Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program:
Buněčná a vývojová biologie

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Characterization of TRAIL-induced, receptor-specific signaling in cancer cells

Charakterizace TRAILem indukované, receptor-specifické signalizace v nádorových buňkách

Diplomová práce

Školitel:
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Praha, 2013

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 2.5.2013

Podpis

Na tomto místě bych chtěl poděkovati zejména Dr. Ladislavu Anděrovi za příkladné vedení práce, podnětné diskuze a rady. Dík patří také všem ostatním členům laboratoře Buněčné signalizace a apopózy Ústavu molekulární genetiky Akademie věd, zejména pak Janu Švadlenkovi, který mě uvedl do světa tvrdé vědecké práce. V neposlední řadě děkuji svým rodičům a přátelům za podporu při studiu.

Content

List of abbreviations

APC - adenomatous polyposis coli

cFLIP - cellular flice-like inhibitory protein

CREB - cAMP response element-binding protein

D - aspartic acid

DcR1/2 - decoy receptor 1/2

DD - death domain

DED - death effector domain

DISC - death-inducing signaling complex

DR - death receptor

E - glutamic acid

ERK - Extracellular signal-regulated kinase

FADD - Fas-associated protein with death domain

G - glycine

H - histidine

I - isoleucine

IAP - inhibitor of apoptosis

IkB - inhibitor kappa B

IKK - IkB kinase

JNK - c-Jun N-terminal kinase

K - lysine

MAPK - mitogen-activated protein kinase

mDR5 - mouse death receptor 5

MK2 - MAP kinase-activated protein kinase 2

MOMP - mitochondrial outer membrane permeabilization

MSK-1 - Mitogen- and stress-activated protein kinase-1

mTOR - mammalian target of rapamycin

N - asparagine

NF-κB - nuclear factor kappa B

NGF - nerve growth factor

NK - natural killer

NSCLC - non-small cell lung carcinoma

OPG - osteoprotegerin

PI3K - phosphoinositide 3-kinase

PKB - protein kinase B

PKC - protein kinase C

PLAD - preligand assebly domain

PTEN - Phosphatase and tensin homolog

R - arginine

RANK - Receptor Activator of Nuclear Factor kappa B

RIP - receptor-interacting protein

ROCK1 - Rho-associated, coiled-coil containing protein kinase 1

S - serine

TAK1 - TGFβ-activated kinase 1

TNF - tumor necrosis factor

TRADD - TNF receptor type 1-associated death domain protein

TRAF2 - TNF receptor-associated factor 2

TRAIL - TNF-related apoptosis-inducing ligand

XIAP - X-linked inhibitor of apoptosis

Abstract

TNF-related apoptosis-inducing ligand (TRAIL) is a member of TNF family expressed mainly by hematopoietic cells. TRAIL brought significant attention mainly for its ability to trigger apoptosis in a number of cancer cells. In addition to apoptosis, TRAIL can induce several other signaling pathways such as activation of MAP kinases or canonical NF-κB signaling. Human TRAIL can bind to five receptors but only two of them (death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5) can trigger TRAIL-mediated apoptotic and non-apoptotic signaling in target cells. Both receptors are ubiquitously expressed on normal and cancer cells, but the relative contribution of DR4 and DR5 to TRAIL-induced signaling is not well known. Using DR4/DR5-specific variants of TRAIL, we examined how individual receptor contributes to the induction of apoptosis and NF-κB, JNK, p38, ERK1/2 and TAK1 signaling pathways in selected colorectal cells. We found that in DLD-1 cells, apoptosis and activation of JNKs are mainly mediated by DR4-selective ligand. In TRAIL-resistant HT-29 cells, we show that though DISC formation and activation of caspase-8 proceeds mainly via DR4-specific signaling, activation of NF-κB pathway is mainly triggered by DR5 selective ligand. In other cells and analyzed signaling pathways both receptor-specific ligands triggered very similar responses. This thesis thus provides the first systematic insight into DR4/DR5-specific signaling in colorectal cell lines.

