been generated (Zhang et

al., 2009). In contrast to ATP-competitive inhibitors which target the active state of kinases, a second approach targets kinases in their inactive state. This strategy profits from a larger diversity of conformations and therefore greater possibilities to design selective inhibitors. For example, the Bcr-Abl inhibitor Imatinib targets an additional large cavity adjacent to the ATP binding site which is only accessible in the inactive state (Liu and Gray, 2006). The third type of inhibitors inhibits by allosteric mechanisms or by competition with regulatory elements. So far only a few examples have been reported, among them the compound GNF-2 which inhibits Bcr-Abl via an allosteric non-ATP competitive mechanism (Adrian et al., 2006).

Many kinase inhibitors have been successfully applied as sensitisers to TRAIL-induced apoptosis. In general, kinases whose inhibition sensitises to TRAIL-induced apoptosis can be clustered into different groups: kinases involved in the regulation of cell cycle, kinases that are involved in JAK/STAT signalling, and kinases that have been implicated in TRAIL-non-apoptotic signalling pathways, namely ERK, PI3/AKT.

CKII has been implicated in cell cycle control and already been mentioned in section 1.2.2, as phosphorylation of Bid by CKII inhibits cleavage by caspase-8 (Desagher et al., 2001). Therefore, it is not surprising, that CKII inhibition has been reported to sensitise to TRAIL-induced apoptosis (Kim et al., 2008). However, not only phosphorylation of Bid seems to be affected but also enhanced DISC activity (Izeradjene et al., 2005) and down-regulation of cFLIP have been reported upon CKII inhibition with the inhibitor DRB (Llobet et al., 2008). Other than CKII, inhibition of Aurora kinase B and Cyclin-Dependent Kinase 4, which are also involved in cell cycle regulation, has been reported to sensitise to TRAIL via up-regulation of TRAIL-R2 (Li et al., 2009) and down-regulation of survivin, respectively (Retzer-Lidl et al., 2007). However, the exact link, how inhibition of cell cycle regulation induces these changes has not yet been established.

Another signalling pathway that seems to confer TRAIL resistance is the JAK/STAT signalling pathway. It takes part in the regulation of cellular responses to cytokines and growth factors. An overview of JAK/STAT signalling is depicted in figure 7.

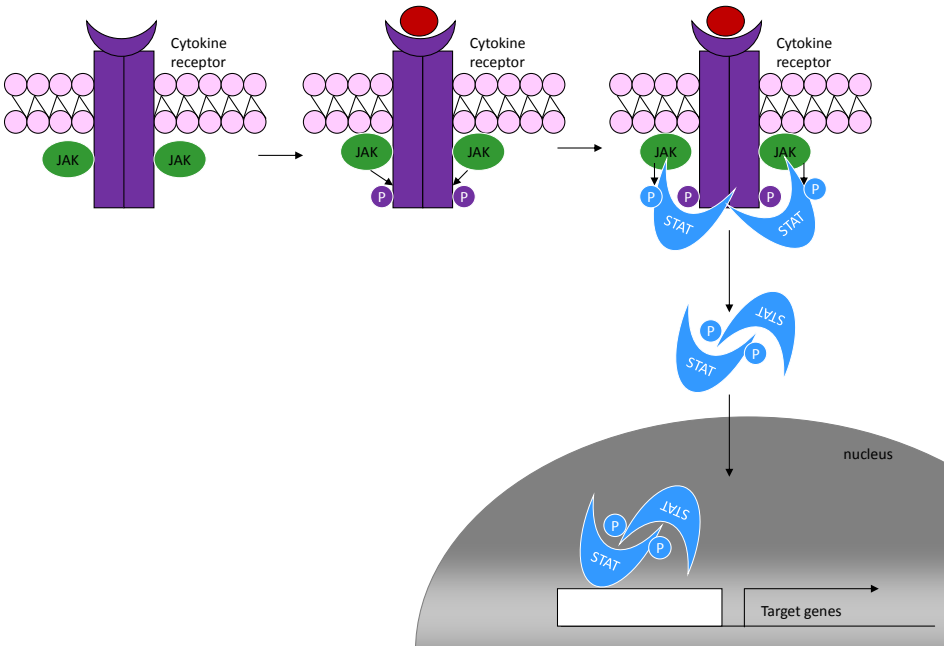


figure 7. Overview of the JAK-STAT signalling pathway.

JAKs have tyrosine kinase activity and bind to the intracellular part of cytokine receptors. The binding of the ligand to the receptor triggers activation of JAKs. They phosphorylate tyrosine residues on the receptor and create sites for interaction with proteins that contain phosphotyrosine-binding SH2 domain. STATs possessing SH2 domains are capable of binding these phosphotyrosine residues and are recruited to the receptors where they are phosphorylated by JAKs. These phosphotyrosines then act as docking sites for SH2 domains of other STATs, leading to their dimerisation. Activated STAT dimers translocate to the nucleus and activate transcription of their target genes.

Employing Janus kinases (JAKs) or and Signal Transducers and Activators of Transcription (STATs), the pathway transduces the signal carried by these extracellular polypeptides to the cell nucleus, where activated STAT proteins modify gene expression. Inhibition of JAK2 with the inhibitor AG490 augmented TRAIL-induced apoptosis and led to a down-regulation of XIAP and survivin in hepatoma cells (Fuke et al., 2007). JAK2 is responsible for activation of STAT3. Consequently, inhibition of STAT3 with a STAT3 inhibitor peptide also led to sensitisation (Kusaba et al., 2007). STATs were originally discovered as targets of JAK, but it has now become apparent that certain stimuli can also activate them independently of JAKs. Accordingly, dephosphorylation of STAT3 by inhibition of ATM using the inhibitor KU-55933 has been shown to sensitise to TRAIL in melanoma cells (Ivanov et al., 2009). Sensitisation was further increased by radiation and correlated with up-regulation of TRAIL-R2 and down-regulation of cFLIP. Noteworthy, blockage of tumour-cell-derived Interleukin-4

(IL-4) has also been shown to sensitise to TRAIL in cancer cells from different tissue origins (Todaro et al., 2008). As IL-4 has been reported to stimulate JAK/STAT signalling (Rolling et al., 1996), inhibition of the JAK/STAT pathway might be the underlying mechanism for IL-4 mediated sensitisation to TRAIL.

The ERK pathway has been shown to be essential for TRAIL-non apoptotic signalling (section 1.5.2).

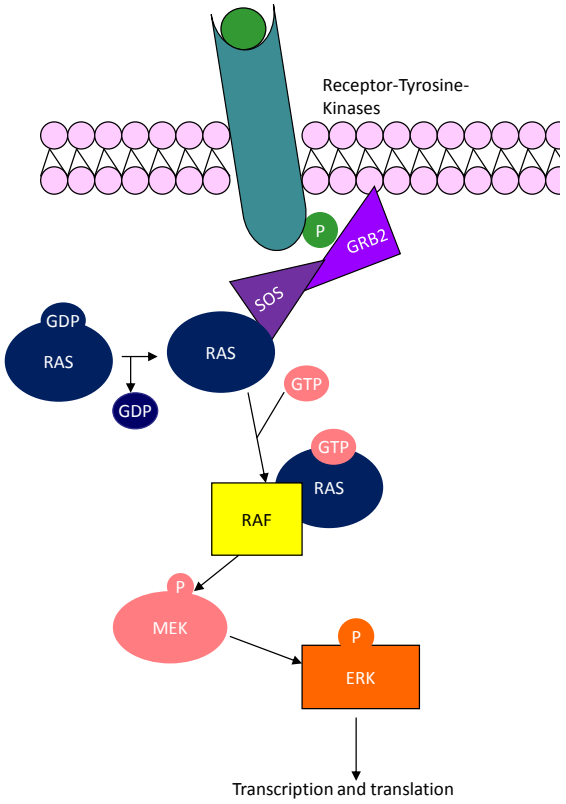


figure 8. Overview of the ERK pathway.

Receptor tyrosine kinases (RTKs) such as the epidermal- growth-factor-receptor (EGFR) are activated by extracellular ligands. Binding of the ligand activates the tyrosine kinase activity of the receptor and it becomes auto-phosphorylated on its tyrosine residues. Adaptor proteins such as GRB2 which contain SH2 domains bind to the phosphotyrosine residues of the activated receptor. GRB2 then binds to the guanine nucleotide exchange factor SOS. When the GRB2-SOS complex binds to phosphorylated RTKs, SOS is activated and promotes the removal of GDP from Ras. Ras can then bind GTP and becomes active. Activated Ras activates the protein kinase activity of RAF kinase. RAF kinase phosphorylates and activates MEK. MEK in turn phosphorylates and activates ERK. ERK then regulates the activity of several transcription factors and affects translation.

It is a complex signal transduction pathway that couples intracellular responses to the binding of growth factors to cell surface receptors, it controls transcription and regulates the cell cycle. Activation of MAPK/ERK promotes cell division in many cell types. An overview of the basic pathway is depicted in the figure 8. Several studies have observed a sensitisation of cells to TRAIL upon inhibition of the MAPK/ERK pathway mostly using the MEK inhibitors U0126 (Grosse-Wilde et al., 2008) or PD98059 (Lee et al., 2005; Lee et al., 2006). Inhibition of the MAPK/ERK pathway affected the expression of cFLIP, XIAP and Bcl-2.

Arguably, the most important pathway when it comes to TRAIL-sensitisation is the PI3K/AKT pathway (figure 9). Three major classes of PI3Ks exist in humans but only the class IA subgroup has been linked to cancer so far. The class IA PI3K are heterodimers and consist of a regulatory subunit (p85 family) and p110 subunit. There are 4 different isoforms of the p110 subunit, namely: α, β, γ and δ. In normal tissues p110α and p110β are ubiquitously expressed, whereas expression of p110γ and p110δ is mostly restricted to leukocytes. Cancer-specific mutations of the α-subunit occur in diverse tumours with frequencies up to 30 % (Samuels et al., 2004). In contrast to this no cancer-specific mutations have been identified in the other three isoforms. However, over-expression of the non-α isoforms induced oncogenic transformation *in vitro*. Furthermore, the β-isoform is expressed at high levels in colon and bladder carcinoma and the δ-isoform in glioblastoma and acute myeloid leukemia (reviewed in Vogt et al., 2007).

Active class IA PI3Ks are capable of phosphorylating phosphatidylinositol(4,5)-bisphosphate (PIP2) to generate phosphatidylinositol(3,4,5)-trisphosphate (PIP3) (reviewed in Shaw and Cantley, 2006). In addition to being activated by RTKs, p110 can also bind directly to Ras which also triggers PI3K activation. PIP3 recruits AKT to the plasma membrane. AKT is then activated by two phosphorylation events; phosphoinositide dependent Kinase (PDK) 1 is also recruited via PIP3 and phosphorylates AKT at residue T308. The identity of PDK2 who phosphorylates AKT at the second activating phosphorylation site S473 remains controversial, several kinases have been implicated in acting as PDK2s among them mTOR, DNA-dependent protein kinase **(**DNA-PK) and ATM (reviewed in Dong and Liu, 2005). Active AKT controls cell survival, cell cycle, cell growth and metabolism through phosphorylation of a plethora of substrates. It blocks apoptosis at different stages of the TRAIL-receptor pathway either directly by phosphorylation or indirectly by inducing gene transcription or translation via different downstream effectors.

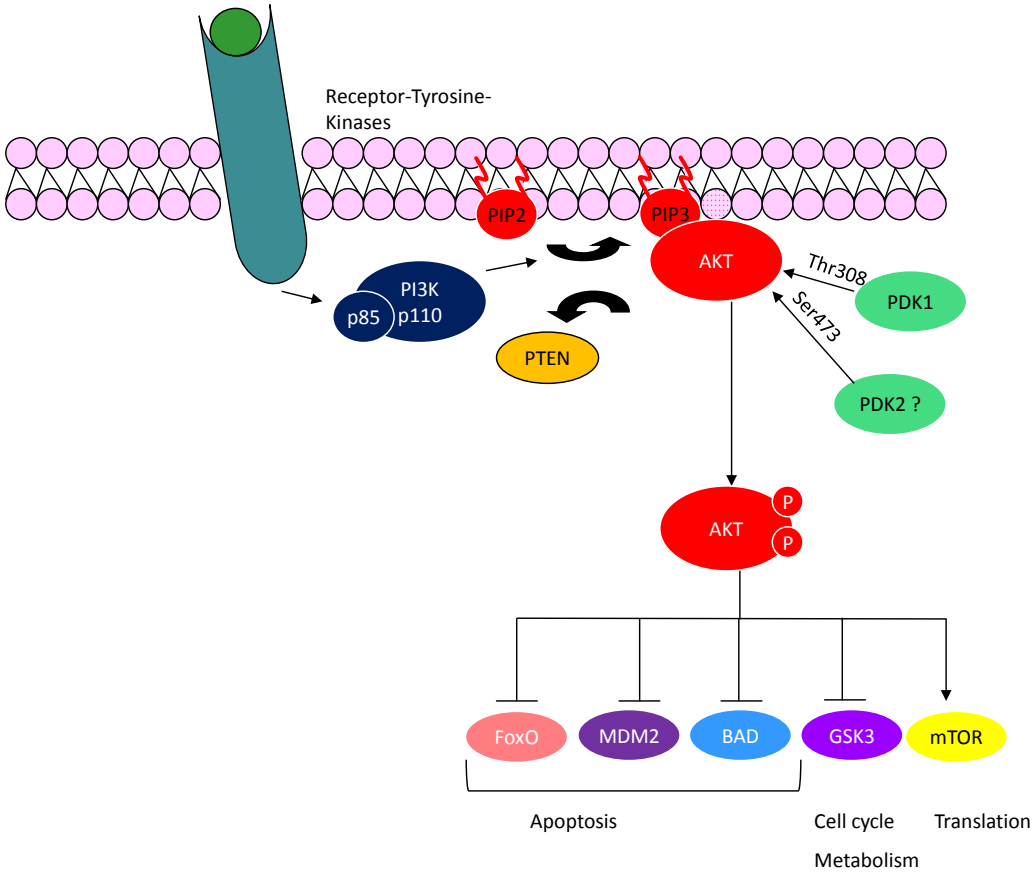


figure 9. Overview of the PI3K/AKT pathway.

The catalytic subunits of class I PI3K can be activated by upstream receptors, e.g. RTKs. PI3K catalysis the generation of PIP3 from PIP2. This can be reversed by the action of lipid phosphatase PTEN. PIP3 recruits AKT and PDK1. PDK1 activates AKT via phosphorylation. A second kinase referred to as PDK2 also phosphorylates AKT which is necessary for complete activation of AKT which controls cell survival, cell cycle, cell growth and metabolism through phosphorylation of a plethora of substrates (of which the most important ones in the context of this thesis are depicted in the figure). Adapted from Shaw et al. (Shaw and Cantley, 2006).

Directly regulated are for example: Caspase-9, which is inactivated by phosphorylation at residue S196 by AKT (Cardone et al., 1998), Bad, whose phosphorylation by AKT keeps it in the cytosol sequestered by 14-3-3 (Datta et al., 1997) and Ped/PEA-15, whose anti-apoptotic action is stabilised after phosphorylation by AKT (Trencia et al., 2003). Indirectly regulated are different members of the Bcl-2 family and IAPs. Considering this, it is not very surprising that inhibitors of AKT such as tribicine or perifosine sensitise to TRAIL-induced apoptosis (Shrader et al., 2007; Tazzari et al., 2008). In line with this inhibition of kinases upstream of AKT, such as PI3K by Wortmannin and LY294002 (Seol et al., 2005), and EGFR by gefinitib (Shrader et al., 2007) also induced TRAIL sensitisation. Depending on the cellular system, a

down-regulation of cFLIP, XIAP, cIAP1, cIAP2, surviving, Bcl-2, Mcl-1 has been reported to correlate with inhibition of the PI3K/AKT pathway (Alladina et al., 2005; Kim et al., 2004; Panka et al., 2001; Wang et al., 2008). Also inhibition of other kinases could ultimately be linked back to inhibition of the PI3K/AKT pathway. In this respect sensitisation of leukaemia cells to TRAIL by inhibition of DNA-PK by the inhibitor DMNB was shown to be mediated via AKT (Kim et al., 2009). The same applies for Protein Kinase C ε (PKCε) which has been reported in a number of studies to confer TRAIL-resistance (Felber et al., 2007; Shankar et al., 2008; Shinohara et al., 2001). The most recent study by Shankar et al. (2008) suggest that sensitisation to TRAIL by inhibition of PKCε is mediated downstream via an inhibition of AKT. Intriguingly, also the TRAIL-sensitising effect of the proteasome inhibitor bortezomib can also at least partly be attributed to inhibition of the PI3K/AKT pathway (Chen et al., 2008). Furthermore, physiological processes like heat shock or detachment have been shown to augment TRAIL-induced apoptosis dependent on the down-regulation of PI3K/AKT signalling (Lane et al., 2008; Pespeni et al., 2007). Also microRNAs which have been recently discovered as class of post-transcriptional genetic regulators, seem to influence TRAIL sensitivity via influencing the PI3K/AKT pathway. microRNA-221 & 222 regulate TRAIL resistance and enhance tumourigenicity through PTEN down-regulation (Garofalo et al., 2009).

Taking all this into consideration, inhibition of PI3K/AKT pathway is a promising approach for a successful application in combination to TRAIL. Several inhibitors of the PI3K/AKT pathway are in clinical trials at the moment an overview of which is given in table 1. So far, the efficacy and toxicity of TRAIL in combination with PI3K/AKT inhibitors has not been tested *in v*ivo, but considering the apparent synergy between inhibition of this pathway and stimulators of the TRAIL pathway, it seems to be only a matter of time for this combination to be studied in further preclinical and clinical investigations.

Sorafenib is a positive example of a kinase inhibitor that has already made it into the clinic as a novel therapeutic for the treatment of advanced renal cell carcinoma and advanced primary liver cancer. Sorafenib is a multikinase inhibitor. It has been designed to inhibit the ERK pathway but later studies showed that it also targets vascular endothelial growth factor receptors (VEGFR)-2 and -3 which are upstream of PI3K/AKT. Therefore Sorafenib inhibits both of the important survival pathways (Wilhelm et al., 2004). A number of studies show the

synergism between TRAIL and Sorafenib (Ricci et al., 2007) and this combination is already in clinical trials.

Table 1: PI3K/AKT pathway inhibitors in clinical development for cancer treatment (Engelman, 2009).

Inhibitor	Company	Phase of clinical trial
PI3K inhibitors		
XL147	Exelixis	Phase I
PX866	Oncothyreon	Phase I
GCD0941	Genentech/Piramed/Roche	Phase I
BKM120	Novartis	Phase I
CAL101	Calistoga Pharmaceuticals	Phase I
AKT inhibitors		
Perifosine	Keryx	Phase I/II
GSK690693	GSK	Phase I
VQD002	Vioquest	Phase I
MKK2206	Merck	Phase I

1.6. TRAIL as a therapeutic agent

Currently, several companies pursue TRAIL-R-targeted therapies in clinical trials using TRAIL-R agonists alone or in combination with other anti-cancer therapeutics. This chapter will introduce a variety of TRAIL-R agonists developed so far, discuss new approaches invented to improve the targeting of TRAIL-R agonists to the tumour site and will summarise the available data about their effects on primary tumours *in vitro* and in clinical trials.

1.6.1. TRAIL-Receptor agonists and their toxicities

In order to trigger the TRAIL-mediated apoptotic pathway soluble recombinant versions of TRAIL as well as agonistic antibodies targeting TRAIL-R1 and TRAIL-R2, respectively can be applied. Ideally, these agonists should on the one hand have high anti-tumour activity, but at the same time low toxicity for normal cells to ensure a safe and efficient application as anti-cancer drug in the clinics.

Agonistic TRAIL-R specific monoclonal antibodies

It is still a matter of debate whether TRAIL-R3 and TRAIL-R4 truly act as decoy receptors and whether their overexpression protects cancer cells from TRAIL-induced apoptosis (Buchsbaum et al., 2006). However, to overcome a potential safeguarding effect of TRAIL-R3 and -R4, agonistic monoclonal antibodies specifically targeting TRAIL-R1 or -R2 have been developed in the hopes of gaining a more effective anti-tumour effect. Additionally, these monoclonal antibodies have an increased half-life (14-21 days) when compared to recombinant forms of TRAIL (about 30 min in non-human primates). However, one has to bear in mind that these benefits might potentially come along with a higher toxicity for normal cells.

The TRAIL-R2-specific antibody TRA-8 for instance has been reported to kill leukaemia cells, astrocytoma and engrafted breast cells while sparing normal human astrocytes, B and T cells as wells as primary human hepatocytes (Buchsbaum et al., 2003; Ichikawa et al., 2001).

Due to the formation of higher order complexes and the recruitment and activation of innate immune cells, additional cross-linking of TRAIL-R-specific antibodies by Fc-receptor-expressing immune cells can lead to a higher efficiency in the anti-tumour response (Takeda et al., 2004). A combination of TRAIL-R-specific antibodies with CD40- and 4-1BB-specific antibodies was able to completely eradicate syngenic tumours without any observed toxicity in mice (Uno et al., 2006). In this model, anti-TRAIL-R antibodies on the one hand kill TRAIL-sensitive tumour cells and on the other hand recruit Fc-receptor expressing cells such as DCs and macrophages via the constant region of the antibody. These antigen-presenting cells (APCs) subsequently engulf the apoptotic tumour cells, process tumour antigens and present them to surrounding T cells. Concomitant stimulation with anti-CD40 and anti-4-1BB antibodies induces further APC activation in order to efficiently stimulate surrounding cytotoxic T cells. Being properly activated, CTLs are then able to kill the TRAIL-resistant tumour burden expressing tumour-associated antigens.

Yet again, besides leading to increased anti-tumour responses, cross-linking of TRAIL-R specific antibodies may also result in higher toxicity for normal cells, including primary human hepatocytes (Mori et al., 2004). Furthermore, it has to be considered that TRAIL-receptor targeting therapies employing TRAIL-R specific antibodies carry the risk of

developing uncontrolled autoimmune responses. The Fc-Part of the antibody may bind to appropriate Fc-receptors of APCs thereby leading to their activation.

Recombinant TRAIL

In contrast to TRAIL-R specific antibodies, recombinant forms of TRAIL allow for the activation of TRAIL-R1 and TRAIL-R2 at the same time. This might be a promising strategy as the expression profile of TRAIL-receptors on tumours is mostly unknown. So far, a variety of soluble TRAIL versions has been generated, each encoding the extracellular domain of human TRAIL that is amino-terminally fused to an oligomerisation motif, e.g. a poly-histidine tag (His-TRAIL) (Pitti et al., 1996), a FLAG-epitope (Schneider, 2000), a leucine zipper (Walczak et al., 1999) or an isoleucine zipper motif (Ganten et al., 2006). These additional tags improve receptor oligomerisation which is necessary to successfully transmit the death signal. Yet again, as has been discussed for TRAIL-R specific antibodies, the ability of recombinant TRAIL to form higher-order complexes might coincide with increased toxicity for normal cells (Koschny et al., 2007b; Lawrence et al., 2001).

It seems that two main factors determine TRAIL sensitivity of normal human cells, i.e. the form of the recombinant TRAIL used and the model system chosen. Highly oligomerised forms of TRAIL, e.g. cross-linked FLAG-TRAIL were reported to induce killing of primary human hepatocytes, keratinocytes and astrocytes in some model systems (reviewed in Koschny et al., 2007b). However, it is still a matter of debate which of the model systems most reliably resembles physiological conditions. The studies by Ganten *et al.* in primary human hepatocytes shed new light on this matter (Ganten et al., 2005). Here, freshly isolated primary human hepatocytes at day one of *in vitro* culture were efficiently killed by highly aggregated forms of TRAIL. However, on day four of *in vitro* culture, on which the phenotype of primary human hepatocytes resembles normal liver tissue, the primary human hepatocytes turned out to be TRAIL resistant. These results correspond to the ones obtained in an elegant *in vivo* study by Hao *et al.* in which orthotopically xenotransplanted human liver cells in mice did not show toxicity upon treatment with non-tagged TRAIL (Hao et al., 2004). Furthermore, application of TRAIL alone or in combination with chemotherapeutics *in vivo*, as has been shown in mice, cynomologues monkeys and chimpanzees did not lead to any signs of toxicity (Ashkenazi et al., 1999). However, one has to bear in mind that toxicity could potentially occur under certain sensitising conditions like viral hepatitis or in a pro-inflammatory milieu (Liang et al., 2007; Mundt et al., 2005). A recent study indeed reported

toxicity of DATR , a recombinant soluble human TRAIL mutant (DATR) which was explored by Chengdu Diao Pharmaceutical Group (Zou et al., ,2010). Rodents and crab-eating macaques were used to estimate potential adverse effects of DATR following a single dose administration. The median lethal dose (LD50) of intravenous injection to rats and mice was determined as 262.0 and 1018.0 mg/kg, respectively. Data suggested that liver, renal and haematological systems might be the target effectors of toxic effect induced by DATR. However, the dosage is excessively high and does not reflect the concentration which has been used in animal models when anti-tumour activity of TRAIL was observed.

As non-tagged TRAIL shows the lowest toxicity for normal cells *in vitro* when compared to highly oligomerised forms of TRAIL, e.g. His- or FLAG- TRAIL, and nevertheless shows considerable killing activity, this form of human soluble TRAIL was chosen for clinical development (see below). Studies comparing recombinant version of TRAIL to TRAIL-R specific antibodies are still missing today. However, for the CD95 system and TNF-R system it is known that the killing potential of the recombinant cognate ligand is superior to the respective antibody (Schlosser et al., 2000). Despite having a much lower half-life than TRAIL-R-specific antibodies, the same might also apply for recombinant TRAIL. Accordingly, Apo2L/TRAIL, which is already in phase II clinical trials has a high anti-tumour activity *in vivo* due to significant tumour penetration (Kelley and Ashkenazi, 2004; Koschny et al., 2007a).

Taking all this into consideration, the data obtained so far suggest TRAIL-R agonists, including TRAIL-R specific antibodies and soluble recombinant TRAIL, as promising novel biotherapeutic drug for the treatment of cancer.

1.6.2. **Potency of TRAIL in primary tumours**

A variety of studies which investigated the effect of TRAIL on tumour cell lines so far yielded very promising results. In contrast to this, the effect in primary tumour cells seems to be more diverse. Pre-clinical studies applying TRAIL to freshly isolated human myeloma cells show that TRAIL can efficiently induce apoptosis in these otherwise chemotherapy resistant cells (Gazitt, 1999; Mitsiades et al., 2001). However, TRAIL could not do so in acute lymphoblastic leukaemia, acute myelogenous leukaemia, acute promyelocytic leukaemia and in primary B cell acute or chronic lymphocytic leukaemia (Clodi et al., 2000; MacFarlane et al., 2002). The factors that determine TRAIL resistance of primary tumour cells could only be

revealed for a few cancer types. For example, Riccioni *et al.* (Riccioni et al., 2005) reported a correlation between TRAIL-resistance and the expression of decoy receptors in myeloid leukaemia. Furthermore, it could be shown that TRAIL resistance in primary glioblastoma is dependent on the expression of the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome TEN) and cFLIP (Panner et al., 2005). Expression of wild type PTEN and low levels of cFLIP rendered the cells TRAIL-sensitive, whereas the expression of mutated PTEN together with high levels cFLIP confers TRAIL resistance. However, the expression levels of cFLIP seem to be irrelevant for (oligo) astrocytoma specimen (Koschny et al., 2007a) as well as in isolated tumour cells form medullablastoma, meningeoma, esthesioneuroblastoma and soft tissue sarcoma, all of which are TRAIL resistant (Clayer et al., 2001). Intriguingly, for pancreatic cancer and cholangiocarcinoma cells TRAIL treatment has been observed to enhance migration and metastatic spread *in vitro* and *in vivo* (Ishimura et al., 2006; Trauzold et al., 2006).

Taken together, as most primary tumour cells – unlike cancer cell lines- are TRAIL-resistant and TRAIL treatment was even counterproductive in some cases, the application of TRAIL as a single agent needs to be questioned. It is of major importance to carefully characterise the tumour specimen with regard to its TRAIL sensitivity prior to treatment in order to be able to administer a tailored therapy specific to the patient's sensitivity profile. For this purpose, it is necessary to develop biomarkers and appropriate sensitivity tests (McCarthy et al., 2005). As the expression of O-glycosylating enzymes seems to correlate with TRAIL sensitivity, these enzymes might be valuable markers to predict the prospect of success of a TRAIL-based therapy (Wagner et al., 2007). The expression of the O-glycosylating enzyme GALNT3 for instance correlates with TRAIL sensitivity in colorectal cancer (CRC) and the expression of GALNT14 with TRAIL sensitivity in non small cell lung cancer (NSCLC) , pancreatic cancer and melanoma cell lines. Thus, specific O-glycosylating enzymes could potentially be used as predictive biomarkers for responsiveness to TRAIL-based cancer therapy.

1.6.3. **Clinical development of TRAIL-R agonists (TRAs)**

On the basis of the promising pre-clinical findings concerning TRAIL-R targeting approaches, TRAIL receptor agonists (TRAs) are being developed by several companies. The progress of one recombinant ligand, one anti-TRAIL-R1, five anti-TRAIL-R2 antibodies and a Ad5-TRAIL gene therapy in clinical trials will be summarised.

Table 2: summarises the clinical development of TRAs.

Name	Description	Combination	Phase
rhApo2L/TRAIL (PRO1762, AMG-951)	Recombinant TRAIL binds to TRAIL-R1 and TRAIL-R2	-	Phase II (NHL, NSCLC)
		Irinotecan and Cetuximab	Phase I (CRC)
		Rituximab	Phase I/II (NHL)
		Bevacizumab	Phase II (NSCLC)
		Paclitaxel, Carboplatin and Bevacizumab (PCB)	PhaseI/II (NSCLC)
Mapatumumab (HGS-ETR1)	Human monoclonal antibody targeting TRAIL-R1	-	Phase II (NHL, CRC, NSCLC, MM)
		Bortezomib	Phase II (MM)
		Paclitaxel and Cisplatin	Phase I/II (advanced solid tumours)
		Gemcitabin and Cisplatin	Phase I/II (advanced solid tumours)
		Paclitaxel and Carboplatin	Phase I/II (advanced solid tumours)
Lexatumumab (HGS-ETR2)	Human monoclonal antibody targeting TRAIL-R2	-	Phase I (advanced solid tumours)
		FOLFIRI	Phase Ib (advanced solid tumours)
		Gemcitabin, Pemetrexed und Doxorubicin	Phase Ib (advanced solid tumours)
CS-1008	Monoclonal antibody, humanised form of the murine TRAIL-R antibody TRA-8	-	Phase I (advanced solid tumors and lymphomas)
		Gemcitabin	Phase Ib (pancreatic cancer)
LBY135	Chimeric monoclonal antibody targeting TRAIL-R2	-	Phase I/II
		Capecitabin	Phase I (advanced solid tumours)

Apomab	Human monoclonal antibody targeting TRAIL-R2	-	Phase II (NHL, NSCLC)
		Rituximab	PhaseI/II (NHL)
		Bevacizumab	PhaseI/II(NSCLC)
AMG-655	Human monoclonal antibody targeting TRAIL-R2	-	Phase II (pancreatic cancer, NSCLC,CRC, Soft tissue sarcoma)
		mFOLFOX6 and Bevacizumab	Phase I/II (CRC)
		Doxorubicin	Phase I/II (soft tissue sarcoma)
		Gemcitabin	Phase I/II (pancreatic cancer)
		Paclitaxel and Carboplatin	Phase I/II (NSCLC)
		Panitumumab	Phase I/II (CRC)
Ad5-TRAIL	Recombinant adenoviral TRAIL; binds to TRAIL-R1 und TRAIL-R2	-	Phase I

The first company to develop TRAs was Human Genome Science (HGS). They developed two fully humanised monoclonal antibodies activating TRAIL-R1 and -R2, respectively: Mapatumumab (HGS-ETR1) and Lexatumumab (HGS-ETR2), which are the most advanced TRAs in clinical trials. Both antibodies have been very successful in pre-clinical studies and induced apoptosis across a wide range of human tumour cell lines as well as in primary cells isolated from solid haematological malignancies. In all studies conducted so far, Mapatumumab was generally well tolerated, with the maximum tolerated dose yet to be reached. It has yielded stable disease as best clinical response in a phase Ia setting (Tolcher et al., 2007). In contrast, phase Ib studies in which Mapatumumab was tested in combination with either gemcitabine-cisplatin or paclitaxel-cisplatin have yielded partial responses (28% and 23%, respectively) (Chow et al., 2006; Hotte et al., 2005; Hotte et al., 2008). In this case a dose limiting toxicity could be observed for one patient. Another study tested Mapatumumab in combination with paclitaxel and carboplatin in solid tumours. Mapatumumab was well tolerated up to a dosage of 20 mg/kg. Five out of 27 patients showed a partial response and 12

patients yielded stable disease (Leong et al., 2009). Mapatumumab's activity could also be validated in three Phase II studies with patients suffering from Non-Hodgkin's lymphoma (NHL), CRC, and NSCLC. For NHL, Mapatumumab as a single agent has yielded 3 objective clinical responses in patients suffering from NHL (Younes et al., 2005). However, phase II studies in CRC (Trarbach et al., ,2010) and NSCLC (Greco et al., 2008) have produced stable disease as best response in 32% and 29% of the cases, respectively. The mono-therapy was well tolerated with only one drug-related serious adverse event recorded. Another phase II study is currently investigating the efficiency and safety of Mapatumumab in combination with bortezomib in patients suffering from advanced multiple myeloma (study number: HGS 1012-C1055).

The results for Lexatumumab resemble those obtained for Mapatumumab. In phase Ia clinical study several patients have reached stable disease with Lexatumumab as a monotherapeutic agent, but no response of the tumour has yet been recorded (Patnaik et al., 2006; Plummer et al., 2007; Wakelee et al., 2009). In contrast, combinations of Lexatumumab with FOLFIRI or doxorubicin were well tolerated and induced tumour shrinkage and partial response in wide range of cancer types (Sikic et al., 2007). Several grade 3 toxicities, among them elevated liver enzymes, were related to Lexatumumab treatment and maximum tolerated dose was set 20 mg/kg. Nevertheless, Lexatumumab could safely be administered, making further evaluations with regard to combinational therapy warranted. Noteworthy, a pre-clinical study showed a complete regression of various tumour cell line xenografts *in vivo* upon treatment with Lexatumumab and the Smac-mimetic SM-164 (Petrucci et al., 2007).

The humanised anti-TRAIL-R2 antibody CS-1008 (Tigatuzumab) was developed for treatment of solid tumours and lymphoma by Daiichi Sankyo. It exhibits high-anti-tumour activity against astrocytoma and leukaemia cells *in vitro* and against engrafted breast cancer cells *in vivo* (Yada et al., 2008). A phase I study of CS-1008, for advanced solid tumours or lymphomas showed that CS-1008 was well tolerated, and the maximum tolerated dose was not reached (Saleh et al., 2008). The high number of patients with stable disease in this phase I trial suggests anti-tumour activity

Novartis has produced the TRAIL-R2 specific antibody LBY135, which is able to induce apoptosis in 50% of a panel of 40 human colon cancer cell lines with an IC50 of < 10 nM. The anti-tumour activity of LBY135 could be proven in human CRC xenograft models in

mice (Buchsbaum et al., 2003; Ichikawa et al., 2001). In a phase I trial of LBY135, alone and in combination with capecitabine in advanced solid tumours, LBY135 is well tolerated and has shown signs of clinical activity (Sharma et al., 2008)

The fully humanised TRAIL-R2 targeting antibody Apomab was developed by Genentech. Today, it is in phase I and phase II clinical trials for solid tumours. Preliminary results of the phase Ia study revealed that Apomab was safe and well tolerated and yielded 52 % stable disease. Two dose limiting toxicities occurred comprising asymptomatic transaminitis and pulmonary embolism in one patient each (Camidge D., 2007). In 2007, a phase II study was initiated, evaluating Apomab as monotherapeutic agent for sarcoma and in combination with avastatin against NSCLC. More studies evaluating the effect of Apomab in combination with the CD20 targeting antibody rituximab or with bevacizumab as a first line treatment for NSCLC are planned.

Another fully humanised monoclonal antibody against TRAIL-R2 referred to as AMG 655 is developed by Amgen. In phase Ib clinical trials, it showed anti-tumour effects against CRC and NSCLC, in which it led to metabolic partial responses or partial responses, respectively. So far, neither dose limiting toxicities nor severe side effects were recorded when AMG 655 was applied at doses of 20 mg/kg every two weeks. However, 9 of 11 patients showed adverse effects including hypomagnesaemia, fever and fatigue (LoRusso et al., 2007). In a second study the safety and efficacy of AMG 655 plus modified FOLFOX6 and bevacizumab for the first-line treatment of patients with metastatic colorectal cancer was evaluated (Saltz et al., 2009). Out of 12 patients the best overall tumour responses were: 5 partial responses (2 unconfirmed, both underwent resection); 6 stable disease.

The only recombinant form of TRAIL so far tested in clinical trials is an untagged version of human TRAIL, referred to as rhAPO2L/TRAIL that is developed by Genentech in cooperation with Amgen. Pharmacokinetics and safety studies (phase Ib/II) were carried out in patients suffering from low-grade NHL. Preliminary results have proven Apo2L/TRAIL to be safe and active either alone or in combination with Rituximab. To date no dose limiting toxicities have been reported; of the five patients investigated, two showed complete response, one partial response and two stable disease. More NHL patients are being recruited for further dose optimisation (Herbst et al., 2006). Another Phase Ib study of rhApo2L/TRAIL plus irinotecan and cetuximab or FOLFIRI in metastatic CRC patients

indicated that rhApo2L/TRAIL can be safely combined with irinotecan-based regimens(Yee et al., 2009). A phase II study of rhApo2L/TRAIL with FOLFIRI should provide more information on safety, efficacy, and a potential diagnostic for rhApo2L/TRAIL.

Ad5-TRAIL is a recombinant form of TRAIL which is expressed adenovirally. Consequently, no recombinat protein is administered in Ad5-TRAIL-therapy but adenovirus which induces the expression of membrane-bound TRAIL in infected cells. Ad5-TRAIL is being evaluted in clinical trials Phase I for prostate cancer. So far it is well tolerated without any dose limiting toxicities or side effects (Griffith et al., 2007a).

Looking back on the pre-clinical and clinical data summarized in this chapter, targeting the TRAIL-receptors with the different TRAIL-R agonists developed represents a promising approach for anti-cancer therapy in the future. Currently, the use of TRAIL-R agonists is restricted to tumours which are TRAIL sensitive in the first place or tumours that can be sensitised by co-treatment with other anti-cancer drugs. Therefore, it is essential to improve the understanding of the mechanisms that confer TRAIL-resistance to the remaining tumour types to be able to overcome our current limitations in cancer treatment by rational drug identification and design.

2. Aims and Objectives

With its unique ability of killing tumour cells while sparing normal cells, TRAIL represents a promising tool for cancer-treatment. For a sensible application of TRAIL in combination with other drugs, it will be key to understand the biochemical mechanism responsible for resistance to TRAIL-induced cell death and for sensitisation by DNA-damaging drugs and other cancer therapeutics. The BH3-only protein Bid is a key player at the crossroad of life and death and it is phosphorylated in an ATM-dependent manner following DNA damage turning it into a pro-survival molecule (Kamer et al., 2005). Therefore, perturbations of Bid phosphorylation and /or ATM activity might play a role in TRAIL sensitivity. With Bid being a pivotal player in TRAIL-induced apoptosis this modification might be involved in TRAIL resistance and its breakage. While the phosphorylation status of Bid had no detectable impact on TRAIL-sensitivity in the model system used in this study, the interesting discovery was made that the ATM-inhibitor KU-55993 (Hickson et al., 2004) sensitises HeLa cells to TRAIL- induced apoptosis. Agents that sensitise to TRAIL induced apoptosis are very interesting in two aspects. First of all they might provide a new opportunity for combinational treatment with TRAIL. Second, analysing the mechanism of action might reveal new information on how TRAIL-resistance evolves. Therefore the aim of this thesis was to reveal the mechanism underlying KU-55933 mediated sensitisation to TRAIL-induced apoptosis.

3. Materials and Methods

3.1. Materials

3.1.1. Cell Lines

Name	Description	Medium	Source
A549	Lung cancer cell line	DMEM+ 10 % FCS	German Resource Centre for Biological Material (DSMZ), Bayreuth; Germany
DLD1	Colon carcinoma cell line	DMEM+ 10 % FCS	Kindly provided by O. Kranenburg, UMC Utrecht; Netherlands
DLD1p	Colon carcinoma cell line	RPMI+ 10 % FCS	Kindly provided by B. Burgering UMC Utrecht; Netherlands (Kops et al., 2002)
DL23	DLD1 cells stably transfected with 4-HT inducible active Foxo3a	RPMI+ 10 % FCS	Kindly provided by B. Burgering UMC Utrecht; Netherlands (Kops et al., 2002)
HCT116	Colon carcinoma cell line	DMEM+ 10 % FCS	Kindly provided by B.Vogelstein, Howard Hughes Medical Institute Baltimore; USA (Zhang et al., 2000)
HCT116 Bax -/-	HCT116 knockout for Bax	DMEM+ 10 % FCS	Kindly provided by B.Vogelstein, Howard Hughes Medical Institute Baltimore; USA (Zhang et al., 2000)
HeLa	Cervix carcinoma cell line	DMEM+ 10 % FCS	German Resource Centre for Biological Material (DSMZ), Bayreuth; Germany

MCF-7	Breast cancer cell line	DMEM+ 10 % FCS	German Resource Centre for Biological Material (DSMZ), Bayreuth; Germany
L6	Lymphoblastoid cell line isolated from AT patient	RPMI+ 10 % FCS	Kindly provided by Y. Shiloh, Tel Aviv University; Israel (Taylor et al., 2002)
XhoC3	Murine embryonal cell line	DMEM+ 10 % FCS+ Pyruvate-β-Mercapto-ethanol	Kindly provided by J. Brost, NKI Amsterdam; Netherlands (Kast et al., 1989)

3.1.2. **Media**

All media were purchased from Gibco/Invitrogen. DMEM (Dulbecco's Modified Eagle Medium) and RPMI (Roswell Park Memorial Institute) both contained the more stable Glutamax^{TM} as Glutamine source. All media were supplemented with 10% fetal calf serum (FCS) (Gibco/Invitrogen) before use. Cells were generally cultured in the absence of antibiotics.

For transfection experiments RPMI without FCS was used.

3.1.3. **Antibodies**

For Western blot analysis the following primary antibodies were used:

Antibody	**Isotype**	**Source**
AKT (pan) (C67E7)	rabbit	Cell Signaling
ATM	IgG1	Rockland
Bad	rabbit	Cell Signaling
Bak	IgG1	BD Pharmingen
Bax	Rat	BD Pharmingen
Bid	rabbit	BD Pharmingen

Caspase-3 (AF605)	goat	R & D systems
Caspase-8 (C15)	mIgG2b	Axxora
Caspase-9	mIgG1	MBL
cFLIP (NF-6)	mIgG1	Axxora
FADD	mIgG1	Transduction Laboratories
FLAG (M2)	mIgG1	Sigma
FoxO1 (C29H4)	rabbit	Cell Signaling
FoxO3a	rabbit	Cell Signaling
GSK3α	rabbit	Cell Signaling
Gsk3β (27C10)	rabbit	Cell Signaling
Mouse Bid	rabbit	Kindly provided by A. Gross
mTor	rabbit	Cell Signaling
P70S6	Rabbit	Cell Signaling
PARP	mIgG1	BD Pharmingen
Phopsho-Bad (S136)	rabbit	Cell Signaling
Phospho GSK3α/β (S21/9)	rabbit	Cell Signaling
Phospho-AKT (S473)	IgG2b	Cell Signaling
Phospho-ATM (S1981)	rabbit	Rockland
Phospho-Bid (S78)	rabbit	Kindly provided by A. Gross
Phospho-FoxO1(T24)/FoxO3a (T32)/FoxO4(T28) (4G6)	rabbit	Cell Signaling
Phospho-mTor (S2448)	rabbit	Cell Signaling
Phospho-P70S6	IgG2b	Cell Signaling
PI3 Kinase p110 γ	rabbit	Millipore
PI3 Kinase p110α (C73F8)	rabbit	Cell Signaling
PI3 Kinase p110β (C33D4)	rabbit	Cell Signaling
PI3 Kinase p110δ (AW103)	IgG1	Upstate
TRAIL-R1 (TR1-PSC-1139)	rabbit	Axxora
TRAIL-R2 (TR2-PSC-2019)	rabbit	Axxora
XIAP	rabbit	Axxora
β-actin	mIgG1	Sigma

Secondary horseradish peroxidase (HRP)–conjugated antibodies for Western Blot analysis were purchased from Southern Biotech and Santa Cruz Biotechnologies:

Antibody	Antigen	Serum	Company
anti-mIgG1-HRP	mIgG1	Goat	Southern Biotech
anti-mIgG2b-HRP	mIgG2b	Goat	Southern Biotech
anti-goat IgG-HRP	goat IgG	Rabbit	Santa Cruz Biotechnologies
anti-rabbit IgG-HRP	rabbit IgG	Goat	Southern Biotech

For flow cytometric analysis the following antibodies were used:

Antibody	**Antigen**	**Isotype**	**Company**
HS101	TRAIL-R1	mIgG1	Axxora
HS201	TRAIL-R2	mIgG1	Axxora
HS301	TRAIL-R3	mIgG1	Axxora
HS402	TRAIL-R4	mIgG1	Axxora

Biotinylated secondary goat Fab anti-mouse antibodies were purchased from Southern Biotechnology and streptavidin-phycoerythrin (Strep-PE) was obtained from BD Pharmingen.