Abstrakt

TNF-related apoptosis-inducing ligand (TRAIL) je protein patřící do TNF rodiny exprimovaný hlavně hematopetickými buňkami. TRAIL je zajímavý zejména svou schopností vyvolat apoptózu v mnohých nádorových buňkách. TRAIL je také schopen iniciovat několik dalších signálních drah, jako například MAP kinázovou signalizaci, či aktivaci NF-κB dráhy. Lidský TRAIL váže celkem pět receptorů, z nichž pouze dva jsou schopny iniciovat apoptotickou a neapoptotickou signalizaci v cílových buňkách. Oba receptory jsou široce koexprimovány na zdravých i nádorových buňkách. Jaký je relativní příspěvek jednotlivých receptorů k TRAILem indukované signalizaci není dobře prostudováno. V této práci jsme pomocí DR4/DR5-specifických variant ligandu TRAIL prozkoumali, jakým způsobem se tyto jednotlivé receptory zapojují do TRAILem indukované aktivace apoptózy a drah NF-κB, JNK, p38, ERK1/2 a TAK1 ve vybraných kolorektálních nádorových buňkách. Ukazujeme, že v DLD-1 buňkách apoptóza a JNK kináza jsou aktivovány zejména DR4-specifický ligandem. V rezistentní linii HT-29, ačkoliv apoptotický komplex DISC byl formován zejména DR4-specifickým ligandem, dráha NF-κB byla indukována zejména DR5 selektivním ligandem. V ostatních prozkoumaných případech vyvolaly oba ligandy srovnatelné odpovědi. Tato práce nabízí první systematický pohled na DR4/DR5 specifickou signalizaci v kolorektálních nádorových buňkách.

Introduction

TRAIL is a cytokine with therapeutic potential that can selectively induce apoptosis in numerous cancer cell lines but generally not in untransformed cells. In addition to apoptosis, TRAIL can induce several non-apoptotic pathways such as MAP kinases or NF-kB, which can lead to enhanced proliferation of cells resistant to TRAIL-induced apoptosis. Upon binding to its two death receptors, DR4 and DR5, TRAIL triggers assembly of the multiprotein "death-inducing signaling complex" (DISC). Formation of the DISC ultimately leads to the activation of initiator caspase-8, which triggers apoptosis either via activation of effector caspases or via mitochondrial outer membrane permeabilization. The TRAIL-induced apoptotic signaling is well characterized. However, the mechanisms by which TRAIL triggers non-apoptotic pathways are much less clear.

Many cancer cells co-express both DR4 and DR5 on their surface. However, the relative contributions of DR4 and DR5 receptors to TRAIL-induced signaling are poorly characterized. In a cell type specific manner, the TRAIL signaling may be initiated exclusively via DR4 or DR5 receptor despite the presence of both receptors. The biochemical basis for the preferential usage of either DR4 or DR5 is unknown.

In the theoretical part of this Thesis, we summarize current knowledge of TRAIL-induced signaling pathways and critically review the work concerning DR4/DR5-specific TRAIL signaling. In the experimental part, we characterized TRAIL-induced apoptosis, MAP kinases, Akt and NF-kB signaling in three colorectal cancer cell lines. Most importantly, we also examined how DR4 or DR5 alone contribute to the induction of these pathways.

1 Literature review

1.1 Apoptosis

The term apoptosis was introduced in 1972 by J.F.Kerr and his co-workers as a "mechanism of controlled cell destruction"(Kerr et al., 1972), reflecting an indispensable role of cell death in metazoan development and physiology. The apoptotic machinery is genetically encoded and apoptotic cell death can be triggered by various stimuli. As a tightly controlled process, apoptosis is involved in embryonic development, continuous renewal of various tissues or maintenance of the immune system. Deregulated apoptosis is connected with a number of serious diseases including neurological disorders, autoimmune diseases, and perhaps most importantly, cancer (Elmore, 2007; Hanahan and Weinberg, 2011).

Physiological hallmarks of apoptotic cells include morphological changes such as cell shrinkage, chromatin condensation and membrane "blebbing", ultimately leading to cell disintegration. Remains of apoptotic cells are engulfed by surrounding cells or specific phagocytes as macrophages and discarded in a non-inflammatory way from the tissue. From the biochemical perspective, the central executors of apoptosis are caspases, cysteinyl proteases activated by various means, which can specifically cleave over five hundred of proteins in human cells, including a number of structural proteins, transcription factors, kinases and other enzymes (Fridman et al., 2013).

There are two principal pathways of apoptosis in mammals. So called "intrinsic" pathway, which is triggered by intracellular sensors of apoptotic stimuli, and the "extrinsic" pathway, which can be initiated by membrane “death” receptors from the TNFR (tumor necrosis factor receptors) family (Figure 1.). Both pathways eventually converge to activate the effector caspases-3 and -7, which are main executors of cell destruction (Elmore, 2007).