3.1.4. **Recombinant proteins**

Protein	**Description**	**Source**
iz-TRAIL	Isoleucine zipper tagged human TRAIL	(Ganten et al., 2006)
Murine iz-TRAIL	Isoleucine zipper tagged murine TRAIL	(Ganten, Haas et al. 2004).
moTAP-TRAIL	The moTAP tag consists of a 3 x FLAG-tag, followed by a precision site and an AviTag.	Produced and kindly provided by S. Prieske

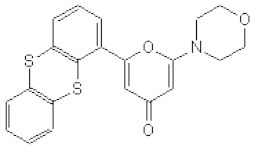
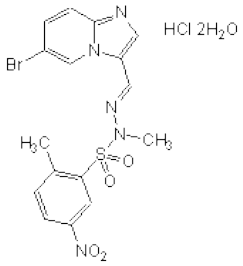
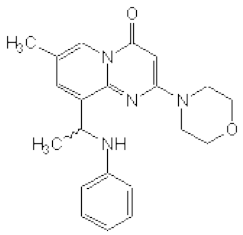
3.1.5. **Chemicals**

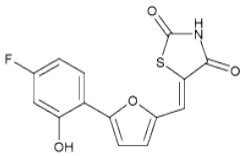
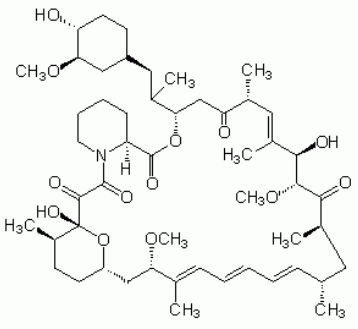
Chemical	**Manufacturer**
10 x Trypsin	Gibco/Invitrogen, Karlsruhe; Germany
Acetic acid (HOAc)	J. T. Baker Chemicals, U.K
Agarose	Sigma-Aldrich, Munich; Germany
Bacto-Trypton	AppliChem, Darmstadt; Germany
Bacto-Yeast	AppliChem, Darmstadt; Germany
Bicine	Gerbu, Gaiberg; Germany
Biotin	Pierce, Rockford; United States
Bis Tris	MB Biomedicals, Solon; United States
Bovine serum albumin (BSA)	Serva, Heidelberg; Germany
Calcium Chloride (CaCl2)	Sigma-Aldrich, Munich; Germany
Chloroform	Merck, Darmstadt; Germany
Chloroquine	Sigma-Aldrich, Munich; Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Munich; Germany
Disodium hydrogenphosphate	Merck, Darmstadt; Germany
Dharmafect	Dharmacon, Chicago,United States
DNA ladder: SmartLadder	Eurogentec, Southampton, UK
Ethanol absolute (EtOH)	Merck, Darmstadt; Germany
Ethylendiamintetraacetate (EDTA)	Roth, Karlsruhe; Germany
Formaldehyde	J.T. Baker, Deventer; Netherlands
Glycerin	Roth, Karlsruhe; Germany
Glycine	AppliChem, Darmstadt; Germany
HEPES	Gerbu, Gaiberg; Germany
Hydrochloric acid (HCl)	J. T. Baker Chemicals, U.K
Isopropyl alcohol	Merck, Darmstadt; Germany
LB Broth	MoBio Laboratories; United States
Lipofectamine	Invitrogen, Karlsruhe, Germany
Luria Broth (LB) Agar	MoBio Laboratories; United States
Magnesiumchloride ($MgCl_2$)	Merck, Darmstadt; Germany
MES	Roth, Karlsruhe; Germany
Methanol	Fluka, Seelze; Germany

Methanol (MeOH)	J. T. Baker Chemicals, U.K
Milk powder	Roth, Karlsruhe; Germany
MTT	Sigma-Aldrich, Munich; Germany
PEG (Polyethylenglycol 1500)	Roth, Karlsruhe; Germany
Pipes	Sigma-Aldrich, Munich; Germany
Ponceau S	AppliChem, Darmstadt; Germany
Potassium acetate (KOAc)	Merck, Darmstadt; Germany
Potassium chloride (KCl)	Merck, Darmstadt; Germany
Potassium dihydrogenphosphate (KH_2PO_4)	Merck, Darmstadt; Germany
Potassium hydrogencarbonat ($KHCO_3$)	Merck, Darmstadt; Germany
Propidium iodide	Sigma-Aldrich, Munich; Germany
Protease Inhibitors (Complete 50x tablets)	Sigma-Aldrich, Munich; Germany
Protein ladder: SeeBlue®plus2	Invitrogen, Karlsruhe; Germany
Qentix-Western Blot Signal Enhancer	Pierce, Rockford; United States
Sodium acetate (NaOAc)	Merck, Darmstadt; Germany
Sodium azide (NaN_3)	Merck, Darmstadt; Germany
Sodium chloride (NaCl)	Sigma-Aldrich, Munich; Germany
Sodium citrate	Sigma-Aldrich, Munich; Germany
Sodium dodecylsulfate (SDS)	Sigma-Aldrich, Munich; Germany
Sodium hydrogencarbonate ($NaHCO_3$)	Merck, Darmstadt; Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt; Germany
SuperSignal West Dura Extended Duration Substrate	Pierce, Rockford; United States
SuperSignal West Femto Extended Duration Substrate	Pierce, Rockford; United States
TCEP® Bond Breaker	Pierce, Rockford; United States
Tris-Hydrochloride (Tris-HCl)	Sigma-Aldrich, Munich; Germany
Triton X-100	AppliChem, Darmstadt; Germany
Trizma Base	Sigma-Aldrich, Munich; Germany
Trypan blue	Invitrogen, Karlsruhe, Germany
Tween 20	AppliChem, Darmstadt; Germany

Western Lightning®–ECL	PerkinElmer, Massachusetts; USA
β-Mercaptoethanol	Merck, Darmstadt; Germany

3.1.6. **Inhibitors**

Inhibitor	**Chemical name/structure**	**Target**	**Source**
KU-55933	2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one 	ATM	Calbiochem
PIK-75	N-((1E)-(6-Bromoimidazo[1,2-a]pyridin-3-yl)methylene)-N′-methyl-N″-(2-methyl-5-nitrobenzene)sulfonohydrazide 	PI3 Kinase p110α	Calbiochem
TGX-221	(±)-7-Methyl-2-(morpholin-4-yl)-9-(1-phenylaminoethyl)-pyrido[1,2-a]-pyrimidin-4-one 	PI3 Kinase p110β	Calbiochem

AS252424	5-[5-(4-Fluoro-2-hydroxyphenyl)-furan-2-ylmethylene)]-thiazolidine-2,4-dione 	PI3 Kinase p110 γ	Enzo
Rapamycin		mTORC1	Calbiochem
SMAC 59		XIAP, cIAP1 and cIAP2	Kind gift from D. Delia

3.1.7. **Common buffers and solutions**

Common Buffers and solutions are listed below. Additional buffers are mentioned in the respective paragraphs.

PBS	137 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4
LDS sample buffer (4x)	1170 mM sucrose 560 mM Tris Base 420 mM Tris-HCl 280 mM LDS 1.61 mM EDTA 0.75 ml 1% Serva Blue G250 0.25 ml 1% Phenolred

Reducing sample buffer (RSB) (4x)	LDS sample buffer (4x) Added freshly before use: 25 mM TCEP
Blocking Milk	1 x PBS 5 % Milk powder 0.05 % Tween-20
Cell lysis buffer	30 mM Tris-HCl pH 7.5 150 mM NaCl 10 % glycerol 1 % Triton X-100 Prior to use 1 x Complete protease inhibitors (Sigma) were added.
Crystal Violet solution	1 % crystal violet 50 % EtOH
FACS-Buffer	1 x PBS 5 % FCS
LB-Medium	10 g Bacto Trypton 5 g Yeast Extract 10 g NaCl Ad 1L deionised H_2O pH 7.0
MES SDS Running Buffer (20x)	50 mM MES 50 mM Trizma Base 1 mM EDTA

	0.1 % (w/v) SDS pH 7.3
Nicoletti buffer	0.1 % (v/v) Triton X-100, 0.1 % (w/v) sodium citrate 50 µg/ml propidium iodide (PI)
PI solution	1 µg/ml propidium iodide 1 x PBS
SOB-Medium	2 % Bacto Trypton 0.5 % Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgSO4 pH 7.0
SOC-Medium	2 % Bacto Trypton 0.5 % Yeast Extract 10 mM NaCl 2.5 mM KCL 10 mM $MgSO_4$ 10 mM $MgCl_2$ pH 7.0
Solution for cell fixation	1x PBS 10 % Formaldehyde
Stripping buffer	50 mM glycine HCl pH 2.3

TAE-Buffer (50x) **(Tris-Acetate-EDTA)**	2 M Trizma Base 2 M Acetic acid 50 mM EDTA (pH 8)
TB-buffer	10 mM Pipes, 55 mM $MnCl_2$ 15 mM $CaCl_2$ 250 mM KCl
Transfer Buffer (20X)	25 mM Bicine 25 mM Bis-Tris 1 mM EDTA Dilute to 1x with water, add 10 % Methanol
Tris Acetate SDS Running buffer (20x)	50 mM Tricine 50 mM Tris Base 0.1 % SDS pH 8.24
Wash Buffer (WB)	1 x PBS 0.05 % Tween-20

3.1.8. **Consumables**

Name	**Company**
Cell Culture Petri dishes	TPP, Trasadingen; Switzerland
Cell Culture test Plates (6-, 12-, 24-well)	TPP, Trasadingen; Switzerland
Cryogenic vials	Nunc, Wiesbaden; Germany
Combi tips	Eppendorf, Hamburg; Germany
Cuvettes	Greiner Bio-One, Flacht; Germany
Dialysis Tube	Roth, Karlsruhe; Germany
Falcon tubes (15 ml and 50 ml)	TPP, Trasadingen; Switzerland
Filters for solutions (0.22 µm)	Schleicher & Schuell; UK
Glassware	Schott, Mainz; Germany
Hybond ECL Nitrocellulose Membrane	Amersham Bioscience; UK
NuPAGE® 4-12 % Bis-Tris Gels	Invitrogen, Karlsruhe; Germany
NuPAGE® 3-8 % Tris-Acetate Gels	Invitrogen, Karlsruhe; Germany
PCR tubes (12-well strips)	StarLab, Ahrensburg, Germany
Pipette tips (0.1-10, 1-200, 101-1000 µl)	StarLab, Ahrensburg, Germany
Plastic pipettes (5 ml, 10 ml and 25 ml)	Becton Dickinson, Heidelberg; Germany
Polypropylene round bottom tube (10 ml)	Becton Dickinson, Heidelberg; Germany
PS-Test Tubes for FACS	Greiner Bio-One, Flacht; Germany
Round and flat bottom 96-well test plates	TPP, Trasadingen; Switzerland
Safe-Lock Reaction Tubes (1,5ml, 2 ml)	Eppendorf, Hamburg; Germany
Sealing foil	Roche, Mannheim; Germany
Single-Use Needles	Becton Dickinson, Heidelberg; Germany
Single-Use Scalpel	Feather, Osaka; Japan
Single-Use Syringe (5 ml, 30 ml, 50 ml)	Terumo, Eschborn; Germany
Sterile filter (0.22 µm and 0,45 µm pore size)	Millipore, Billerica; United States
Tissue Culture flasks (25, 75 and 150 cm^2)	TPP, Trasadingen; Switzerland
Whatman paper	Schleicher & Schuell; UK
X-Ray film HyperfilmTM ECL	Amersham Bioscience; UK

3.1.9. **Instruments**

Instrument	**Manufacturer**
Äkta Prime	Amersham Pharmacia Biotech, Germany
Biofuge Stratos	Heraeus, Hanau, Germany
Biohazard safety cabinet class II	Scanlaf, Lynge, Denmark
Blotting equipment X cell IITM	Novex, Bergisch Gladbach; Germany
Confocal microscope (SP5 inverted)	Leica, Wetzlar, Germany
Cryo 1°C Freezing container	Nalgene Labware, Nee rijse; Belgium
Electrophoresis chamber Cell protean II	Biorad, Munich; Germany
Flow Cytometer FACSCalibur	Becton Dickinson, Heidelberg; Germany
Freezer -20°C	Liebherr, Biberach; Germany
Freezer -80°C	New Brunswick Scientific Co; USA
Fridge, profi line	Liebherr, Ochsenhausen; Germany
GelSystem Flexi 4040	Biostep, Jahnsdorf; Germany
Hyper Processor X-Ray film Developer	Amersham Bioscience; UK
Heating Block Thermo Mixer Compact	E Eppendorf, Hamburg; Germany
Incubator Polymax 1040	Heidolph, Schwabach; Germany
Incubator Stericult 2000 Forma	Scientific, Scotia; United States
Magnetic stirrer MR3000	Heidolph, Schwabach; Germany
Microscope Axiovant 25	Zeiss, Jena; Germany
Microwave	AEG, Nuremberg; Germany
Mithras Luminometer LB 940	Berthold Technologies, Germany
Multichannel pipettes	Micronic Systems; United States
Multifuge 3S-R	Heraeus, Hanau, Germany
Multiskan Ascent	Thermo Labsystems, Vantaa; Finnland
Multistepper	Eppendorf, Hamburg; Germany
Multitron Incubator Shaker	Appropriate Technical Resources; USA
NanoDrop Spectrophotometer ND-1000	NanoDrop Technologies, USA
PAGE chamber X Cell II^{TM}	Novex, Invitrogen, Karlsruhe; Germany
pH Meter	Mettler, Giessen; Germany
Photometer Ultrospec 3100 pro	Amersham, Freiburg; Germany
Pipetman	Integra Bioscience, Fernwald; Germany

Pipettes (10 µl, 100 µl, 200 µl, 1 ml)	Gilson, Bad Camber; Germany
Power supply PowerEASE 500	BioRad, Hercules; United States
Power supply PoerPac 1000	Novex, Invitrogen, Karlsruhe; Germany
PCR cycler Peltier Thermal cycler 200	MJ research Inc., Watertown;USA
See-Saw Rocker	Stuart, Staffordshire, UK
Sonifier	Branson Ultrasonics Corporation, USA
Table Centrifuge Biofuge	Heraeus, Hanau, Germany
Thermomixer compact	Eppendorf, Hamburg; Germany
Varifuge 3O-R	Heraeus, Hanau, Germany
Vortex	Heidolph, Schwabach; Germany
Water bath	B. Braun, Melsungen; Germany

3.2. Methods

3.2.1. Cellular biology methods

Cell culture and passaging of cells

Suspension cells were cultured in 75 cm^2 cell culture flasks in RPMI supplemented with 10 % FCS at 37°C in a humidified atmosphere with 5 % CO2 and split every 2 to 3 days with a number of 1-5 x 10^5 cells/ml so that the number of cells did not exceed 1 x 10^6 cells/ml.

All adherent cell lines were cultured in 75 cm^2 or 150 cm^2 cell culture flasks in DMEM + Glutamax or RPMI + Glutamax , depending on the cell line with different supplements (see section 3.1.1) and 10 % FCS at 37°C in a humidified atmosphere with 5 % CO_2. Cells were split at 80 % confluence by washing with 1x PBS followed detachment with 3-5 ml 1x Trypsin/EDTA for 1-5 minutes. The trypsinisation was stopped by adding fresh medium containing 10 % FCS (10 ml). Detached cells were centrifuged (1400 rpm, 4min) and resuspended in fresh medium containing 10 % FCS. Cells were diluted 1:10 or 1:20 depending on the growth rate. Cells were cultured for a maximum of 15 passages as cells may change their phenotype in long-term cultures.

For counting of cells, a sterile aliquot of cells was mixed with trypan blue in a 1:2 dilution. Trypan blue penetrates cells with reduced membrane integrity and therefore stains dead cells. The number of living cells was then estimated in an improved Neubauer haemocytometer

under the microscope. Living cells in four large squares were counted, and the mean was used to calculate living cells per ml according to the following formula: Cells/mL= (mean of number of cells per big square) x dilution x 10^4

Long term storage of cell lines

For long-term storage, cells were kept in liquid nitrogen. To freeze eukaryotic cell lines, confluent adherent cells were detached as described above. Detached cells or relatively dense suspension cultures were spun down. After centrifugation, cells were resuspended in pre-cooled (+4ºC) FCS containing 10 % DMSO and aliquoted into cryotubes (5 x 10^6 - 1 x 10^7 cells/ml). DMSO was used as a cryoprotectant because it prevents the formation of ice crystals which would otherwise lyse the cells during thawing. Cells were slowly cooled to -80ºC and then transferred to liquid nitrogen for long-term storage at -196ºC.

To take frozen cells into culture, frozen vials were thawed at 37°C in a water bath and cells were rapidly transferred into pre-warmed (37°C) medium containing 10 % FCS. To remove traces of DMSO, cells were centrifuged and resuspended in new medium before transferring them to the cell culture flask. Experiments were performed after passaging the cells at least twice to reduce cellular stress.

Cell viability assay

Cell viability was quantified by MTT-Assay. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction only takes place when mitochondrial reductase enzymes are active, and therefore conversion can directly be related to the percentage of living cells by comparing the absorbance of an untreated medium control to the absorbance of a sample treated with an apoptotic stimulus.

1 x 10^4 cells per well were seeded in a 96-well format on the first day of the experiment. The next day cells were incubated with the cell death inducing agent. On the third day, 25 µl of MTT (2.5 mg/ml in PBS) solution per well were added to the medium and incubated for at least 2 h at 37°C in 5 % CO_2. Subsequently the medium was taken off and 100 µl of isopropanol and acetic acid (95:5/v:v) were added to each well. After shaking and mixing for 15 min the absorbance was measured at 450 nm using the Multiskan Ascent (Thermo Labsystems, Egelsbach, Germany). The percentage of viable cells was calculated as follows:

100 x (absorption of treated cells - absorption of Triton X-100 lysed cells)/ absorption of medium treated cells - absorption of Triton X-100 lysed cells).

Quantification of Apoptosis

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified as described (Nicoletti et al., 1991). Briefly, 0.5 x 10^5 cells were seeded in 24-well plates. On the next day they were incubated with or without apoptotic stimulus in 1 ml medium at 37°C for 24 h or 48 h. Living and dead cells were harvested in the same tube, washed twice with PBS and then resuspended in 300 µl "nicoletti buffer"(see buffers). After 24 h incubation at 4°C apoptosis was quantitatively determined as cells containing nuclei with subdiploid DNA content using flow cytometry.

Long-term survival assays

5 x 10^5 HeLa or DLD1 cells were seeded in 6-well plates. HeLa or DLD1 cells were treated with KU-55933, PIK75 or DMSO as control for 1 hr before addition of iz-TRAIL. Dead cells were washed off with PBS after 24 h. Surviving cells were cultured for 4 additional days in medium without any further death stimulus. After 5 days cells were washed twice with PBS, fixed with 10 % formaldehyde in PBS for 30 min at room temperature and stained with crystal violet (1 % in 50 % ethanol).

3.2.2. Molecular biology methods

DNA amplification by polymerase chain reaction (PCR)

For amplification of plasmid or cDNA, polymerase chain reactions (PCRs) were performed. Depending on the purpose, different polymerases were used. Polymerases with proof-reading activity, like Pfu (Fermentas Life Sciences) and KapaHiFi (KAPA Biosystems) were used for preparative PCRs while Taq polymerase (Fermentas Life Sciences) was used for analytic PCRs. For one PCR reaction primers, DNA template, polymerase buffer, nucleotides and DNA polymerase were mixed as follows:

Forward Primer (10 pmol/µl)	1 µl
Reverse Primer (10 pmol/µl)	1 µl
10x polymerase buffer	5 µl
dNTP Mix (each 10 mM)	1 µl

Template DNA (plasmid, cDNA)	10-100 ng
Polymerase	1 µl (2.5 U)
H_2O add	50 µl

The melting temperature of primers used for PCR was calculated using Oligo Property Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html), where they were also checked for self complementarity. In an ideal situation, the GC content should be 50 % and there is no self-complementarity or hairpin formation. The annealing temperature ranged from 50ºC to 60ºC according to the primers used. The elongation time was calculated according to the length of the amplicon (60 sec/1000bp). Primers used for cloning of full length Bid:

Forward: 5' CAC CAT GGA CTG TGA GGT 3' length: 18 GC content 56% melting Temperature 56°C

Reverse: 5' TCA GTC CAT CCC ATT TCT 3' length: 18 GC content 47 % melting Temperature: 53 °C

The scheme of the PCR is shown below.

Step	Temperature	Duration	
Denaturation	95ºC	3 min	
Denaturation	95ºC	35 sec	30 cycles
Annealing	50-60ºC	35 sec	
Elongation	72ºC	60sec /1000 bp	
Final elongation	72ºC	10 min	
Cool-down	to 4 ºC	∞	

DNA digestion and restriction analysis

For restriction analysis and subsequent cloning of amplified PCR products into defined vectors, plasmid DNA or PCR products were digested with restriction enzymes. For restriction analysis, plasmid DNA was digested with restriction enzymes that cut the plasmid DNA at defined restriction sites. All restriction enzymes used in this thesis were purchased for NEB (Frankfurt, Germany). The length of the fragments after enzymatic digestion provides information about the location of the restriction sites and the size of the plasmid. Restriction maps are usually available for commercially available plasmids according to which the size of the restriction fragments can be predicted. After DNA digestion, plasmid fragments were supplemented with DNA loading buffer, loaded onto an agarose gel in 1 x TAE buffer and subjected to gel electrophoresis. The percentage of the agarose gels was chosen according to the size of the DNA- higher percentages for larger DNA fragments and ranged from 0.5 % - 2 %. Due to the applied electric current, the negatively charged DNA molecules move through the matrix at different rates, depending on their size, towards the positive anode. A DNA ladder (Smart Ladder, Eurogentec) was loaded in parallel to the DNA samples and was used to assess the size of the DNA. After gel-electrophoresis, the gel was stained with ethidium bromide to visualize the DNA in ultra-violet light.

Gel extraction of DNA fragments

For the isolation of the DNA fragment(s) separated by electrophoresis, the QIAquick Gel Extraction Kit from Qiagen was used. Briefly, the agarose containing the DNA was dissolved and applied to a QIAquick column. Afterwards the DNA fragment was washed and eluted with H_2O.

TOPO® PCR cloning

For instant cloning of PCR fragment without restriction digest, the Directional TOPO® cloning kit from Invitrogen (K4900-01) was used. The topoisomerase cleaves the duplex DNA allowing for the incorporation of the PCR product which in turn releases the topoisomerase which was covalently bound to the TOPOvector. For directional TOPO® cloning, the four bases CACC were added to the forward primer to allow site directed (GTGG) integration into the TOPO® vector. The PCR proof-reading Pfu Polymerase or KapaHiFi create blunt-end PCR products and were employed to generate PCR products. The PCR products were integrated into the vector following the manufacturer´s instruction.

Preparation of competent *E. coli*

To generate chemically competent *E. coli* for transformation of ligation reactions or plasmids, a frozen bacterial stock (*E. coli* Top 10 F´, Invitrogen) was streaked out on a plain LB plate and incubated at 37ºC overnight to obtain single colonies. A single colony was picked and inoculated in 5 ml LB medium and grown overnight at 37ºC. 3 ml of this culture were inoculated in 250 ml SOB medium until the OD600 reached 0.5. The bacteria were placed on ice immediately and centrifuged at 3000 rpm for 10 min. The supernatant was removed and the bacteria were resuspended in 80 ml ice-cold TB-buffer and incubated for 10 min at 4ºC. Afterwards, the solution was centrifuged again at 3000 rpm for 10 min, the supernatant was removed and the bacteria were resuspended in 10 ml ice-cold TB-buffer. DMSO was then added to a final concentration of 7 %. After 10 min incubation at 4ºC, the bacteria were aliquoted at 200 µl, immediately frozen in liquid nitrogen and then stored at -80ºC.

Transformation of competent *E. coli*

An aliquot of chemically competent *E. coli* Top 10 F´ was slowly thawed on ice and 10-100 ng plasmid DNA or half of the ligation reaction were added to the bacteria followed by incubation on ice for 30 min. Afterwards, bacteria were subjected to heat-shock at 42ºC for 90 sec and subsequently incubated on ice for 2 min. This treatment increases the DNA uptake by the bacteria. 200 µl SOC medium were added and cells were incubated at 37ºC for 60 min. This incubation time is essentials as it enables the bacteria to express the antibiotic resistance gene encoded by the plasmid. Afterwards, bacteria were streaked out on LB-agar-plates containing the respective antibiotic agent and selected overnight at 37ºC. On the next day, a single colony was inoculated in 5 ml LB-medium containing the respective antibiotic agent. 4 ml of the bacterial culture were used to isolate the plasmid using QIAprep Miniprep Kit from Qiagen. Additionally, glycerol stocks were prepared. 700 µl of the culture were transferred to a cryotube and supplemented with 300 µl of 50 % sterile glycerol and stored at -80 ºC.

Site directed Mutagenesis

To create Bid mutants that were either not phosphorylatable or mimicked a phosphorylation the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's instruction. The procedure is based on three different steps. In the first step the template DNA is denatured and mutagenic primers which contain the desired mutation are annealed. Then the primers are extended using PfuUltra DNA polymerase to create mutated

DNA strands. In the second step parental methylated and hemimethylated DNA is digested with Dpn I. Dpn I endonuclease (target sequence:5´-Gm6ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation containing synthesised DNA. In the last step the nicked vector DNA incorporating the desired mutations is then transformed into ultracompetent bacteria. The reaction contained:

10× reaction buffer	5 µl
pcDNA3.1 Bid wt (as template)	10 ng
oligonucleotide primer #1	125 ng
oligonucleotide primer #2	125 ng
dNTP mix	1 µl
QuikSolution (contained in the Kit)	3 µl
PfuUltra HF DNA polymerase (2.5 U/µl)	1 µl
ad H_2O	50 µl

The cycling parameters used:

Step	Temperature	Duration	
Denaturation	95ºC	1 min	
Denaturation	95ºC	50 sec	18 cycles
Annealing	60ºC	50 sec	
Elongation	68ºC	60sec/1000 bp	
Final elongation	68ºC	7 min	
Cool-down	to 4 ºC		

The template used was pcDNA3.1 containing wt Bid. The mutagenesis primers used were as follows.

BidS78A	P1: 5´ GAA GAA TAG AGG CAG ATT CTG AAG **CTC** AAG AAG ACA TCA CC G 3’
	P2: 5´ CGG ATG ATG TCT TCT TGA GCT TCA GAA TCT GCC TCT ATT CTT C 3’

BidS78E	P1: 5´ GAA GAA TAG AGG CAG ATT CTG AAG **AAC** AAG AAG ACA T CAT CCG 3’ P2: 5´ CGG ATG ATG TCT TCT TGT TCT TCA GAA TCT GCC TCT ATT CTT C 3’

mRNA quantification by Quantitative Real-Time PCR (qPCR)

Isolation of total RNA

Total RNA was isolated from cells using TRIZOL (Invitrogen). Briefly, 5 x 10^5 – 1 x 10^6 cells were detached from the plates as described before, the cell pellet was transferred into a 1.5 ml test tube, centrifuged and the supernatant removed. The cell pellet was then thoroughly resuspended in 1 ml TRIZOL and incubated for 5 min at RT under the fume hood. Subsequently, 500 µl chloroform were added and the solution was mixed by vortexing for 15 sec followed by incubation for 3 min at RT and centrifugation at 13 000 rpm (4ºC) for 15 min. After centrifugation a phase separation could be observed. The upper aqueous phase containing the RNA was transferred to a new test tube and 500 µl isopropanol were added followed by incubation at RT for 10 min to precipitate the RNA. After a centrifugation step (13 000 rpm, 4ºC, 15 min), the supernatant was removed and 300 µl ethanol (70 %) were added to wash the RNA pellet. After centrifugation at 13 000 rpm (4ºC) for 10 min, the supernatant was removed, the RNA pellet air-dried for 5 min and subsequently dissolved in RNAse-free water. The RNA was stored at -80°C until further use.

Reverse transcription

After isolation, mRNA was reverse transcribed into cDNA using RevertAid™ cDNA synthesis kit according to the manufacturer’s instruction (Fermentas Life Sciences). Briefly, 3 µg RNA were mixed with 1 µl Oligo$(dT)_{18}$ primer and H_2O to a final volume of 12 µl. This mixture was incubated at 70ºC for 5 min followed by incubation on ice for 2 min. Subsequnetly, 1 µl Ribolock™ Ribonuclease inhibitor (20 U), 5 µl reaction buffer and 2 µl dNTP mix (10 mM) were added. The mixture was incubated at 37ºC for 5 min. Afterwards 1 µl RevertAid™ H Minus M-MuLV RT (200 U) was added followed by an incubation at 42ºC for 1 h. Then the reaction was stopped by inactivation of the enzyme at 70°C for 10 min. The cDNA was stored at -20°C or kept on ice for immediate use for qPCR analysis.

Quantitative Real-Time PCR (qPCR)

The amount of gene specific mRNA was quantified using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The Universal Probe Library Assay Design Centre (https://www.roche-applied-science.com) was used to generate primers and probes specific for each gene of interest. The Mastermix Absolute qPCR ROX mix (ABgene) was used for the amplification of cDNA. For each qPCR reaction 4.4 µl of the cDNA were used in a total volume of 13 µl. The qPCR reaction was performed as followed:

Initiation 50° C for 2 min

Enzyme activation 95° C for 15 min

Denaturation 95° C for 15 sec
Annealing/Extension 60° C for 60 sec
} 40 cycles

Cycle threshold C(t) values were recorded and analysed using SDS Softwarev2.3 and SDRQ Manager. After normalisation to the house keeping gene GAPDH, relative differences in mRNA levels were assessed based on the C(t) values.

Primers and Probes for qPCR:

XIAP for	GCT TGC AAG AGC TGG ATT TT	Probe 25 (Roche)
rev	TGG CTT CCA ATC CGT GAG	
cFLIP for	CTT CGC TCC CAA AAT TGA GT	Probe 50 (Roche)
rev	TCC ACA AAT CTT GGC TCT TTA CT	

siRNA-mediated knock-down (KD) of target genes

For all knockdown experiments On-Target-plus siRNA (Dharmacon) was used. Each gene was targeted by a pool of 4 single siRNA sequences to reduce off-target effects. An siRNA sequence targeting Renilla Luciferase (Rluc) was used as control (Elbashier et al., 2001). For siRNA transfections in a 6-well format, 2.5 µl siRNA (20 µM) and 1.5 µl Dharmafect Reagent 1 were used per well. siRNA and transfection reagent were incubated with 196 µl RPMI for 30 min at RT. Cells were detached from the cell culture flask as described before and resuspended in DMEM containing 10 % FCS to a concentration of 100 000 cells/ 800 µl.

Per 6-well, 800 µl of the cell solution were added to 200 µl transfection mix (final volume 1 ml) followed by an incubation for 48 h – 96 h depending on the stability of the target protein at 37°C in a humidified atmosphere with 5 % CO2.

siRNA pools used:

Protein	**Gene ID**	**On-Target-plus SMART pool (Dharmacon)**
AKT1	207	L-003000
ATM	472	L-003201
Bak	578	L-003305
cFLIP	8837	L-003772
FoxO1	2308	L-003006
FoxO3a	2309	L-003007
GSK3α	2931	L-003009
GSK3β	2932	L-003010
mTor	2475	L-003008
PI3K p110 α	5290	L-003018
PI3K p110 β	5291	L-003019
PI3K p110 δ	5293	L-006775
PI3K p110γ	5294	L-005274

esiRNA mediated knockdown of endogenous Bid and re-expression of Bid mutants

Endoribonuclease prepared siRNA (esiRNA) for the knockdown of endogenous Bid in the untranslated region was kindly provided by F. Bucholz (MPI Dresden; Germany) (Yang et al., 2002). One day before transfection 6 x 10^5 HeLa cells were plated per 6-well so that they were 80% confluent at the time of transfection. 1 µg Plasmid DNA (pcDNA3.1Bidwt, BidS78A, BidS78E) and 40 pmol Bid esiRNA were diluted in 250µl Opti-MEM I without serum. 3 µl Lipofectamine 2000 were diluted in 250 µl Opti-MEM I without serum and incubated for 5 min at room temperature. After 5 minutes incubation both solution were combined and incubated at RT for 20 min. Then the DNA-esiRNA –Lipofectamine mix was added to each well and incubated at 37°C for 48 h.

3.2.3. **Biochemical methods**

Preparation of cell lysates

Adherent cells were trypsinised to detach the adherent cells from the plates. Detached cells as well as the suspension cell lines were harvested by centrifugation at 1400 rpm for 5 min at 4°C and washed twice with PBS. The resulting cell pellets were then resuspended in 50 µl lysis buffer supplemented with Complete™ protease inhibitors (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions to prevent protein degradation by proteases. After 30 min incubation on ice, lysates were centrifuged at 13 000 rpm at 4 °C for 15 min to remove the nuclei and the protein containing supernatant was taken off and stored at -20°C.

BCA assay – determination of protein content

To determine the protein concentration of cell lysates, the bicinchoninic acid (BCA)-containing protein assay was used (Pierce, Rockford, IL, USA). Therefore, 1 µl lysate was incubated in 200 µl BCA solution at 60ºC for 20 min, followed by measuring light absorption at 540 nm. In an alkaline medium, proteins reduce Cu^{2+} to Cu^{1+} which forms a blue-coloured complex with bicinchoninic acid. Larger polypeptides or proteins, but not single amino acids and dipeptides, will react to produce the light blue to violet complex that absorbs light at 540 nm. A standard curve was created according to manufacturer's instruction and the protein content in the cell lysates was calculated accordingly.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For the separation of proteins by SDS-PAGE, lysates were supplemented with four-fold concentrated standard reducing sample buffer (4xRSB) and incubated at 75ºC for 10 min. 100 µg protein per lane were then separated on 4–12 % NUPAGE Bis-Tris gradient gels or 3-8 % Tris-Acetate gels (Novex, San Diego, CA, USA) in MES or TA buffer, respectively, according to the manufacturer's instructions. A marker containing proteins of defined sizes was used to assess the size of the proteins (SeeBlue® Plus2, Gibco/Invitrogen, Karlsruhe, Germany). For the separation of relatively small proteins Bis-Tris gradient gels and 1x MES running buffer was used while Tris-Acetate gels and 1x TA running buffer was applied to separate larger proteins (>150 kDa). Gels were run at 200V for 45 min.

Western blot analysis

The proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by the method of Towbin *et al.* (Towbin et al., 1979a). The transfer was carried out at 30 V for 2 h. Afterwards, the membrane was shortly washed with deionised H_2O and stained with Ponceau-S to control for equal blotting. The membranes were then treated with the Western Blot signal enhancer Qentix (Thermo Scientific PIERCE Biotechnology, Rockford, USA), blocked for 1 h in 5 % blocking buffer, washed with washing buffer and incubated overnight with the primary antibody PBS-T supplemented with 5 % BSA. After 3 x 5 min washes in wash buffer the blots were incubated with HRP-conjugated isotype-specific secondary antibody diluted 1: 20 000 in PBS-T for at least 1 h. Subsequently, the blots were washed again (3 x 5 min) and then developed by enhanced chemoluminescence following the manufacturer's protocol (Amersham Pharmacia Biotech, Uppsala, Sweden). For weak signals, SuperSignal West Dura (Pierce/Thermo Scientific) or SuperSignal West Femto (Pierce/Thermo Scientific) was used as detection agent, while ECL Western Blotting substrate (Pierce/Thermo Scientific) was applied when strong signals were expected. For the use of further primary antibodies, blots were stripped with stripping buffer at RT for 15 min. Afterwards blots were washed in washing buffer and then blocked using blocking buffer for at least 1 h.

Immunoprecipitation of TRAIL-receptor signalling complexes

For the precipitation of receptor signalling complexes, 1x 10^7 cells were seeded in a 150 cm^2 cell culture plate overnight. On the next day, the medium was removed and 10 ml prewarmed (37°C) DMEM containing 10 % FCS and 1 µg/ml moTAP--TRAIL was added to the cells. After incubation for 10 min the supernatant was removed and cells were immediately washed with ice-cold PBS. Cells were then scraped from the plates at 4ºC and transferred to a 15 ml Falcon tube with ice-cold PBS followed by a centrifugation step at 1300 rpm (4ºC) for 3 min. The supernatant was removed and the cells were resuspended in 900 µl ice-cold lysisbuffer (without Triton) and transferred to a 1.5 ml Eppendorf tube. 100 µl 10 % Triton-X- 100 (4ºC) were added, the tube was mixed and incubated on ice for 45 min. Afterwards, the lysate was centrifuged at 13 000 rpm (4ºC) for 20 min to remove nuclei and cell debris. The supernatant was transferred to a new 1.5 ml Eppendorf tube and the protein content was determined by the BCA assay. Cell lysates were then adjusted to contain the same protein amount per ml. 30 µl of the adjusted cell lysates were removed and stored at -20ºC (= lysates before IP). M2 beads

(15 µl bead volume) were added to all adjusted cell lysates followed by over-night incubation at 4ºC in an overhead shaker. For the precipitation of non-stimulated receptors, moTAP-iz-TRAIL was added post-lysis to the unstimulated cells at an end concentration of 1 µg/ml. To control for unspecific binding to the anti-FLAG M2 beads, a "beads only" control was included. On the next day, the tubes were centrifuged at 7 000 rpm (4ºC) for 3 min, the supernatant was removed and the beads were washed 5 times with ice-cold lysisbuffer. Afterwards, 30 µl 2 x RSB were added followed by an incubation at 80ºC for 10 min to prepare the lysates for separation by SDS-PAGE.

TRAIL receptor surface staining by flow cytometry

For the analysis of surface-expressed receptors, cells were detached from the plates and washed with ice-cold FACS-buffer (1 x PBS, 5 % FCS). After centrifugation (3 min, 1200 rpm, 4°C) 1 x 10^5 cells were incubated in 100 µl of FACS-buffer containing 5 µg/ml antibody of TRAIL-R1 (HS101), TRAIL-R2 (HS201) or an mIgG1-control antibody respectively on ice for 30 min. Afterwards, cells were centrifuged and washed three times with 200 µl ice-cold FACS buffer. Then 100 µl biotinylated secondary goat anti-mouse antibodies (5 µg/ml in FACS buffer) were added and incubated on ice for 20 min. Subsequently, cells were centrifuged and washed three times with 200 µl ice-cold FACS-buffer. In a third step, cells were incubated with Streptavidin-PE (1:200 in FACS-buffer) for 20 min on ice. Subsequently, cells were centrifuged, washed 3 times with 200 µl ice-cold FACS-buffer, and then analysed by flow cytometry with a FACS Calibur.

Immunofluorescence and Confocal microscopy

For immunocytochemistry, 3 x 10^5 cells were seeded in 6-well plates on sterile coverslips. On the next day, cells were either left untreated or subjected to treatment with PIK75 for 6 h. Dead cells were then washed away three times with PBS. Cells were fixed for 10 min in 3 % formaldehyde in PBS. Subsequently, cells were washed again three times with PBS before cells were permeabilised with 0.2 % Triton X-100 in PBS for 5 min. Unspecific binding sites were blocked by incubation with 1 % BSA for 1h. Then cells were incubated in 20 µl primary rabbit anti-FoxO3a antibody (rabbit, Cell signalling) (1:500 in 1 % BSA/PBS) and incubated overnight at 4°C in the dark. On the next day, cells were washed three times with PBS and then incubated with the secondary fluorescently labelled antibody (Alexa-488-anti-rabbit, Invitrogen) (1:400 in 1% BSA/PBS) for 1h at 4°C in the dark. Cells were washed again three times in PBS. Then DAPI containing mounting solution (ProLong Gold antifade reagent with

DAPI, Invitrogen) was put on a microscopic slide and the cells fixed to the coverslip were put face down onto the slide. Sides of the coverslips were fixed with nail varnish to prevent movement. Cells were visualized confocal microscopy (SP5 inverted confocal microscope, Leica).

PI3 Kinase assay

p110α was immunoprecipitated overnight using anti-PI3 Kinase p110α antibody (C73F8 Cell Signaling) and Protein G beads and subsequently incubated for 5 min together either with DMSO as control, KU-55933 (1 µM), PIK-75 (1 µM) or TGX-221 (1 µM) and the substrate PIP2 (1 µg/µl) in kinase buffer (20 mM TrisHcl pH 7.5, 100 mM NaCl, 1 mM EGTA) (Whitman et al., 1985). ATP (10 µM) was added to the mix and incubated at 37 °C for 2 h. Subsequently, Kinase-Glo® reagent was added according to the manufacturer's instruction and incubated at RT in the dark for 10 min before the luminescence was recorded with an integration time of 0.1 s.

3.2.4. Statistical analysis

Data were calculated as mean and standard deviation (SD). Comparisons of results between treated and control groups were made by the Student's *t* tests. $P \leq 0.05$ between groups was considered significant.

4. Results

4.1. DNA damage-induced Bid phosphorylation in human cells

To corroborate the findings that DNA damaged-induced phosphorylation of murine full-length Bid by Ataxia telangiectasia mutated (ATM) at serine residue S78 may affect its pro-apoptotic function, it was tested whether this phosphorylation of endogenous Bid also occurs in human cells following DNA damage. To investigate the functional role of DNA damage-mediated Bid phosphorylation at S78 in human cells the cervix carcinoma cell line HeLa was treated with the DNA damaging drug etoposide for 0-120 min (figure 10). At a concentration of 10 µM etoposide induced the phosphorylation of Bid already after 15 min, with the signal peaking at 60 min and then slowly decreasing again. So far a phosphorylation of Bid has only been shown in murine cells or in human cells in which murine Bid was overexpressed (Kamer et al., 2005). Thus, this result shows that DNA-damage-induced phosphorylation of human Bid occurs on the endogenous level which indicates that Bid phosphorylation upon DNA damage is conserved among different species.

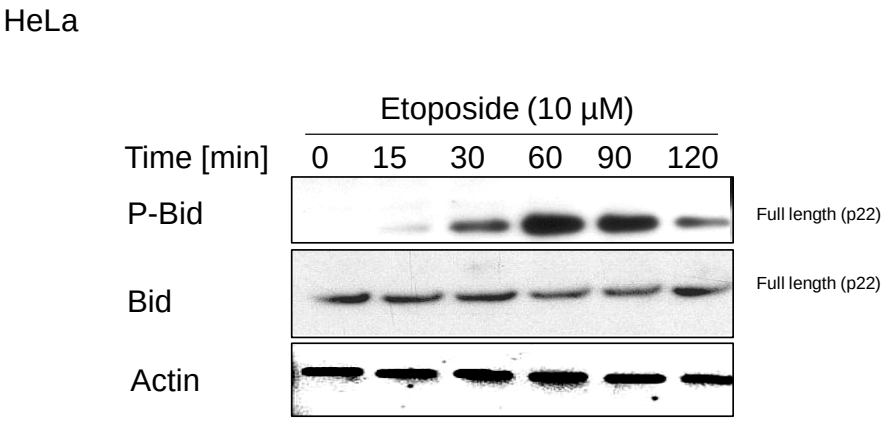


figure 10. DNA damage-induced phosphorylation of full length human Bid.

HeLa cells were treated with 10 µM etoposide for 0, 15, 30, 60, 90 and 120 min. 100 µg protein of the total cell lysates were applied in each lane. The resulting Western blot is shown using an S78 specific Phospho-Bid antibody, a Bid antibody and an anti-Actin antibody which served as loading control.

4.2. Phosphorylation of Bid in TRAIL-induced apoptosis

HeLa cells die upon TRAIL treatment in a concentration dependent manner; a dose-response curve of HeLa cells after 24 h TRAIL treatment is depicted in (figure 11).