1.1.1 The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is in mammalian cells regulated by proteins from the Bcl-2 family, which form a complex signaling network controlling mitochondrial outer membrane permeabilization (MOMP), a crucial step in the execution of apoptosis. MOMP enables the release of several important pro-apoptotic proteins, such as

cytochrome c, Smac/DIABLO or AIF from the mitochondrial intermembrane space into the cytosol. Cytochrome c forms together with its receptors Apaf1 and dATP the "apoptosome", a wheel-like cytoplasmic complex serving as a platform for the activation of procaspase-9. Once activated at the apoptosome, caspase-9 cleaves and activates executioner caspase-3 and caspase-7. Release of the IAP antagonist Smac/DIABLO from mitochondria is essential for unlocking the block imposed on the caspases 3 and 9 by their inhibitors from the IAP (Inhibitors of Apoptosis Proteins) family (namely XIAP) and thus ensuring smooth and efficient activation of apoptotic signaling (Tait and Green, 2010) (Figure 1.)

Pro-apoptotic proteins Bax and Bak from the Bcl-2 family have been identified as direct mediators of MOMP. They form pores of yet unknown structure in the mitochondrial outer membrane and allow the release of previously mentioned apoptotic factors from the mitochondrial intermembrane space. The pore-forming activity of Bax and Bak proteins is tightly controlled by the anti-apoptotic members of the Bcl-2 family. In healthy cells, these anti-apoptotic proteins such as Bcl-2, Bcl-xL or Mcl-1 directly interact with Bax and Bak to keep them in the inactive state. Upon apoptotic stimulus, members of the BH3-only proteins such as Bim, Bad, tBid, Puma, Noxa or others are expressed, released or activated and can a) competitively inhibit anti-apoptotic Bcl-2 proteins and b) some of them as tBid or Bim can directly enhance pro-apoptotic, channel-forming activities of Bax and Bak. BH3-only proteins can be activated at transcriptional or posttranscriptional level by plethora of inputs such as oncogene activation, DNA damage, viral infection or growth factors/cytokines withdrawal (Youle and Strasser, 2008)

1.1.2 The extrinsic apoptotic pathway

Main triggers of the extrinsic apoptotic pathway are cell surface "death receptors" from the TNFR family. There are six death receptors binding three different ligands that could be distinguished by the presence of the protein-protein interaction region in their intracellular parts called the Death Domain (DD). Upon binding of their respective ligands, these receptors can trigger apoptosis but they can also activate non-apoptotic signaling leading to cell proliferation or migration. Receptors TNFR1and DR3 are mainly involved in regulation of proliferation, survival and differentiation, while Fas/CD95, TRAIL-R1/DR4, TRAIL-R2/DR5 and DR6 are more known as inducers of apoptosis.

Upon activation of Fas, DR4 or DR5, the adaptor protein "Fas-associated protein with death domain" (FADD), is recruited to the receptors via its death domain and initiates formation of a complex where procaspase-8 is activated - so called death inducing signaling complex (DISC) (Chahrazade and Walczak, 2011).

Depending on cell type and the cellular conditions, the extrinsic apoptosis can be executed directly by mitochondria-independent (type I signaling) and/or via Bax/Bak-dependent MOMP activated by of the truncated BH3-only protein tBid (type II signaling) (Figure 3) (Gonzalvez and Ashkenazi, 2010).