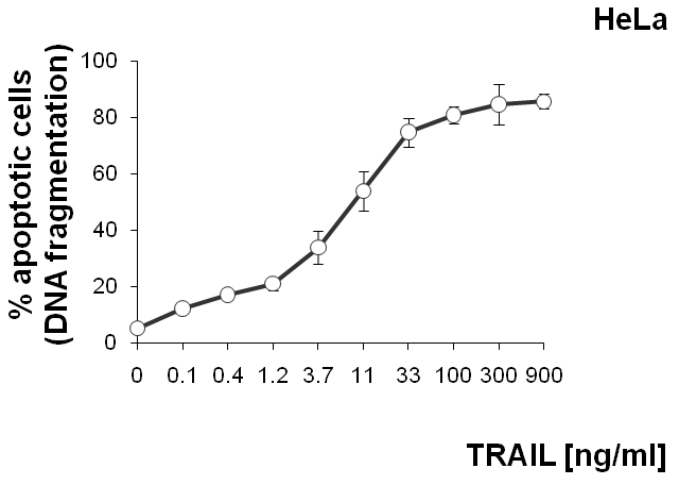


figure 11. TRAIL-induced apoptosis in HeLa cells.

HeLa cells were treated with increasing concentrations of iz-TRAIL and analysed for their subdiploid DNA content after 24 h using flow cytometry. Values are mean ± SD of three independent experiments.

To examine the role of Bid phosphorylation in TRAIL-induced apoptosis, HeLa cells were treated for 30 min to 6 h with 100 ng/ml iz-TRAIL, a concentration at which about 80 % of the cells undergo apoptosis (figure 12). Etoposide treated HeLa cells were used as a positive control to show the phosphorylation of full-length Bid. Interestingly, iz-TRAIL treatment alone induced the phosphorylation of tBid after 1.5 h with the signal getting stronger over time. This event seems to happen shortly after Bid cleavage which was already detectable after 1 h. At this time the amount of full length Bid present in the lysates of TRAIL-treated cells is already decreased and tBid became detectable. This is consistent with the data obtained for caspase-8, which is responsible for Bid cleavage and its inhibitor cFLIP. After 30 min the active cleavage fragment of caspase-8 p18 was already detectable and $cFLIP_L$ was almost completely cleaved. The cleavage of Bid precedes the activation of caspase-9. Fully cleaved caspase-3 appeared later, starting after 3 hours, indicating that Hela cells are type II cells, i.e. cells which depend on the amplification loop via the mitochondria and the action of the apoptosome to mediate caspase-3 activation (see figure 6). However, some cleavage of the caspase-3 target PARP was already detectable earlier, after 1.5 h, hinting at some active

caspase-3 present in the sample which might have been activated via the direct death receptor pathway. This early caspase-3 activity might also be responsible for early cleavage of caspase-9, which possesses a caspase-3 cleavage site (Zou et al., 2003).

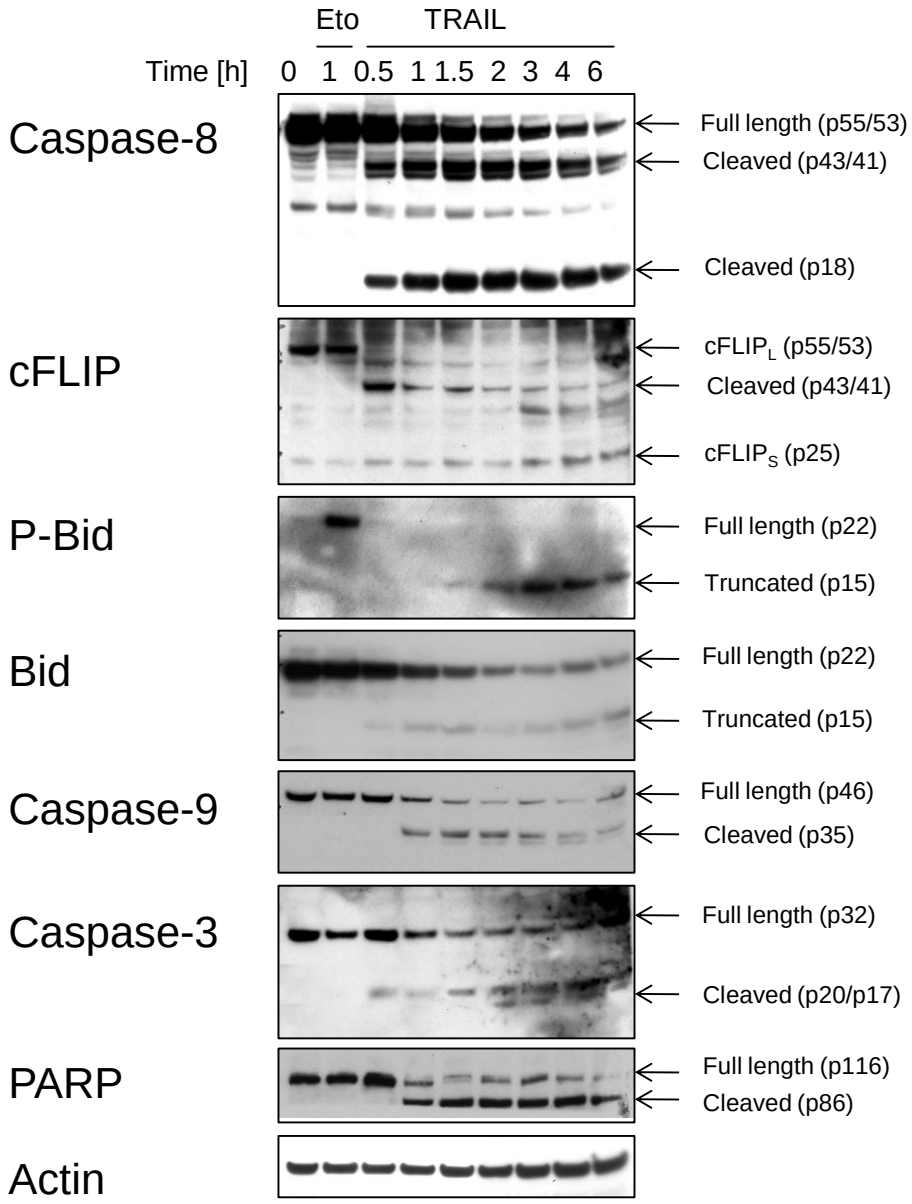


figure 12. TRAIL-induced Bid phosphorylation in HeLa cells.

HeLa cells were either left untreated, treated with etoposide (10 µM, 1h) or treated with 100 ng/ml iz-TRAIL for the indicated times. 100 µg protein of the total cell lysates were applied in each lane. The resulting Western blot was probed with the indicated antibodies.

Taken together, these results show for the first time that TRAIL induces the phosphorylation of tBid at the residue S78. This residue has previously been shown to become phosphorylated

in an ATM-dependent manner upon DNA-damage (Kamer et al., 2005). Phosphorylation clearly occurs after Bid cleavage, however from these data it is hard to tell whether it occurs upstream or downstream of mitochondrial activation as cleavage of caspase-9 was already detectable after 1 h and therefore precedes phosphorylation. Early cleavage of caspase-9 might be caused by activation of the mitochondria or by activation of caspase-3 via the direct death receptor pathway, which can then in turn activate caspase-9.

To analyse whether this TRAIL-induced phosphorylation of tBid is restricted to human cells or whether it is conserved in other species, the same experiments were carried out using the TRAIL-sensitive mouse cell line XhoC3. A dose-response curve of XhoC3 cells after treatment with murine iz-TRAIL treatment for 24 h is depicted in figure 13.

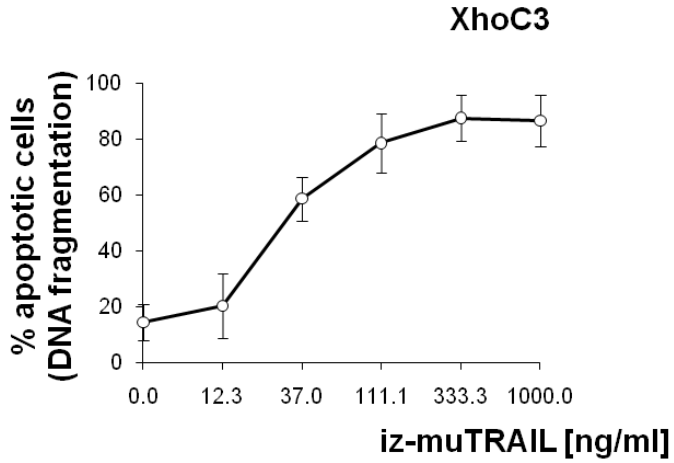


figure 13. XhoC3 cells are sensitive to treatment with murine iz-TRAIL.

XhoC3 cells were treated with increasing concentrations of mu iz-TRAIL and analysed for their subdiploid DNA content after 24 h. Values are mean ± SD of three independent experiments.

XhoC3 cells were treated with 100 ng/ml murine iz-TRAIL and investigated for phosphorylated Bid and other members of the TRAIL-R pathway (figure 14). Like in human cells, also in the murine XhoC3 cells phosphorylation of full length Bid could be observed after etoposide treatment. Importantly, also TRAIL-induced tBid-phosphorylation could be observed after 1 h. Some phosphorylation of full length Bid also occurred, but decreased over time corresponding to the cleavage of full length Bid into tBid. Cleavage of caspase-9 and caspase-3 occurred later at 3 h and 4 h, respectively. Yet again, some PARP cleavage was already detectable after 3 h indicating that there was some active caspase-3 present in the sample although it was not yet detectable by Western blot. These data suggest that TRAIL-

induced phosphorylation of tBid is conserved among different species. Since caspase-9 and caspase-3 became activated much later and clearly after the phosphorylation of tBid, phosphorylation of tBid upon TRAIL treatment can be placed upstream of the mitochondria in the murine system.

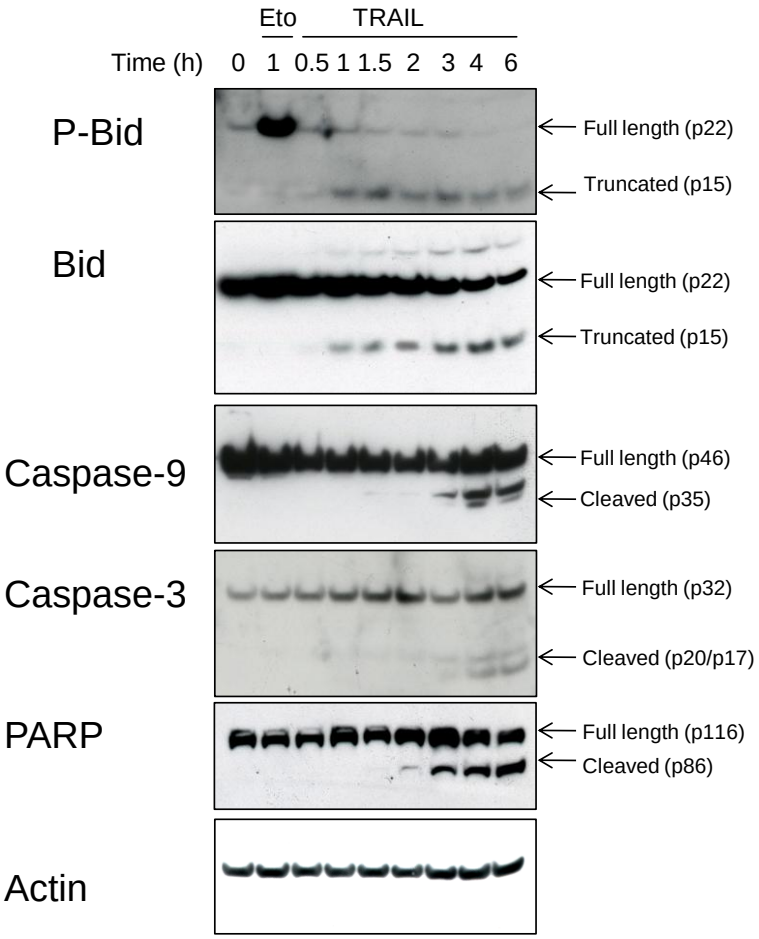


figure 14. TRAIL-induced Bid phosphorylation in murine Xhoc3 cells.

XhoC3 cells were either left untreated, treated with etoposide (10 µM, 1h) or treated with 100 ng/ml iz-TRAIL for the indicated times. 100 µg protein of the total cell lysates were applied in each lane. The resulting Western blot was probed with the indicated antibodies.

4.3. TRAIL-induced tBid phosphorylation is ATM-independent

DNA damage-induced phosphorylation of Bid at residue S78 is ATM-dependent (Kamer et al., 2005; Zinkel et al., 2005). To investigate whether this also applies to TRAIL-induced phosphorylation of tBid, ATM was knocked down in HeLa cells. Cells were then treated with TRAIL and phosphorylation of tBid was investigated by Western blot. figure 15 shows a representative Western Blot prepared from HeLa cells lysates, which were transfected with

siRNA targeting Rluc or ATM respectively. Cells were treated with 100 ng/ml iz-TRAIL for various times or with etoposide for 1 h, which served as positive control for DNA damage-induced phosphorylation of Bid by ATM.

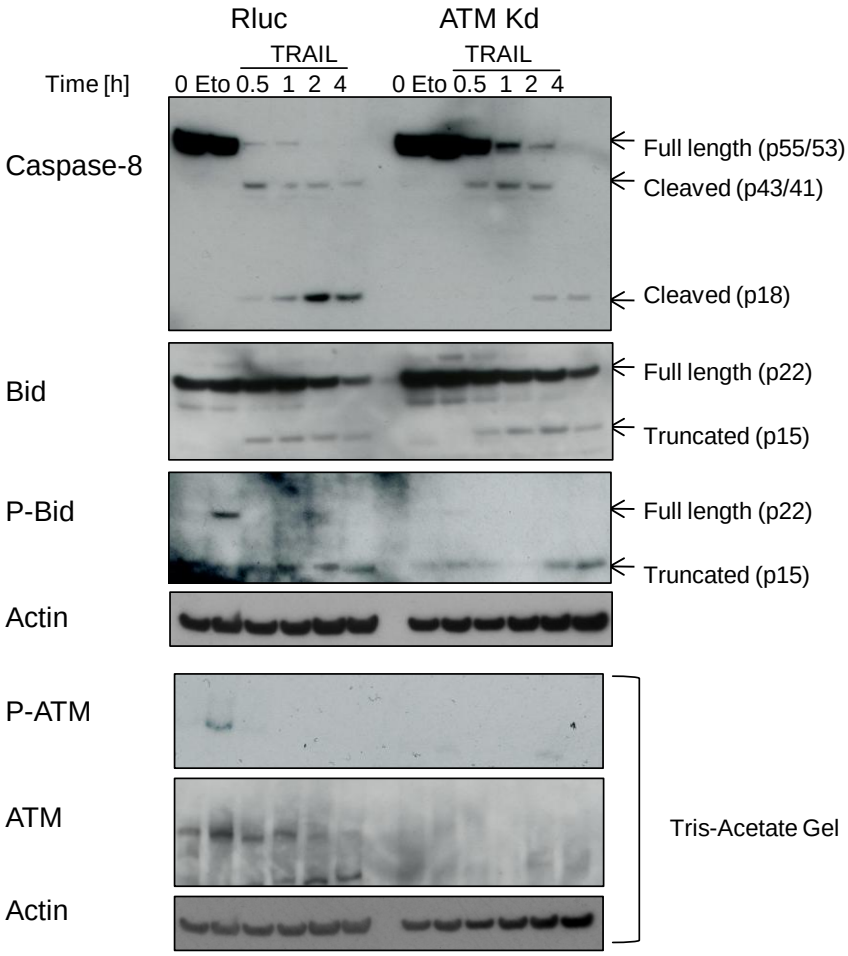


figure 15. TRAIL-induced phosphorylation of tBid is independent of ATM.

HeLa cells were transfected with siRNA targeting ATM or Rluc as control for 72 hours. Cells were either left untreated, treated with etoposide (10 µM, 1hr) or treated with 100 ng/ml iz-TRAIL for the indicated times. 100 µg protein of the total cell lysates were applied in each lane. A Bis-Tris gel was used to resolve small proteins, a Tris-Acetate gel was used to resolve proteins >150 kDa. The resulting Western blot was probed with the indicated antibodies.

HeLa cells transfected with siRNA targeting Rluc and treated with etoposide showed phosphorylated forms of full length Bid. The TRAIL-induced phosphorylation of tBid became visible after 2 h. These results are consistent with the ones obtained in non-transfected Hela cells (section 4.2). In contrast to this, HeLa cells transfected with siRNA targeting ATM did not show Bid phosphorylation upon etoposide treatment whereas TRAIL-induced

phosphorylation of tBid was still detectable. The lower part of figure 15 shows a Western blot run in parallel, prepared from the same cell lysates using a Tris-Acetate gel. With the use of Tris-Acetate gels, which are more suitable for the separation of proteins with high molecular weight, it was possible to detect the 380 kDa protein ATM, which became cleaved upon TRAIL-treatment. The knockdown of ATM was very efficient as almost no ATM and no active ATM could be detected in the cell lysates by Western blot. However, the active phosphorylated form of ATM could only be detected in the etoposide-treated sample and not in TRAIL-treated cells. This further supports that ATM, responsible for DNA-damage-induced phosphorylation of Bid (Kamer et al., 2005), does not appear to be involved in TRAIL-induced phosphorylation of tBid.

Taken together, these results indicate that ATM is the kinase which is responsible for DNA-damage-induced phosphorylation of Bid, as it can clearly be inhibited by ATM knockdown. However, ATM does not seem to be involved in TRAIL-induced phosphorylation of Bid as it still occurs in the absence of ATM. Hence, a different kinase must be responsible for TRAIL-induced phosphorylation of tBid.

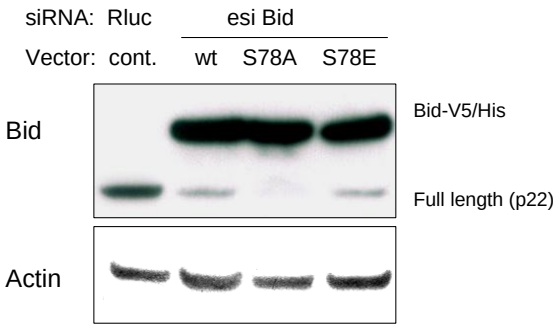
4.4. The role of TRAIL-induced tBid phosphorylation

To investigate the role of TRAIL-induced tBid phosphorylation Bid mutants were created that can either not be phosphorylated (Bid S78A) or that mimic its phosphorylation (Bid S78E). The apoptotic outcome upon TRAIL treatment was then investigated in HeLa cells expressing either Bid wt, Bid S78A or Bid S78E. As potential changes in the apoptotic outcome upon introduction of the different Bid mutants might be masked by endogenous Bid expression, endogenous Bid was silenced using esiRNA targeting the untranslated region of Bid in parallel with the re-introduction of the different Bid mutants.

As shown in figure 16a endogenous Bid was efficiently knocked down using esiRNA and re-expression levels of the three different Bid proteins was comparable. Knockdown of endogenous Bid in HeLa cells induced TRAIL resistance, again indicating that HeLa cells are type II cells (figure 16b). Reintroduction of Bid wt or the Bid mutants rendered HeLa cells TRAIL sensitive again. Interestingly, no difference in the apoptotic outcome between wild type Bid and the different Bid mutants could be detected when these cells were treated with TRAIL for 24 h (figure 16c).

This indicates that TRAIL-induced phosphorylation of tBid might be an epiphenomenon and might not be decisive for the apoptotic outcome of TRAIL stimulation. Alternatively, its importance may not be detectable under the conditions employed here. One possibility is that it could be masked by the given expression pattern of Bcl-2 family members in HeLa cells.

a)



b) c)

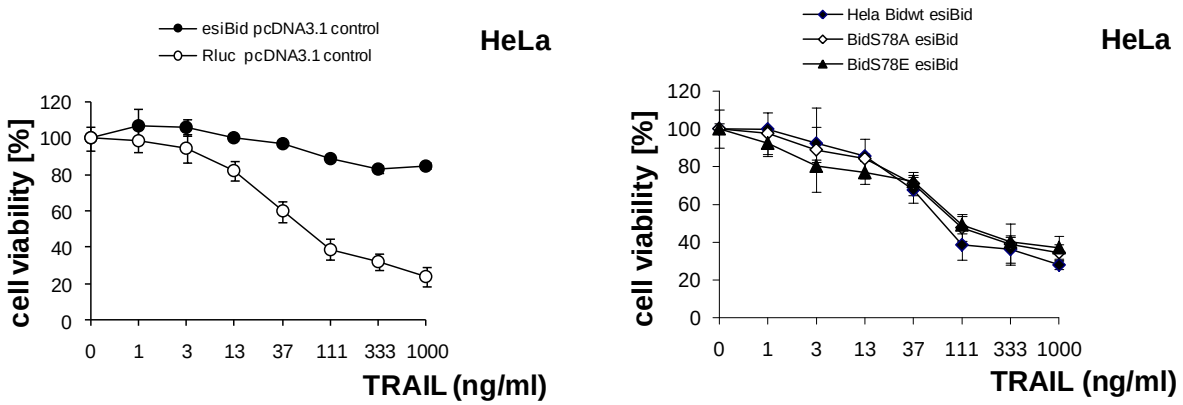


figure 16. Re-introduction of a non-phosphorylatable form of Bid does not change the apoptotic outcome of TRAIL stimulation in HeLa cells.

HeLa cells were transfected with esiRNA targeting endogenous Bid or Rluc and co-transfected with Bid wt, Bid S78a, Bid S78E or the empty vector control. (a) Lysates were prepared and subjected to Western blot analysis to control for the knockdown of endogenous Bid and expression of the different mutants.(b,c) After 36 h cells were treated with increasing concentrations of TRAIL for 24 h. Then cell viability was measured by MTT-assay.

4.5. HeLa and DLD1 cells can be sensitised to TRAIL-induced apoptosis by the ATM inhibitor KU-55933.

The results from section 4.3 indicated that the kinase ATM is most likely not involved in TRAIL-induced tBid phosphorylation. In oder to gain a second independent assessment, I intented to test pharmacologically whether tBid phosphorylation was dependent on ATM in parallel to the ATM knockdown experiments. For this, the well characterised ATM inhibitor KU-55933 (Hickson et al., 2004) seemed to be a suitable tool. However, ATM deficiency can result in resistance to CD95L and TRAIL-mediated killing due to up-regulation of cFLIP (Stagni et al., 2008), which would interfere with the evaluation whether phosphorylation of tBid was independent of ATM. Therefore the effect of KU-55933 on TRAIL apoptosis sensitivity had to be determined first. figure 17a shows a dose-response curve of HeLa cells upon KU-55933 treatment. The concentration of 10 µM was only slightly toxic on its own and is used in most studies to specifically inhibit ATM (Hickson et al., 2004). Therefore this concentration was used in combination with TRAIL to analyse its effect on TRAIL sensitivity. Strikingly, KU-55933 did not render cells resistant to TRAIL but rather exerted the opposite effect. It potently sensitised HeLa cells to TRAIL-induced apoptosis (figure 17 b). At a concentration of 1.2 ng/ml TRAIL was already capable of inducing apoptosis in 70 % of the cells in the presence of KU-55933 as compared to 20 % apoptosis in cells that were treated with TRAIL alone. The sensitisation to TRAIL-induced apoptosis was concentration-dependent (figure 17c) with the maximal sensitisation to be observed at 10 µM. The lowest concentration of 1 µM KU-55933 was not sufficient to sensitise the cells to TRAIL-induced apoptosis. In addition, clonogenic assays were conducted to explore whether KU-55933 had an effect on long-term survival following TRAIL-treatment. Indeed, KU-55933 and TRAIL synergistically suppressed colony formation of HeLa cells while KU-55933 alone did not interfere with the survival of the cells (figure 17d). Thus, HeLa cells which are relatively sensitive to TRAIL-induced apoptosis can be further sensitised to TRAIL by co-application of KU-55933.

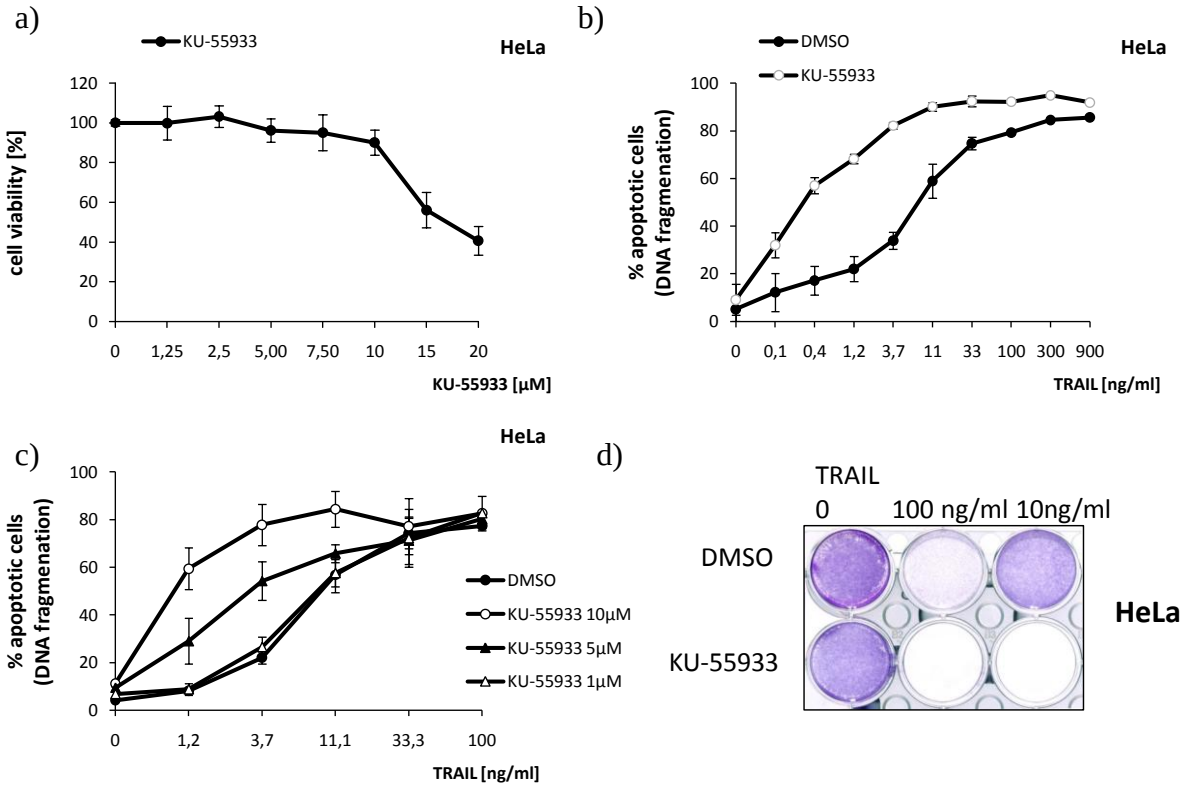


figure 17. KU-55933 and TRAIL co-treatment sensitises HeLa cells to TRAIL-induced apoptosis and reduces clonogenic survival.

(a) HeLa cells were treated with increasing concentrations of KU-55933 for 24 h. Cell viability was then measured by MTT-assay. (b) HeLa cells were treated with increasing concentrations of iz-TRAIL with or without pre-incubation with KU-55933 (10 µM, 1h) and analysed for their subdiploid DNA content after 24 h. (c) HeLa cells were treated with increasing concentrations of iz-TRAIL with or without pre-incubation with KU-55933 (10 µM, 5µM and 1µM) and analysed for their subdiploid DNA content after 24 h. Values are mean ± SD of three independent experiments. (d) HeLa cells were treated with either DMSO or KU-55933 (10 µM) alone or in combination with increasing concentrations of iz-TRAIL for 24 h. Dead cells were washed away and fresh medium was added every second day. Cell viability was visualised by crystal violet at day 5. One representative of three independent experiments is shown.

In line with this, cancer cell lines of different tissue origin can be further sensitised to TRAIL-induced apoptosis by co-treatment with KU-55933, e.g. the breast cancer cell line MCF-7 and the lung cancer cell line A549 (figure 18).

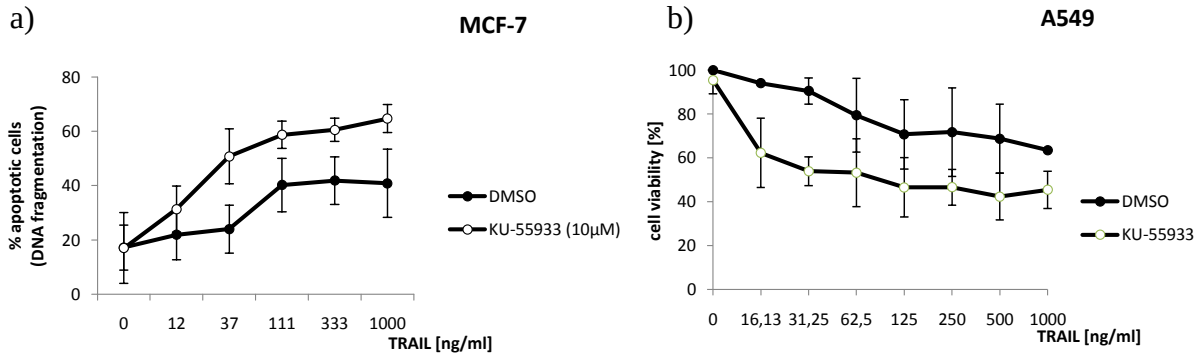
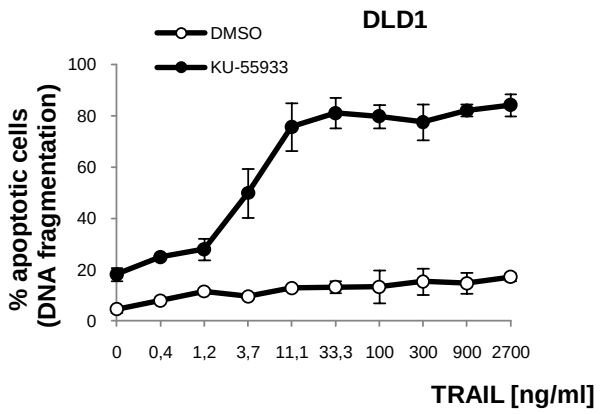


figure 18. KU-55933 and TRAIL co-treatment sensitises the breast cancer cell line MCF-7 and the lung adenocarcinoma epithelial cell line A549 to TRAIL-induced apoptosis.

(a) MCF-7 cells were treated with increasing concentrations of iz-TRAIL with or without pre-incubation with KU-55933 (10 µM) and analysed for their subdiploid DNA content after 24 h. Values are mean ± SD of two independent experiments. (b) A549 cells were treated with increasing concentrations of iz-TRAIL with or without pre-incubation with KU-55933 (10 µM). Cell viability was quantified by MTT assay after 24 h Values are mean ± SD of two independent experiments.

However, most primary tumour cells are TRAIL-resistant. It is therefore important to test whether a given drug cannot only further sensitise cells that are already TRAIL sensitive but whether it can also break tumour cell resistance to TRAIL. To test this, the TRAIL-resistant human colon carcinoma cell line DLD1 was used. DLD1 cells are resistant to TRAIL when applied at very high concentrations (figure 19a). However, when this cell line was pre-treated with KU-55933, at a concentration which itself was only slightly toxic, 80 % of the cells became TRAIL-sensitive. Furthermore, KU-55933 and TRAIL acted synergistically and led to a reduction in long-term survival, as shown in a clonogenic assay (figure 19b). Taken together, KU-55933 can efficiently sensitise TRAIL-resistant DLD1 cells to TRAIL-induced apoptosis.

a)



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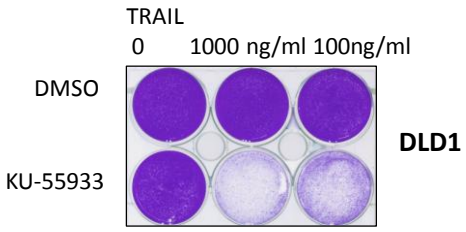


figure 19. KU-55933 and TRAIL co-treatment sensitises the TRAIL-resistant DLD1 cells to TRAIL-induced apoptosis and reduces clonogenic survival.

(a) DLD1 cells were treated with increasing concentrations of iz-TRAIL with or without pre-incubation with KU-55933 (20 µM) and analysed for their subdiploid DNA content after 24 h. Values are mean ± SD of three independent experiments. (b) DLD1 cells were treated with either DMSO or KU-55933 (20 µM) alone or in combination with increasing concentrations of iz-TRAIL for 24 h. Dead cells were washed away and fresh medium was added every second day. Cell viability was visualized by crystal violet at day 5. One representative of three independent experiments is shown.

4.6. KU-55933 mediated sensitisation to TRAIL-induced apoptosis is independent of ATM inhibition

The TRAIL sensitising effect of the ATM inhibitor KU-55933 has only recently been shown in melanoma cells (Ivanov et al., 2009). However, AT cells which have been isolated from patients suffering from Ataxia telangiectasia that lack functional ATM are generally resistant to death receptor-mediated apoptosis (Stagni et al., 2008). Therefore the finding that an ATM-specific inhibitor sensitises tumour cells to TRAIL-induced apoptosis is surprising and in fact counterintuitive. Thus, the question arose whether the observed effect was truly due to inhibition of ATM.

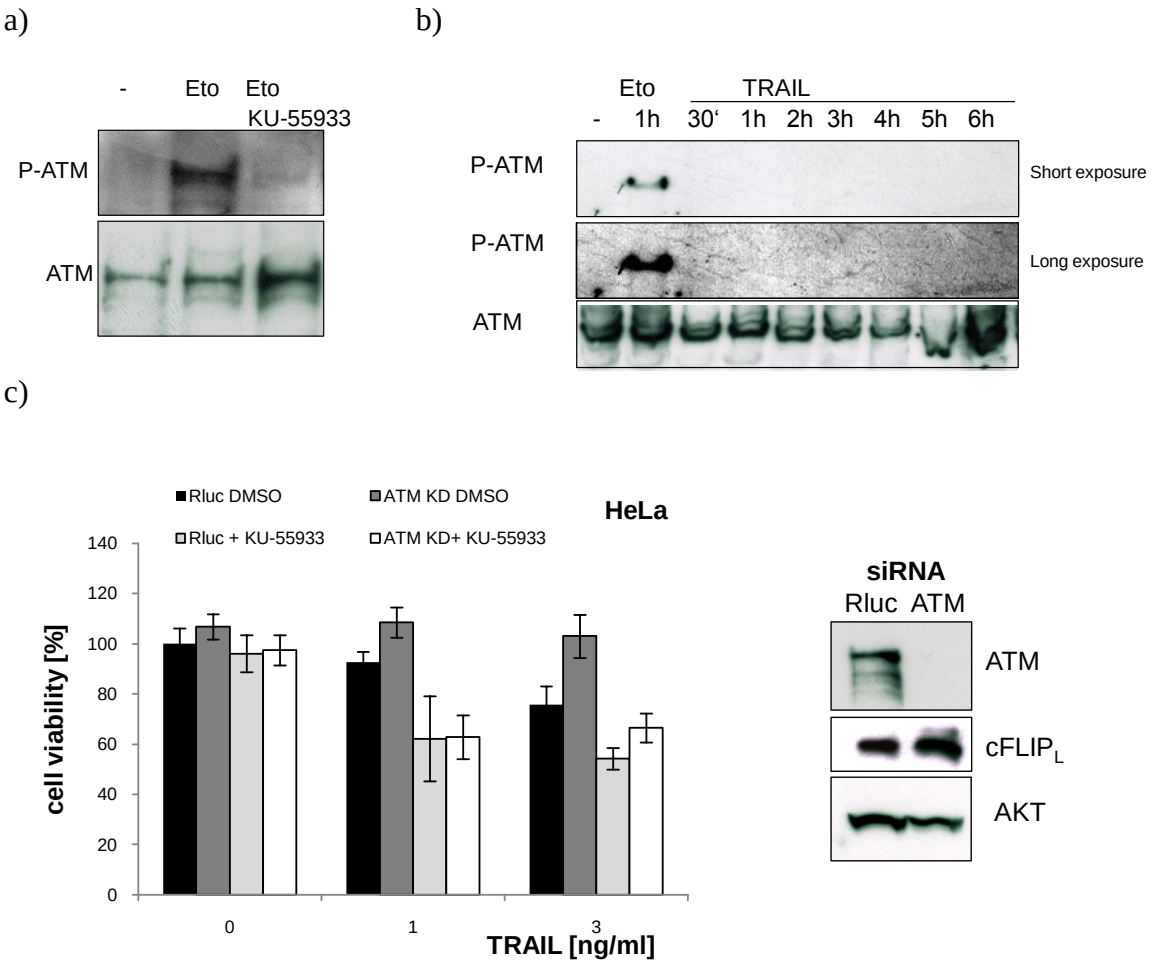


figure 20. The sensitisation to TRAIL-induced apoptosis by KU-55933 is independent of ATM.

(a) HeLa cells were left untreated or stimulated with etoposide (10 µM) or with etoposide and KU-55933 (10 µM) for 1 h. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Tris-Acetate gel. One representative result of three independent experiments is shown. (b) HeLa cells were left untreated, stimulated with etoposide (10 µM) for 1 h as positive control or stimulated with 2 ng/ml iz-TRAIL for the indicated time points. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Tris-Acetate gel. One representative result of three independent experiments is shown. (c) HeLa cells were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or ATM. After 72 h control and ATM KD cells were incubated with 1 ng/ml or 3 ng/ml iz-TRAIL in the presence or absence of KU-55933 (10 µM). Cell viability was quantified by MTT-assay. Efficiency of knockdown was analysed by Western blot. AKT was used as loading control. Values are mean ± SD of three independent experiments.

As already mentioned, ATM becomes activated upon DNA damage. KU-55933 efficiently blocked the autophosphorylation of ATM which is necessary for ATM activation upon DNA damage induced with etoposide (figure 20a). However, TRAIL treatment alone at low concentrations which are sufficient for sensitisation by KU-55933, did not lead to an activation of ATM whereas ATM phosphorylation was detected upon etoposide treatment which was included as positive control (figure 20 b). If sensitisation to TRAIL-induced apoptosis by KU-55993 (figure 17 and figure 19) were due to inhibition of ATM, it could only be facilitated by the inhibition of basal ATM activity. This mechanism was suggested by Ivanov et al. (Ivanov et al., 2009). To test this, ATM was knocked down transiently using siRNA (figure 20c). If the observed effect were due to inhibition of basal ATM activity, a knockdown of ATM should also sensitise cells to TRAIL-induced apoptosis. However, ATM knockdown did not sensitise HeLa cells to TRAIL but rather induced a more resistant phenotype, resembling the situation in AT cells. Accordingly and in line with Stagni *et al.* (Stagni et al., 2008), a slight up-regulation of cFLIP could be detected which might account for this effect (figure 20c). Furthermore, treatment of ATM-knockdown cells with KU-55933 could still sensitise the cells to TRAIL-induced apoptosis indicating that sensitisation to TRAIL-induced cell death mediated by KU-55933 is not due to the inhibition of basal activity of ATM. To corroborate this finding, L6 cells, which are a lymphoblastic cell line isolated from an AT patient and therefore completely lack ATM activity, were analysed. As shown in figure 21 L6 cells could also be sensitised to TRAIL-induced apoptosis by co-treatment with KU-55933. However in this case a pre-incubation with KU-55933 for 8 hours was necessary to observe the sensitising effect of KU-55993. Taken together these results demonstrate that KU-55933-mediated sensitisation to TRAIL-induced apoptosis is independent of ATM inhibition. Instead, they suggest that KU-55933 acts on a target different from ATM to enable TRAIL-induced apoptosis.

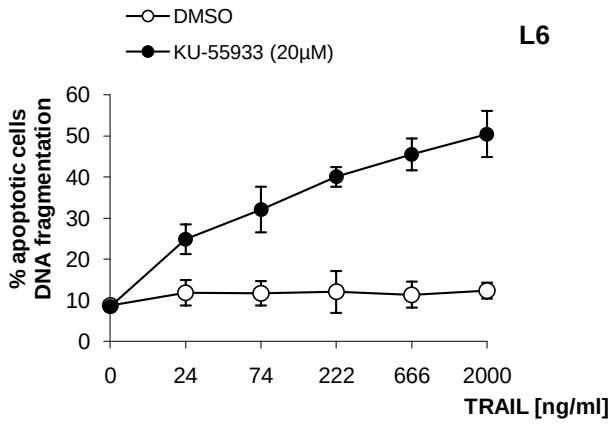


figure 21. KU-55933 sensitises the TRAIL-resistant AT cell line L6 to TRAIL-induced apoptosis.

L6 cells were treated with increasing concentrations of iz-TRAIL with or without pre-incubation with KU-55933 (20 µM) for 8h and analysed for their subdiploid DNA content after 24h. Values are mean ± SD of three independent experiments.

4.7. KU-55933 sensitises to TRAIL-induced apoptosis by inhibiting PI3K p110α

KU-55933 is a kinase inhibitor designed to act as an ATP-competitive inhibitor at the ATP binding site of ATM (Hickson et al., 2004). It is therefore most likely that the cellular target of KU-55933 responsible for sensitisation to TRAIL-induced apoptosis is another kinase. As ATM belongs to the PI3-Kinase related Kinases (PIKK) family it appeared most likely that KU-55933 were to sensitise cells to TRAIL-induced apoptosis by inhibiting one of the different PIKK family members. Interestingly, and in line with this hypothesis, inhibition of PI3 kinase itself was shown to sensitise cells to TRAIL-induced apoptosis (Alladina et al., 2005; Kandasamy and Srivastava, 2002; Opel et al., 2008). Therefore, the effects of down-regulation and inhibition of the four isoforms of the catalytic subunit p110 of PI3 kinase on TRAIL-induced apoptosis were tested. The response to TRAIL was not altered upon knockdown of the subunits β, γ, and δ in comparison to the control (figure 22). In contrast, knockdown of p110α drastically sensitised the cells to TRAIL-induced apoptosis even though knockdown was incomplete.

a)

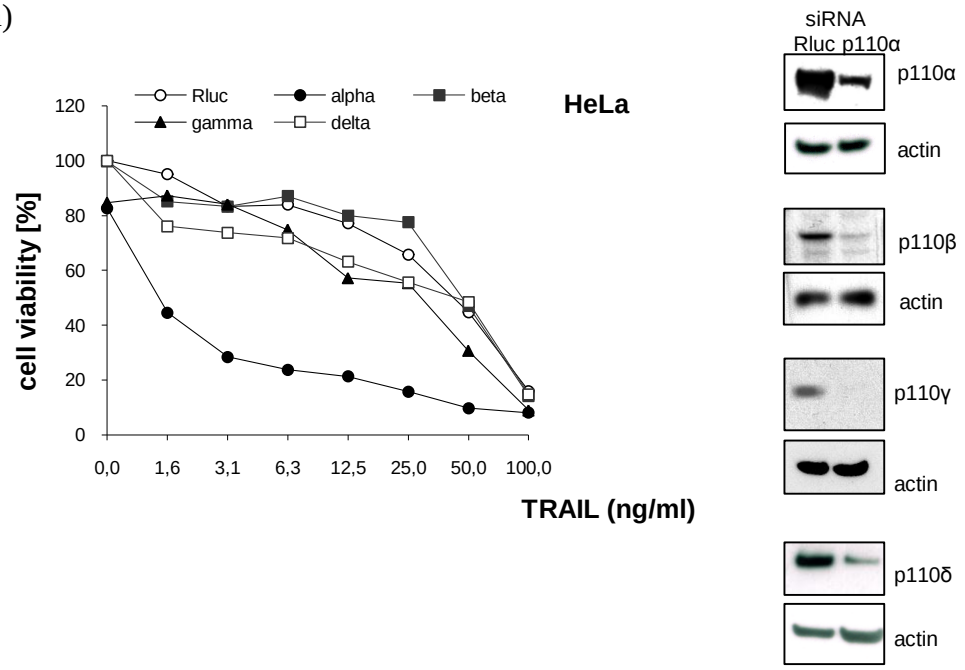


figure 22. HeLa cells can be sensitised to TRAIL-induced apoptosis by knockdown of p110α.

HeLa cells were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or one of the 4 isoforms of PI3K p110 α, β, γ and δ. After 72 hours control and KD cells were incubated with the indicated concentrations of iz-TRAIL. Cell viability was quantified by MTT assay after 24 h. Efficiency of knockdown was analysed by Western Blot. Actin was used as loading control. One representative result of three independent experiments is shown.

As an independent assessment, these isoforms were inhibited pharmacologically using the isoform specific inhibitors PIK75 (inhibits p110α), TGX-221 (inhibits p110β) and As252424 (inhibits p110γ), inhibitors which had previously been used to investigate the role of different isoform of PI3K (Kim et al., 2007). A specific inhibitor for p110δ was not commercially available. To find out whether any of these inhibitors can sensitise to TRAIL-induced apoptosis subtoxic concentration needed to be determined (figure 23a, b and c). In line with the knockdown experiment, only co-treatment with the p110α specific inhibitor PIK75, applied at subtoxic concentrations, led to an increase in TRAIL-induced apoptosis whereas the inhibitors specific for p110β and p110γ, applied at subtoxic concentrations, had no effect (figure 23d).

To test whether KU-55933 might interfere with the PI3K pathway KU-55933 treated cells were investigated for phosphorylation of AKT, which was taken as surrogate for PI3K

activity. KU-55933 was not only able block to ATM activation (figure 20) but also interfered with the PI3K/AKT pathway. It reduced basal AKT phosphorylation already drastically after 30 min. No phosphorylation of AKT was detectable anymore after 1h of treatment (figure 24). PIK75 treatment induced rapid disappearance of Phospho-AKT with some Phospho-AKT becoming detectable again after 1 h (figure 24).

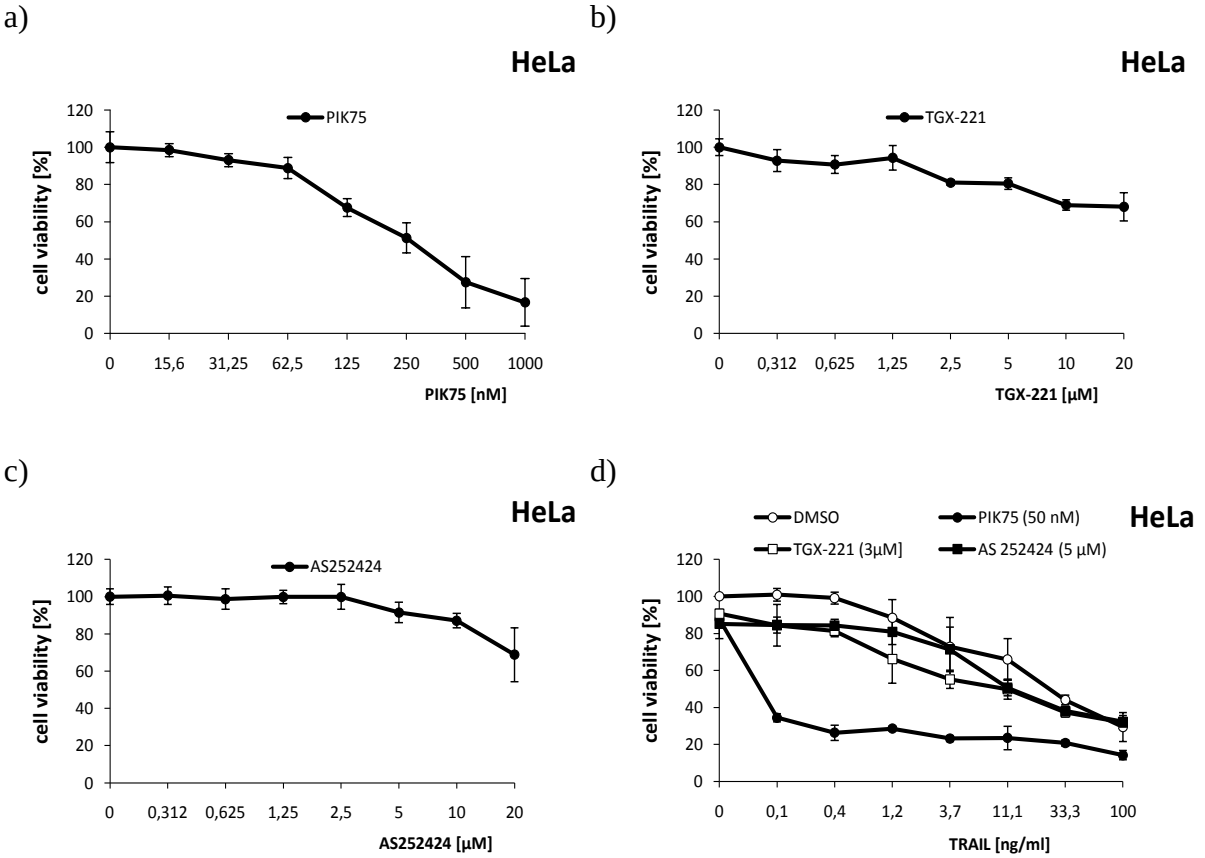


figure 23. HeLa cells can be sensitised to TRAIL- induced apoptosis by the p110 α specific inhibitor PIK75.

(a), (b) and (c) HeLa cells treated with increasing concentration of PIK75, TGX-221 and As252424, respectively for 24 h. Then cell viability was measured by MTT-assay (d) HeLa cells were pre-incubated for 1 h either with DMSO as control, PIK75 (50 nM), TGX-221 (1 µM) or AS 252424 (3 µM). Subsequently, increasing concentrations of iz-TRAIL were added. Cell viability was quantified by MTT-assay after 24 h. Values are mean ± SD of three independent experiments.

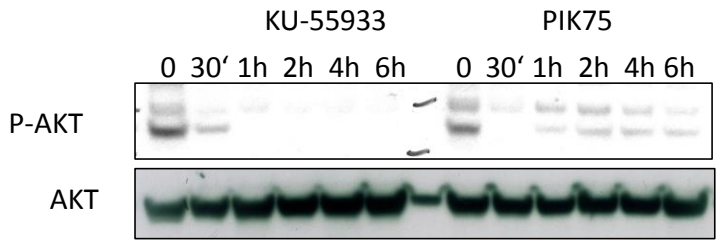


figure 24. KU-55933 inhibits phosphorylation of AKT.

HeLa cells were stimulated with KU-55933 (10 µM) or PIK75 (50 nM) for the indicated time points. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of three independent experiments is shown.

As only the knockdown and inhibition of p110α, and not the other PI3K isoforms, sensitised to TRAIL-induced apoptosis, it seemed likely that KU-55933 worked via inhibition of p110α. To test this kinase assay was performed. In this assay immunoprecipitated p110α was incubated with its substrate PIP_2 and ATP either alone, with PIK75, KU-55933 or TGX-221 which was used as negative control. Subsequently ATP-consumption was measured using the Kinase-Glo® reagent. This reagent generates a luminescent signal which is correlated with the amount of ATP present and inversely correlated with kinase activity. If the kinase is active, ATP will be consumed and the luminescent signal will be low. If the kinase activity is blocked, the ATP will not be consumed resulting in a higher luminescent signal.

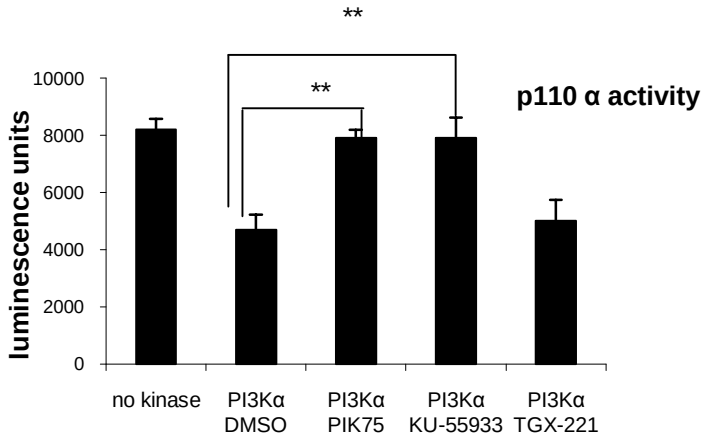


figure 25. KU-55933 directly inhibits PI3 Kinase p110α.

Immunoprecipitated p110α was incubated for 5 min either with DMSO as control, KU-55933 (1 µM), PIK75 (1 µM), or TGX-221 (1 µM) and the substrate PIP2 (1 µg/µl) in kinase buffer. The kinase assay was performed as described in Materials and Methods (section 3.2.3). Values are mean ± SD of three independent experiments.

As shown in figure 25, p110α alone reduced the amount of ATP left in the sample by 50 %. As expected, this ATP-consumption was almost completely blocked by the addition of PIK75. KU-55933 was also able to significantly block kinase activity almost to the same extent as PIK75. In contrast to this, TGX-221 did not significantly affect the activity of p110α. This result shows that KU-55933 is able to interfere with the PI3K pathway via direct inhibition of p110α.

DLD1 cells are chemotherapy resistant and have an activating mutation in the PIK3CA gene and are therefore hallmarked by strong activation of the PI3K/AKT pathway (Samuels et al., 2005). Remarkably, these cells can also be sensitised to TRAIL-induced apoptosis by co-treatment with PIK75 (figure 26a), as PIK75 acts as an ATP- competitive inhibitor of p110α. Additionally, their long-term survival was reduced as only very few clones survived treatment with TRAIL in combination with PIK75 (figure 26b).

a) b)

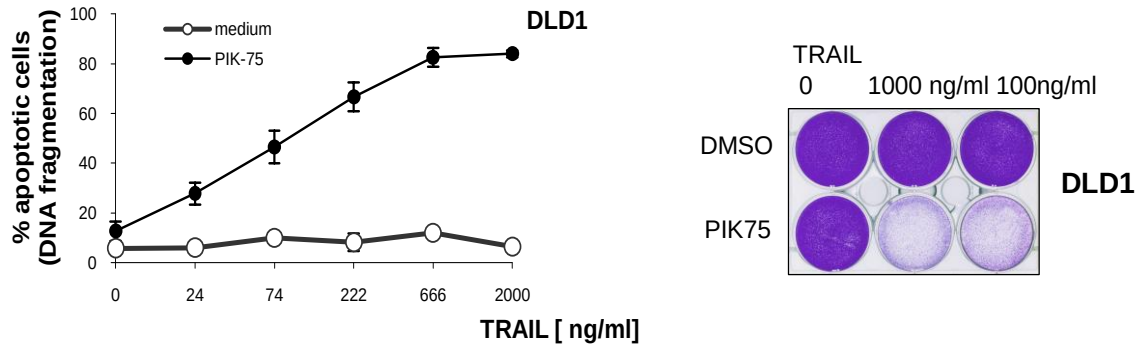


figure 26. PIK75 and TRAIL co-treatment sensitises TRAIL-resistant DLD1 cells to TRAIL-induced apoptosis and reduces clonogenic survival.

(a) DLD1 cells were treated with increasing concentrations of iz-TRAIL with or without preincubation with PIK75 (100 nM) and analysed for their subdiploid DNA content after 24 h. Values are mean ± SD of three independent experiments. (b) DLD1 cells were treated with either DMSO or PIK75 (100 nM) alone or in combination with increasing concentrations of iz-TRAIL for 24 h. Dead cells were washed away and fresh medium was added every second day. Cell viability was visualized by crystal violet at day 5. One representative of three independent experiments is shown.

4.8. Molecular changes facilitating TRAIL sensitisation by KU-55933/PIK75

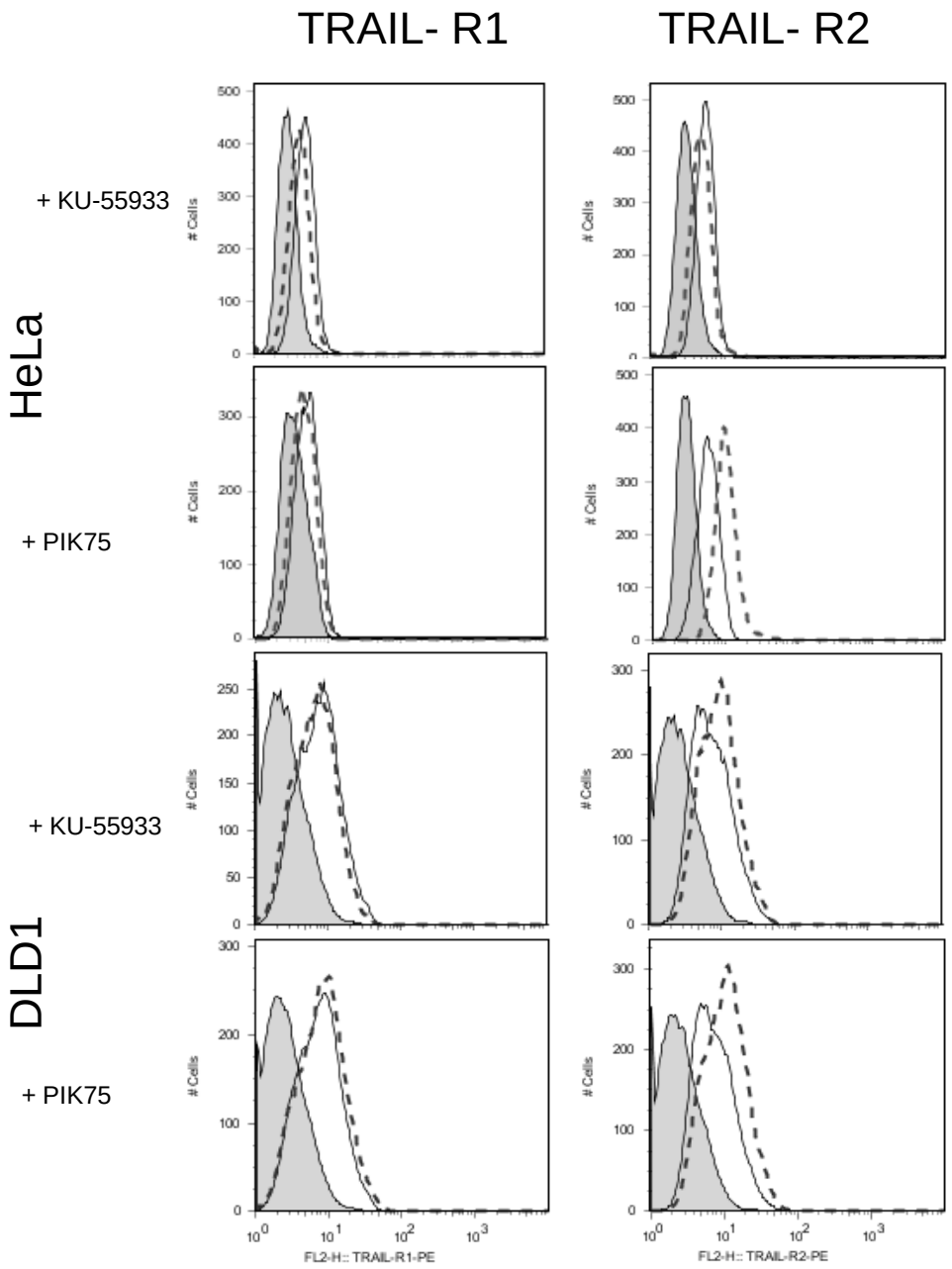
So far it has been demonstrated that KU-55933-mediated sensitisation to TRAIL-induced apoptosis works via the inhibition of PI3K p110α. However, the mechanism underlying this sensitisation has not been investigated. Regulation of the TRAIL-Rs as well as intracellular factors might be responsible for the sensitisation. Often, sensitisation to TRAIL-induced apoptosis correlates with up-regulation of TRAIL-R1 and TRAIL-R2. An up-regulation of TRAIL-R2 upon inhibition of the PI3K pathway was reported by two independent studies (Rychahou et al., 2005; Tazzari et al., 2008). Ivanov *et al.* (2009) also claimed that up-regulation of TRAIL-R2 was important for KU-55933-mediated sensitisation to TRAIL-induced apoptosis. Thus, it was examined whether TRAIL-receptors become up-regulated upon KU-55933 treatment and whether this is necessary for sensitisation to TRAIL- induced cell death in this cell system.

TRAIL-Rs were stained using specific antibodies and analysed using flow cytometry. In HeLa cells, KU-55933 treatment did not enhance but even slightly decreased the surface expression of TRAIL-R1 and TRAIL-R2 (figure 27a). Treatment with PIK75 also led to a slight down-regulation of TRAIL-R1 but at the same time enhanced surface expression of TRAIL-R2. Treatment with KU-55933 or PIK75 did not change the surface expression of TRAIL-R1 in TRAIL-resistant DLD1 cells. However the surface expression of TRAIL-R2 is slightly up-regulated by both treatments. TRAIL-R3 and TRAIL-R4 could not be detected on the surface of untreated or sensitised HeLa or DLD1 cells (data not shown). As an up-regulation of TRAIL-R2 was observed on DLD1 cells after KU-55933 or PIK75 treatment, it was investigated whether this up-regulation was indeed the reason for the sensitisation to TRAIL-induced apoptosis by KU-55933 and PIK75.

To asses this, a “wash kill” experiment was performed as previously described by our laboratory (Ganten et al., 2005). If up-regulation of TRAIL-R2 by KU-55933 or PIK75 was responsible for sensitisation, binding of additional TRAIL to these new TRAIL-Rs on the cell surface would be necessary for apoptosis induction. DLD1 were incubated with TRAIL for 30 min to occupy all present TRAIL-receptors on the cells surface. Unbound TRAIL was washed off after 30 min and cells were treated with KU-55933 or PIK75, either alone or with additional TRAIL. As shown in figure 27b, no significant differences in TRAIL-induced

apoptosis were observed between KU-55933- or PIK75- treated cells when unbound TRAIL was removed and not replaced and cells which were further incubated in the presence of TRAIL. Thus, receptor up-regulation is not essential for the sensitisation of DLD1 cells to TRAIL-induced apoptosis.

a)



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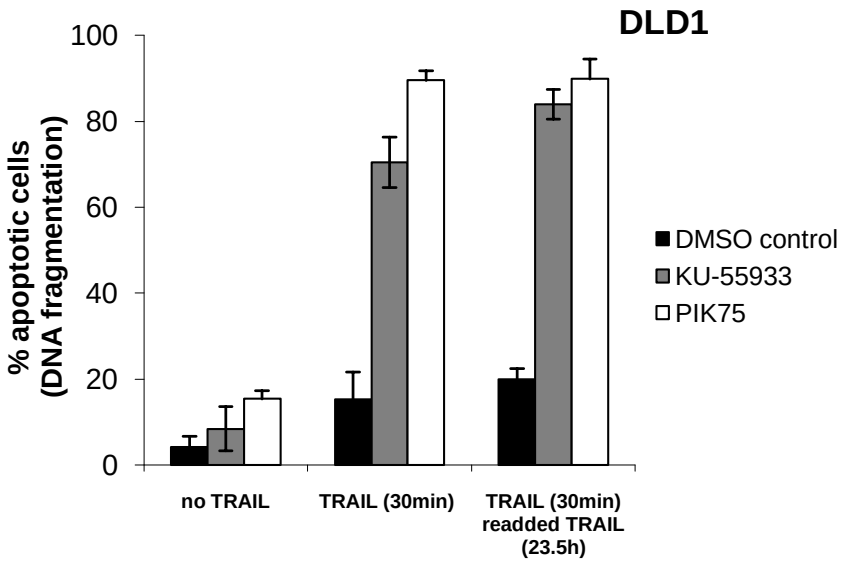


figure 27. Surface expression of TRAIL-R1 and TRAIL-R2 changes upon KU-55933 or PIK75 treatment but is not essential for sensitisation.

(a) Surface expression analysis of TRAIL-R1 and TRAIL-R2 on HeLa and DLD1 cells was performed 1 h after treatment with DMSO, KU-55933 or PIK75 inhibitor in comparison to an isotype-matched control mIgG1 monoclonal antibody (tinted grey). TRAIL-R expression of control treated cells is shown as black solid line. TRAIL-R expression of KU-55933 or PIK75 treated cells is shown as grey dashed line. Only PI–negative cells were counted, to exclude non-specific staining of dead cells,. One representative result out of 3 independent experiments is shown. (b) DLD1 cells were incubated with 1 µg/ml iz-TRAIL for 30 min and then washed 5 times with medium. Cells were then cultured either in medium containing KU-55933 (20 µM) or PIK75 (100 nM) in the absence or in the presence of 1 µg/ml iz-TRAIL. Control cells were washed 5 times without any addition of TRAIL. After 24 h cells were analysed for their subdiploid DNA content. One of three experiments with comparable results is shown.

As the up-regulation of TRAIL-Rs turned out not to be essential for KU-55933 and PIK75 mediated sensitisation to TRAIL-induced apoptosis, intracellular components have to be responsible for the observed effect. Therefore, the regulation of known components of the death receptor pathway upon treatment with KU-55933 and PIK75 was investigated by Western Blot (figure 28).

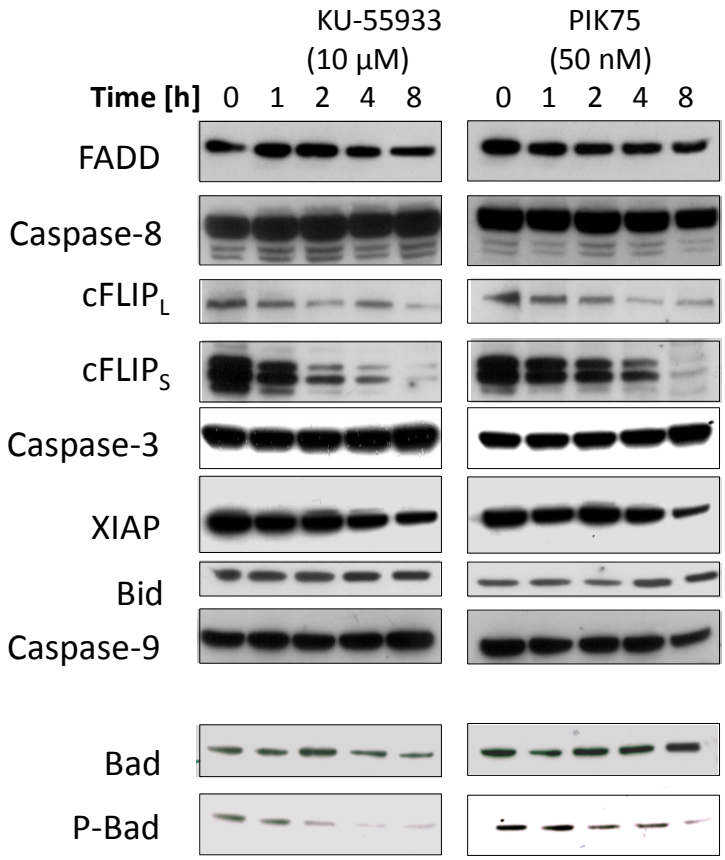


figure 28. Treatment with KU-55933 and PIK75 leads to a down-regulation of cFLIP and XIAP in HeLa cells.

HeLa cells were stimulated with KU-55933 (10 µM) or PIK75 (50 nM) for the indicated time points. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of two independent experiments is shown.

Expression of FADD, caspase-8, Bid, caspase-9 and caspase-3 remained unchanged upon stimulation with KU-55933 or PIK75. However, cFLIP$_L$ and cFLIP$_S$ as well as XIAP were down-regulated upon stimulation with either KU-55933 or PIK75. Furthermore, a reduction in the phosphorylation of Bad could be detected, which has been described before to take place upon inhibition of the PI3K-pathway (Kang et al., 2004). Down-regulation of XIAP expression was mediated by transcriptional regulation as qPCR analysis showed a decrease of mRNA expression for XIAP following treatment with KU-55933 or PIK75 (figure 29). mRNA levels for cFLIP were also down-regulated upon KU-55933 treatment, whereas in the case of PIK75 treatment even a slight increase in the levels of cFLIP mRNA was detected. As the protein was clearly down-regulated upon stimulation with KU-55933 and PIK75 (figure

28), cFLIP expression seems to be regulated rather at the post-transcriptional level, possibly by enhanced ubiquitination and degradation (Poukkula et al., 2005) .

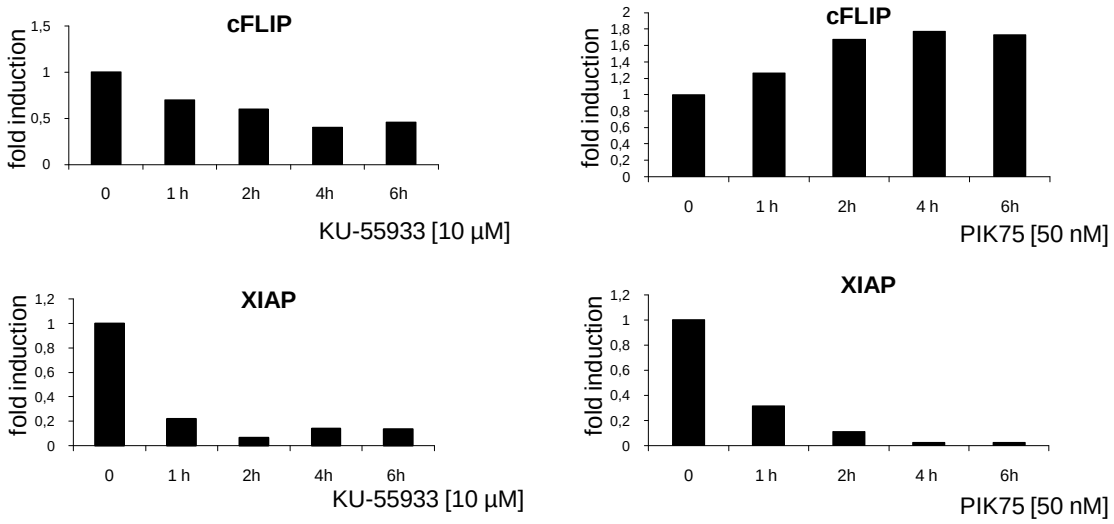


figure 29. Expression of XIAP is down-regulated on mRNA level upon treatment with KU-55993 and PIK-75.

HeLa cells were stimulated with KU-55933 (10 µM) or PIK75 (50 nM) for the indicated time points. RNA was isolated and the expression of cFLIP and XIAP was analysed by qPCR. GAPDH was used for normalisation. One of two experiments with comparable results is shown.

As cFLIP levels were slightly up-regulated upon ATM knockdown (figure 20), the observed down-regulation of cFLIP by KU-55933 could only be explained if an inhibition of p110α at the same time overrode this effect. Indeed, this seems to be the case as shown in figure 30. HeLa cells in which p110α was knocked down clearly showed a decrease of $cFLIP_L$. This effect could also be observed in HeLa cells in which p110α and ATM were silenced at the same time. In contrast to this a knockdown for ATM alone again shows a slight up-regulation of $cFLIP_L$ when compared to control cells.

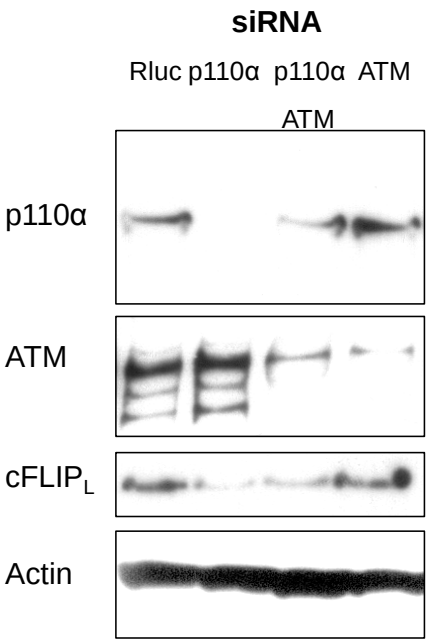


figure 30. Concomitant Knockdown of p110α and ATM leads to down-regulation of cFLIP.

HeLa cells were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or p110α, ATM or p110α and ATM. After 72 h control cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of two independent experiments is shown.

As a reduction in cFLIP levels possibly leads to a difference in the formation or composition of the DISC, analysis of the native DISC was performed in DLD1 cells with and without pre-treatment with KU-55933 or PIK75. To control for differential expression of caspase-8, FADD and cFLIP total cell lysates (TCL) were analysed for the respective proteins (figure 31).

KU-55933- and PIK75-treated cells recruited slightly less cFLIP to the DISC. This resulted in increased caspase-8 cleavage, with the p43/41 cleavage fragments only being detectable in KU-55933- and PIK75-treated cells.

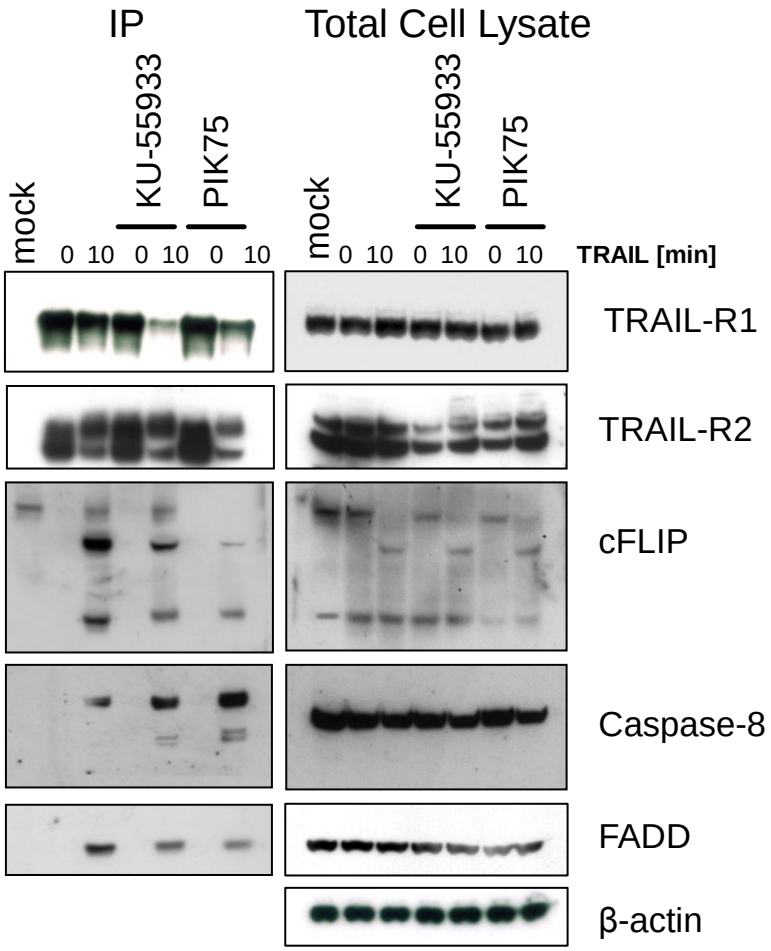


figure 31. Treatment with KU-55933 or PIK75 leads to stronger DISC formation in DLD1 cells.

DLD1 cells were pre-treated with KU-55933 and PIK75 for 4 h and either stimulated with 1 µg/ml FLAG-TRAIL for 10 min or left unstimulated before cell lysis. TRAIL was added to unstimulated control lysates at a final concentration of 1 µg/ml. To check for unspecific binding to the beads a negative control containing beads only (Mock) was included. Expression of TRAIL-R1, TRAIL- R2, caspase-8, FADD/MORT1, and cFLIP, lysates was analysed by Western blot. Actin was included as loading control. One representative result out of at least two independent experiments is shown.

As the changes at the DISC, albeit detectable, are not very dramatic, the down-regulation of cFLIP is probably not the only factor which is essential for the sensitisation. To investigate at which other stage the TRAIL-apoptosis pathway was influenced by KU-55933 or PIK75 treatment, DLD1 cells were treated with TRAIL alone or in combination with KU-55933 or PIK75 and subjected to Western blot analysis. DLD1 cells do not only show a quicker and enhanced cleavage of caspase-8 and caspase-9 when co-treated with TRAIL and KU-55933 or PIK75, respectively, but also a stronger activation of caspase-3 (figure 32). Although

caspase-3 levels are slighty lower in KU-55933 treated samples, the fully activated caspase-3 cleavage fragment p12 is only detectable in KU-55933 or PIK75 co-treated samples. As this final cleavage step is essential for the protease activity of caspase-3, cleavage of the caspase-3 substrate PARP can also only be observed in these samples. XIAP inhibits the full activation of caspase-3 (Riedl et al., 2001), which has been shown to be down-regulated on protein and mRNA levels upon treatment with KU-55933 and PIK75 (figure 28 and figure 29). Hence, XIAP is most likely the factor inhibiting full cleavage and activation in DLD1 cells which are treated with TRAIL alone.

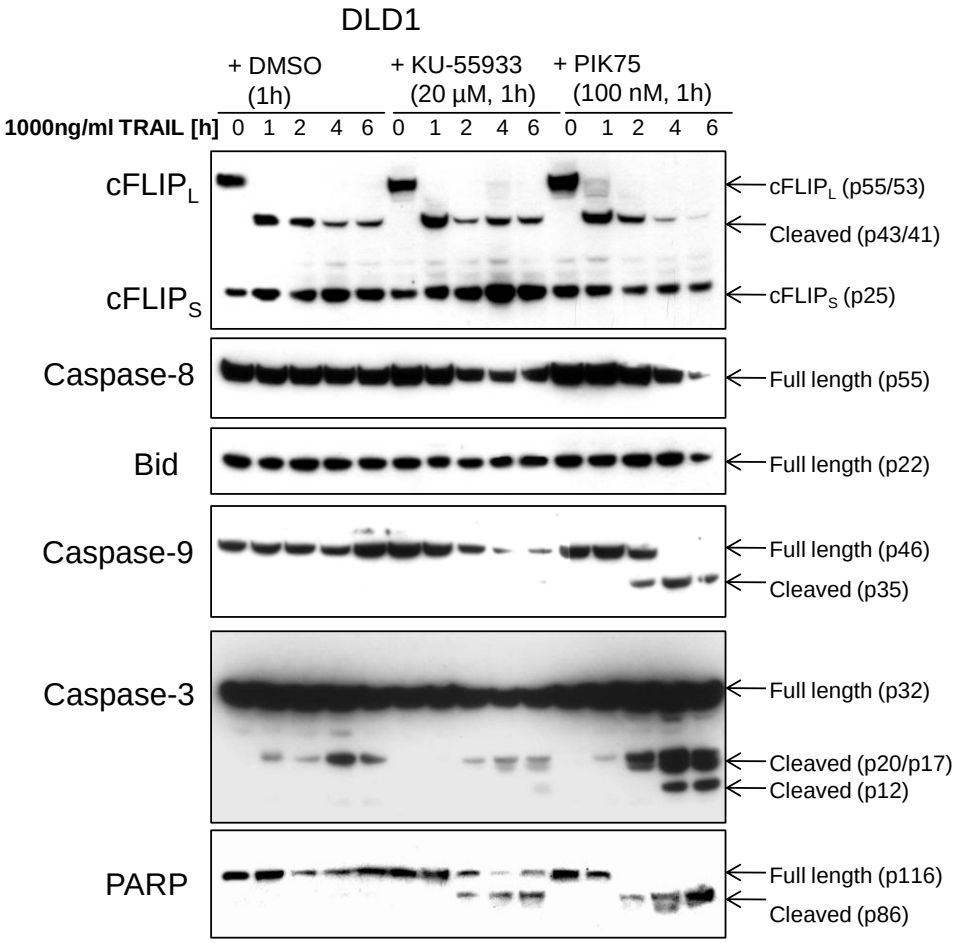
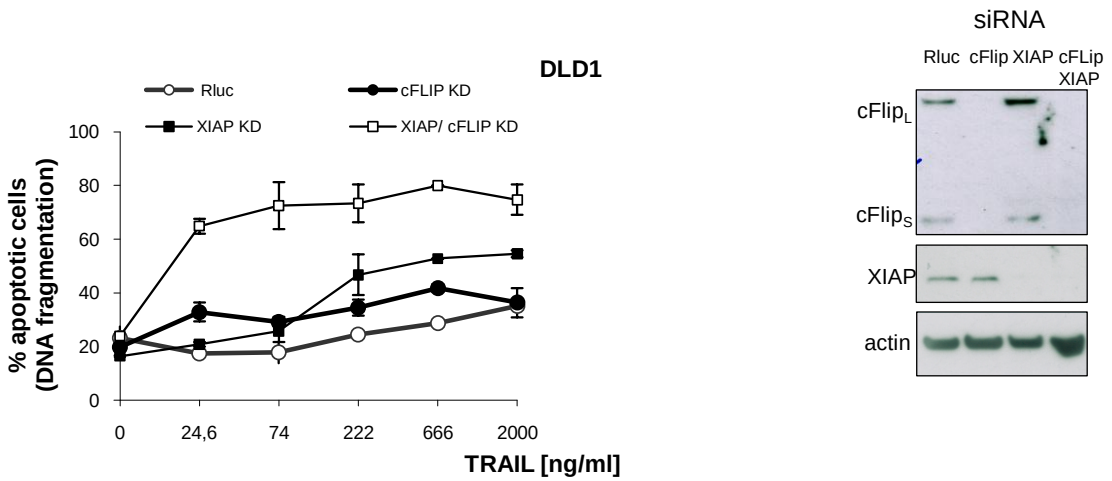


figure 32. Full activation of caspase-3 and PARP cleavage is only detectable in cells which are co-treated with TRAIL and KU-55933 or TRAIL and PIK-75, respectively.

HeLa cells were stimulated with 1µg/ml iz-TRAIL for the indicated time points after 1 h preincubation with DMSO, KU-55933 (20 µM) or PIK75 (100 nM). Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of two independent experiments is shown.

a)



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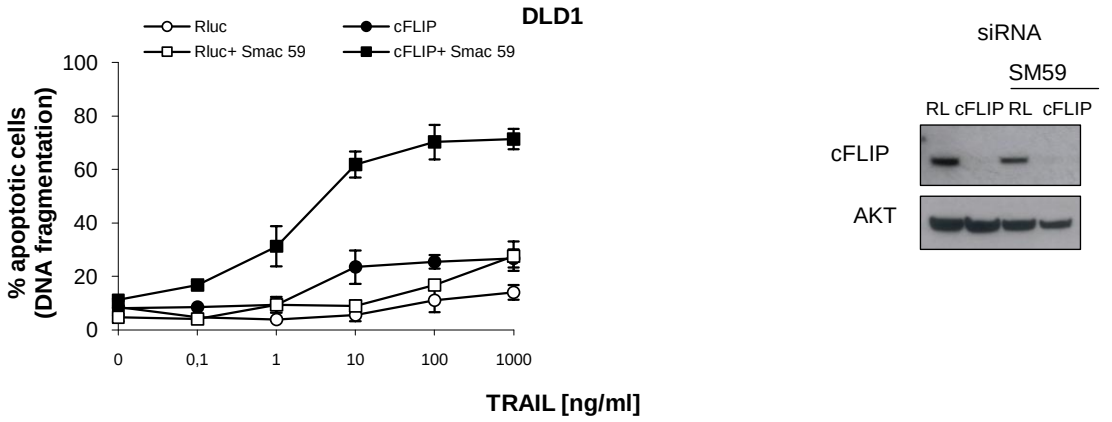


figure 33. Concomitant down-regulation of cFLIP and XIAP is sufficient to sensitise DLD1 cells to TRAIL-induced apoptosis.

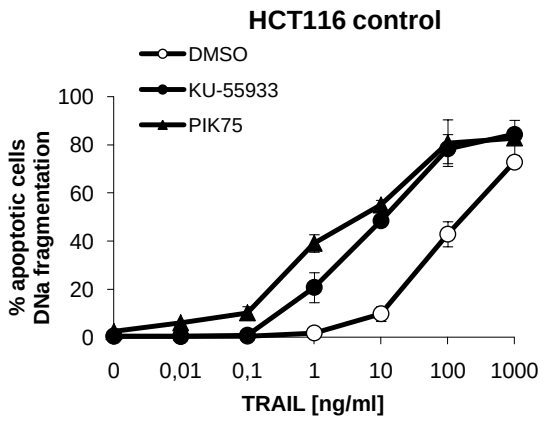
(a) DLD1 cells were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or cFLIP, XIAP or cFLIP and XIAP. After 48 h control and KD cells were incubated with increasing concentrations of iz-TRAIL for 24 h and then analysed for subdiploid DNA content. (b) DLD1 cells were transfected with siRNA either targeting Rluc as control or cFLIP. After 48 h control and KD cells were incubated with or without SMAC59 (10 nM) and increasing concentrations of iz-TRAIL for 24 h and then analysed for their subdiploid DNA content. Values are mean ± SD of three independent experiments. The efficiency of the knockdown of the different proteins was controlled by Western blot.

To evaluate the importance of the two factors cFLIP and XIAP concerning the sensitisation to TRAIL-induced apoptosis, the expression of cFLIP, XIAP or cFLIP and XIAP was silenced using siRNA in DLD1 cells (figure 33a). Knockdown of either cFLIP or XIAP alone was not

sufficient to sensitise DLD1 cells to TRAIL-induced apoptosis. However, concomitant knockdown of both proteins efficiently sensitised DLD1 cells to apoptosis. Accordingly, DLD1 cells could also be sensitised by knockdown of cFLIP in combination with SMAC mimetics which block XIAP (figure 33b).

These results clearly show that down-regulation of cFLIP and XIAP both is sufficient and necessary for sensitisation of DLD1 cells to TRAIL-induced apoptosis. However, a contribution of pro- or anti-apoptotic factors that act on the mitochondria and are regulated upon KU-55933/PIK75 treatment cannot be excluded. For example a regulation of phosphorylation of Bad has been observed in KU-55933 and PIK75 treated cells (figure 28)To test for a potential involvement of the mitochondria in the sensitisation mediated by KU-55933 and PIK75, HCT116 Bax-/- cells were used in which Bak was additionally knocked down to completely take out the action of the mitochondria. HCT116 control cells are TRAIL sensitive but can further be sensitised by co-treatment with KU-55933 or PIK-75 (figure 34a). In contrast to this, TRAIL-induced apoptosis is completely blocked in HCT116 Bax-/- Bak KD (figure 34b). However these cells can still be sensitised by co-treatment with KU-55933 or PIK75, almost to the same extent as the control cells. This indicates that regulation of pro-apoptotic mitochondrial events only marginally contributes to sensitisation by KU-55933 or PIK75 and that they are in fact not required for it.

a)



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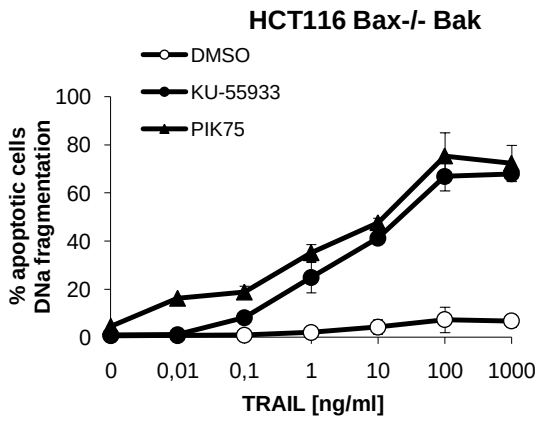

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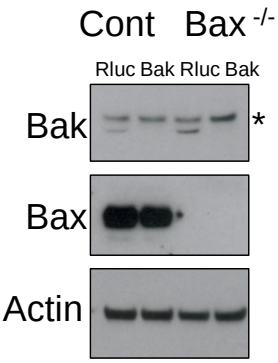


figure 34. Regulation of pro- or anti-apoptotic factors on mitochondrial levels is only marginally involved in the sensitisation to TRAIL by KU-55933 or PIK75.

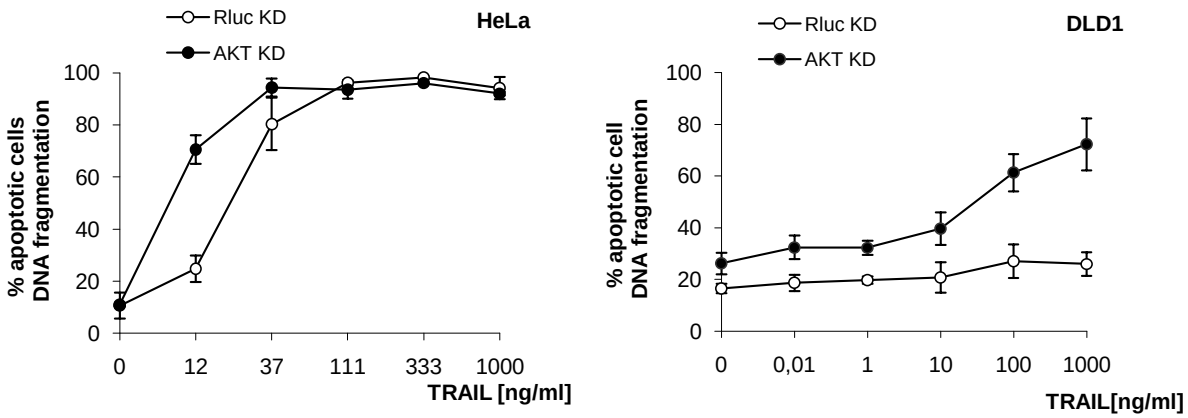
(a), (b) HCT116 control cells and HCT116 Bax-/- Bak KD cells were incubated with increasing concentrations of iz-TRAIL for 24 h and then analysed for their subdiploid DNA content. Values are mean ± SD of three independent experiments. (c) As control for the knockdown lysates of the control and KD cells were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western Blot. KD efficiency of Bak was controlled by Western blot. The asterix indicates an unspecific band. One representative result out of two independent experiments is shown.