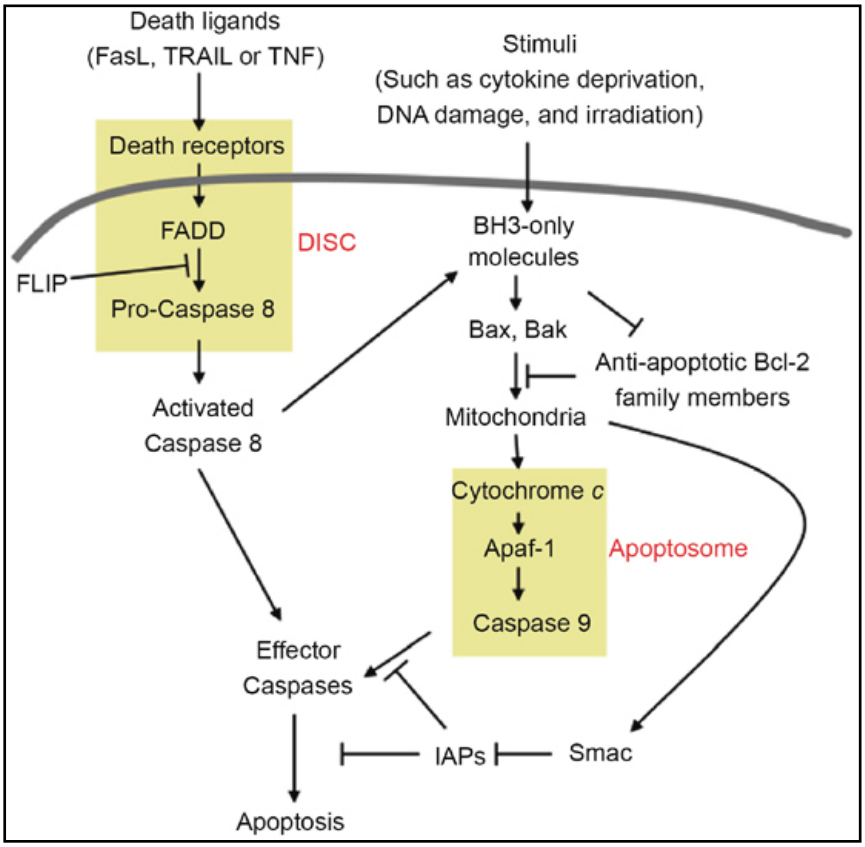

Figure 1. - Schematic representation of the main mammalian apoptotic pathways. The extrinsic pathway is activated by death ligands and proceeds via assembly of the DISC. The intrinsic pathway is activated by intracellular proteins from the Bcl-2 family upon various stresses. Both pathways eventually converge on activation of the effector caspases. (from (Xu and Shi, 2007)).

1.2 TRAIL-induced signaling

TNF-related apoptosis-inducing ligand (TRAIL) was discovered in 1996 as a new member of the TNF family. TRAIL is a transmembrane type II ligand specifically expressed on natural killer (NK) cells, activated B-cells, cytotoxic T-lymphocytes (CTLs) and dendritic cells, suggesting its role in the immune system modulation (Allen and El-Deiry, 2012). The definitive role of TRAIL signaling in human physiology is not yet fully comprehended.

Five receptors for TRAIL have been discovered so far in human. Two of them, DR4 and DR5, are able to initiate apoptotic signaling specifically in various cancer cell lines, but not in non-transformed cells. Therefore, targeting DR4 and DR5 by various means, ranging from their natural ligand TRAIL to antibodies and small molecules could be possibly applicable in a future cancer therapy. Activation of DR4/DR5 leads to assembly of the DISC, which initiates proteolytical cascade leading to cell disintegration. In addition to this apoptotic signaling, TRAIL can also activate several other pathways such as MAP kinases or NF-κB (Gonzalvez and Ashkenazi, 2010). These pathways are referred to as "non-apoptotic" TRAIL signaling.

1.2.1 TRAIL ligand

Human TRAIL is a type II transmembrane protein composed of 281 amino acids. TRAIL mRNA is expressed in many tissues including the spleen, thymus, placenta, lung, colon and small intestine (Wiley et al., 1995), at protein levels, TRAIL expression is restricted to immune cells such as NK cells, CTLs, B-cells and dendritic cells. For its potential toxicity, TRAIL expression is tightly regulated and can be induced by lipopolysacharides, interferons, or transcription factors involved in apoptosis such as p53 or FOXO (Allen and El-Deiry, 2012).

The recombinant human TRAIL (rhTRAIL) consisting of its C-terminal, extracellular part (96/114-281) is commonly used in research and is explored as a potential anti-cancer therapeutic. The structure of rhTRAIL has been solved in the complex with DR5. The overall structure is similar to that of other TNF family members and consists of two antiparallel B-sheets. TRAIL subunits associate to form a homotrimeric complex, assembly of which is dependent on a zinc-binding site formed by cysteine 230 at contact sites of each subunit (Figure 3). Mutation of cysteine 230 disrupts

TRAIL stability and biological activity. In the unbound TRAIL, the receptor interacting region is formed by loops that are intrinsically unstructured (Hymowitz et al., 1999).