4.9. Down-regulation of cFLIP and XIAP downstream of AKT is facilitated by activation of FoxO3a

Inhibition of PI3 kinase leads to inhibition of the kinase AKT. Consequently, knockdown of AKT1 can also sensitise HeLa and DLD1 cells to TRAIL-induced apoptosis (figure 35).

However, knockdown of AKT1 cannot sensitise DLD1 cells to the same extent as inhibition of p110 α (figure 26).

a) b)



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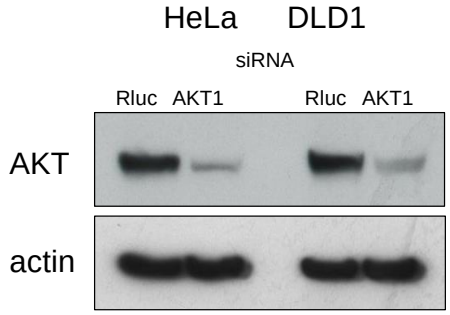


figure 35. HeLa cells and DLD1 cells can be sensitised to TRAIL-induced apoptosis by knockdown of AKT1.

HeLa cells (a) or DLD1 cells (b) were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or AKT1. After 72 h control and KD cells were incubated with 0.01 -100 ng/ml iz-TRAIL and then analysed for their subdiploid DNA content. Values are mean ± standard deviation of two independent experiments. (c) Efficiency of knockdown was analysed by Western blot. Actin was used as loading control. One representative result of two independent experiments is shown.

This suggests that the inhibition of the other AKT isoforms - AKT2 and AKT3 might also be involved in sensitising cells to TRAIL-induced apoptosis. These different isoforms have been described to have redundant or non-redundant functions depending on the context. Active AKT regulates cell survival, cell-cycle progression, cell growth and cell metabolism through the phosphorylation of a diverse set of substrates. So far it is not clear which signalling cascade triggered by AKT is responsible for mediating TRAIL resistance in HeLa and DLD1

cells. Therefore, the substrates with the best known relevance to human cancer have been investigated concerning their influence on TRAIL-sensitivity, namely GSK3, FOXO and mTOR signalling.

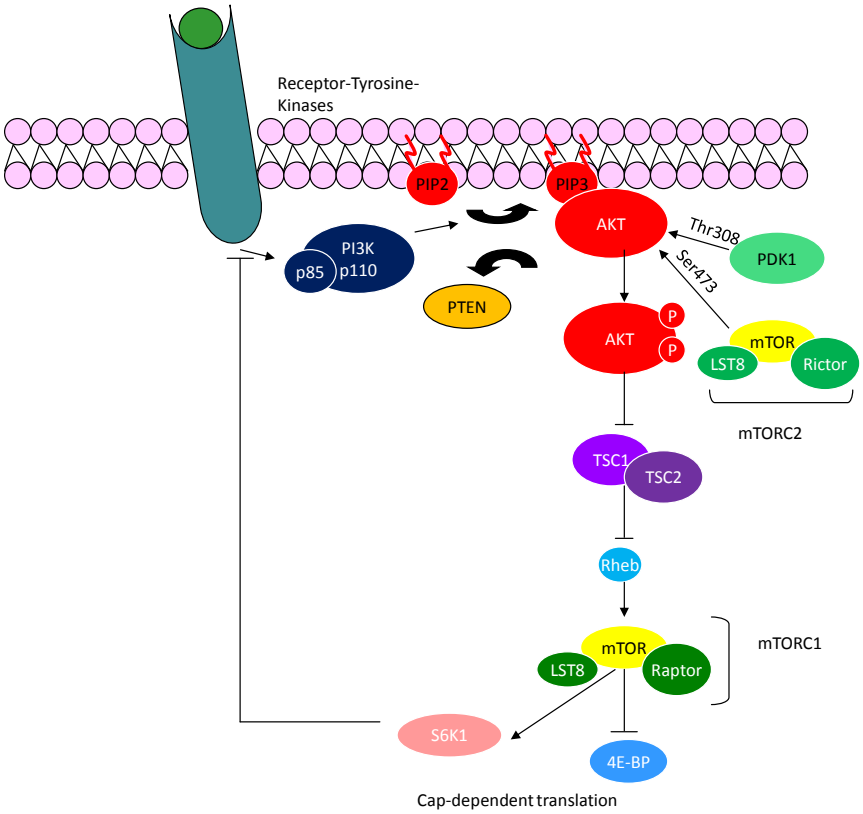


figure 36. mTOR signalling to translation initiation.

Growth factors trigger activation of PI3K and AKT (as described before). mTOR in a complex with LST8 and Rictor can act as PDK2 and phosphorylate AKT at S473. AKT phosphorylates TSC2 and destabilises the TSC1/TSC2 complex and thus promotes the activation of mTOR by Rheb. mTOR in complex with LST8 and Raptor meditates phosphorylation of S6K1 and 4E-BP which in turn induce Cap-dependent translation. Adapted from Mamane et al. (Mamane et al., 2006).

Signalling by mTOR regulates Cap-dependent translation. Active AKT indirectly activates mTOR via phosphorylation. An overview about mTOR signalling is shown in figure 36. As expected inhibition of AKT using PIK75 leads to a loss of phosphorylation of mTOR (figure 37).

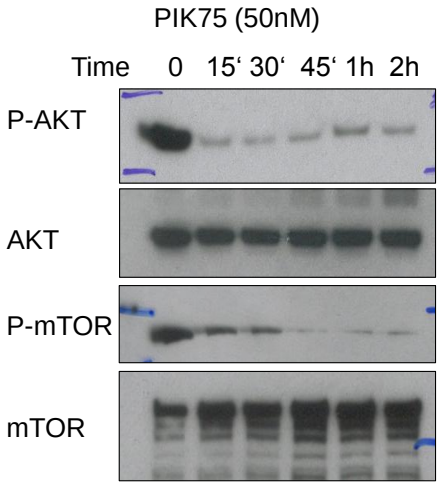
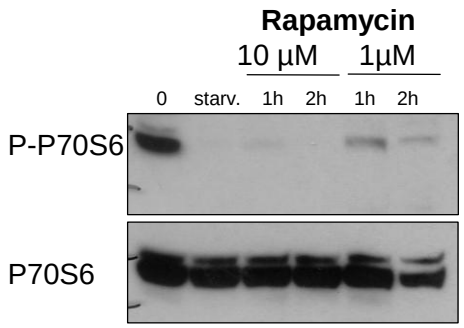


figure 37. Inhibition of p110α by PIK75 leads to loss of phosphorylation of mTOR.

HeLa cells were stimulated with PIK75 (50 nM) for the indicated time points. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of three independent experiments is shown.

If the observed sensitisation to TRAIL using KU-55933 or PIK75 worked via the inhibition of the mTOR pathway, then inhibition of mTOR would be expected to sensitise the cells to TRAIL as well. mTOR is part of two different and mutually exclusive mTOR complexes referred to as mTOR complex (mTORC)1 and mTORC2. The well-described inhibitor of the mTOR Complex 1 rapamycin was used to study the effects of mTOR inhibition on TRAIL-induced apoptosis. The phosphorylation of the downstream target of mTOR P70S6 was used as surrogate for mTOR activity. Starvation, used as a positive control, led to a complete loss of phosphorylation of P70S6 (figure 38a). Similarly, a concentration of 10 µM rapamycin was sufficient to inhibit mTOR activity in HeLa and DLD1 cells (figure 38c). Although mTOR is clearly efficiently inhibited at 10 µM rapamycin, the treatment was not toxic at this concentration in HeLa and DLD1 cells (figure 38b, d). Furthermore co-treatment with rapamycin and TRAIL did not result in sensitisation to TRAIL-induced apoptosis in HeLa cells and DLD1 cells (figure 38b, d).Thus, it is not very likely that KU-55933 or PIK75 exert their TRAIL-sensitising effects via the inhibition of mTORC1.

a)



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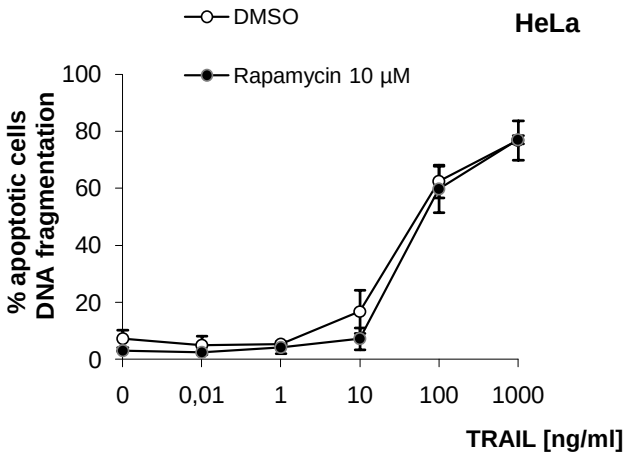

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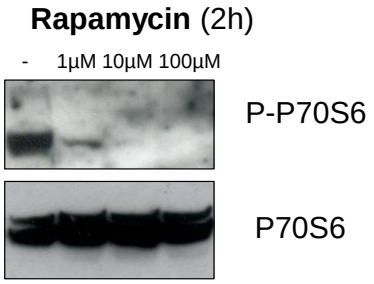

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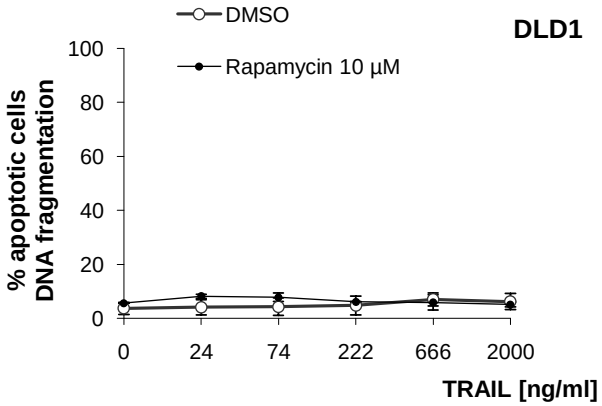


figure 38. Treatment with rapamycin leads to inhibition of mTOR activity but does not sensitise to TRAIL-induced apoptosis.

(a,c) HeLa or DLD1 cells were stimulated with rapamycin at the indicated concentrations and for the indicated times. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of two independent experiments is shown.(b,d) HeLa or DLD1 cells were treated with increasing concentrations of iz-TRAIL with or without preincubation with rapamycin (10 µM) and analysed for their subdiploid DNA content after 24h. Values are mean ± standard deviation of three independent experiments.

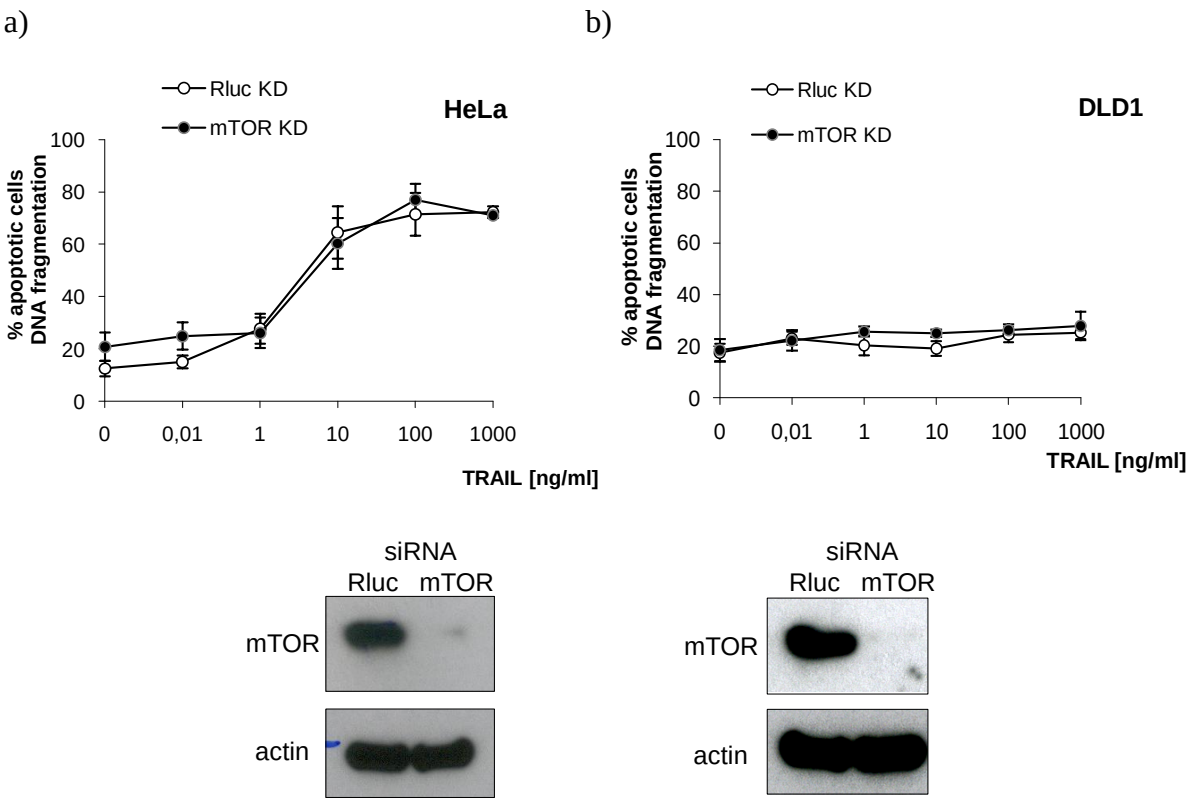


figure 39. Knockdown of mTOR does not sensitise to TRAIL- induced apoptosis.

(a,b) HeLa or DLD1 cells were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or mTOR. After 48 h control and KD cells were incubated with increasing concentrations of iz-TRAIL for 24 h and then analysed for subdiploid DNA content. Values are mean ± SD of two independent experiments. The efficiency of the knockdown of the different proteins was controlled by Western blot.

However, as rapamycin only inhibits mTORC1 there is still the possibility that mTORC2 could be involved in the sensitisation process. To test this, mTOR was knocked down in HeLa and DLD1 cells, which equally affects mTORC1 and mTOR2. However, although the knockdown of mTOR was very efficient in both cell lines as shown in figure 39, neither cell line was sensitised to TRAIL by knockdown of mTOR. Taken together, these results suggest that PIK75 and KU-55933 most likely exert their TRAIL-sensitising effects independently of the mTOR pathway. Besides mTOR, AKT regulates GSK3 signalling. Active AKT phosphorylates GSK3 and thereby inhibits it. When the AKT pathway is inhibited GSK3 becomes active. GSK3 is involved in different physiologically pathways itself- ranging from

metabolism, cell cycle, gene expression, development to oncogenesis. The regulation of GSK3 by the PI3K/AKT pathway is depicted in figure 40. One of its major functions is the regulation of Wnt signalling. Together with APC and Axin active GSK3 forms the ß-catenin destruction complex and blocks ß-catenin translocation to the nucleus. The phosphorylation of GSK3 was not as strongly affected by inhibition of the PI3K/AKT pathway as for example phosphorylation of mTOR (figure 37). Nevertheless, phosphorylation of GSK3 was diminished after 15-45 min of PIK75 treatment before it got back to basal levels after 1 h (figure 41). Although GSK3 phosphorylation is not that strongly diminished, it is still possible that the reduction of GSK3 phosphorylation has an effect on TRAIL sensitivity. If the activation of GSK3 via inhibition of AKT were to be responsible for TRAIL sensitisation via KU-55933 and PIK75, knockdown of GSK3 would be expected to block the sensitising effects of KU-55933 and PIK75.

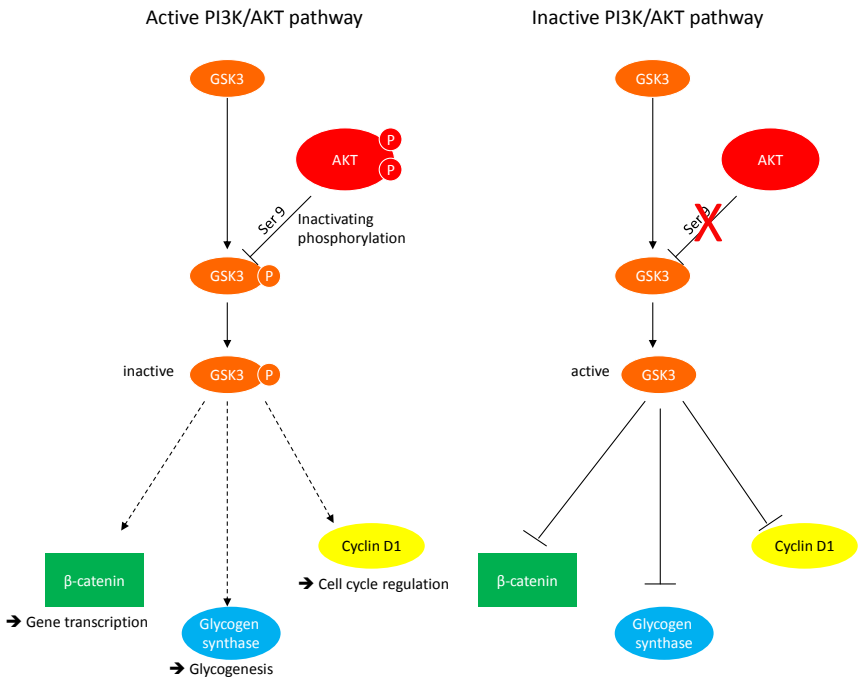


figure 40. Regulation of GSK3 activity by the PI3K/AKT pathway.

Both isoforms of GSK3 are constitutively active in resting cells. But their actions are tightly controlled. Inhibitory phosphorylation sites are present in both isoforms. Active AKT phosphorylates GSK3 at these inhibitory phosphorylation sites. Inhibition of GSK3 activity induces gene transcription via β-catenin, the cell cycle progression and glycogenesis. Is the PI3K/AKT pathway inhibited, GSK3 is active and is responsible for degradation of β-catenin and inhibition of Glycogen Synthase and Cyclin D. Adapted from Beurel and Jope (Beurel and Jope, 2006).

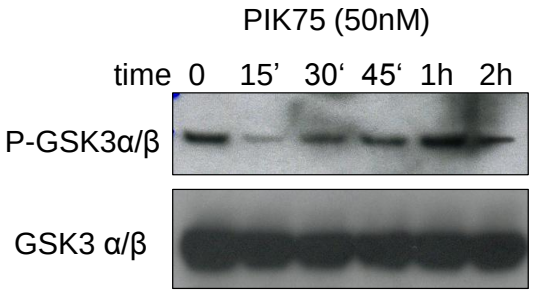


figure 41. Inhibition of p110α by PIK75 leads to diminished phosphorylation of GSK3.

HeLa cells were stimulated with PIK75 (50 nM) for the indicated time points. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of three independent experiments is shown.

As shown in figure 42 knockdown of both of the GSK3 isoforms either alone or together was not sufficient to block KU-55933 or PIK75-induced sensitisation to TRAIL-induced apoptosis. Remarkably, silencing of the GSK3β isoforms rather induces a slight sensitisation to TRAIL-induced apoptosis. Taken together, these data indicate that activation of GSK3 via inhibition of the PI3K/AKT pathway is also not involved in the sensitisation to TRAIL mediated by KU-55933 or PIK75.

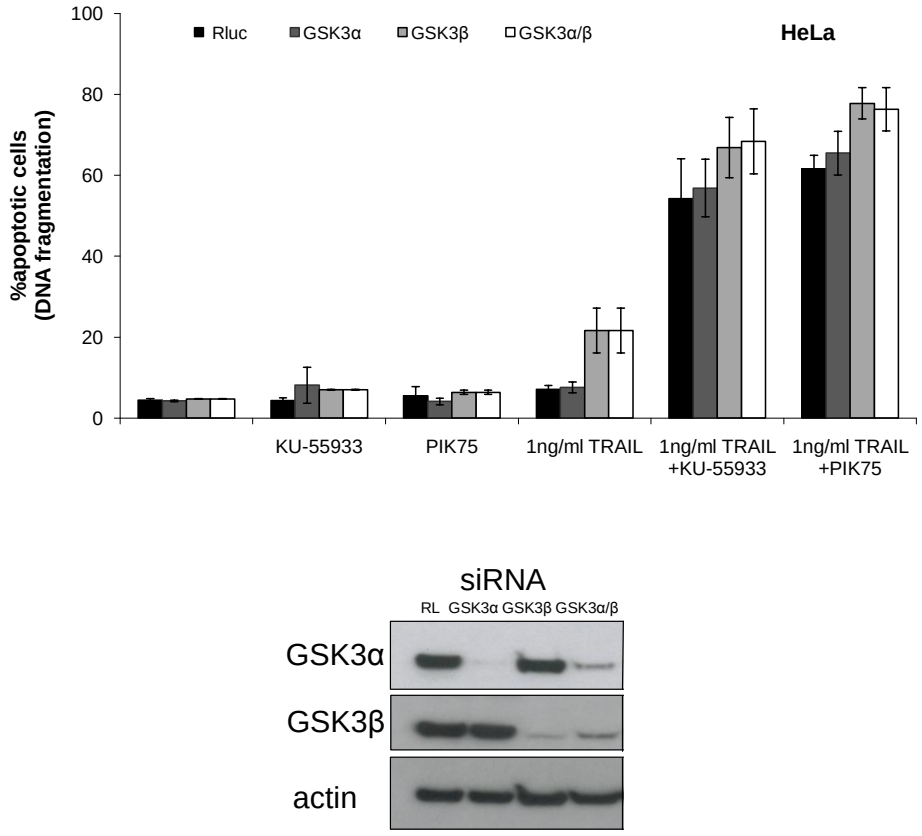


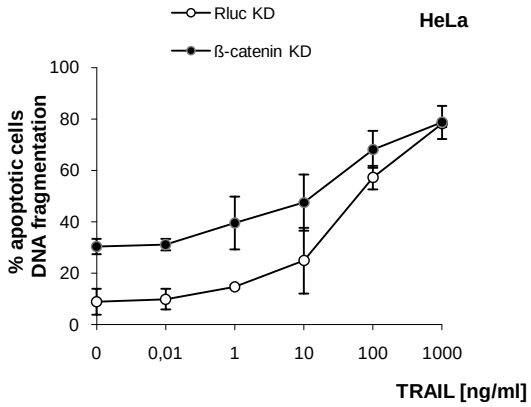
figure 42. Knockdown of GSK3α or GSK3β does not block KU-55933 or PIK75 induced sensitisation to TRAIL- induced apoptosis.

HeLa were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or GSK3α, GSK3β or GSK3α and β. After 72 h control and KD cells were incubated with 1ng/ml of iz-TRAIL for 24 h in presence or absence of KU-559933 (10µM) or PIK75 (50 nM) and then analysed for their subdiploid DNA content. Values are mean ± SD of two independent experiments. The efficiency of the knockdown of the different proteins was controlled by Western blot.

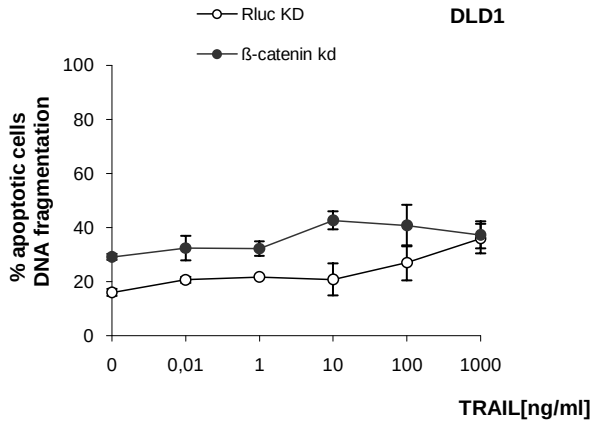
This is in line with the results obtained for the knockdown of β-catenin which is one of the most prominent downstream targets of GSK3. As described above, inhibition of AKT leads to the activation of GSK3 which in turn is responsible for the destruction of β-catenin. Therefore, if active GSK3 were responsible for the sensitisation observed, knockdown of β-catenin would also sensitise the cells to TRAIL-induced apoptosis. Knockdown of β-catenin was very efficient after 48h and was already quite toxic on its own (figure 43). It induced about 30 % of apoptosis in HeLa and DLD1 cells, which might not be so surprising as β-catenin mainly induces survival signals. However, although being quite toxic on its own, there was no synergistic effect of knockdown of β-catenin and additional TRAIL-treatment.

This provides further support for the conclusion that GSK3 and β-catenin are not involved in TRAIL sensitisation in HeLa and DLD1 cells by inhibition of the PI3K /AKT pathway.

a)



b)



c)

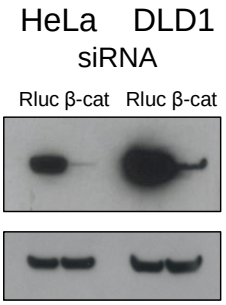


figure 43. Knockdown of β-catenin does not sensitise to TRAIL- induced apoptosis.

(a,b) HeLa or DLD1 cells were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or β-catenin. After 48 h control and KD cells were incubated with increasing concentrations of iz-TRAIL for 24 h and then analysed for their subdiploid DNA content. Values are mean ± standard deviation of two independent experiments. (c) The efficiency of the knockdown of the different proteins was controlled by Western blot.

Another important function of active AKT is the inhibition of FoxO transcription factors. As shown in figure 44 phosphorylation of FoxO1 and FoxO3a was markedly decreased after p110α inhibition and the expression level of FoxO1 was increased.

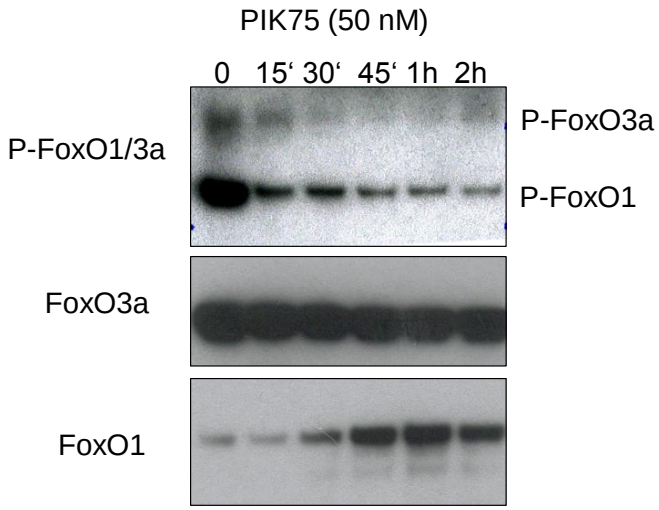


figure 44. Inhibition of p110α by PIK75 leads to diminished of phosphorylation of FoxO1 and Foxo3a.

HeLa cells were stimulated with PIK75 (50 nM) for the indicated time points. Cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of three independent experiments is shown.

Phosphorylation of FoxO transcription factors by AKT leads to the exclusion of FoxO from the nucleus and their translocation in the cytosol. Upon inhibition of the PI3K/AKT pathway FoxO does not become phosphorylated anymore and can therefore remain in the nucleus to act as a transcription factor as depicted in figure 45.

Translocation of FoxO3a into the nucleus could be observed after PIK75 treatment as shown in figure 46. When cells were untreated the FoxO3a was detectable in the nucleus and in the cytosol. In contrast to this, after 6 h of PIK75 treatment FoxO3a was only detectable in the nucleus as the staining for FoxO3a completely overlaps with the DAPI staining.

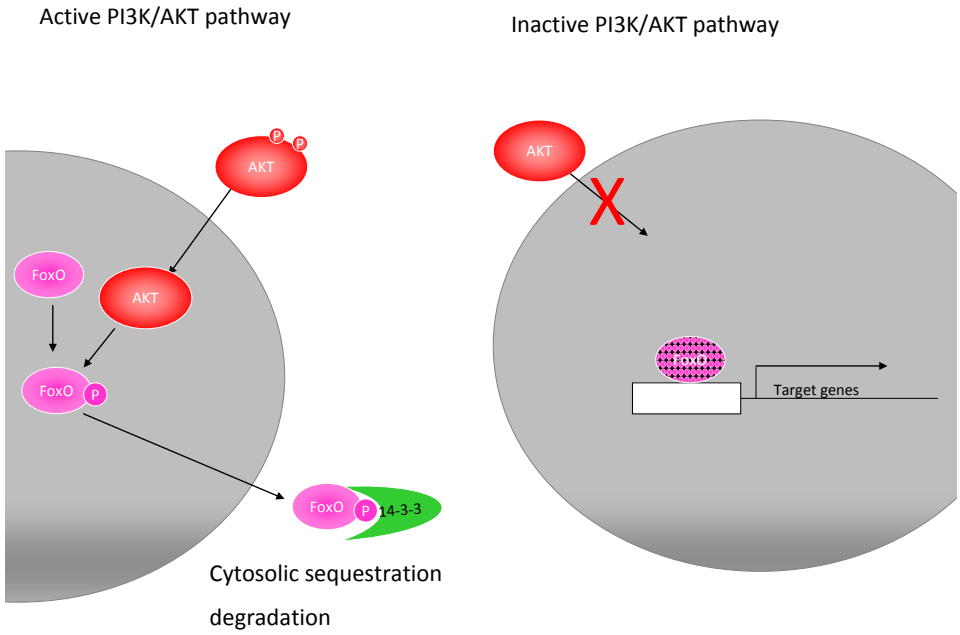


figure 45. Nuclear Export of FoxO controlled by the PI3K/AKT pathway.

FoxO1, FoxO3a and FoxO4 have three conserved amino acid residues which are targets for phosphorylation by AKT. Phosphorylation of FoxOs leads to interaction with 14-3-3 proteins and the nuclear export of the FoxO-14-3-3 complex. Inhibition of the PI3K/AKT pathway leads to dephosphorylation of FoxOs and target gene activation.

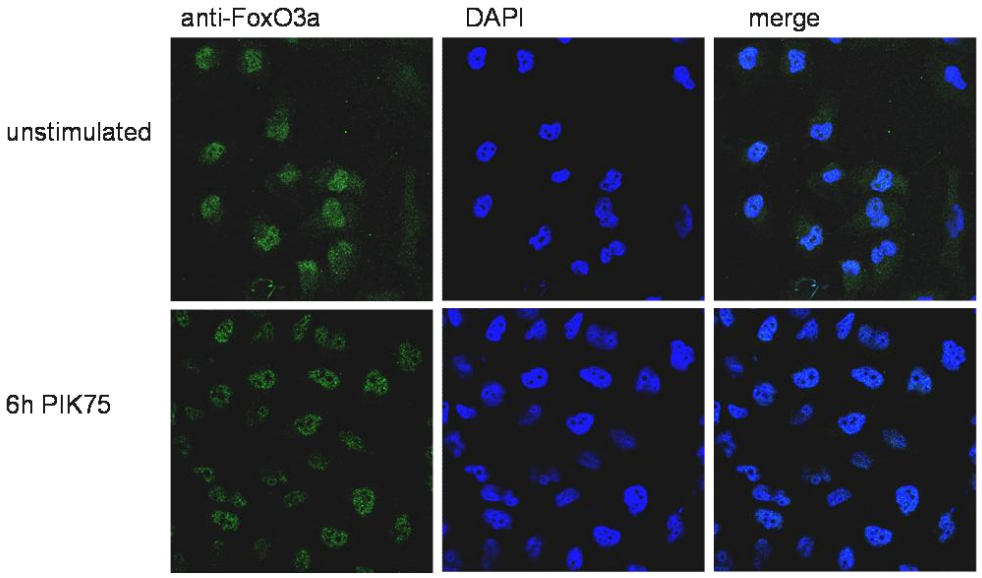


figure 46. Treatment with PIK75 enhances nuclear localisation of FoxO3a.

HeLa cells were left untreated or treated with PIK75 for 6h. Then they were subjected to staining with a FoxO3a specific antibody (green fluorescence); nuclei were revealed by DAPI staining. Localisation of FoxO3a was examined by confocal microscopy.

If sensitisation to TRAIL by KU-55933 or PIK75 worked via FoxO1 or FoxO3a, their knockdown should block sensitisation. As shown in figure 47 knockdown of FoxO1 in HeLa cells worked very well. However, no detectable difference in TRAIL-sensitivity could be observed between control cells and FoxO1 knockdown cells. Accordingly, no blockage of KU-55933 or PIK75 mediated sensitisation to TRAIL could be observed, either. Unfortunately, the knockdown of FoxO3a did not work very well (figure 47). Although optimisation experiments were carried out using higher amounts of siRNA for longer times with different FoxO3a targeting siRNA pools, no reliable knockdown of Foxo3a could be achieved (data not shown). Therefore a different approach was taken to study the influence of FoxO3a on TRAIL-induced apoptosis. DLD1 cells were used which express an inducible version of non-phosphorylatable, and therefore active FoxO3a (generated and kindly provided by Prof. Burgering (Kops et al., 2002)). These cells, referred to as DL23 cells, as well as the parental DLD1 cell line used for transfection (here referred to as DLD1p) were very TRAIL sensitive (figure 48), whereas the DLD1 cells that had been used in the work described here so far were TRAIL-resistant (figure 19).

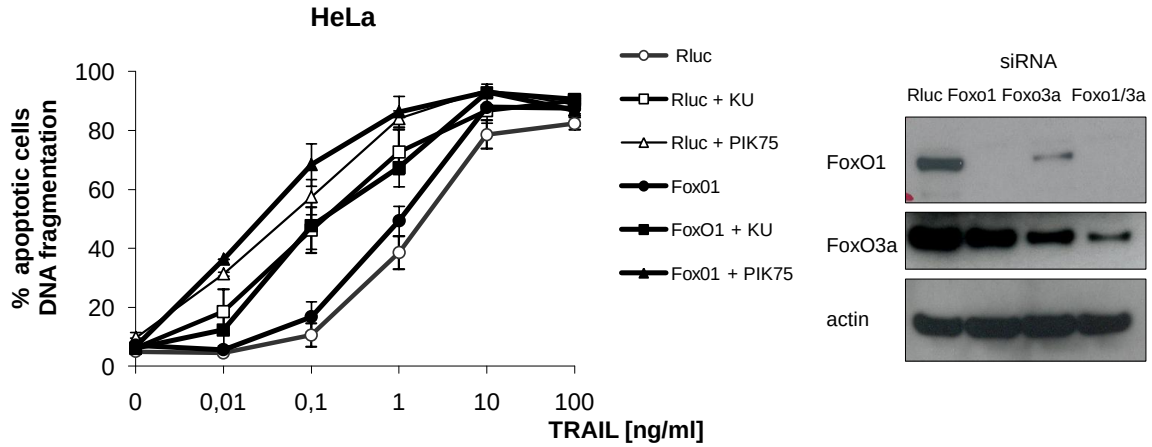


figure 47. Knockdown of FoxO1 does not block KU-55933 or PIK75 induced sensitisation to TRAIL- induced apoptosis.

HeLa were transfected with siRNA either targeting Renilla luciferase (Rluc) as control or FoxO1. After 72 h control and KD cells were incubated with increasing concentrations of iz-TRAIL for 24 h and then analysed for subdiploid DNA content. Values are mean ± standard deviation of two independent experiments. The efficiency of the knockdown of the different proteins was controlled by Western blot.

It has been described before that there are two different DLD1 cell lines which differ in their sensitivity to death ligand-induced apoptosis (Zhang et al., 2005). This difference in sensitivity is caused by differential expression of caspase-8. Indeed the TRAIL-resistant DLD1 cells used in the previous experiments have very low levels of caspase-8 whereas the TRAIL-sensitive DLD1p cells and DL23 cells expressed rather high levels of caspase-8 (figure 48b). FoxO3a expression in DL23 cells can be induced by Hydroxy-Tamoxifen (4-HT) treatment. figure 49a shows that a concentration of 20 nM 4-HT was sufficient to induce expression of HA-tagged FoxO3a. The highest expression of FoxO3a after 24 hours could be achieved with 100 nM of 4-HT. This concentration was used to investigate whether expression of active FoxO3a influenced TRAIL-sensitivity. As DLD1p cells and DL23 were *per se* very TRAIL-sensitive, TRAIL was titrated in a very low concentration range to detect a possible sensitisation by FoxO3a. Treatment with 4-HT did not influence TRAIL sensitivity of DLD1p cells, excluding a sensitising effect of 4-HT itself on TRAIL-induced apoptosis (figure 49).

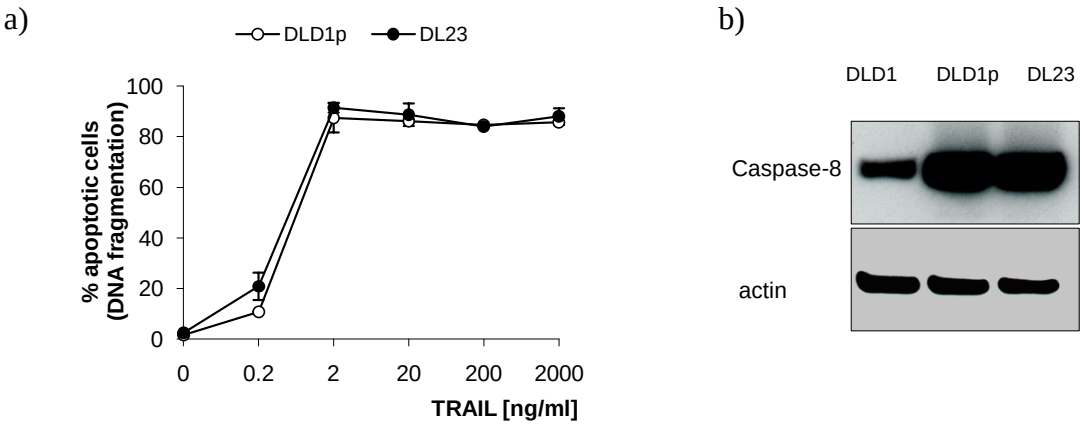


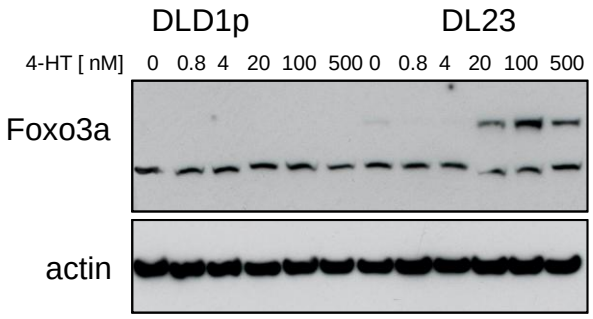
figure 48. DL23 and DLD1p cells are very TRAIL sensitive due to high levels of caspase-8.

(a) DL23 and DLD1p cells were treated with increasing concentrations of iz-TRAIL for 24 h and then analysed for their subdiploid DNA content. Values are mean ± standard deviation of two independent experiments. (b) DLD1, DLD1p and DL23 cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of two independent experiments is shown.

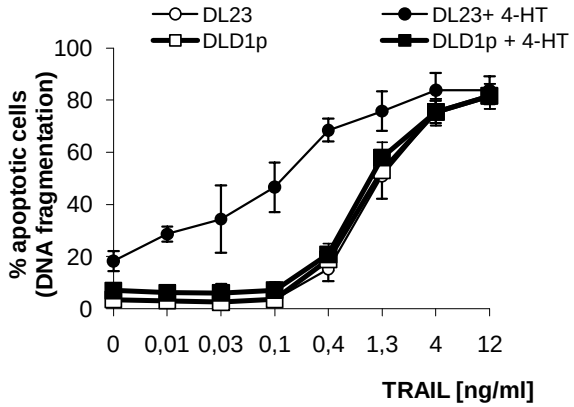
DL23 cells died in a similar manner upon TRAIL-treatment as the parental DLD1p cells. When DL23 were treated with 4-HT for 24 h to induce active FoxO3a, cells became TRAIL-sensitive. 4-HT treatment alone was only slightly toxic. Furthermore, treatment with 4-HT led to a rapid decrease in cFLIP levels and a slower reduction of XIAP (figure 49). Taken

together these results suggest that the TRAIL-sensitisation by KU-55933 and PIK75 most likely works via the activation of the transcription factor FoxO3a. Expression of active FoxO3a did not only sensitise to TRAIL-induced apoptosis but also triggered reduction of cFLIP and XIAP, the same molecular changes that are responsible for TRAIL sensitisation mediated by KU-55933 and PIK75.

a)



b)



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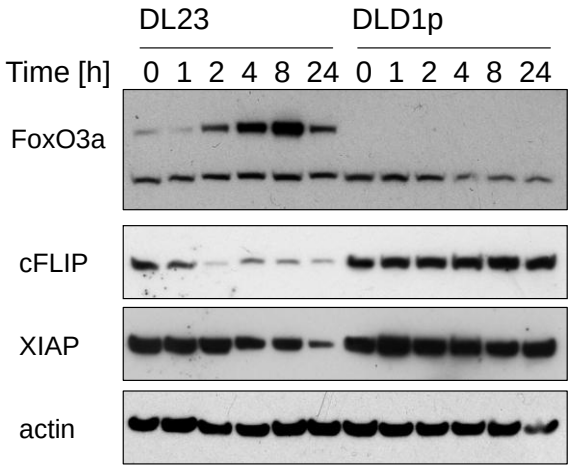


figure 49. Constitutively active FoxO3a sensitises to TRAIL-induced apoptosis and induces down-regulation of cFLIP and XIAP.

(a),(c) DLD1p cells and DL23 were treated with increasing amounts of 4-HT for 24 h (a) or for the indicated times with 100 nM 4-HT (c). Then cells were lysed and 50 µg of protein were analysed by SDS-PAGE using a Bis-Tris gel and subsequent Western blot. One representative result of two independent experiments is shown. (b) DLD1p and DL23 were either left untreated or treated with 4-HT (100 nM) for 24 h. Then cells were treated with increasing amount of iz-TRAIL and analysed for subdiploid DNA content. Values are mean ± standard deviation of three independent experiments.

5. Discussion

5.1. TRAIL-induced phosphorylation of tBid

Bid is a key molecule in apoptosis as it acts as a linker between the death receptor pathway and the mitochondrial pathway. For a long time Bid was thought to be a killer molecule but recent evidence suggested that it also has a pro-survival role (Zinkel et al., 2005). Its phosphorylation by the kinase ATM at residue S78 turned out to be essential for Bid's role as a pro-survival molecule (Kamer et al., 2005). With Bid being a pivotal player in death receptor-induced apoptosis this work set out to investigate the effects of perturbation of phosphorylation of Bid and ATM activity on TRAIL-induced apoptosis.

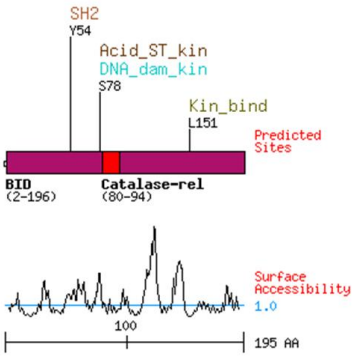
DNA damage-induced phosphorylation of Bid by ATM at residue S78 had only been observed for murine Bid in murine cells or in human cells in which murine Bid was overexpressed (Kamer et al., 2005). In this thesis it is now shown that this phosphorylation also occurred on endogenous human Bid in human cells (see figure 10) suggesting a conserved mechanism among different species. Surprisingly, TRAIL treatment alone without any further DNA damage already led to phosphorylation of tBid as detected with an S78 Phospho-Bid specific antibody in the TRAIL-sensitive HeLa cell line. As shown in figure 12, this phosphorylation occurred after activation of caspase-8 and cleavage of full length Bid into tBid, suggesting that the cleavage of Bid may be essential for phosphorylation of tBid following TRAIL stimulation. This phosphorylation could also be detected in the mouse cell line XhoC3, again indicating a conserved mechanism (see figure 14). Espistatic analysis in the XhoC3 cells also indicated that phosphorylation of tBid occurred upstream of the activation of the mitochondria.

As ATM has been reported to be the kinase phosphorylating Bid upon DNA damage (Kamer et al., 2005) it might also be involved in TRAIL-induced phosphorylation of Bid. However, no active phosphorylated ATM could be detected on Western blot level when cells were treated with TRAIL, whereas etoposide-treated cells used as positive control exhibited activated ATM. This already indicated that ATM was responsible for DNA-damage induced phosphorylation of Bid but not for TRAIL-induced tBid phosphorylation. Additionally, siRNA targeting ATM was used to virtually exclude the involvement of ATM in TRAIL-induced phosphorylation of tBid. Using this siRNA-based approach it could be shown that

DNA damage-induced phosphorylation of Bid vanished upon specific knockdown of ATM, but TRAIL-induced tBid phosphorylation was still detectable (see figure 15). This led to the conclusion that a different kinase than ATM was responsible for TRAIL-induced phosphorylation of tBid. Potential candidates are the kinases DNA-PK and CKII that are predicted to phosphorylate Bid at residue S78 with a much higher likelihood than ATM by the Motif Scanner Scansite (http://scansite.mit.edu/). A screen shot of a search scanning for kinases that phosphorylate Bid (Protein ID: P55957) at residue S78 is shown in figure 50.

Motif Scan Graphic Results: P55957

Description: RecName: Ful=BH3-interacting domain death agonist; AltName: Full=p22 BID; Short=BID; Contains: RecName: Full=BH3-interacting domain death agonist p15; AltName: Full=p15 BID; Contains: RecName: Full=BH3-interacting domain death agonist p13;
Motifs scanned: All
Stringency: Medium
Show domains: Yes



DNA damage kinase group (DNA_dam_kin)				
DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S78	0.3123	0.009 %	RIEADSESQEDIIRN	3.570
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S78	0.5860	2.323 %	RIEADSESQEDIIRN	3.570
Acidophilic serine/threonine kinase group (Acid_ST_kin)				
Casein Kinase 2			Gene Card CSNK2B	
Site	Score	Percentile	Sequence	SA
S78	0.5054	0.938 %	RIEADSESQEDIIRN	3.570

figure 50. Screenshot of a search for kinases which potentially phosphorylate Bid at S78 with the Motif Scanner Software Scansite.

Similar to ATM, DNA-PK is also involved in the DNA damage response to DNA double strand breaks (reviewed in Shiloh, 2003). So far it has not been implicated in the phosphorylation of Bid. However, two recent studies linked DNA-PK activity to TRAIL-induced apoptosis. A study performed by Kim et al. (2009) observed a sensitisation of K562 cells to TRAIL when cells were treated with a DNA-PK inhibitor or when DNA-PK was knocked down. In contrast to this, Solier et al. (2009) showed that DNA-PK became activated following TRAIL stimulation and found that the use of a DNA-PK inhibitor together with TRAIL did not change the apoptotic outcome. Furthermore, the activation of DNA-PK was only detectable after cytochrome *c* release from the mitochondria. However it is still possible

that basal levels of active DNA-PK are present which might be involved in the phosphorylation of Bid or tBid upstream of cytochrome c release from the mitochondria.

As mentioned in the introduction, CKII has already been described to phosphorylate Bid (Desagher et al., 2001; Olsen et al., 2006). This phosphorylation is constitutive and reduces its cleavage by caspase-8 (Degli Esposti et al., 2003; Desagher et al., 2001). However, the phosphorylation site focused on in this study was residue T59 (Degli Esposti et al., 2003). Therefore, it would be interesting to investigate whether inhibition of CKII by the specific inhibitor DRB might also affect TRAIL-induced Bid phosphorylation at residue S78. If this were the case it should be examined whether it is functionally involved in the sensitisation to TRAIL induced by DRB treatment (Kim et al., 2008). One argument in favour of the hypothesis that CKII might also be involved in phosphorylation of tBid after TRAIL treatment might be that Casein Kinase II is a constitutively active kinase. Our collaboration partner Atan Gross made the observation that induced expression of tBid also led to the phosphorylation of tBid without any further stimulus (unpublished observation). Taking this into consideration the most likely scenario would be that Bid undergoes a conformational change when it is cleaved into tBid. This results in an increased accessibility of the phosphorylation site S78 which would then become phosphorylated by a constitutively active kinase.

The solution structure of Bid has been resolved by two different group in parallel by NMR spectroscopy already in 1999 (Chou et al., 1999; McDonnell et al., 1999). The study by Mc Donnell *et al.* modelled the structure of tBid based on the structure of Bid and compared both structures. The study by Chou et al. even went one step further; they also resolved the structure of tBid. Both studies found that Bid does not undergo any dramatic conformational changes upon cleavage to tBid. Nevertheless, they observed marked changes in the character of Bid surfaces comprising hydrophobic exposure and surface charge. The residue S78 is directly adjacent to the helix 3, in a region that showed significant changes in the chemical shift, as shown in figure 51.

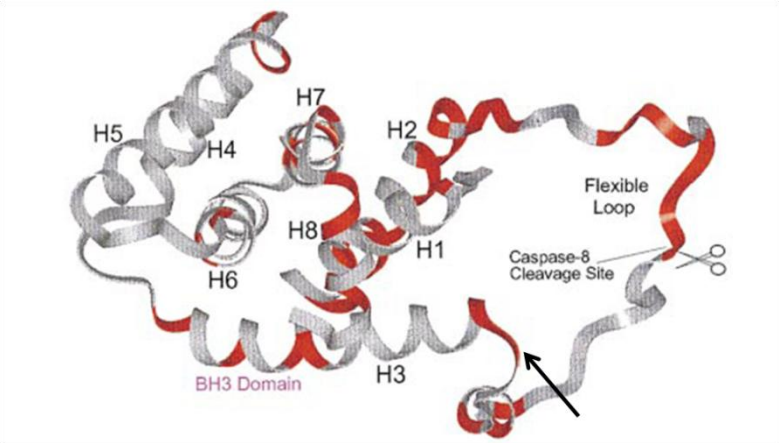


figure 51. Ribbon diagram of BID highlighting the residues whose local chemical environments are changed due to caspase-8 cleavage.

In this representation, residues of which NH chemical shift changes are greater than 0.1 and 0.02 ppm in 15N and 1H dimension, respectively are coloured in red. The position of S78 is approximately indicated by the arrow. Figure adapted from Chou et al. (Chou et al., 1999).

At the moment the role of this TRAIL-induced phosphorylation of tBid is still unclear. A post-translational modification of tBid might alter the affinity for binding to Bcl-2 family members or affect targeting to the mitochondria as has been shown for N-myristoylated tBid (Zha et al., 2000). In the system used in this study in which endogenous Bid was knocked down using esiRNA and in which the different Bid mutant were re-expressed, no difference could be detected in the apoptotic outcome upon TRAIL treatment (figure 16). Therefore, one might conclude that TRAIL-induced tBid phosphorylation is an epiphenomenon and is not associated with a functional role in TRAIL-induced apoptosis. However, as phosphorylation of Bid at the same residue in the context of DNA damage has a major impact on cellular fate (Zinkel et al., 2005), it is more likely that this phosphorylation event has functional consequences on TRAIL-induced apoptosis. It is possible that these consequences were not detectable in the cell line used in this study. Different anti-apoptotic Bcl-2 family members may be differentially affected by the action of phosphorylated versus non-phosphorylated tBid. In addition, there is still a controversy about whether tBid directly acts on pro-apoptotic Bcl-2 family members or whether it indirectly activates them by neutralising anti-apoptotic members. Perhaps the phosphorylation of tBid differentiates between these different actions of tBid. This is an intriguing hypothesis but to test it an experimental system that evaluates the differential binding capacities of phosphorylated tBid versus tBid to Bax and Bak and all anti-apoptotic Bcl-2 proteins would have to be established.

5.2. Sensitisation to TRAIL-induced apoptosis by the ATM-inhibitor KU-55933

As mentioned in the introduction, TRAIL selectively kills about 50% of tumour cell lines while sparing the majority of normal cells from apoptosis. This unique feature makes TRAIL a promising tool for anti-cancer therapy. Unfortunately, most primary tumour cells turned out to be resistant to TRAIL, which casts doubt over the potential of TRAIL to be used as a single agent to treat cancer. However, a variety of conventional and targeted cancer drugs can sensitise many primary tumour cells to TRAIL-mediated apoptosis. To find and characterise new agents that sensitise to TRAIL is not only interesting with regard to cancer therapy but also makes it possible to unravel mechanisms and pathways conveying TRAIL resistance. With improved understanding of the mechanisms that confer TRAIL-resistance one might be able to overcome current limitations in cancer treatment by rational drug identification and design, as well as develop biomarker driven patient selection criteria. As already mentioned in the results section the ATM inhibitor KU-55933 was intended to be used as an independent assessment to test whether ATM was involved in tBid phosphorylation. KU-55933 was identified as an ATP-competitive inhibitor for ATM in a small molecule compound library screen (Hickson et al., 2004; Hollick et al., 2007). There, KU-55933 was shown to inhibit ATM with an IC50 value of 13 nmol/L whereas the IC50 value for other members of the phosphoinositide 3-kinase-related kinase (PIKK) family like DNA-PK or PI3K was determined as 2,5 µmol/L and 16,6 µmol/L respectively. KU-55933 inhibits the phosphorylation of a range of ATM targets upon activation of ATM using ionizing radiation in HeLa cells. KU-55933 also sensitises these cells to apoptosis caused by chemotherapeutics which induce DNA double strand breaks (Hickson et al., 2004). KU-55933 might be useful in the treatment of HIV infection as inhibition of ATM prevents retroviral replication (Lau et al., 2005). Using KU-55933 to specifically target DNA-damage repair could also have implications for anti-cancer therapy. It has been successfully applied as a single agent to kill senescent, otherwise chemotherapy-resistant breast, lung, and colon carcinoma cells, which are hallmarked by constitutive activation of ATM (Crescenzi et al., 2008). Accordingly, pancreatic tumour cell lines with a mutation in the Fanconi anaemia pathway which results in constitutive activation of ATM could also be killed by KU-55933 (Kennedy et al., 2007). Besides its potential application in HIV therapy, and potential use as single agent in cancer

therapy mentioned in the introduction, it has been shown that co-treatment with KU-55933 can sensitise breast carcinoma cells to ionizing radiation (Cowell et al., 2005).

This thesis showed at 10 µM, a concentration which is used in most studies to efficiently inhibit ATM, KU-55933 further sensitised TRAIL-sensitive HeLa cells to TRAIL-induced apoptosis in a concentration dependent-manner (figure 17). This result corresponds to the observations presented in a recent study which showed that KU-55933 can be used in combination with TRAIL to further enhance sensitivity of cells to TRAIL-induced apoptosis in melanoma cells (Ivanov et al., 2009). Remarkably, co-treatment with KU-55933 could even break TRAIL resistance in the colon carcinoma cell line DLD1 (figure 19). Furthermore, the long-term survival of HeLa as well as DLD1 cells was significantly diminished upon co-treatment with KU-55933 and TRAIL (figure 17 and figure 19).

These results showed that a combination of TRAIL and KU-55933 might potentially be a new treatment option for tumours cells that are resistant to TRAIL as a single agent.

5.3. Sensitisation to TRAIL-induced apoptosis mediated by KU-55933 is independent of ATM inhibition

As AT cells have been reported to be resistant to death receptor-induced apoptosis due to elevated levels of cFLIP (Stagni et al., 2008), the finding that the use of an ATM inhibitor sensitised to TRAIL-induced apoptosis was quite surprising. Therefore the question arose whether the observed effect was truly due to inhibition of ATM. As already mentioned, the dosage of 10 µM KU-55933 was used in most studies to efficiently inhibit ATM. At this concentration KU-55933 was not toxic for HeLa cells but was sufficient to completely abrogate the activation of ATM upon stimulation with etoposide (figure 20). In contrast to this, ATM did not become activated upon TRAIL stimulation in HeLa cells in this study, although it has recently been reported that TRAIL leads to an activation of ATM (Solier et al., 2009). However, this activation seems to be a late event in TRAIL-induced apoptosis and occurs, similar to the activation of DNA-PK, after cytochrome c release from the mitochondria. Cytochrome *c* release is generally considered to be the point of no return, after which cells are doomed to die. Considering this, it is very unlikely that inhibition of active ATM downstream of cytochrome *c* release were responsible for the KU-55933-mediated TRAIL sensitisation.

Ivanov et al. (Ivanov et al., 2009) claim that KU-55933 sensitised to TRAIL-induced apoptosis by blocking the basal activity of ATM. However, no basal activity of ATM was detected in this study in HeLa cells by Western blot, not even after very long exposures times (figure 20). Therefore, the observed sensitisation by KU-55933 could only be due to an inhibition of the basal activity of ATM which was below the detection limit. If this were the case, a knockdown of ATM should have the same effect as KU-55933 treatment. However, when ATM was knocked down cells became rather more TRAIL-resistant. Nevertheless, KU-55933 was still able to sensitise these ATM knockdown cells. In line with this the same result was obtained in AT cells which lack functional ATM (figure 21). Taken together, these results indicate that KU-55933-mediated sensitisation to TRAIL-induced apoptosis is independent of ATM inhibition.

5.4. Sensitisation to TRAIL-induced apoptosis by inhibition of the PI3K catalytic subunit p110 α

As ATM belongs to the PIKK family and the inhibition of the PI3K/AKT pathway has been shown to sensitise to TRAIL-induced apoptosis in various tumour cell types, it seemed most likely that KU-55933 might work via the inhibition of PI3K. Indeed, when the four different isoforms of the PI3K p110 subunit α, β, γ and δ were knocked down, it was found that knockdown of p110α sensitised HeLa cells to TRAIL-induced apoptosis whereas suppression of the other isoforms had no effect (figure 22). Correspondingly, pharmacological inhibition of p110α with the specific inhibitor PIK75 had the same effect (figure 23). Both, PIK75 as well as KU-55933 interfered with the phosphorylation of AKT, which was taken as surrogate for PI3K activity (figure 24). Furthermore, using a kinase assay a direct inhibition of p110α by KU-55933 could be shown (figure 25). These results demonstrate that KU-55933 was not as specific as previously thought. Thus, results derived solely from the use of KU-55933 might have to be reconsidered taking into account the potential additional inhibition of the PI3K pathway.

Moreover, this study underlines the importance of the PI3K/AKT survival pathway for TRAIL sensitivity. Genetic studies showed that tumour cells which contain an activating somatic mutation in PI3K are relatively TRAIL-resistant (Samuels et al., 2005). Several studies exist which make use of the inhibition of the PI3K/AKT pathway to sensitise to TRAIL-induced apoptosis. They either target PI3K directly by using LY294002 (Martelli et

al., 2003), inhibit receptor tyrosine-kinases which function upstream of PI3K with gefitinib (Shrader et al., 2007) or target mTOR activity which is downstream of AKT using rapamycin (Panner et al., 2005).

Although several studies reported that PI3K inhibition sensitises to TRAIL-induced apoptosis, so far only one of them has investigated the importance of the different isoforms which have non-redundant functions (reviewed in Ihle and Powis, 2009) and might therefore differentially affect TRAIL-induced apoptosis. The study by Opel et al. (Opel et al., 2008) only focussed on the two ubiquitously expressed isoforms α and β and finds that knockdown of PI3K subunits p110α and/or p110β sensitised glioblastoma cells to TRAIL-induced apoptosis. As the α-isoform seems to be the most dominant regulator of cell growth (Knight et al., 2006) and mutations in p110α gene (PIK3CA) occur in diverse tumours with frequencies of up to 32 % (Samuels et al., 2004), specifically targeting the α-isoform of p110 by the specific inhibitor PIK75 to sensitise to TRAIL, as has been shown for the first time in this study, might be advantageous in comparison to broader inhibitors. This might reduce unwanted side effects as the different isoforms have non-redundant functions.

PIK75 was developed by Hayakawa *et al.* (Hayakawa et al., 2007) who aimed to increase the stability of their previously developed p110α inhibitor which was specific but unstable in solution and ineffective in vivo. As a result of this study PIK75 is very selective for p110α (IC50: 30 nM), stable in solution and has also been shown to be effective *in vivo*. In a HeLa cervical cancer xenograft model in which PIK75 was applied daily for 2 weeks, PIK75 was well tolerated and was able to suppress tumour growth by 62 % without any toxicity. The inhibitor LY294002 targets all isoforms and has been shown to be toxic under certain conditions, e.g. in a mouse xeno-transplant model of ovarian cancer (Hu et al., 2000). In this model, LY294002 significantly inhibited growth and ascites formation of ovarian carcinoma but two of the 12 mice in the treatment group died. Additionally, 80 % of the LY294002-treated mice developed dry and scaly skin, possibly due to hyperkeratosis in the epidermis as a result of increasing LY294002-induced apoptosis.

Mutations in PIK3CA or KRAS, which in turn results in hyperactivity of PI3K p110α, are very frequent in metastatic colorectal carcinoma (13.6% and 29.0%, respectively) and tumours bearing these mutations are often chemotherapy-resistant (Sartore-Bianchi et al., 2009). Additionally, the EGFR-targeting monoclonal antibodies panitumumab and cetuximab

which are currently used to treat metastatic colorectal cancer are ineffective in patients who have tumours with PIK3CA and KRAS mutations (Amado et al., 2008; Lievre et al., 2006; Sartore-Bianchi et al., 2009). Furthermore, mutation of PIK3CA led to increased cell motility and metastasis in breast and colon cancer models (Guo et al., 2007; Pang et al., 2009). Both colorectal cancer cell lines used in this study, the TRAIL-resistant DLD1 cell line and the HCT116 cell line, carry activating mutations in the PIK3CA and the KRAS genes (Samuels et al., 2005). The influence of the PIK3CA mutations in these cell lines on TRAIL sensitivity has already been revealed in an elegant study by Samuels et al. (Samuels et al., 2005). Here, the authors generated DLD1 and HCT116 cells in which either the wild-type or mutant alleles of PIK3CA gene were disrupted using a gene targeting approach. This resulted in DLD1 and HCT116 cells that expressed either wt p110α or mutant constitutively active p110α. DLD1 and HCT116 cells that only expressed mutant p110α were TRAIL-resistant at the applied concentration of TRAIL reflecting the phenotype of the parental cell lines. In contrast to this, DLD1 cells and HCT116 cells only expressing the wt p110α allele became TRAIL sensitive. Considering this, the authors came to the conclusion that TRAIL-based therapies in patients with a mutation in the PIK3CA gene or with constitutive AKT activation caused by other mutations are to not likely to be useful.

This thesis now shows that although TRAIL might not be a treatment option when applied as a single agent, combination of TRAIL with a p110α inhibitory drug, e.g. PIK75 or KU-55933 represents a promising treatment option, as it can efficiently kill these chemotherapy resistant cells with great metastatic potential *in vitro*. The next step to establish the combination of TRAIL and PI3K p110α inhibition as potential cancer treatment will be to determine the efficacy and the toxicity of this combination *in vivo*. A potential candidate for a future combination treatment with TRAIL is the p110α specific inhibitor GDC-0941 which is currently developed by Genentech/Piramed (Folkes et al., 2008). This compound is orally available and now in clinical trials. It has already yielded promising results as a single agent in ovarian xenografts (Raynaud et al., 2009) or in combinations with trastuzumab and pertuzumab in breast cancer models (Junttila et al., 2009; Yao et al., 2009).

5.5. Molecular changes facilitating TRAIL-sensitisation by KU-55933/ PIK75

After PI3K p110α had been identified as the target of KU-55933, the question arose how p110α activity conveys TRAIL resistance. Many studies about TRAIL sensitisation suggest up-regulation of TRAIL-Rs to be the underlying mechanism. However, in our laboratory the observation was made that although up-regulation of TRAIL-Rs often correlates with sensitisation to TRAIL and sometimes contributes to sensitisation, it is rarely the decisive, causative factor for sensitisation. Indeed, up-regulation of TRAIL-R1 and-R2 has been reported to be important for sensitising tumour cells to TRAIL-induced apoptosis when the PI3K/AKT pathway is inhibited (Rychahou et al., 2005; Tazzari et al., 2008). Also, Ivanov et al. (Ivanov et al., 2009) claimed that sensitisation to TRAIL-induced apoptosis in melanoma cells by KU-55933 was mediated by up-regulation of TRAIL-R2. This study shows that up-regulation of TRAIL-R2 coincided with, but was not essential for sensitisation to TRAIL-induced apoptosis of HeLa and DLD1 cells (figure 27). Therefore, intracellular regulatory mechanisms must exist that facilitate sensitisation to TRAIL mediated by KU-55933 and PIK75. Regulation most likely occurs at three different levels: at the DISC, at the mitochondria, or at the level of caspase-3 activation.

Many intracellular factors have been linked to TRAIL sensitisation upon inhibition of the PI3K/AKT pathway, among them: cFLIP, cIAP1, cIAP2, Survivin, Bcl-2, Bad, Bim, and XIAP. However, most studies only show a correlation between the regulation of the respective molecule and rarely present evidence that the respective factor is indeed decisive for facilitating TRAIL sensitisation.

To find out which molecules were regulated intracellularly that might facilitate TRAIL sensitisation, members of the direct apoptotic pathway were investigated on the protein level. A strong down-regulation of both, $cFLIP_L$ and $cFLIP_S$ short on protein level could be observed (figure 28), which had been described before upon inhibition of the PI3K pathway (Bortul et al., 2003; Han et al., 2007; Kang et al., 2004; Panka et al., 2001). As the effects on the transcriptional level were only marginal, it is likely that post-transcriptional mechanisms were involved in cFLIP down-regulation. Previously, ubiquitin–mediated degradation has been shown to be involved in the post-transcriptional regulation of $cFLIP_L$ and $cFLIP_S$ (Poukkula et al., 2005). Only recently a study reported a reduced half-life of cFLIP after

inhibition of AKT which correlated with increased ubiquitination by the E3 ligase atrophin-interacting protein 4 (AIP4) (Panner et al., 2009). It will be interesting to address how p110α inhibition negatively regulates cFLIP stability. cFLIP forms part of the DISC and inhibits the activation of caspase-8. Therefore, changes of the DISC composition that come along with down-regulation of cFLIP have been investigated. Though, changes at the DISC level were detectable, they were not dramatic (figure 31). It was suspected that the down-regulation of cFLIP alone might not be sufficient to break TRAIL-resistance in DLD1 cells and other factors might also be involved. A kinetic in DLD1 cells revealed that full-activation of caspase-3 only occurred in cells that were co-treated with TRAIL and KU-55933 or PIK-75. cIAP-1 and cIAP-2, survivin and XIAP all belong to the IAP family and a correlation between down-regulation upon inhibition of the PI3K pathway and increased TRAIL sensitivity has been described for each of them (Han et al., 2007; Kim et al., 2004; Shrader et al., 2007; Tazzari et al., 2008). XIAP, whose expression was shown to be regulated by AKT directly, via phosphorylation (Dan et al., 2004) and indirectly via transcription (Takeuchi et al., 2005), seemed to be the most likely candidate in the system used in this study. Indeed, XIAP was strongly down-regulated on the protein (figure 28) and mRNA levels in HeLa cells (figure 29). Using siRNA-mediated knockdown of cFLIP and XIAP it could be demonstrated that the concomitant suppression of cFLIP and XIAP was both required and sufficient for TRAIL apoptosis sensitisation by KU-55933- or PIK75-mediated inhibition of p110α. Down-regulation of cFLIP resulted in enhanced caspase-8 activation at the DISC and down-regulation of XIAP facilitated full activation of caspase-3. In line with this, cells could also be sensitised by knockdown of cFLIP and treatment with SMAC mimetics (figure 33), a drug class developed to block the activity of XIAP (Mastrangelo et al., 2008), whereas combination of TRAIL with SMAC mimetics alone was not sufficient to sensitise these cells.

Although the concomitant down-regulation of cFLIP and XIAP was sufficient to sensitise to TRAIL-induced apoptosis this did not exclude that a regulation of proteins acting at mitochondria might contribute to the observed sensitisation mediated by KU-55933 or PIK75. In this regard, a loss of phosphorylation of the pro-apoptotic Bcl-2 family member Bad has been observed upon treatment with KU-55933- or PIK-75. Phosphorylated Bad is usually sequestered in the cytosol but upon loss of phosphorylation Bad can induce apoptosis via the mitochondrial pathway by binding to and counteracting the anti-apoptotic function of Bcl-2, Bcl-XL and Bcl-w (reviewed in Danial, 2008), as illustrated in figure 52.

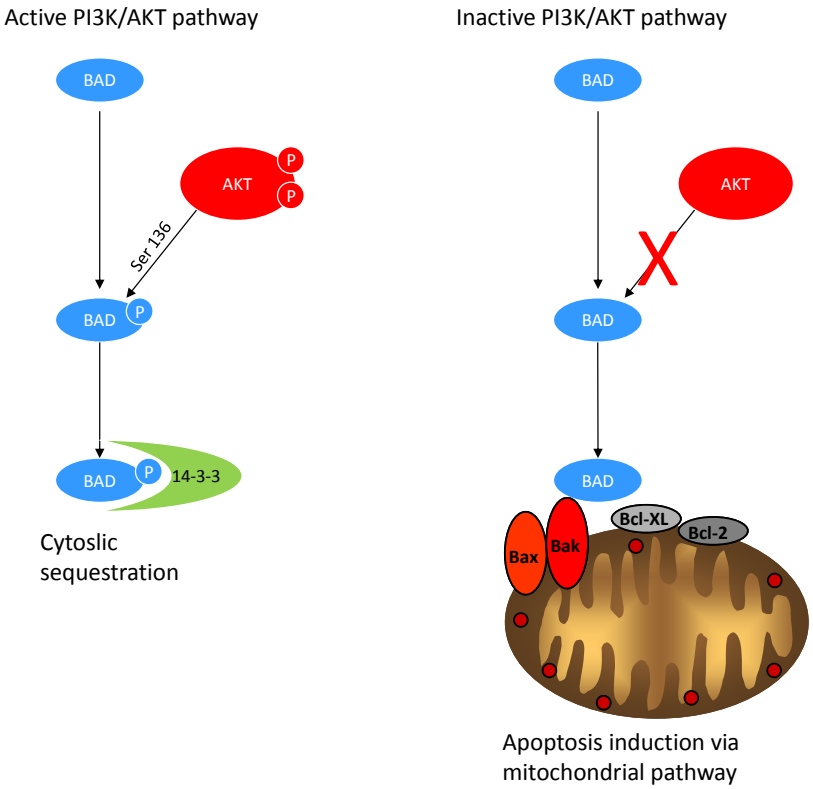


figure 52. Regulation of Bad activity by the PI3K/AKT pathway.

Activation of the AKT by survival signals leads to phosphorylation of BAD at S136, which allows its association with 14-3-3 proteins and sequestration in the cytosol. Upon inhibition of the PI3K/AKT pathway phosphorylation of Bad is lost and Bad can induce apoptosis via the mitochondrial pathway.

A correlation between the phosphorylation status of Bad and TRAIL-sensitivity has already been reported by two independent studies (Kang et al., 2004; Martelli et al., 2003). However, data shown in these studies are only correlative and do not show that loss of phosphorylation was decisive for sensitisation to TRAIL. Similarly, down-regulation of Bcl-2 upon inhibition of the PI3K/AKT was observed (Alladina et al., 2005; Han et al., 2007). Yet again the data only establish correlation and do not show a decisive role for Bcl-2 in the sensitisation process.

To investigate the contribution of apoptotic regulators that act at the mitochondria on KU-55933- and PIK75-mediated sensitisation to TRAIL, HCT116 Bax-/- cells in which, in addition, Bak was knocked down were employed (figure 34). HCT116 cells are type II cells. Taking out Bax and Bak completely takes out the mitochondrial pathway in these cells and thereby also blocks TRAIL-induced apoptosis which in these cells is entirely dependent on the action of tBid on the mitochondria. However, when co-treated with KU-55933 or PIK75

the HCT116 Bax-/- Bak–KD cells could still be sensitised to TRAIL virtually to the same extent as the control cells. This means, that although Bcl-2 family members are certainly regulated upon treatment with KU-55933 and PIK75, and might contribute when mitochondria are present, they are not the decisive factors that allow for sensitisation by KU-55933 and PIK75 to occur.

Taken together the results indicate that treatment with KU-55933 or PIK75 leads to simultaneous down-regulation of cFLIP and XIAP which together facilitates sensitisation to TRAIL-induced apoptosis. A regulation of Bcl-2 family members occurs but is not required for TRAIL sensitisation. The use of KU-55933 or PIK75 in combination with TRAIL might be advantageous when compared to other sensitising agents, like e.g. SMAC mimetics. By down-regulating both, cFLIP and XIAP, KU-55933 and PIK75 do not only target resistance at the level of caspase-3 activation but also at the DISC.

5.6. Down-regulation of cFLIP and XIAP is facilitated by activation of FoxO3a downstream of AKT

A plethora of studies has shown that inhibition of PI3K leads to sensitisation to TRAIL-induced apoptosis. PI3K mainly seems to exert its TRAIL-sensitising effect by acting on its downstream effector AKT. Several studies have shown that cells expressing a dominant negative version of AKT are sensitised to TRAIL whereas cells over-expressing active AKT become more TRAIL-resistant (Kandasamy and Srivastava, 2002; Thakkar et al., 2001). Correspondingly, a study using the AKT inhibitor perefosine also observed a correlation between TRAIL sensitisation and down-regulation of cFLIP and XIAP (Tazzari et al., 2008) ; the same changes that were observed here after treatment with KU-55933 and PIK75. In line with this it could be shown here that HeLa cells as well as DLD1 cells could be sensitised to TRAIL by knockdown of AKT1 (figure 35). The isoforms AKT1 in comparison with the two other isoforms, AKT2 and AKT3, has been shown to be predominantly activated by mutated p110α (Samuels et al., 2005).

The involvement of AKT in the TRAIL sensitisation process triggered by inhibition of PI3K seems to be a well-accepted fact. The knockdown of AKT1 already served as a positive control for the first siRNA screen performed in 2003 in search of modulators of TRAIL-induced apoptosis (Aza-Blanc et al., 2003). However AKT phosphorylates a variety of substrates and thereby triggers or inhibits different pathways. So far the pathway which

allows for TRAIL sensitisation upon PI3K inhibition downstream of AKT has not been identified.

Through the phosphorylation of various substrates AKT regulates the four intersecting biological processes: cells survival, cell progression, cell growth and cell metabolism. Among the substrates which have been linked to cancer are mTOR, GSK3 and FoxO, all of which have been implicated in TRAIL sensitivity and resistance.

mTOR signalling is one of the major pathways which becomes activated downstream of AKT. mTOR regulates cap-dependent mRNA translation and integrates extracellular signals and translational control. It does so by phosphorylating 4E-BP and S6K1 which then can no longer bind to and inhibit eukaryotic initiation factors eIF4E and eIF3, respectively, which can in turn initiate cap-dependent translation. An overview about the mTOR signalling pathway is given in figure 36. mTOR stands for mammalian target of rapamycin. Rapamycin was discovered as a product of the bacterium Streptomyces hygroscopicus in a soil sample from the Easter Island— an island also known as "Rapa Nui", hence the name (Vezina et al., 1975). Originally developed as an anti-fungal agent, rapamycin was found to exert its anti-proliferative effect due to inhibition of mTOR. Rapamycin or rapamycin analogues (rapalogues) have shown activity against many types of cancer in phase I and II trials. Specifically, partial responses and stable disease have been observed in NSCLC, breast, cervical, and uterine carcinomas, as well as sarcoma, mesothelioma, mantle cell lymphoma, and glioblastoma (LoPiccolo et al., 2008). However, the observation that prolonged rapamycin treatment leads to an enhanced PI3K/AKT activation via an S6K1 negative-feedback loop might complicate the use of rapamycin in cancer treatment (Sun et al., 2005).

A few studies have already investigated whether inhibition of translation via mTOR inhibition sensitises to TRAIL-induced apoptosis with contradicting results. A study by Panner *et al.* (2005) reported that resistance to TRAIL is associated with reduced expression of $cFLIP_S$ and decreased S6K1 phosphorylation in glioblastoma cells. $cFLIP_S$ mRNA was poorly translated as it sedimented with light polysomes in TRAIL-sensitive glioblastomas, but co-sedimented with heavy polysomes in TRAIL-insensitive glioblastoma multiforme, thus indicating a translational regulation. Accordingly, rapamycin treatment or siRNA-mediated knockdown of S6K sensitised cells to TRAIL. In a second study the same group reported other anti-apoptotic proteins apart from $cFLIP_S$ also to be controlled via translational

regulation. Among them were cIAP2, XIAP, Survivin and Bcl-2 (Panner et al., 2006). Interestingly, a third study showed that inhibition of the AKT pathway led to increased ubiquitination of cFLIP. However, the same group did not see a reduction of cFLIP after rapamycin treatment (Panner et al., 2009). Noteworthy, all three studies were conducted in the same cell system – glioblastoma multiforme. An independent study also using glioblastoma found that co-treatment with rapamycin could not sensitise to TRAIL (Opel et al., 2008).

Another group investigated the effect of rapamycin in TRAIL-resistant human mesothelioma multicellular spheroids (Barbone et al., 2008; Wilson et al., 2008). They observed a sensitisation of these spheroids to TRAIL by rapamycin treatment as well as by knockdown of S6K1. As this sensitisation could be ablated by Bid knockdown, it probably occurred at the mitochondrial level (Barbone et al., 2008).

As cFLIP and XIAP have been reported to be down-regulated upon mTOR inhibition, the mTOR pathway seemed to be a possible candidate for conveying the TRAIL-sensitising effect by KU-55933 and PIK75 downstream of AKT. In HeLa and DLD1 cells phosphorylation of mTOR was greatly diminished after inhibition of p110α (figure 37). Rapamycin treatment did not affect TRAIL-induced apoptosis although phosphorylation of the mTOR target S6K1 was efficiently inhibited (figure 38). As rapamycin treatment is only able to inhibit the activity of mTOR in complex with LST8 and Raptor, referred to as mTORC1, this experiment did not exclude a potential role for mTORC2 (mTOR in complex with LST8 and Rictor) in TRAIL sensitisation. The only inhibitor known so far which also inhibits the activity of mTORC2 is PI-103. Yet, PI-103 also inhibits p110α and is, therefore, not suitable to dissect the effects downstream of PI3K/AKT (Knight et al., 2006). However, a knockdown of mTOR equally affects mTORC1 and mTORC2. Yet again, no effect on TRAIL sensitivity could be observed in this study after knockdown of mTOR. Taken together these results excluded the mTOR pathway as the downstream mediator of TRAIL sensitisation upon inhibition of p110α.

Another molecule downstream of AKT is GSK3, a multi-functional kinase involved in diverse physiological pathways ranging from metabolism, cell cycle, gene expression and development to oncogenesis (reviewed in Rayasam et al., 2009). AKT acts as a major negative regulator of GSK3 activity by phosphorylating GSK3 at inhibitory sites (depicted in figure 40). However, several other kinases can also phosphorylate these sites.

GSK3 is implicated in oncogenesis as a component of the Wnt pathway (Rayasam et al., 2009). As mentioned in section 4.9, active GSK3 exists in a complex with APC and Axin which phosphorylates β-catenin and targets it for degradation. Once GSK3 is inactivated, β-catenin accumulates and translocates to the nucleus, where it induces target genes like c-myc. Aberrant Wnt signalling has been reported in a wide range of cancers.

GSK3 can have opposing effects on apoptosis, either strongly inhibiting or promoting apoptosis (reviewed in Beurel and Jope, 2006). For example, GSK3$\beta^{-/-}$ mice died during embryonic development due to massive hepatocyte apoptosis, which led to the concept that GSK3 inhibits apoptosis (Hoeflich et al., 2000). This observation seems to be the direct opposite of the finding of Pap and Cooper that overexpression of GSK3 was sufficient to induce apoptosis (Pap and Cooper, 1998). Through more recent studies using GSK3 inhibitors like lithium evidence accumulated that the death-stimulus dictates whether GSK3 acts as a pro- death or pro-survival molecule (reviewed in Beurel and Jope, 2006). GSK3 acts pro-apoptotically when the intrinsic mitochondrial pathway is triggered but in an anti-apoptotic manner in death-receptor induced apoptosis. Accordingly, three independent studies report that inhibition of GSK with lithium or knockdown of GSK3 sensitised to TRAIL-induced apoptosis. The earliest study by Liao et al. showed that inhibition of GSK3 with lithium or the knockdown of GSK3β breaks TRAIL resistance of prostate cancer cells (Liao et al., 2003). The role of the α-isoforms has not been addressed in this study. TRAIL sensitisation was associated with increased proteolytic processing of caspase-8 and its downstream target Bid. In line with this, using an siRNA-based approach, another study found that the knockdown of isoform GSK3β but not GSK3α sensitised cells to TRAIL-induced apoptosis which was correlated with an up-regulation of TRAIL-R2 (Rottmann et al., 2005). The most recent publication on that matter showed GSK3 in a complex with DDX3 and cIAP-1 which were associated with death receptors (Sun et al., 2008). GSK3 restrained apoptotic signalling by inhibiting formation of the DISC and caspase-8 activation. Stimulated death receptors seem to surmount the anti-apoptotic complex by causing GSK3 inactivation and cleavage of DDX3 and cIAP-1 to enable progression of the apoptotic signalling cascade. In cells resistant to death receptor stimulation the anti-apoptotic complex remains functional. This resistance could be overcome by GSK3 inhibitors. In this study both isoforms were found to be in the anti-apoptotic complex.

In contrast an siRNA screen conducted by Aza-Blanc et al. (Aza-Blanc et al., 2003) in HeLa cells found the α-isoform of GSK3 to be necessary for TRAIL-induced apoptosis as its knockdown induced a more resistant phenotype.

Based on the data described above, it seemed unlikely that KU-55933- or PIK75-induced TRAIL sensitisation worked via GSK3. As already mentioned, AKT usually phosphorylates and thereby inactivates GSK3. Inhibition of the PI3K/AKT pathway therefore leads to activation GSK3. If active GSK3 were necessary for sensitisation, inhibition or knockdown of GSK3 would rather block TRAIL sensitisation than induce it. In line with this hypothesis, phosphorylation of GSK3 was not significantly altered upon inhibition of p110α (figure 41). Furthermore, knockdown of either isoforms did not block TRAIL sensitisation by KU-55933 or PIK75. On the contrary, knockdown of the β-isoforms rather induced a more TRAIL-sensitive phenotype, which has been described in the studies mentioned above (figure 42).

A knockdown of β-catenin, which reflects the situation of activated GSK3 in Wnt signalling, did not influence TRAIL-induced apoptosis either. Therefore a down-regulation of β-catenin targets like the soluble TRAIL-receptor OPG (De Toni et al., 2008), is not involved in sensitisation mediated by inhibition of the PI3K/AKT pathway. These results are in line with a recent publication which showed that although it has been widely accepted that active PI3K signalling feeds positively into the Wnt pathway by AKT-mediated inhibition of GSK3, compartmentalisation of GSK3 by Axin prohibits cross-talk between the PI3K and Wnt pathways. Thus, Wnt-mediated transcriptional activity is not modulated by activation of the PI3K/AKT pathway (Ng et al., 2009).

Taken together GSK3 does not seem to be the pivotal player mediating KU-55933 or PIK75-induced TRAIL sensitisation downstream of AKT.

The next important factors regulated by AKT are the Forkhead Box O (FoxO) transcription factors. FoxO transcription factors regulate multiple signalling pathways and play a role in a number of physiological and pathological processes including cancer (reviewed in Maiese et al., 2008). In mammals, there are four different FoxO family members: FoxO1, FoxO3a, FoxO4 and FoxO6. Direct phosphorylation of FoxO1, FoxO3a and FoxO4 by AKT facilitates interaction of FoxOs with 14-3-3 causing the displacement of the complex from the nucleus into the cytoplasm (figure 45). Translocation of FoxOs into the cytoplasm results in inhibition of target gene transcription. As might be expected, growth factor withdrawal and inhibition of

PI3K have been shown to result in dephosphorylation of FoxO at its AKT sites and thereby to target gene activation. Accordingly, inhibition of p110α using PIK75 induced a loss of phosphorylation and a translocation of FoxO3a into the nucleus (figure 46).

FoxO has been implicated in the regulation of apoptosis by inducing transcription of e.g. Bim, CD95L and TRAIL (Modur et al., 2002). Besides inducing gene transcription of TRAIL itself, one study has recently linked FoxOs and resistance to TRAIL-induced apoptosis. In this study in activated hepatic stellate cells, concomitant knockdown of FoxO1 and FoxO3a together induced a more TRAIL-resistant phenotype (Park et al., 2009) and thereby identified FoxOs as factors required for TRAIL-induced apoptosis. However, the contribution of the isoforms FoxO1 and FoxO3a has not been addressed separately. TRAIL itself was shown to lead to a dephosphorylation for FoxOs and consequently translocation of FoxOs to the nucleus. The authors then claimed that up-regulation of cFLIP was responsible for inducing FoxO-mediated TRAIL resistance. However, the data in support of the interpretation were not convincing. The data rather showed that knockdown of FoxOs inhibited the cleavage of cFLIP and TRAIL-induced down-regulation of cFLIP, which occurred in the course of TRAIL-induced apoptosis. FoxO knockdown alone did not lead to an increase of cFLIP which might have been expected if cFLIP was the factor essential for the observed TRAIL-resistance induced by FoxO. Furthermore, the authors did not investigate whether an activation of FoxOs by additional inhibition of PI3K leads to a sensitisation to TRAIL-induced apoptosis as has been addressed in this thesis.

A second study which investigated cell death induced by over-expression of constitutively active FoxO3a reported a down-regulation of cFLIP in Human Umbilical Vein Endothelial Cells (HUVECs) (Skurk et al., 2004). This down-regulation proved to be essential for FoxO3a-induced cell death which depended on the activation of the death-receptor-induced apoptosis. To date, a role of FoxOs as modulators of TRAIL sensitivity in cancer cells has not been studied.

As cFLIP down-regulation is one of the changes occurring upon inhibition of p110α, it was possible that FoxO transcription factors convey TRAIL sensitisation upon treatment with KU-55933 or PIK75. To evaluate the contribution of the two different FoxO transcription factors 1 and 3a, both of them were addressed separately. Similarly to GSK3, AKT usually phosphorylates and thereby inactivates FoxOs. Inhibition of the PI3K/AKT pathway leads to

activation of FoxOs. Therefore, if active FoxO1 were necessary for sensitisation, inhibition or knockdown of FoxO1 would be expected to block TRAIL sensitisation. Knockdown of FoxO1 worked very well but neither did it influence TRAIL-induced apoptosis itself nor was it able to block KU-55933- or PIK75- mediated sensitisation to TRAIL (figure 47). Therefore, a role for FoxO1 in the sensitisation of cancer cells to TRAIL could be excluded. The role of FoxO3a was approached differently. A tool which has widely been used to study the role of FoxO3a in different contexts is an inducible, non-phosphorylatable mutant version of FoxO3a. This version can no longer be inactivated by AKT and therefore mimics the situation upon inhibition of PI3K. DL23 cells, which are DLD1 cells stably transfected with the 4-HT inducible mutant were provided by Prof. Burgering (Kops et al., 2002). These cells, as well as the parental cell line were very TRAIL-sensitive in comparison to the DLD1 cells previously used in this study. Such a difference between different DLD1 cells has already been noticed before and has been associated with differential caspase-8 levels (Zhang et al., 2005). Indeed, the TRAIL-resistant DLD1 cells were marked by a much lower caspase-8 expression than DL23 cells or their parental DLD1 cells. Induction of constitutively active FoxO3a in DL23 sensitised these cells to TRAIL-induced apoptosis and led to a concomitant down-regulation of cFLIP and XIAP (figure 49), the same molecular changes which occurred upon blockage of p110α. Down-regulation of XIAP by FoxO3a activity has been described previously. Interestingly, this study, which also used an inducible constitutively active FoxO3a, showed that TNF stimulation resulted in apoptosis instead of pro-inflammatory signalling (Lee et al., 2008).

Taken together, these results show for the first time that active FoxO3a can sensitise cancer cells to TRAIL-induced apoptosis and uncover which of the many pathways downstream of activated AKT is responsible for TRAIL resistance and whose activation by PI3K inhibitors is decisive for sensitisation to TRAIL-induced apoptosis. Although many different factors are inhibited/activated by inhibition of PI3K/AKT, activation of FoxO3a is sufficient induce the down-regulation of cFLIP and XIAP which has been proven to be pivotal for TRAIL sensitisation by inhibition of p110α.

6. Conclusion and Outlook

The results of this study suggest that the combination of TRAIL and KU-55933 or TRAIL and PIK75 respectively could be a promising option for cancer therapy, in particular for cancer types which are hallmarked by mutations of KRAS or PI3KCA, including but not limited to cancers of the colon, breast, ovary, lung and pancreas. This study revealed FoxO3a as the decisive modifier of TRAIL-induced apoptosis in cancer cells. As AKT triggers many different pathways which play an important role in other processes that also affect normal physiology, it would be advantageous to target FoxO3A directly. Possibly a small molecule leading to activation of FoxO3a in a more direct manner can be identified by rational drug design.

The next step in advancing the combination of TRAIL with KU-55933 and /or TRAIL with PIK75, respectively will be to evaluate its toxicity and the efficacy of the agents alone and in combination in normal cells and in primary cancer cells. Addiotionally, an *in vivo* study should be performed to find out whether the application of TRAIL and KU-55933/PIK75 is feasible and effective in vivo. As mutations in KRAS or in the PI3K/AKT pathway occur in almost 80 % of colon carcinomas, a colon carcinoma model would be ideal to study the effect of the different combinations. Although there are very good genetic models for benign adenoma (Sansom et al., 2006), no genetic mouse model is available for spontaneous tumours that arise in the intestine, become invasive, and metastasise to organs such as liver, lungs, and lymph nodes, as they do in humans. Because metastasis is responsible for most colon cancer mortality and PIK3CA has been identified as a driver of metastasis (Guo et al., 2007; Pang et al., 2009), it would be important to study the effect of TRAIL in combination with KU-55933 or PIK75 on metastasis. DLD1 and HCT116 cells used in this study have already been employed for orthotopic transplantation models (Samuels et al., 2005) and have great metastatic potential (Guo et al., 2007). Noteworthy, a panel of DLD1 cells as well as HCT116 exists which express wild type versions versus mutant version of different genes as has been described in section 5.4. Among the target genes are e.g. KRAS, p53 and PTEN. The use of these cells would provide the opportunity to study the efficacy of TRAIL in combinations with KU-55933 or PIK75 in the presence or absence of specific mutations. Thus, it might be possible to determine a certain mutation pattern that renders a particular tumour treatable or

not treatable with TRAIL in combination with KU-55933 or PIK75. It can also be envisaged that it will be possible in the future to use mutation patterns and/or correlating expression profiles as a prognostic tool to predict which tumours are treatable with the combination of TRAIL and KU-55933 or PIK75.

Currently, the use of TRAIL-based cancer therapy is restricted to tumours which are TRAIL sensitive in the first place or tumours that can be sensitised by co-treatment with other anti-cancer drugs. In this respect it would be interesting to study the surviving clones that do not die upon treatment with TRAIL and KU-55933/PIK75. These clones probably up-regulate certain survival pathways that allow them to escape the induction of cell death. If these pathways were to be identified and the escape mechanism could be interfered with by combining such interference with TRAIL and PI3K inhibitors, it could be possible to limit the tumour's possibilities of creating a therapy resistant variant even before this materialises. This strategy may help in overcoming current limitations in cancer therapy.

7. List of Abbreviations

4-HT	Hydroxy-tamoxifen
AIP4	Atrophin-interacting protein 4
APC	Antigen presenting cell
ATG	Autophagy related genes
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
Bcl	B cell lymphoma
BH	Bcl-2 homology
Bid	BH3- interacting domain death agonist
CAD	Caspase activated DNase
CARD	Caspase recruitment domain
cDNA	Complementary DNA
cFLIP	Cellular FLICE-like inhibitory protein
CK	Casein kinase
CRC	Colon carcinoma
CRD	Cysteine rich domain
CRR	Cysteine rich repeats
DC	Dendritic cell
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DEN	Diethylnitrosamine
DISC	Death-inducing signalling complex
DMEM	Dulbecco’s modified Eagle's medium
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
E.coli	Escherichia coli
EAE	Experimental autoimmune encephalomyelitis
EAT	Experimental autoimmune thyroiditis
EDTA	ethylenediaminetetraacetic acid

EGFR	Epidermal growth factor receptor
esiRNA	Endoribonuclease-prepared siRNA
ERK	extracellular signal-regulated kinase
FADD	Fas-associated protein with death domain
FCS	Fetal calf serum
HDACi	Histone deacetylase inhibitors
HRP	Horse-raddish peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of caspase activated DNase
IKK	IκB-kinase
IL	Interleukin
INF	Interferon
JAK	Janus kinase
JNK	C-Jun N-terminal kinase
KD	Knockdown
LPS	Lipopolysaccharide
LS	Large subunit
MEFs	Mouse embryonic fibroblasts
MOMP	Mitochondrial outer-membrane permeabilisation
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells
NHL	Non-Hodgkin lymphoma
NSCLC	Non-small cell lung cancer
OPG	Osteoprotegerin
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PI3K	Phosphoinositide 3-kinases
PIKK	phosphoinositide 3-kinase-related kinases
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate

PKB	Protein kinase B
PLAD	Pre-ligand assembly domain
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of NF-κB
RIP1	Receptor Interacting Protein 1
Rluc	Renilla luciferase
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SL	Small subunit
STAT	Signal Transducers and Activator of Transcription
tBid	Truncated Bid
TCL	Total cell lysates
TCR	T cell receptor
TM	Transmembrane
TNF	Tumour necrosis factor
TRA	TRAIL-receptor agonist
TRAF-2	TNF-receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand- receptor
VDAC	Voltage dependent anion channel
VEGFR	Vascular endothelial growth factor receptor
Wt	Wild type
XIAP	X-linked inhibitor apoptosis protein

8. List of Figures

9. References

Adrian, F.J., Q. Ding, T. Sim, A. Velentza, C. Sloan, Y. Liu, G. Zhang, W. Hur, S. Ding, P. Manley, J. Mestan, D. Fabbro, and N.S. Gray. 2006. Allosteric inhibitors of Bcr-abl-dependent cell proliferation. *Nat Chem Biol*. 2:95-102.

Akritopoulou-Zanze, I., and P.J. Hajduk. 2009. Kinase-targeted libraries: the design and synthesis of novel, potent, and selective kinase inhibitors. *Drug Discov Today*. 14:291-7.

Alladina, S.J., J.H. Song, S.T. Davidge, C. Hao, and A.S. Easton. 2005. TRAIL-induced apoptosis in human vascular endothelium is regulated by phosphatidylinositol 3-kinase/Akt through the short form of cellular FLIP and Bcl-2. *J Vasc Res*. 42:337-47.

Amado, R.G., M. Wolf, M. Peeters, E. Van Cutsem, S. Siena, D.J. Freeman, T. Juan, R. Sikorski, S. Suggs, R. Radinsky, S.D. Patterson, and D.D. Chang. 2008. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol*. 26:1626-34.

Ashkenazi, A., R.C. Pai, S. Fong, S. Leung, D.A. Lawrence, S.A. Marsters, C. Blackie, L. Chang, A.E. McMurtrey, A. Hebert, L. DeForge, I.L. Koumenis, D. Lewis, L. Harris, J. Bussiere, H. Koeppen, Z. Shahrokh, and R.H. Schwall. 1999. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest*. 104:155-62.

Aza-Blanc, P., C.L. Cooper, K. Wagner, S. Batalov, Q.L. Deveraux, and M.P. Cooke. 2003. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol Cell*. 12:627-37.

Badovinac, V.P., K.A. Messingham, T.S. Griffith, and J.T. Harty. 2006. TRAIL deficiency delays, but does not prevent, erosion in the quality of "helpless" memory CD8 T cells. *J Immunol*. 177:999-1006.

Bai, L., H.M. Ni, X. Chen, D. DiFrancesca, and X.M. Yin. 2005. Deletion of Bid impedes cell proliferation and hepatic carcinogenesis. *Am J Pathol*. 166:1523-32.

Baliga, B., and S. Kumar. 2003. Apaf-1/cytochrome c apoptosome: an essential initiator of caspase activation or just a sideshow? *Cell Death Differ*. 10:16-8.

Barbone, D., T.M. Yang, J.R. Morgan, G. Gaudino, and V.C. Broaddus. 2008. Mammalian target of rapamycin contributes to the acquired apoptotic resistance of human mesothelioma multicellular spheroids. *J Biol Chem*. 283:13021-30.

Barnhart, B.C., E.C. Alappat, and M.E. Peter. 2003. The CD95 type I/type II model. *Semin Immunol*. 15:185-93.

Barry, M., J.A. Heibein, M.J. Pinkoski, S.F. Lee, R.W. Moyer, D.R. Green, and R.C. Bleackley. 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol*. 20:3781-94.

Beurel, E., and R.S. Jope. 2006. The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog Neurobiol*. 79:173-89.

Bortul, R., P.L. Tazzari, A. Cappellini, G. Tabellini, A.M. Billi, R. Bareggi, L. Manzoli, L. Cocco, and A.M. Martelli. 2003. Constitutively active Akt1 protects HL60 leukemia cells from TRAIL-induced apoptosis through a mechanism involving NF-kappaB activation and cFLIP(L) up-regulation. *Leukemia*. 17:379-89.

Brincks, E.L., A. Katewa, T.A. Kucaba, T.S. Griffith, and K.L. Legge. 2008. CD8 T cells utilize TRAIL to control influenza virus infection. *J Immunol*. 181:4918-25.

Buchsbaum, D.J., T. Zhou, W.E. Grizzle, P.G. Oliver, C.J. Hammond, S. Zhang, M. Carpenter, and A.F. LoBuglio. 2003. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. *Clin Cancer Res*. 9:3731-41.

Buchsbaum, D.J., T. Zhou, and A.F. Lobuglio. 2006. TRAIL receptor-targeted therapy. *Future Oncol*. 2:493-508.

Camidge D., H.R., Gordon M., Eckhardt S., Kurzroc R., Durbin B., Ing J., Ling J., Sager J., Mendelsohn D. 2007. ASCO annual Meeting Proceedings Part I *Journal of Clinical Oncology*. 25:3582.

Cardone, M.H., N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, and J.C. Reed. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science*. 282:1318-21.

Chan, C.W., E. Crafton, H.N. Fan, J. Flook, K. Yoshimura, M. Skarica, D. Brockstedt, T.W. Dubensky, M.F. Stins, L.L. Lanier, D.M. Pardoll, and F. Housseau. 2006. Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. *Nat Med*. 12:207-13.

Chan, F.K., H.J. Chun, L. Zheng, R.M. Siegel, K.L. Bui, and M.J. Lenardo. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science*. 288:2351-4.

Chao, Y., E.N. Shiozaki, S.M. Srinivasula, D.J. Rigotti, R. Fairman, and Y. Shi. 2005. Engineering a dimeric caspase-9: a re-evaluation of the induced proximity model for caspase activation. *PLoS Biol*. 3:e183.

Chen, K.F., P.Y. Yeh, K.H. Yeh, Y.S. Lu, S.Y. Huang, and A.L. Cheng. 2008. Down-regulation of phospho-Akt is a major molecular determinant of bortezomib-induced apoptosis in hepatocellular carcinoma cells. *Cancer Res*. 68:6698-707.

Chen, M., H. He, S. Zhan, S. Krajewski, J.C. Reed, and R.A. Gottlieb. 2001. Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. *J Biol Chem*. 276:30724-8.

Chipuk, J.E., L. Bouchier-Hayes, and D.R. Green. 2006. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ*. 13:1396-402.

Chou, J.J., H. Li, G.S. Salvesen, J. Yuan, and G. Wagner. 1999. Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell*. 96:615-24.

Chow, L.Q., S.G. Eckhardt, D.L. Gustafson, C. O'Bryant, S. Hariharan, S. Diab, N.L. Fox, A. Corey, K. Padavic, M. Brown, and R.B. Cohen. 2006. HGS-ETR1, an antibody targeting TRAIL-R1, in combination with paclitaxel and carboplatin in patients with advanced solid malignancies: Results of a phase 1 and PK study. *Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings Part I.* . 24:2515

Cirman, T., K. Oresic, G.D. Mazovec, V. Turk, J.C. Reed, R.M. Myers, G.S. Salvesen, and B. Turk. 2004. Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. *J Biol Chem*. 279:3578-87.

Clayer, M., S. Bouralexis, A. Evdokiou, S. Hay, G.J. Atkins, and D.M. Findlay. 2001. Enhanced apoptosis of soft tissue sarcoma cells with chemotherapy: A potential new approach using TRAIL. *J Orthop Surg (Hong Kong)*. 9:19-22.

Clodi, K., D. Wimmer, Y. Li, R. Goodwin, U. Jaeger, G. Mann, H. Gadner, and A. Younes. 2000. Expression of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors and sensitivity to TRAIL-induced apoptosis in primary B-cell acute lymphoblastic leukaemia cells. *Br J Haematol*. 111:580-6.

Cohen, J.J., and R.C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol*. 132:38-42.

Corazza, N., S. Jakob, C. Schaer, S. Frese, A. Keogh, D. Stroka, D. Kassahn, R. Torgler, C. Mueller, P. Schneider, and T. Brunner. 2006. TRAIL receptor-mediated JNK activation and Bim phosphorylation critically regulate Fas-mediated liver damage and lethality. *J Clin Invest*. 116:2493-9.

Cordier, S.M., K. Papenfuss, and H. Walczak. 2009. From Biochemical Principles of Apoptosis Induction by TRAIL to Application in Tumour Therapy. *Results Probl Cell Differ*.

Cowell, I.G., B.W. Durkacz, and M.J. Tilby. 2005. Sensitization of breast carcinoma cells to ionizing radiation by small molecule inhibitors of DNA-dependent protein kinase and ataxia telangiectsia mutated. *Biochem Pharmacol*. 71:13-20.

Crescenzi, E., G. Palumbo, J. de Boer, and H.J. Brady. 2008. Ataxia telangiectasia mutated and p21CIP1 modulate cell survival of drug-induced senescent tumor cells: implications for chemotherapy. *Clin Cancer Res*. 14:1877-87.

Cretney, E., J.L. McQualter, N. Kayagaki, H. Yagita, C.C. Bernard, I.S. Grewal, A. Ashkenazi, and M.J. Smyth. 2005. TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L suppresses experimental autoimmune encephalomyelitis in mice. *Immunol Cell Biol*. 83:511-9.

Cretney, E., A. Shanker, H. Yagita, M.J. Smyth, and T.J. Sayers. 2006. TNF-related apoptosis-inducing ligand as a therapeutic agent in autoimmunity and cancer. *Immunol Cell Biol*. 84:87-98.

Cretney, E., K. Takeda, H. Yagita, M. Glaccum, J.J. Peschon, and M.J. Smyth. 2002. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol*. 168:1356-61.

Dai, Y., M. Liu, W. Tang, Y. Li, J. Lian, T.S. Lawrence, and L. Xu. 2009. A Smac-mimetic sensitizes prostate cancer cells to TRAIL-induced apoptosis via modulating both IAPs and NF-kappaB. *BMC Cancer*. 9:392.

Dan, H.C., M. Sun, S. Kaneko, R.I. Feldman, S.V. Nicosia, H.G. Wang, B.K. Tsang, and J.Q. Cheng. 2004. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *J Biol Chem*. 279:5405-12.

Danial, N.N. 2008. BAD: undertaker by night, candyman by day. *Oncogene*. 27 Suppl 1:S53-70.

Datta, S.R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M.E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*. 91:231-41.

De Toni, E.N., S.E. Thieme, A. Herbst, A. Behrens, P. Stieber, A. Jung, H. Blum, B. Goke, and F.T. Kolligs. 2008. OPG is regulated by beta-catenin and mediates resistance to TRAIL-induced apoptosis in colon cancer. *Clin Cancer Res*. 14:4713-8.

Degli-Esposti, M.A., W.C. Dougall, P.J. Smolak, J.Y. Waugh, C.A. Smith, and R.G. Goodwin. 1997a. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity*. 7:813-20.

Degli-Esposti, M.A., P.J. Smolak, H. Walczak, J. Waugh, C.P. Huang, R.F. DuBose, R.G. Goodwin, and C.A. Smith. 1997b. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med*. 186:1165-70.

Degli Esposti, M., G. Ferry, P. Masdehors, J.A. Boutin, J.A. Hickman, and C. Dive. 2003. Post-translational modification of Bid has differential effects on its susceptibility to cleavage by caspase 8 or caspase 3. *J Biol Chem*. 278:15749-57.

Desagher, S., A. Osen-Sand, S. Montessuit, E. Magnenat, F. Vilbois, A. Hochmann, L. Journot, B. Antonsson, and J.C. Martinou. 2001. Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. *Mol Cell*. 8:601-11.

Diehl, G.E., H.H. Yue, K. Hsieh, A.A. Kuang, M. Ho, L.A. Morici, L.L. Lenz, D. Cado, L.W. Riley, and A. Winoto. 2004. TRAIL-R as a negative regulator of innate immune cell responses. *Immunity*. 21:877-89.

Dong, L.Q., and F. Liu. 2005. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. *Am J Physiol Endocrinol Metab*. 289:E187-96.

Druker, B.J., M. Talpaz, D.J. Resta, B. Peng, E. Buchdunger, J.M. Ford, N.B. Lydon, H. Kantarjian, R. Capdeville, S. Ohno-Jones, and C.L. Sawyers. 2001. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 344:1031-7.

Ehrhardt, H., S. Fulda, I. Schmid, J. Hiscott, K.M. Debatin, and I. Jeremias. 2003. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. *Oncogene*. 22:3842-52.

Ehrlich, S., C. Infante-Duarte, B. Seeger, and F. Zipp. 2003. Regulation of soluble and surface-bound TRAIL in human T cells, B cells, and monocytes. *Cytokine*. 24:244-53.

Ellis, R.E., J.Y. Yuan, and H.R. Horvitz. 1991. Mechanisms and functions of cell death. *Annu Rev Cell Biol*. 7:663-98.

Emery, J.G., P. McDonnell, M.B. Burke, K.C. Deen, S. Lyn, C. Silverman, E. Dul, E.R. Appelbaum, C. Eichman, R. DiPrinzio, R.A. Dodds, I.E. James, M. Rosenberg, J.C. Lee, and P.R. Young. 1998. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem*. 273:14363-7.

Engelman, J.A. 2009. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 9:550-62.

Fadok, V.A., D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, and P.M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*. 148:2207-16.

Fedorov, O., S. Muller, and S. Knapp. The (un)targeted cancer kinome. *Nat Chem Biol*. 6:166-169.

Felber, M., J. Sonnemann, and J.F. Beck. 2007. Inhibition of novel protein kinase C-epsilon augments TRAIL-induced cell death in A549 lung cancer cells. *Pathol Oncol Res*. 13:295-301.

Finnberg, N., J.J. Gruber, P. Fei, D. Rudolph, A. Bric, S.H. Kim, T.F. Burns, H. Ajuha, R. Page, G.S. Wu, Y. Chen, W.G. McKenna, E. Bernhard, S. Lowe, T. Mak, and W.S. El-Deiry. 2005. DR5 knockout mice are compromised in radiation-induced apoptosis. *Mol Cell Biol*. 25:2000-13.

Fischer, U., C. Stroh, and K. Schulze-Osthoff. 2006. Unique and overlapping substrate specificities of caspase-8 and caspase-10. *Oncogene*. 25:152-9.

Folkes, A.J., K. Ahmadi, W.K. Alderton, S. Alix, S.J. Baker, G. Box, I.S. Chuckowree, P.A. Clarke, P. Depledge, S.A. Eccles, L.S. Friedman, A. Hayes, T.C. Hancox, A. Kugendradas, L. Lensun, P. Moore, A.G. Olivero, J. Pang, S. Patel, G.H. Pergl-Wilson, F.I. Raynaud, A. Robson, N. Saghir, L. Salphati, S. Sohal, M.H. Ultsch, M. Valenti, H.J. Wallweber, N.C. Wan, C. Wiesmann, P. Workman, A. Zhyvoloup, M.J. Zvelebil, and S.J. Shuttleworth. 2008. The identification of 2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin -4-yl-thieno[3,2-d]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. *J Med Chem*. 51:5522-32.

Fuke, H., K. Shiraki, K. Sugimoto, J. Tanaka, T. Beppu, K. Yoneda, N. Yamamoto, K. Ito, M. Masuya, and Y. Takei. 2007. Jak inhibitor induces S phase cell-cycle arrest and augments TRAIL-induced apoptosis in human hepatocellular carcinoma cells. *Biochem Biophys Res Commun*. 363:738-44.

Fulda, S., E. Meyer, and K.M. Debatin. 2002. Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. *Oncogene*. 21:2283-94.

Ganten, T.M., R. Koschny, T.L. Haas, J. Sykora, M. Li-Weber, K. Herzer, and H. Walczak. 2005. Proteasome inhibition sensitizes hepatocellular carcinoma cells, but not human hepatocytes, to TRAIL. *Hepatology*. 42:588-97.

Ganten, T.M., R. Koschny, J. Sykora, H. Schulze-Bergkamen, P. Buchler, T.L. Haas, M.B. Schader, A. Untergasser, W. Stremmel, and H. Walczak. 2006. Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. *Clin Cancer Res*. 12:2640-6.

Garcia-Saez, A.J., J. Ries, M. Orzaez, E. Perez-Paya, and P. Schwille. 2009. Membrane promotes tBID interaction with BCL(XL). *Nat Struct Mol Biol*. 16:1178-85.

Garofalo, M., G. Di Leva, G. Romano, G. Nuovo, S.S. Suh, A. Ngankeu, C. Taccioli, F. Pichiorri, H. Alder, P. Secchiero, P. Gasparini, A. Gonelli, S. Costinean, M. Acunzo, G. Condorelli, and C.M. Croce. 2009. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell*. 16:498-509.

Gazitt, Y. 1999. TRAIL is a potent inducer of apoptosis in myeloma cells derived from multiple myeloma patients and is not cytotoxic to hematopoietic stem cells. *Leukemia*. 13:1817-24.

Golks, A., D. Brenner, C. Fritsch, P.H. Krammer, and I.N. Lavrik. 2005. c-FLIPR, a new regulator of death receptor-induced apoptosis. *J Biol Chem*. 280:14507-13.

Greco, F.A., P. Bonomi, J. Crawford, K. Kelly, Y. Oh, W. Halpern, L. Lo, G. Gallant, and J. Klein. 2008. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. *Lung Cancer*. 61:82-90.

Griffith, T., B. Konety, F. Joudi, H. Aubert, M. Cohen, T. Ratliff, and R. Williams. 2007a. Phase I study of Ad5-TRAIL in men with clinically organ confined prostate cancer. *AACR Meeting Abstracts*. 2007:4870-.

Griffith, T.S., H. Kazama, R.L. VanOosten, J.K. Earle, Jr., J.M. Herndon, D.R. Green, and T.A. Ferguson. 2007b. Apoptotic cells induce tolerance by generating helpless CD8+ T cells that produce TRAIL. *J Immunol*. 178:2679-87.

Grosse-Wilde, A., O. Voloshanenko, S.L. Bailey, G.M. Longton, U. Schaefer, A.I. Csernok, G. Schutz, E.F. Greiner, C.J. Kemp, and H. Walczak. 2008. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest*. 118:100-10.

Guo, F., C. Sigua, J. Tao, P. Bali, P. George, Y. Li, S. Wittmann, L. Moscinski, P. Atadja, and K. Bhalla. 2004. Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells. *Cancer Res*. 64:2580-9.

Guo, X.N., A. Rajput, R. Rose, J. Hauser, A. Beko, K. Kuropatwinski, C. LeVea, R.M. Hoffman, M.G. Brattain, and J. Wang. 2007. Mutant PIK3CA-bearing colon cancer cells display increased metastasis in an orthotopic model. *Cancer Res*. 67:5851-8.

Halaas, O., R. Vik, A. Ashkenazi, and T. Espevik. 2000. Lipopolysaccharide induces expression of APO2 ligand/TRAIL in human monocytes and macrophages. *Scand J Immunol*. 51:244-50.

Han, H., S.W. Shin, C.Y. Seo, H.C. Kwon, J.Y. Han, I.H. Kim, J.Y. Kwak, and J.I. Park. 2007. 15-Deoxy-delta 12,14-prostaglandin J2 (15d-PGJ 2) sensitizes human leukemic HL-60 cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through Akt downregulation. *Apoptosis*. 12:2101-14.

Hao, C., J.H. Song, B. Hsi, J. Lewis, D.K. Song, K.C. Petruk, D.L. Tyrrell, and N.M. Kneteman. 2004. TRAIL inhibits tumor growth but is nontoxic to human hepatocytes in chimeric mice. *Cancer Res*. 64:8502-6.

Hayakawa, M., K. Kawaguchi, H. Kaizawa, T. Koizumi, T. Ohishi, M. Yamano, M. Okada, M. Ohta, S. Tsukamoto, F.I. Raynaud, P. Parker, P. Workman, and M.D. Waterfield. 2007. Synthesis and biological evaluation of sulfonylhydrazone-substituted imidazo[1,2-a]pyridines as novel PI3 kinase p110alpha inhibitors. *Bioorg Med Chem*. 15:5837-44.

Heinrich, M.C., C.L. Corless, G.D. Demetri, C.D. Blanke, M. von Mehren, H. Joensuu, L.S. McGreevey, C.J. Chen, A.D. Van den Abbeele, B.J. Druker, B. Kiese, B. Eisenberg, P.J. Roberts, S. Singer, C.D. Fletcher, S. Silberman, S. Dimitrijevic, and J.A. Fletcher. 2003.

Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol*. 21:4342-9.

Held, J., and K. Schulze-Osthoff. 2001. Potential and caveats of TRAIL in cancer therapy. *Drug Resist Updat*. 4:243-52.

Hengartner, M.O., and H.R. Horvitz. 1994. C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell*. 76:665-76.

Herbst, R.S., D.S. Mendolson, S. Ebbinghaus, M.S. Gordon, P. O'Dwyer, G. Lieberman, J. Ing, R. Kurzrock, W. Novotny, and G. Eckhardt. 2006. A phase I safety and pharmacokinetic (PK) study of recombinant Apo2L/TRAIL, an apoptosis-inducing protein in patients with advanced cancer. *Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings Part I.* . 24.

Herr, I., P. Schemmer, and M.W. Buchler. 2007. On the TRAIL to therapeutic intervention in liver disease. *Hepatology*. 46:266-74.

Hickson, I., Y. Zhao, C.J. Richardson, S.J. Green, N.M. Martin, A.I. Orr, P.M. Reaper, S.P. Jackson, N.J. Curtin, and G.C. Smith. 2004. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res*. 64:9152-9.

Hoeflich, K.P., J. Luo, E.A. Rubie, M.S. Tsao, O. Jin, and J.R. Woodgett. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature*. 406:86-90.

Hollick, J.J., L.J. Rigoreau, C. Cano-Soumillac, X. Cockcroft, N.J. Curtin, M. Frigerio, B.T. Golding, S. Guiard, I.R. Hardcastle, I. Hickson, M.G. Hummersone, K.A. Menear, N.M. Martin, I. Matthews, D.R. Newell, R. Ord, C.J. Richardson, G.C. Smith, and R.J. Griffin. 2007. Pyranone, thiopyranone, and pyridone inhibitors of phosphatidylinositol 3-kinase related kinases. Structure-activity relationships for DNA-dependent protein kinase inhibition, and identification of the first potent and selective inhibitor of the ataxia telangiectasia mutated kinase. *J Med Chem*. 50:1958-72.

Hopkins-Donaldson, S., J.L. Bodmer, K.B. Bourloud, C.B. Brognara, J. Tschopp, and N. Gross. 2000. Loss of caspase-8 expression in neuroblastoma is related to malignancy and resistance to TRAIL-induced apoptosis. *Med Pediatr Oncol*. 35:608-11.

Horak, P., D. Pils, G. Haller, I. Pribill, M. Roessler, S. Tomek, R. Horvat, R. Zeillinger, C. Zielinski, and M. Krainer. 2005a. Contribution of epigenetic silencing of tumor necrosis factor-related apoptosis inducing ligand receptor 1 (DR4) to TRAIL resistance and ovarian cancer. *Mol Cancer Res*. 3:335-43.

Horak, P., D. Pils, A. Kaider, A. Pinter, K. Elandt, C. Sax, C.C. Zielinski, R. Horvat, R. Zeillinger, A. Reinthaller, and M. Krainer. 2005b. Perturbation of the tumor necrosis factor--related apoptosis-inducing ligand cascade in ovarian cancer: overexpression of FLIPL and deregulation of the functional receptors DR4 and DR5. *Clin Cancer Res*. 11:8585-91.

Hornstein, M., M.J. Hoffmann, A. Alexa, M. Yamanaka, M. Muller, V. Jung, J. Rahnenfuhrer, and W.A. Schulz. 2008. Protein phosphatase and TRAIL receptor genes as new candidate tumor genes on chromosome 8p in prostate cancer. *Cancer Genomics Proteomics*. 5:123-36.

Hotte, S.J., H.W. Hirte, E.X. Chen, L.H. Le, A. Corey, M. Maclean, A. Iacobucci, N.L. Fox, and A.M. Oza. 2005. HGS-ETR1 , a Fully Human Monoclonal Antibody to the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Death Receptor 1 (TRAIL-R1) in Patients with Advanced Solid Cancer: Results of a Phase 1 Trial. *Journal of Clinical Oncology, 2005 ASCO Annual Meeting Proceedings.* . 23:3052.

Hotte, S.J., H.W. Hirte, E.X. Chen, L.L. Siu, L.H. Le, A. Corey, A. Iacobucci, M. MacLean, L. Lo, N.L. Fox, and A.M. Oza. 2008. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. *Clin Cancer Res*. 14:3450-5.

Hu, L., C. Zaloudek, G.B. Mills, J. Gray, and R.B. Jaffe. 2000. In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). *Clin Cancer Res*. 6:880-6.

Hymowitz, S.G., H.W. Christinger, G. Fuh, M. Ultsch, M. O'Connell, R.F. Kelley, A. Ashkenazi, and A.M. de Vos. 1999. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell*. 4:563-71.

Ichikawa, K., W. Liu, L. Zhao, Z. Wang, D. Liu, T. Ohtsuka, H. Zhang, J.D. Mountz, W.J. Koopman, R.P. Kimberly, and T. Zhou. 2001. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med*. 7:954-60.

Ihle, N.T., and G. Powis. 2009. Take your PIK: phosphatidylinositol 3-kinase inhibitors race through the clinic and toward cancer therapy. *Mol Cancer Ther*. 8:1-9.

Inoue, S., N. Harper, R. Walewska, M.J. Dyer, and G.M. Cohen. 2009. Enhanced Fas-associated death domain recruitment by histone deacetylase inhibitors is critical for the sensitization of chronic lymphocytic leukemia cells to TRAIL-induced apoptosis. *Mol Cancer Ther*. 8:3088-97.

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

Ishimura, N., H. Isomoto, S.F. Bronk, and G.J. Gores. 2006. Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells. *Am J Physiol Gastrointest Liver Physiol*. 290:G129-36.

Ivanov, V.N., H. Zhou, M.A. Partridge, and T.K. Hei. 2009. Inhibition of ataxia telangiectasia mutated kinase activity enhances TRAIL-mediated apoptosis in human melanoma cells. *Cancer Res*. 69:3510-9.

Izeradjene, K., L. Douglas, A. Delaney, and J.A. Houghton. 2005. Casein kinase II (CK2) enhances death-inducing signaling complex (DISC) activity in TRAIL-induced apoptosis in human colon carcinoma cell lines. *Oncogene*. 24:2050-8.

Janssen, E.M., N.M. Droin, E.E. Lemmens, M.J. Pinkoski, S.J. Bensinger, B.D. Ehst, T.S. Griffith, D.R. Green, and S.P. Schoenberger. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 434:88-93.

Jeremias, I., I. Herr, T. Boehler, and K.M. Debatin. 1998. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. *Eur J Immunol*. 28:143-52.

Jost, P.J., S. Grabow, D. Gray, M.D. McKenzie, U. Nachbur, D.C. Huang, P. Bouillet, H.E. Thomas, C. Borner, J. Silke, A. Strasser, and T. Kaufmann. 2009. XIAP discriminates between type I and type II FAS-induced apoptosis. *Nature*. 460:1035-9.

Joy, A.M., C.E. Beaudry, N.L. Tran, F.A. Ponce, D.R. Holz, T. Demuth, and M.E. Berens. 2003. Migrating glioma cells activate the PI3-K pathway and display decreased susceptibility to apoptosis. *J Cell Sci*. 116:4409-17.

Junttila, T.T., R.W. Akita, K. Parsons, C. Fields, G.D. Lewis Phillips, L.S. Friedman, D. Sampath, and M.X. Sliwkowski. 2009. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer Cell*. 15:429-40.

Kahraman, A., F.J. Barreyro, S.F. Bronk, N.W. Werneburg, J.L. Mott, Y. Akazawa, H.C. Masuoka, C.L. Howe, and G.J. Gores. 2008. TRAIL mediates liver injury by the innate immune system in the bile duct-ligated mouse. *Hepatology*. 47:1317-30.

Kamer, I., R. Sarig, Y. Zaltsman, H. Niv, G. Oberkovitz, L. Regev, G. Haimovich, Y. Lerenthal, R.C. Marcellus, and A. Gross. 2005. Proapoptotic BID is an ATM effector in the DNA-damage response. *Cell*. 122:593-603.

Kandasamy, K., and R.K. Srivastava. 2002. Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in non-small cell lung cancer cells. *Cancer Res*. 62:4929-37.

Kang, Y.C., K.M. Kim, K.S. Lee, S. Namkoong, S.J. Lee, J.A. Han, D. Jeoung, K.S. Ha, Y.G. Kwon, and Y.M. Kim. 2004. Serum bioactive lysophospholipids prevent TRAIL-induced apoptosis via PI3K/Akt-dependent cFLIP expression and Bad phosphorylation. *Cell Death Differ*. 11:1287-98.

Karapetis, C.S., S. Khambata-Ford, D.J. Jonker, C.J. O'Callaghan, D. Tu, N.C. Tebbutt, R.J. Simes, H. Chalchal, J.D. Shapiro, S. Robitaille, T.J. Price, L. Shepherd, H.J. Au, C. Langer,

M.J. Moore, and J.R. Zalcberg. 2008. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med.* 359:1757-65.

Kast, W.M., R. Offringa, P.J. Peters, A.C. Voordouw, R.H. Meloen, A.J. van der Eb, and C.J. Melief. 1989. Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. *Cell*. 59:603-14.

Kaufmann, T., L. Tai, P.G. Ekert, D.C. Huang, F. Norris, R.K. Lindemann, R.W. Johnstone, V.M. Dixit, and A. Strasser. 2007. The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. *Cell*. 129:423-33.

Kayagaki, N., N. Yamaguchi, M. Nakayama, H. Eto, K. Okumura, and H. Yagita. 1999a. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs. *J Exp Med*. 189:1451-60.

Kayagaki, N., N. Yamaguchi, M. Nakayama, A. Kawasaki, H. Akiba, K. Okumura, and H. Yagita. 1999b. Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. *J Immunol*. 162:2639-47.

Kayagaki, N., N. Yamaguchi, M. Nakayama, K. Takeda, H. Akiba, H. Tsutsui, H. Okamura, K. Nakanishi, K. Okumura, and H. Yagita. 1999c. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J Immunol*. 163:1906-13.

Kelley, S.K., and A. Ashkenazi. 2004. Targeting death receptors in cancer with Apo2L/TRAIL. *Curr Opin Pharmacol*. 4:333-9.

Kennedy, R.D., C.C. Chen, P. Stuckert, E.M. Archila, M.A. De la Vega, L.A. Moreau, A. Shimamura, and A.D. D'Andrea. 2007. Fanconi anemia pathway-deficient tumor cells are hypersensitive to inhibition of ataxia telangiectasia mutated. *J Clin Invest*. 117:1440-9.

Kerr, J.F., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 26:239-57.

Kim, H.R., K. Kim, K.H. Lee, S.J. Kim, and J. Kim. 2008. Inhibition of casein kinase 2 enhances the death ligand- and natural kiler cell-induced hepatocellular carcinoma cell death. *Clin Exp Immunol*. 152:336-44.

Kim, M.J., H.B. Kim, J.H. Bae, J.W. Lee, S.J. Park, D.W. Kim, S.I. Park, C.D. Kang, and S.H. Kim. 2009. Sensitization of human K562 leukemic cells to TRAIL-induced apoptosis by inhibiting the DNA-PKcs/Akt-mediated cell survival pathway. *Biochem Pharmacol*. 78:573-82.

Kim, S., A. Garcia, S.P. Jackson, and S.P. Kunapuli. 2007. Insulin-like growth factor-1 regulates platelet activation through PI3-Kalpha isoform. *Blood*. 110:4206-13.

Kim, S., J. Kang, J. Qiao, R.P. Thomas, B.M. Evers, and D.H. Chung. 2004. Phosphatidylinositol 3-kinase inhibition down-regulates survivin and facilitates TRAIL-mediated apoptosis in neuroblastomas. *J Pediatr Surg*. 39:516-21.

Klionsky, D.J., and S.D. Emr. 2000. Autophagy as a regulated pathway of cellular degradation. *Science*. 290:1717-21.

Knight, Z.A., B. Gonzalez, M.E. Feldman, E.R. Zunder, D.D. Goldenberg, O. Williams, R. Loewith, D. Stokoe, A. Balla, B. Toth, T. Balla, W.A. Weiss, R.L. Williams, and K.M. Shokat. 2006. A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell*. 125:733-47.

Kops, G.J., R.H. Medema, J. Glassford, M.A. Essers, P.F. Dijkers, P.J. Coffer, E.W. Lam, and B.M. Burgering. 2002. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol*. 22:2025-36.

Koschny, R., T.M. Ganten, J. Sykora, T.L. Haas, M.R. Sprick, A. Kolb, W. Stremmel, and H. Walczak. 2007a. TRAIL/bortezomib cotreatment is potentially hepatotoxic but induces cancer-specific apoptosis within a therapeutic window. *Hepatology*. 45:649-58.

Koschny, R., H. Walczak, and T.M. Ganten. 2007b. The promise of TRAIL--potential and risks of a novel anticancer therapy. *J Mol Med*. 85:923-35.

Krueger, A., I. Schmitz, S. Baumann, P.H. Krammer, and S. Kirchhoff. 2001. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J Biol Chem*. 276:20633-40.

Kusaba, M., K. Nakao, T. Goto, D. Nishimura, H. Kawashimo, H. Shibata, Y. Motoyoshi, N. Taura, T. Ichikawa, K. Hamasaki, and K. Eguchi. 2007. Abrogation of constitutive STAT3 activity sensitizes human hepatoma cells to TRAIL-mediated apoptosis. *J Hepatol*. 47:546-55.

LA, O.R., L. Tai, L. Lee, E.A. Kruse, S. Grabow, W.D. Fairlie, N.M. Haynes, D.M. Tarlinton, J.G. Zhang, G.T. Belz, M.J. Smyth, P. Bouillet, L. Robb, and A. Strasser. 2009. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature*. 461:659-63.

Lamhamedi-Cherradi, S.E., S.J. Zheng, K.A. Maguschak, J. Peschon, and Y.H. Chen. 2003. Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL-/- mice. *Nat Immunol*. 4:255-60.

Lane, D., A. Cartier, C. Rancourt, and A. Piche. 2008. Cell detachment modulates TRAIL resistance in ovarian cancer cells by downregulating the phosphatidylinositol 3-kinase/Akt pathway. *Int J Gynecol Cancer*. 18:670-6.

Lau, A., K.M. Swinbank, P.S. Ahmed, D.L. Taylor, S.P. Jackson, G.C. Smith, and M.J. O'Connor. 2005. Suppression of HIV-1 infection by a small molecule inhibitor of the ATM kinase. *Nat Cell Biol*. 7:493-500.

Lawrence, D., Z. Shahrokh, S. Marsters, K. Achilles, D. Shih, B. Mounho, K. Hillan, K. Totpal, L. DeForge, P. Schow, J. Hooley, S. Sherwood, R. Pai, S. Leung, L. Khan, B. Gliniak, J. Bussiere, C.A. Smith, S.S. Strom, S. Kelley, J.A. Fox, D. Thomas, and A. Ashkenazi. 2001. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med*. 7:383-5.

Lee, H.Y., S.W. Youn, J.Y. Kim, K.W. Park, C.I. Hwang, W.Y. Park, B.H. Oh, Y.B. Park, K. Walsh, J.S. Seo, and H.S. Kim. 2008. FOXO3a turns the tumor necrosis factor receptor signaling towards apoptosis through reciprocal regulation of c-Jun N-terminal kinase and NF-kappaB. *Arterioscler Thromb Vasc Biol*. 28:112-20.

Lee, M.W., J.H. Bach, H.J. Lee, D.Y. Lee, W.S. Joo, Y.S. Kim, S.C. Park, K.Y. Kim, W.B. Lee, and S.S. Kim. 2005. The activation of ERK1/2 via a tyrosine kinase pathway attenuates trail-induced apoptosis in HeLa cells. *Cancer Invest*. 23:586-92.

Lee, T.J., J.T. Lee, J.W. Park, and T.K. Kwon. 2006. Acquired TRAIL resistance in human breast cancer cells are caused by the sustained cFLIP(L) and XIAP protein levels and ERK activation. *Biochem Biophys Res Commun*. 351:1024-30.

Leong, S., R.B. Cohen, D.L. Gustafson, C.J. Langer, D.R. Camidge, K. Padavic, L. Gore, M. Smith, L.Q. Chow, M. von Mehren, C. O'Bryant, S. Hariharan, S. Diab, N.L. Fox, R. Miceli, and S.G. Eckhardt. 2009. Mapatumumab, an antibody targeting TRAIL-R1, in combination with paclitaxel and carboplatin in patients with advanced solid malignancies: results of a phase I and pharmacokinetic study. *J Clin Oncol*. 27:4413-21.

Leverkus, M., M.R. Sprick, T. Wachter, T. Mengling, B. Baumann, E. Serfling, E.B. Brocker, M. Goebeler, M. Neumann, and H. Walczak. 2003. Proteasome inhibition results in TRAIL sensitization of primary keratinocytes by removing the resistance-mediating block of effector caspase maturation. *Mol Cell Biol*. 23:777-90.

Li, H., H. Zhu, C.J. Xu, and J. Yuan. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*. 94:491-501.

Li, J., M.G. Anderson, L.A. Tucker, Y. Shen, K.B. Glaser, and O.J. Shah. 2009. Inhibition of Aurora B kinase sensitizes a subset of human glioma cells to TRAIL concomitant with induction of TRAIL-R2. *Cell Death Differ*. 16:498-511.

Li, L., R.M. Thomas, H. Suzuki, J.K. De Brabander, X. Wang, and P.G. Harran. 2004. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science*. 305:1471-4.

Liang, X., Y. Liu, Q. Zhang, L. Gao, L. Han, C. Ma, L. Zhang, Y.H. Chen, and W. Sun. 2007. Hepatitis B virus sensitizes hepatocytes to TRAIL-induced apoptosis through Bax. *J Immunol*. 178:503-10.

Liao, X., L. Zhang, J.B. Thrasher, J. Du, and B. Li. 2003. Glycogen synthase kinase-3beta suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer. *Mol Cancer Ther*. 2:1215-22.

Lievre, A., J.B. Bachet, D. Le Corre, V. Boige, B. Landi, J.F. Emile, J.F. Cote, G. Tomasic, C. Penna, M. Ducreux, P. Rougier, F. Penault-Llorca, and P. Laurent-Puig. 2006. KRAS

mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res*. 66:3992-5.

Lin, Y., A. Devin, A. Cook, M.M. Keane, M. Kelliher, S. Lipkowitz, and Z.G. Liu. 2000. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. *Mol Cell Biol*. 20:6638-45.

Liu, Y., and N.S. Gray. 2006. Rational design of inhibitors that bind to inactive kinase conformations. *Nat Chem Biol*. 2:358-64.

Llobet, D., N. Eritja, M. Encinas, N. Llecha, A. Yeramian, J. Pallares, A. Sorolla, F.J. Gonzalez-Tallada, X. Matias-Guiu, and X. Dolcet. 2008. CK2 controls TRAIL and Fas sensitivity by regulating FLIP levels in endometrial carcinoma cells. *Oncogene*. 27:2513-24.

LoPiccolo, J., G.M. Blumenthal, W.B. Bernstein, and P.A. Dennis. 2008. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat*. 11:32-50.

LoRusso, P., D. Hong, E. Heath, R. Kurzrock, D. Wang, M. Hsu, L. Goyal, J. Wiezorek, C. Storgard, and R. Herbst. 2007. **First-in-human study of AMG 655, a pro-apoptotic TRAIL receptor-2 agonist, in adult patients with advanced solid tumors**. *Journal of Clinical Oncology, 2007 ASCO Annual Meeting Proceedings Part I*. 25:3534.

Luo, W., J. Li, D. Zhang, L. Song, X.M. Yin, D. Desai, S. Amin, J. Chen, and C. Huang. Bid Mediates Anti-Apoptotic COX-2 Induction through the IKKbeta/NFkappaB Pathway Due to 5-MCDE Exposure. *Curr Cancer Drug Targets*.

MacFarlane, M., N. Harper, R.T. Snowden, M.J. Dyer, G.A. Barnett, J.H. Pringle, and G.M. Cohen. 2002. Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. *Oncogene*. 21:6809-18.

MacFarlane, M., S. Inoue, S.L. Kohlhaas, A. Majid, N. Harper, D.B. Kennedy, M.J. Dyer, and G.M. Cohen. 2005. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. *Cell Death Differ*. 12:773-82.

Maduro, J.H., E.G. de Vries, G.J. Meersma, B.M. Hougardy, A.G. van der Zee, and S. de Jong. 2008. Targeting pro-apoptotic trail receptors sensitizes HeLa cervical cancer cells to irradiation-induced apoptosis. *Int J Radiat Oncol Biol Phys*. 72:543-52.

Maiese, K., Z.Z. Chong, and Y.C. Shang. 2008. OutFOXOing disease and disability: the therapeutic potential of targeting FoxO proteins. *Trends Mol Med*. 14:219-27.

Makhov, P., K. Golovine, R.G. Uzzo, J. Rothman, P.L. Crispen, T. Shaw, B.J. Scoll, and V.M. Kolenko. 2008. Zinc chelation induces rapid depletion of the X-linked inhibitor of apoptosis and sensitizes prostate cancer cells to TRAIL-mediated apoptosis. *Cell Death Differ*. 15:1745-51.

Mamane, Y., E. Petroulakis, O. LeBacquer, and N. Sonenberg. 2006. mTOR, translation initiation and cancer. *Oncogene*. 25:6416-22.

Mariani, S.M., and P.H. Krammer. 1998. Differential regulation of TRAIL and CD95 ligand in transformed cells of the T and B lymphocyte lineage. *Eur J Immunol*. 28:973-82.

Martelli, A.M., P.L. Tazzari, G. Tabellini, R. Bortul, A.M. Billi, L. Manzoli, A. Ruggeri, R. Conte, and L. Cocco. 2003. A new selective AKT pharmacological inhibitor reduces resistance to chemotherapeutic drugs, TRAIL, all-trans-retinoic acid, and ionizing radiation of human leukemia cells. *Leukemia*. 17:1794-805.

Martinon, F., and J. Tschopp. 2007. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ*. 14:10-22.

Mason, K.D., C.J. Vandenberg, C.L. Scott, A.H. Wei, S. Cory, D.C. Huang, and A.W. Roberts. 2008. In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. *Proc Natl Acad Sci U S A*. 105:17961-6.

Mastrangelo, E., F. Cossu, M. Milani, G. Sorrentino, D. Lecis, D. Delia, L. Manzoni, C. Drago, P. Seneci, C. Scolastico, V. Rizzo, and M. Bolognesi. 2008. Targeting the X-linked inhibitor of apoptosis protein through 4-substituted azabicyclo[5.3.0]alkane smac mimetics. Structure, activity, and recognition principles. *J Mol Biol*. 384:673-89.

McCarthy, M.M., M. Sznol, K.A. DiVito, R.L. Camp, D.L. Rimm, and H.M. Kluger. 2005. Evaluating the expression and prognostic value of TRAIL-R1 and TRAIL-R2 in breast cancer. *Clin Cancer Res*. 11:5188-94.

McDonnell, J.M., D. Fushman, C.L. Milliman, S.J. Korsmeyer, and D. Cowburn. 1999. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. *Cell*. 96:625-34.

Merino, D., N. Lalaoui, A. Morizot, P. Schneider, E. Solary, and O. Micheau. 2006. Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2. *Mol Cell Biol*. 26:7046-55.

Micheau, O., and D. Merino. 2004. Controlling TRAIL-mediated caspase-3 activation. *Leukemia*. 18:1578-80.

Micheau, O., M. Thome, P. Schneider, N. Holler, J. Tschopp, D.W. Nicholson, C. Briand, and M.G. Grutter. 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem*. 277:45162-71.

Mirandola, P., C. Ponti, G. Gobbi, I. Sponzilli, M. Vaccarezza, L. Cocco, G. Zauli, P. Secchiero, F.A. Manzoli, and M. Vitale. 2004. Activated human NK and CD8+ T cells express both TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors but are resistant to TRAIL-mediated cytotoxicity. *Blood*. 104:2418-24.

Mitsiades, C.S., S.P. Treon, N. Mitsiades, Y. Shima, P. Richardson, R. Schlossman, T. Hideshima, and K.C. Anderson. 2001. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. *Blood*. 98:795-804.

Mitsiades, N., C.S. Mitsiades, V. Poulaki, D. Chauhan, P.G. Richardson, T. Hideshima, N. Munshi, S.P. Treon, and K.C. Anderson. 2002. Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. *Blood*. 99:4079-86.

Mizutani, Y., M. Nakao, O. Ogawa, O. Yoshida, B. Bonavida, and T. Miki. 2001. Enhanced sensitivity of bladder cancer cells to tumor necrosis factor related apoptosis inducing ligand mediated apoptosis by cisplatin and carboplatin. *J Urol*. 165:263-70.

Modur, V., R. Nagarajan, B.M. Evers, and J. Milbrandt. 2002. FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. *J Biol Chem*. 277:47928-37.

Mori, E., M. Thomas, K. Motoki, K. Nakazawa, T. Tahara, K. Tomizuka, I. Ishida, and S. Kataoka. 2004. Human normal hepatocytes are susceptible to apoptosis signal mediated by both TRAIL-R1 and TRAIL-R2. *Cell Death Differ*. 11:203-7.

Mundt, B., F. Kuhnel, L. Zender, Y. Paul, H. Tillmann, C. Trautwein, M.P. Manns, and S. Kubicka. 2003. Involvement of TRAIL and its receptors in viral hepatitis. *FASEB J*. 17:94-6.

Mundt, B., T. Wirth, L. Zender, M. Waltemathe, C. Trautwein, M.P. Manns, F. Kuhnel, and S. Kubicka. 2005. Tumour necrosis factor related apoptosis inducing ligand (TRAIL) induces hepatic steatosis in viral hepatitis and after alcohol intake. *Gut*. 54:1590-6.

Nebbioso, A., N. Clarke, E. Voltz, E. Germain, C. Ambrosino, P. Bontempo, R. Alvarez, E.M. Schiavone, F. Ferrara, F. Bresciani, A. Weisz, A.R. de Lera, H. Gronemeyer, and L. Altucci. 2005. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. *Nat Med*. 11:77-84.

Ng, S.S., T. Mahmoudi, E. Danenberg, I. Bejaoui, W. de Lau, H.C. Korswagen, M. Schutte, and H. Clevers. 2009. Phosphatidylinositol 3-kinase signaling does not activate the wnt cascade. *J Biol Chem*. 284:35308-13.

Nicholson, D.W. 1999. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ*. 6:1028-42.

Nicotera, P., M. Leist, and L. Manzo. 1999. Neuronal cell death: a demise with different shapes. *Trends Pharmacol Sci*. 20:46-51.

Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature*. 364:806-9.

Oh, S., L.P. Perera, M. Terabe, L. Ni, T.A. Waldmann, and J.A. Berzofsky. 2008. IL-15 as a mediator of CD4+ help for CD8+ T cell longevity and avoidance of TRAIL-mediated apoptosis. *Proc Natl Acad Sci U S A*. 105:5201-6.

Olsen, B.B., J. Petersen, and O.G. Issinger. 2006. BID, an interaction partner of protein kinase CK2alpha. *Biol Chem*. 387:441-9.

Oltersdorf, T., S.W. Elmore, A.R. Shoemaker, R.C. Armstrong, D.J. Augeri, B.A. Belli, M. Bruncko, T.L. Deckwerth, J. Dinges, P.J. Hajduk, M.K. Joseph, S. Kitada, S.J. Korsmeyer, A.R. Kunzer, A. Letai, C. Li, M.J. Mitten, D.G. Nettesheim, S. Ng, P.M. Nimmer, J.M. O'Connor, A. Oleksijew, A.M. Petros, J.C. Reed, W. Shen, S.K. Tahir, C.B. Thompson, K.J. Tomaselli, B. Wang, M.D. Wendt, H. Zhang, S.W. Fesik, and S.H. Rosenberg. 2005. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature*. 435:677-81.

Opel, D., M.A. Westhoff, A. Bender, V. Braun, K.M. Debatin, and S. Fulda. 2008. Phosphatidylinositol 3-kinase inhibition broadly sensitizes glioblastoma cells to death receptor- and drug-induced apoptosis. *Cancer Res*. 68:6271-80.

Pan, G., J. Ni, Y.F. Wei, G. Yu, R. Gentz, and V.M. Dixit. 1997a. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*. 277:815-8.

Pan, G., K. O'Rourke, A.M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, and V.M. Dixit. 1997b. The receptor for the cytotoxic ligand TRAIL. *Science*. 276:111-3.

Pang, H., R. Flinn, A. Patsialou, J. Wyckoff, E.T. Roussos, H. Wu, M. Pozzuto, S. Goswami, J.S. Condeelis, A.R. Bresnick, J.E. Segall, and J.M. Backer. 2009. Differential enhancement of breast cancer cell motility and metastasis by helical and kinase domain mutations of class IA phosphoinositide 3-kinase. *Cancer Res*. 69:8868-76.

Panka, D.J., T. Mano, T. Suhara, K. Walsh, and J.W. Mier. 2001. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J Biol Chem*. 276:6893-6.

Panner, A., C.A. Crane, C. Weng, A. Feletti, A.T. Parsa, and R.O. Pieper. 2009. A novel PTEN-dependent link to ubiquitination controls FLIPS stability and TRAIL sensitivity in glioblastoma multiforme. *Cancer Res*. 69:7911-6.

Panner, A., C.D. James, M.S. Berger, and R.O. Pieper. 2005. mTOR controls FLIPS translation and TRAIL sensitivity in glioblastoma multiforme cells. *Mol Cell Biol*. 25:8809-23.

Panner, A., A.T. Parsa, and R.O. Pieper. 2006. Translational regulation of TRAIL sensitivity. *Cell Cycle*. 5:147-50.

Pap, M., and G.M. Cooper. 1998. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J Biol Chem*. 273:19929-32.

Park, S.J., H.Y. Sohn, J. Yoon, and S.I. Park. 2009. Down-regulation of FoxO-dependent c-FLIP expression mediates TRAIL-induced apoptosis in activated hepatic stellate cells. *Cell Signal*. 21:1495-503.

Patino-Garcia, A., M. Zalacain, C. Folio, C. Zandueta, L. Sierrasesumaga, M. San Julian, G. Toledo, J. De Las Rivas, and F. Lecanda. 2009. Profiling of chemonaive osteosarcoma and paired-normal cells identifies EBF2 as a mediator of osteoprotegerin inhibition to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Clin Cancer Res*. 15:5082-91.

Patnaik, A., H. Wakelee, M. Mita, A. Fitzgerald, M. Hill, N. Fox, T. Howard, S. Ullrich, A. Tolcher, and B. Sikic. 2006. HGS-ETR2--A fully human monoclonal antibody to TRAIL-R2: Results of a phase I trial in patients with advanced solid tumors. *Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings Part I. Vol 24*. 24:3012.

Pespeni, M.H., M. Hodnett, K.S. Abayasiriwardana, J. Roux, M. Howard, V.C. Broaddus, and J.F. Pittet. 2007. Sensitization of mesothelioma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by heat stress via the inhibition of the 3-phosphoinositide-dependent kinase 1/Akt pathway. *Cancer Res*. 67:2865-71.

Petit, P.X., P. Dupaigne, F. Pariselli, F. Gonzalvez, F. Etienne, C. Rameau, and S. Bernard. 2009. Interaction of the alpha-helical H6 peptide from the pro-apoptotic protein tBid with cardiolipin. *FEBS J*. 276:6338-54.

Petrucci, E., L. Pasquini, A. Petronelli, E. Saulle, G. Mariani, R. Riccioni, M. Biffoni, G. Ferretti, P. Benedetti-Panici, F. Cognetti, G. Scambia, R. Humphreys, C. Peschle, and U. Testa. 2007. A small molecule Smac mimic potentiates TRAIL-mediated cell death of ovarian cancer cells. *Gynecol Oncol*. 105:481-92.

Pitti, R.M., S.A. Marsters, S. Ruppert, C.J. Donahue, A. Moore, and A. Ashkenazi. 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem*. 271:12687-90.

Plummer, R., G. Attard, S. Pacey, L. Li, A. Razak, R. Perrett, M. Barrett, I. Judson, S. Kaye, N.L. Fox, W. Halpern, A. Corey, H. Calvert, and J. de Bono. 2007. Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. *Clin Cancer Res*. 13:6187-94.

Potten, C., Wilson, J. 2004. Apoptosis: the life and death of cells. New York, Cambridge University Press.

Poukkula, M., A. Kaunisto, V. Hietakangas, K. Denessiouk, T. Katajamaki, M.S. Johnson, L. Sistonen, and J.E. Eriksson. 2005. Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. *J Biol Chem*. 280:27345-55.

Rachner, T.D., P. Benad, M. Rauner, C. Goettsch, S.K. Singh, M. Schoppet, and L.C. Hofbauer. 2009. Osteoprotegerin production by breast cancer cells is suppressed by dexamethasone and confers resistance against TRAIL-induced apoptosis. *J Cell Biochem*. 108:106-16.

Rayasam, G.V., V.K. Tulasi, R. Sodhi, J.A. Davis, and A. Ray. 2009. Glycogen synthase kinase 3: more than a namesake. *Br J Pharmacol*. 156:885-98.

Retzer-Lidl, M., R.M. Schmid, and G. Schneider. 2007. Inhibition of CDK4 impairs proliferation of pancreatic cancer cells and sensitizes towards TRAIL-induced apoptosis via downregulation of survivin. *Int J Cancer*. 121:66-75.

Ricci-Vitiani, L., F. Pedini, C. Mollinari, G. Condorelli, D. Bonci, A. Bez, A. Colombo, E. Parati, C. Peschle, and R. De Maria. 2004. Absence of caspase 8 and high expression of PED protect primitive neural cells from cell death. *J Exp Med*. 200:1257-66.

Ricci, M.S., S.H. Kim, K. Ogi, J.P. Plastaras, J. Ling, W. Wang, Z. Jin, Y.Y. Liu, D.T. Dicker, P.J. Chiao, K.T. Flaherty, C.D. Smith, and W.S. El-Deiry. 2007. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell*. 12:66-80.

Riccioni, R., L. Pasquini, G. Mariani, E. Saulle, A. Rossini, D. Diverio, E. Pelosi, A. Vitale, A. Chierichini, M. Cedrone, R. Foa, F. Lo Coco, C. Peschle, and U. Testa. 2005. TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. *Haematologica*. 90:612-24.

Riedl, S.J., M. Renatus, R. Schwarzenbacher, Q. Zhou, C. Sun, S.W. Fesik, R.C. Liddington, and G.S. Salvesen. 2001. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 104:791-800.

Riedl, S.J., and G.S. Salvesen. 2007. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol*. 8:405-13.

Roberts, A.I., S. Devadas, X. Zhang, L. Zhang, A. Keegan, K. Greeneltch, J. Solomon, L. Wei, J. Das, E. Sun, C. Liu, Z. Yuan, J.N. Zhou, and Y. Shi. 2003. The role of activation-induced cell death in the differentiation of T-helper-cell subsets. *Immunol Res*. 28:285-93.

Rolling, C., D. Treton, S. Pellegrini, P. Galanaud, and Y. Richard. 1996. IL4 and IL13 receptors share the gamma c chain and activate STAT6, STAT3 and STAT5 proteins in normal human B cells. *FEBS Lett*. 393:53-6.

Rottmann, S., Y. Wang, M. Nasoff, Q.L. Deveraux, and K.C. Quon. 2005. A TRAIL receptor-dependent synthetic lethal relationship between MYC activation and GSK3beta/FBW7 loss of function. *Proc Natl Acad Sci U S A*. 102:15195-200.

Roy, S.S., A.M. Ehrlich, W.J. Craigen, and G. Hajnoczky. 2009. VDAC2 is required for truncated BID-induced mitochondrial apoptosis by recruiting BAK to the mitochondria. *EMBO Rep*. 10:1341-7.

Rychahou, P.G., C.A. Murillo, and B.M. Evers. 2005. Targeted RNA interference of PI3K pathway components sensitizes colon cancer cells to TNF-related apoptosis-inducing ligand (TRAIL). *Surgery*. 138:391-7.

Sacks, J.A., and M.J. Bevan. 2008. TRAIL deficiency does not rescue impaired CD8+ T cell memory generated in the absence of CD4+ T cell help. *J Immunol*. 180:4570-6.

Saleh, M.N., I. Percent, T.E. Wood, J. Posey, III, J. Shah, R. Carlisle, S. Wojtowicz-Praga, and A. Forero-Torres. 2008. A phase I study of CS-1008 (humanized monoclonal antibody

targeting death receptor 5 or DR5), administered weekly to patients with advanced solid tumors or lymphomas. *J Clin Oncol (Meeting Abstracts)*. 26:3537-.

Saltz, L., J. Infante, L. Schwartzberg, J. Stephenson, C. Rocha-Lima, F. Galimi, K. Dillingham, M. Hsu, J. Wiezorek, and C. Fuchs. 2009. Safety and efficacy of AMG 655 plus modified FOLFOX6 (mFOLFOX6) and bevacizumab (B) for the first-line treatment of patients (pts) with metastatic colorectal cancer (mCRC). *J Clin Oncol (Meeting Abstracts)*. 27:4079-.

Salvesen, G.S., and V.M. Dixit. 1999. Caspase activation: the induced-proximity model. *Proc Natl Acad Sci U S A*. 96:10964-7.

Salvesen, G.S., and C.S. Duckett. 2002. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol*. 3:401-10.

Samuels, Y., L.A. Diaz, Jr., O. Schmidt-Kittler, J.M. Cummins, L. Delong, I. Cheong, C. Rago, D.L. Huso, C. Lengauer, K.W. Kinzler, B. Vogelstein, and V.E. Velculescu. 2005. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer Cell*. 7:561-73.

Samuels, Y., Z. Wang, A. Bardelli, N. Silliman, J. Ptak, S. Szabo, H. Yan, A. Gazdar, S.M. Powell, G.J. Riggins, J.K. Willson, S. Markowitz, K.W. Kinzler, B. Vogelstein, and V.E. Velculescu. 2004. High frequency of mutations of the PIK3CA gene in human cancers. *Science*. 304:554.

Sansom, O.J., V. Meniel, J.A. Wilkins, A.M. Cole, K.A. Oien, V. Marsh, T.J. Jamieson, C. Guerra, G.H. Ashton, M. Barbacid, and A.R. Clarke. 2006. Loss of Apc allows phenotypic manifestation of the transforming properties of an endogenous K-ras oncogene in vivo. *Proc Natl Acad Sci U S A*. 103:14122-7.

Sarig, R., Y. Zaltsman, R.C. Marcellus, R. Flavell, T.W. Mak, and A. Gross. 2003. BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. *J Biol Chem*. 278:10707-15.

Sartore-Bianchi, A., M. Martini, F. Molinari, S. Veronese, M. Nichelatti, S. Artale, F. Di Nicolantonio, P. Saletti, S. De Dosso, L. Mazzucchelli, M. Frattini, S. Siena, and A. Bardelli.

2009. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. *Cancer Res*. 69:1851-7.

Sato, K., S. Hida, H. Takayanagi, T. Yokochi, N. Kayagaki, K. Takeda, H. Yagita, K. Okumura, N. Tanaka, T. Taniguchi, and K. Ogasawara. 2001. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. *Eur J Immunol*. 31:3138-46.

Saulle, E., A. Petronelli, L. Pasquini, E. Petrucci, G. Mariani, M. Biffoni, G. Ferretti, G. Scambia, P. Benedetti-Panici, F. Cognetti, R. Humphreys, C. Peschle, and U. Testa. 2007. Proteasome inhibitors sensitize ovarian cancer cells to TRAIL induced apoptosis. *Apoptosis*. 12:635-55.

Sayers, T.J., and W.J. Murphy. 2006. Combining proteasome inhibition with TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) for cancer therapy. *Cancer Immunol Immunother*. 55:76-84.

Schlosser, S.F., F. Azzaroli, T. Dao, R. Hingorani, I. Nicholas Crispe, and J.L. Boyer. 2000. Induction of murine hepatocyte death by membrane-bound CD95 (Fas/APO-1)-ligand: characterization of an in vitro system. *Hepatology*. 32:779-85.

Schneider, P. 2000. Production of recombinant TRAIL and TRAIL receptor: Fc chimeric proteins. *Methods Enzymol*. 322:325-45.

Schneider, P., D. Olson, A. Tardivel, B. Browning, A. Lugovskoy, D. Gong, M. Dobles, S. Hertig, K. Hofmann, H. Van Vlijmen, Y.M. Hsu, L.C. Burkly, J. Tschopp, and T.S. Zheng. 2003. Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Biol Chem*. 278:5444-54.

Schuchmann, M., H. Schulze-Bergkamen, B. Fleischer, J.M. Schattenberg, J. Siebler, A. Weinmann, A. Teufel, M. Worns, T. Fischer, S. Strand, A.W. Lohse, and P.R. Galle. 2006. Histone deacetylase inhibition by valproic acid down-regulates c-FLIP/CASH and sensitizes hepatoma cells towards CD95- and TRAIL receptor-mediated apoptosis and chemotherapy. *Oncol Rep*. 15:227-30.

Screaton, G.R., J. Mongkolsapaya, X.N. Xu, A.E. Cowper, A.J. McMichael, and J.I. Bell. 1997. TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. *Curr Biol*. 7:693-6.

Secchiero, P., E. Melloni, F. Corallini, A.P. Beltrami, F. Alviano, D. Milani, F. D'Aurizio, M.G. di Iasio, D. Cesselli, G.P. Bagnara, and G. Zauli. 2008. Tumor necrosis factor-related apoptosis-inducing ligand promotes migration of human bone marrow multipotent stromal cells. *Stem Cells*. 26:2955-63.

Secchiero, P., E. Melloni, M. Heikinheimo, S. Mannisto, R. Di Pietro, A. Iacone, and G. Zauli. 2004. TRAIL regulates normal erythroid maturation through an ERK-dependent pathway. *Blood*. 103:517-22.

Sedger, L.M., M.B. Glaccum, J.C. Schuh, S.T. Kanaly, E. Williamson, N. Kayagaki, T. Yun, P. Smolak, T. Le, R. Goodwin, and B. Gliniak. 2002. Characterization of the in vivo function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice. *Eur J Immunol*. 32:2246-54.

Sedger, L.M., D.M. Shows, R.A. Blanton, J.J. Peschon, R.G. Goodwin, D. Cosman, and S.R. Wiley. 1999. IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. *J Immunol*. 163:920-6.

Seol, J.W., Y.J. Lee, H.S. Kang, I.S. Kim, N.S. Kim, Y.G. Kwak, T.H. Kim, D.W. Seol, and S.Y. Park. 2005. Wortmannin elevates tumor necrosis factor-related apoptosis-inducing ligand sensitivity in LNCaP cells through down-regulation of IAP-2 protein. *Exp Oncol*. 27:120-4.

Shankar, E., U. Sivaprasad, and A. Basu. 2008. Protein kinase C epsilon confers resistance of MCF-7 cells to TRAIL by Akt-dependent activation of Hdm2 and downregulation of p53. *Oncogene*. 27:3957-66.

Sharma, S., E.G. de Vries, J.R. Infante, C. Oldenhuis, L. Chiang, S. Bilic, M. Goldbrunner, J.W. Scott, and H.A. Burris, III. 2008. Phase I trial of LBY135, a monoclonal antibody agonist to DR5, alone and in combination with capecitabine in advanced solid tumors. *J Clin Oncol (Meeting Abstracts)*. 26:3538-.

Shaw, R.J., and L.C. Cantley. 2006. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*. 441:424-30.

Shedlock, D.J., J.K. Whitmire, J. Tan, A.S. MacDonald, R. Ahmed, and H. Shen. 2003. Role of CD4 T cell help and costimulation in CD8 T cell responses during Listeria monocytogenes infection. *J Immunol*. 170:2053-63.

Sheikh, M.S., Y. Huang, E.A. Fernandez-Salas, W.S. El-Deiry, H. Friess, S. Amundson, J. Yin, S.J. Meltzer, N.J. Holbrook, and A.J. Fornace, Jr. 1999. The antiapoptotic decoy receptor TRID/TRAIL-R3 is a p53-regulated DNA damage-inducible gene that is overexpressed in primary tumors of the gastrointestinal tract. *Oncogene*. 18:4153-9.

Sheridan, J.P., S.A. Marsters, R.M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C.L. Gray, K. Baker, W.I. Wood, A.D. Goddard, P. Godowski, and A. Ashkenazi. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science*. 277:818-21.

Shi, Y. 2004. Caspase activation: revisiting the induced proximity model. *Cell*. 117:855-8.

Shiloh, Y. 2003. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer*. 3:155-68.

Shinohara, H., N. Kayagaki, H. Yagita, N. Oyaizu, M. Ohba, T. Kuroki, and Y. Ikawa. 2001. A protective role of PKCepsilon against TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in glioma cells. *Biochem Biophys Res Commun*. 284:1162-7.

Shrader, M., M.S. Pino, L. Lashinger, M. Bar-Eli, L. Adam, C.P. Dinney, and D.J. McConkey. 2007. Gefitinib reverses TRAIL resistance in human bladder cancer cell lines via inhibition of AKT-mediated X-linked inhibitor of apoptosis protein expression. *Cancer Res*. 67:1430-5.

Sikic, B.I., H.A. Wakelee, M. von Mehren, N. Lewis, A.H. Calvert, E.R. Plummer, N.L. Fox, T. Howard, S.F. Jones, and H.A. Burris, III. 2007. A phase Ib study to assess the safety of lexatumumab, a human monoclonal antibody that activates TRAIL-R2, in combination with gemcitabine, pemetrexed, doxorubicin or FOLFIRI. *J Clin Oncol (Meeting Abstracts)*. 25:14006-.

Skurk, C., H. Maatz, H.S. Kim, J. Yang, M.R. Abid, W.C. Aird, and K. Walsh. 2004. The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. *J Biol Chem*. 279:1513-25.

Slee, E.A., C. Adrain, and S.J. Martin. 1999. Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ*. 6:1067-74.

Solier, S., O. Sordet, K.W. Kohn, and Y. Pommier. 2009. Death receptor-induced activation of the Chk2- and histone H2AX-associated DNA damage response pathways. *Mol Cell Biol*. 29:68-82.

Song, G., G.G. Chen, D.K. Chau, J. Miao, and P.B. Lai. 2008a. Bid exhibits S phase checkpoint activation and plays a pro-apoptotic role in response to etoposide-induced DNA damage in hepatocellular carcinoma cells. *Apoptosis*. 13:693-701.

Song, J.H., K. Kandasamy, and A.S. Kraft. 2008b. ABT-737 induces expression of the death receptor 5 and sensitizes human cancer cells to TRAIL-induced apoptosis. *J Biol Chem*. 283:25003-13.

Song, K., Y. Chen, R. Goke, A. Wilmen, C. Seidel, A. Goke, and B. Hilliard. 2000. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. *J Exp Med*. 191:1095-104.

Sprick, M.R., E. Rieser, H. Stahl, A. Grosse-Wilde, M.A. Weigand, and H. Walczak. 2002. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *Embo J*. 21:4520-30.

Stagni, V., M.G. di Bari, S. Cursi, I. Condo, M.T. Cencioni, R. Testi, Y. Lerenthal, E. Cundari, and D. Barila. 2008. ATM kinase activity modulates Fas sensitivity through the regulation of FLIP in lymphoid cells. *Blood*. 111:829-37.

Sun, M., L. Song, Y. Li, T. Zhou, and R.S. Jope. 2008. Identification of an antiapoptotic protein complex at death receptors. *Cell Death Differ*. 15:1887-900.

Sun, S.Y., L.M. Rosenberg, X. Wang, Z. Zhou, P. Yue, H. Fu, and F.R. Khuri. 2005. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res*. 65:7052-8.

Tagscherer, K.E., A. Fassl, B. Campos, M. Farhadi, A. Kraemer, B.C. Bock, S. Macher-Goeppinger, B. Radlwimmer, O.D. Wiestler, C. Herold-Mende, and W. Roth. 2008. Apoptosis-based treatment of glioblastomas with ABT-737, a novel small molecule inhibitor of Bcl-2 family proteins. *Oncogene*. 27:6646-56.

Taieb, J., N. Chaput, C. Menard, L. Apetoh, E. Ullrich, M. Bonmort, M. Pequignot, N. Casares, M. Terme, C. Flament, P. Opolon, Y. Lecluse, D. Metivier, E. Tomasello, E. Vivier, F. Ghiringhelli, F. Martin, D. Klatzmann, T. Poynard, T. Tursz, G. Raposo, H. Yagita, B. Ryffel, G. Kroemer, and L. Zitvogel. 2006. A novel dendritic cell subset involved in tumor immunosurveillance. *Nat Med*. 12:214-9.

Takeda, K., E. Cretney, Y. Hayakawa, T. Ota, H. Akiba, K. Ogasawara, H. Yagita, K. Kinoshita, K. Okumura, and M.J. Smyth. 2005. TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood*. 105:2082-9.

Takeda, K., M.J. Smyth, E. Cretney, Y. Hayakawa, N. Yamaguchi, H. Yagita, and K. Okumura. 2001. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated and IFN-gamma-dependent suppression of subcutaneous tumor growth. *Cell Immunol*. 214:194-200.

Takeda, K., N. Yamaguchi, H. Akiba, Y. Kojima, Y. Hayakawa, J.E. Tanner, T.J. Sayers, N. Seki, K. Okumura, H. Yagita, and M.J. Smyth. 2004. Induction of tumor-specific T cell immunity by anti-DR5 antibody therapy. *J Exp Med*. 199:437-48.

Takeuchi, H., J. Kim, A. Fujimoto, N. Umetani, T. Mori, A. Bilchik, R. Turner, A. Tran, C. Kuo, and D.S. Hoon. 2005. X-Linked inhibitor of apoptosis protein expression level in colorectal cancer is regulated by hepatocyte growth factor/C-met pathway via Akt signaling. *Clin Cancer Res*. 11:7621-8.

Tanaka, Y., C. Mamalaki, B. Stockinger, and D. Kioussis. 1993. In vitro negative selection of alpha beta T cell receptor transgenic thymocytes by conditionally immortalized thymic cortical epithelial cell lines and dendritic cells. *Eur J Immunol*. 23:2614-21.

Taniai, M., A. Grambihler, H. Higuchi, N. Werneburg, S.F. Bronk, D.J. Farrugia, S.H. Kaufmann, and G.J. Gores. 2004. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res*. 64:3517-24.

Taylor, A., F. Shang, T. Nowell, Y. Galanty, and Y. Shiloh. 2002. Ubiquitination capabilities in response to neocarzinostatin and H(2)O(2) stress in cell lines from patients with ataxia-telangiectasia. *Oncogene*. 21:4363-73.

Taylor, R.C., S.P. Cullen, and S.J. Martin. 2008. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol*. 9:231-41.

Tazzari, P.L., G. Tabellini, F. Ricci, V. Papa, R. Bortul, F. Chiarini, C. Evangelisti, G. Martinelli, A. Bontadini, L. Cocco, J.A. McCubrey, and A.M. Martelli. 2008. Synergistic proapoptotic activity of recombinant TRAIL plus the Akt inhibitor Perifosine in acute myelogenous leukemia cells. *Cancer Res*. 68:9394-403.

Thakkar, H., X. Chen, F. Tyan, S. Gim, H. Robinson, C. Lee, S.K. Pandey, C. Nwokorie, N. Onwudiwe, and R.K. Srivastava. 2001. Pro-survival function of Akt/protein kinase B in prostate cancer cells. Relationship with TRAIL resistance. *J Biol Chem*. 276:38361-9.

Tinel, A., and J. Tschopp. 2004. The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science*. 304:843-6.

Todaro, M., Y. Lombardo, M.G. Francipane, M.P. Alea, P. Cammareri, F. Iovino, A.B. Di Stefano, C. Di Bernardo, A. Agrusa, G. Condorelli, H. Walczak, and G. Stassi. 2008. Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. *Cell Death Differ*. 15:762-72.

Tolcher, A.W., M. Mita, N.J. Meropol, M. von Mehren, A. Patnaik, K. Padavic, M. Hill, T. Mays, T. McCoy, N.L. Fox, W. Halpern, A. Corey, and R.B. Cohen. 2007. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol*. 25:1390-5.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 76:4350-4.

Trarbach, T., M. Moehler, V. Heinemann, C.H. Kohne, M. Przyborek, C. Schulz, V. Sneller, G. Gallant, and S. Kanzler. Phase II trial of mapatumumab, a fully human agonistic monoclonal antibody that targets and activates the tumour necrosis factor apoptosis-inducing ligand receptor-1 (TRAIL-R1), in patients with refractory colorectal cancer. *Br J Cancer*.

Trauzold, A., D. Siegmund, B. Schniewind, B. Sipos, J. Egberts, D. Zorenkov, D. Emme, C. Roder, H. Kalthoff, and H. Wajant. 2006. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. *Oncogene*. 25:7434-9.

Trencia, A., A. Perfetti, A. Cassese, G. Vigliotta, C. Miele, F. Oriente, S. Santopietro, F. Giacco, G. Condorelli, P. Formisano, and F. Beguinot. 2003. Protein kinase B/Akt binds and phosphorylates PED/PEA-15, stabilizing its antiapoptotic action. *Mol Cell Biol*. 23:4511-21.

Truneh, A., S. Sharma, C. Silverman, S. Khandekar, M.P. Reddy, K.C. Deen, M.M. McLaughlin, S.M. Srinivasula, G.P. Livi, L.A. Marshall, E.S. Alnemri, W.V. Williams, and M.L. Doyle. 2000. Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *J Biol Chem*. 275:23319-25.

Uno, T., K. Takeda, Y. Kojima, H. Yoshizawa, H. Akiba, R.S. Mittler, F. Gejyo, K. Okumura, H. Yagita, and M.J. Smyth. 2006. Eradication of established tumors in mice by a combination antibody-based therapy. *Nat Med*. 12:693-8.

Valentijn, A.J., and A.P. Gilmore. 2004. Translocation of full-length Bid to mitochondria during anoikis. *J Biol Chem*. 279:32848-57.

Varfolomeev, E., H. Maecker, D. Sharp, D. Lawrence, M. Renz, D. Vucic, and A. Ashkenazi. 2005. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. *J Biol Chem*. 280:40599-608.

Vaux, D.L., and J. Silke. 2005. IAPs--the ubiquitin connection. *Cell Death Differ*. 12:1205-7.

Vezina, C., A. Kudelski, and S.N. Sehgal. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot (Tokyo)*. 28:721-6.

Vilimanovich, U., and V. Bumbasirevic. 2008. TRAIL induces proliferation of human glioma cells by c-FLIPL-mediated activation of ERK1/2. *Cell Mol Life Sci*. 65:814-26.

Villa, P., S.H. Kaufmann, and W.C. Earnshaw. 1997. Caspases and caspase inhibitors. *Trends Biochem Sci*. 22:388-93.

Vogler, M., H. Walczak, D. Stadel, T.L. Haas, F. Genze, M. Jovanovic, J.E. Gschwend, T. Simmet, K.M. Debatin, and S. Fulda. 2008. Targeting XIAP bypasses Bcl-2-mediated resistance to TRAIL and cooperates with TRAIL to suppress pancreatic cancer growth in vitro and in vivo. *Cancer Res*. 68:7956-65.

Vogt, P.K., S. Kang, M.A. Elsliger, and M. Gymnopoulos. 2007. Cancer-specific mutations in phosphatidylinositol 3-kinase. *Trends Biochem Sci*. 32:342-9.

Wagner, K.W., E.A. Punnoose, T. Januario, D.A. Lawrence, R.M. Pitti, K. Lancaster, D. Lee, M. von Goetz, S.F. Yee, K. Totpal, L. Huw, V. Katta, G. Cavet, S.G. Hymowitz, L. Amler, and A. Ashkenazi. 2007. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med*. 13:1070-7.

Wajant, H. 2004. TRAIL and NFkappaB signaling--a complex relationship. *Vitam Horm*. 67:101-32.

Wajant, H., K. Pfizenmaier, and P. Scheurich. 2002. TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. *Apoptosis*. 7:449-59.

Wakelee, H.A., A. Patnaik, B.I. Sikic, M. Mita, N.L. Fox, R. Miceli, S.J. Ullrich, G.A. Fisher, and A.W. Tolcher. 2009. Phase I and pharmacokinetic study of lexatumumab (HGS-ETR2) given every 2 weeks in patients with advanced solid tumors. *Ann Oncol*.

Walczak, H., M.A. Degli-Esposti, R.S. Johnson, P.J. Smolak, J.Y. Waugh, N. Boiani, M.S. Timour, M.J. Gerhart, K.A. Schooley, C.A. Smith, R.G. Goodwin, and C.T. Rauch. 1997. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *Embo J*. 16:5386-97.

Walczak, H., and T.L. Haas. 2008. Biochemical analysis of the native TRAIL death-inducing signaling complex. *Methods Mol Biol*. 414:221-39.

Walczak, H., R.E. Miller, K. Ariail, B. Gliniak, T.S. Griffith, M. Kubin, W. Chin, J. Jones, A. Woodward, T. Le, C. Smith, P. Smolak, R.G. Goodwin, C.T. Rauch, J.C. Schuh, and D.H. Lynch. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med*. 5:157-63.

Wang, S.H., Z. Cao, J.M. Wolf, M. Van Antwerp, and J.R. Baker, Jr. 2005. Death ligand tumor necrosis factor-related apoptosis-inducing ligand inhibits experimental autoimmune thyroiditis. *Endocrinology*. 146:4721-6.

Wang, X., W. Chen, W. Zeng, L. Bai, Y. Tesfaigzi, S.A. Belinsky, and Y. Lin. 2008. Akt-mediated eminent expression of c-FLIP and Mcl-1 confers acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells. *Mol Cancer Ther*. 7:1156-63.

Weckmann, M., A. Collison, J.L. Simpson, M.V. Kopp, P.A. Wark, M.J. Smyth, H. Yagita, K.I. Matthaei, N. Hansbro, B. Whitehead, P.G. Gibson, P.S. Foster, and J. Mattes. 2007. Critical link between TRAIL and CCL20 for the activation of TH2 cells and the expression of allergic airway disease. *Nat Med*. 13:1308-15.

Whitman, M., D.R. Kaplan, B. Schaffhausen, L. Cantley, and T.M. Roberts. 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. *Nature*. 315:239-42.

Wiley, S.R., K. Schooley, P.J. Smolak, W.S. Din, C.P. Huang, J.K. Nicholl, G.R. Sutherland, T.D. Smith, C. Rauch, C.A. Smith, and et al. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 3:673-82.

Wilhelm, S.M., C. Carter, L. Tang, D. Wilkie, A. McNabola, H. Rong, C. Chen, X. Zhang, P. Vincent, M. McHugh, Y. Cao, J. Shujath, S. Gawlak, D. Eveleigh, B. Rowley, L. Liu, L. Adnane, M. Lynch, D. Auclair, I. Taylor, R. Gedrich, A. Voznesensky, B. Riedl, L.E. Post, G. Bollag, and P.A. Trail. 2004. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*. 64:7099-109.

Willis, S.N., J.I. Fletcher, T. Kaufmann, M.F. van Delft, L. Chen, P.E. Czabotar, H. Ierino, E.F. Lee, W.D. Fairlie, P. Bouillet, A. Strasser, R.M. Kluck, J.M. Adams, and D.C. Huang. 2007. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*. 315:856-9.

Wilson, S.M., D. Barbone, T.M. Yang, D.M. Jablons, R. Bueno, D.J. Sugarbaker, S.L. Nishimura, G.J. Gordon, and V.C. Broaddus. 2008. mTOR mediates survival signals in malignant mesothelioma grown as tumor fragment spheroids. *Am J Respir Cell Mol Biol*. 39:576-83.

Wu, G.S., T.F. Burns, E.R. McDonald, 3rd, W. Jiang, R. Meng, I.D. Krantz, G. Kao, D.D. Gan, J.Y. Zhou, R. Muschel, S.R. Hamilton, N.B. Spinner, S. Markowitz, G. Wu, and W.S. el-Deiry. 1997. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet*. 17:141-3.

Wu, G.S., T.F. Burns, Y. Zhan, E.S. Alnemri, and W.S. El-Deiry. 1999. Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. *Cancer Res*. 59:2770-5.

Wu, H., J. Tschopp, and S.C. Lin. 2007. Smac mimetics and TNFalpha: a dangerous liaison? *Cell*. 131:655-8.

Yada, A., M. Yazawa, S. Ishida, H. Yoshida, K. Ichikawa, S. Kurakata, and K. Fujiwara. 2008. A novel humanized anti-human death receptor 5 antibody CS-1008 induces apoptosis in tumor cells without toxicity in hepatocytes. *Ann Oncol*. 19:1060-7.

Yang, D., F. Buchholz, Z. Huang, A. Goga, C.Y. Chen, F.M. Brodsky, and J.M. Bishop. 2002. Short RNA duplexes produced by hydrolysis with Escherichia coli RNase III mediate effective RNA interference in mammalian cells. *Proc Natl Acad Sci U S A*. 99:9942-7.

Yang, E., J. Zha, J. Jockel, L.H. Boise, C.B. Thompson, and S.J. Korsmeyer. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*. 80:285-91.

Yao, E., W. Zhou, S.T. Lee-Hoeflich, T. Truong, P.M. Haverty, J. Eastham-Anderson, N. Lewin-Koh, B. Gunter, M. Belvin, L.J. Murray, L.S. Friedman, M.X. Sliwkowski, and K.P.

Hoeflich. 2009. Suppression of HER2/HER3-mediated growth of breast cancer cells with combinations of GDC-0941 PI3K inhibitor, trastuzumab, and pertuzumab. *Clin Cancer Res*. 15:4147-56.

Yee, L., H.A. Burris, M. Kozloff, Z. Wainberg, M. Pao, S. Skettino, W. Novotny, B. Durbin, J. Weston, and H. Hurwitz. 2009. Phase Ib study of recombinant human Apo2L/TRAIL plus irinotecan and cetuximab or FOLFIRI in metastatic colorectal cancer (mCRC) patients (pts): Preliminary results. *J Clin Oncol (Meeting Abstracts)*. 27:4129-.

Yin, X.M., Dong, Z. . 2003. Essentials of apoptosis: A guide for Basic and Clinical Research. Humana Press, New Yersey.

Youle, R.J. 2007. Cell biology. Cellular demolition and the rules of engagement. *Science*. 315:776-7.

Youle, R.J., and A. Strasser. 2008. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*. 9:47-59.

Younes, A., J.M. Vose, A.D. Zelenetz, M.R. Smith, H. Burris, S. Ansell, J. Klein, E. Kumm, and M. Czuczman. 2005. Results of a Phase 2 Trial of HGS-ETR1 (Agonistic Human Monoclonal Antibody to TRAIL Receptor 1) in Subjects with Relapsed/Refractory Non-Hodgkin's Lymphoma (NHL). *ASH Annual Meeting Abstracts*. 106:489-.

Yuan, J., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz. 1993. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell*. 75:641-52.

Yue, H.H., G.E. Diehl, and A. Winoto. 2005. Loss of TRAIL-R does not affect thymic or intestinal tumor development in p53 and adenomatous polyposis coli mutant mice. *Cell Death Differ*. 12:94-7.

Zerafa, N., J.A. Westwood, E. Cretney, S. Mitchell, P. Waring, M. Iezzi, and M.J. Smyth. 2005. Cutting edge: TRAIL deficiency accelerates hematological malignancies. *J Immunol*. 175:5586-90.

Zha, J., S. Weiler, K.J. Oh, M.C. Wei, and S.J. Korsmeyer. 2000. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science*. 290:1761-5.

Zhang, J., P.L. Yang, and N.S. Gray. 2009. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer*. 9:28-39.

Zhang, L., J. Yu, B.H. Park, K.W. Kinzler, and B. Vogelstein. 2000. Role of BAX in the apoptotic response to anticancer agents. *Science*. 290:989-92.

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

Zhang, X.R., L.Y. Zhang, S. Devadas, L. Li, A.D. Keegan, and Y.F. Shi. 2003. Reciprocal expression of TRAIL and CD95L in Th1 and Th2 cells: role of apoptosis in T helper subset differentiation. *Cell Death Differ*. 10:203-10.

Zheng, S.J., P. Wang, G. Tsabary, and Y.H. Chen. 2004. Critical roles of TRAIL in hepatic cell death and hepatic inflammation. *J Clin Invest*. 113:58-64.

Zinkel, S.S., K.E. Hurov, and A. Gross. 2007. Bid plays a role in the DNA damage response. *Cell*. 130:9-10; author reply 10-1.

Zinkel, S.S., K.E. Hurov, C. Ong, F.M. Abtahi, A. Gross, and S.J. Korsmeyer. 2005. A role for proapoptotic BID in the DNA-damage response. *Cell*. 122:579-91.

Zisman, A., C.P. Ng, A.J. Pantuck, B. Bonavida, and A.S. Belldegrun. 2001. Actinomycin D and gemcitabine synergistically sensitize androgen-independent prostate cancer cells to Apo2L/TRAIL-mediated apoptosis. *J Immunother*. 24:459-71.

Zou, H., R. Yang, J. Hao, J. Wang, C. Sun, S.W. Fesik, J.C. Wu, K.J. Tomaselli, and R.C. Armstrong. 2003. Regulation of the Apaf-1/caspase-9 apoptosome by caspase-3 and XIAP. *J Biol Chem*. 278:8091-8.

Zou, Y., X. Zhang, Y. Mao, G. Lu, M. Huang, and B. Yuan. Acute toxicity of a single dose DATR, recombinant soluble human TRAIL mutant, in rodents and crab-eating macaques. *Hum Exp Toxicol*.