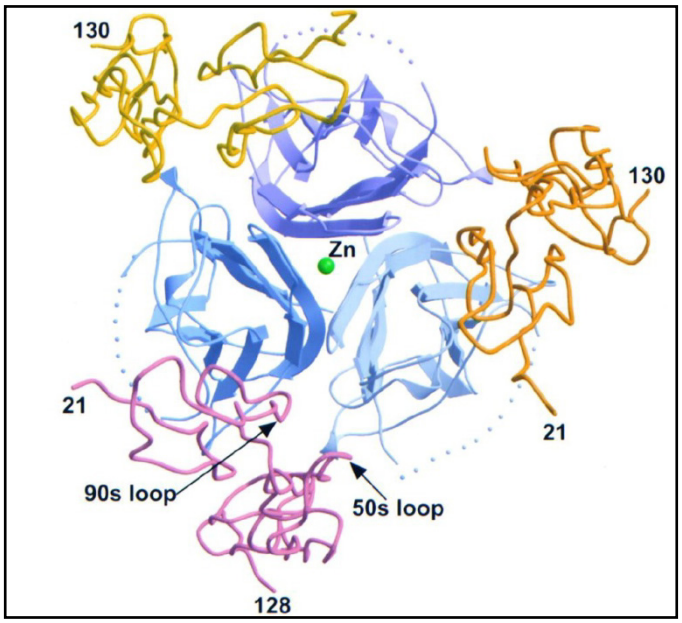

Figure 3. View down the TRAIL-DR5 complex. Zinc-coordinated TRAIL trimer is depicted in blue. DR5 subunits are depicted in yellow, orange and pink respectively. Black arrows emphasize loops mediating interaction with the ligand. From (Hymowitz et al., 1999).

1.2.2 TRAIL receptors

To date, five receptors from the TNF family have been identified for human TRAIL ligand: death receptors DR4 and DR5 (also known as TRAIL-R1 and TRAIL-R2), decoy receptors DcR1 and DcR2 (also known as TRAIL-R3 and TRAIL-R4), and a soluble protein osteoprotegerin (OPG), a molecule involved in bone metabolism (Gonzalvez and Ashkenazi, 2010).

Mice have got only one TRAIL death receptor mDR5, homologous to both DR4 and DR5, three decoy receptors without cytoplasmic domains and also OPG (Yagita et al., 2004). Relatives of DR4 and DR5 and their ligands have been also identified in frogs (*Xenopus*), birds (*Gallus*) and fish (*Danio*). The function of these molecules in non-mammalian organisms is not well defined (Bridgham et al., 2003; Eimon et al., 2006).

Death receptors: DR4 and DR5

Two main pro-apoptotic TRAIL receptors share approximately 55% sequence identity and were discovered by searching for homologs of TNFR1 (Pan et al., 1997; Sheridan et al., 1997). Both receptors are type I transmembrane proteins, 468 and 411 amino acids long, and contain three extracellular cysteine-rich domains (CRDs), which are common feature of the TNFR family members. A short transmembrane sequence is followed by the intracellular domain, which contains highly conserved DD, a six-helical bundle structure approximately 70 amino acids long (Kwon et al., 2012).

The structure has been solved for the TRAIL-DR5 complex (Figure 3). The extracellular part of DR5 consists of three CRDs and has an elongated shape maintained by seven disulfide bridges. The interaction of DR5 with TRAIL is mediated mostly by so called "90s" loop, which contains most of the residues critical for ligand binding, especially glycine 205 and glutamine 236. Mutations of these amino acids significantly weaken TRAIL binding to DR5 (Hymowitz et al., 1999).

Expression analysis of DR4 and DR5 at both mRNA and protein levels documents that both receptors are expressed in a wide range of human tissues (Yagita et al., 2004). However, our knowledge of their function in normal non-transformed cells is still limited. Relatively high levels of DR4 and/or DR5 were found in many cancers including leukemias, colorectal, pancreatic, lung and breast cancers (Holland, 2011). Recently, increased DR5 expression was detected in tumor associated endothelial cells. In these cells, TRAIL treatment led to tumor blood vessels disruption, which ultimately reduced tumor growth in mice (Wilson et al., 2012)

It was originally assumed that TRAIL binding to its receptors induces receptor trimer formation leading of DISC assembly (Hymowitz et al., 1999). This view was first challenged by the finding that death receptors already exist as pre-assembled trimers and this trimerization is mediated through so called "preligand assembly domain" (PLAD) in the CRD1 (Clancy et al., 2005). Also, using fluorescent microscopy and structural modeling, it has been recently shown that TRAIL binding induces formation of organized clusters of DR5 trimers with defined topology (Figure 2) (Valley et al., 2012).

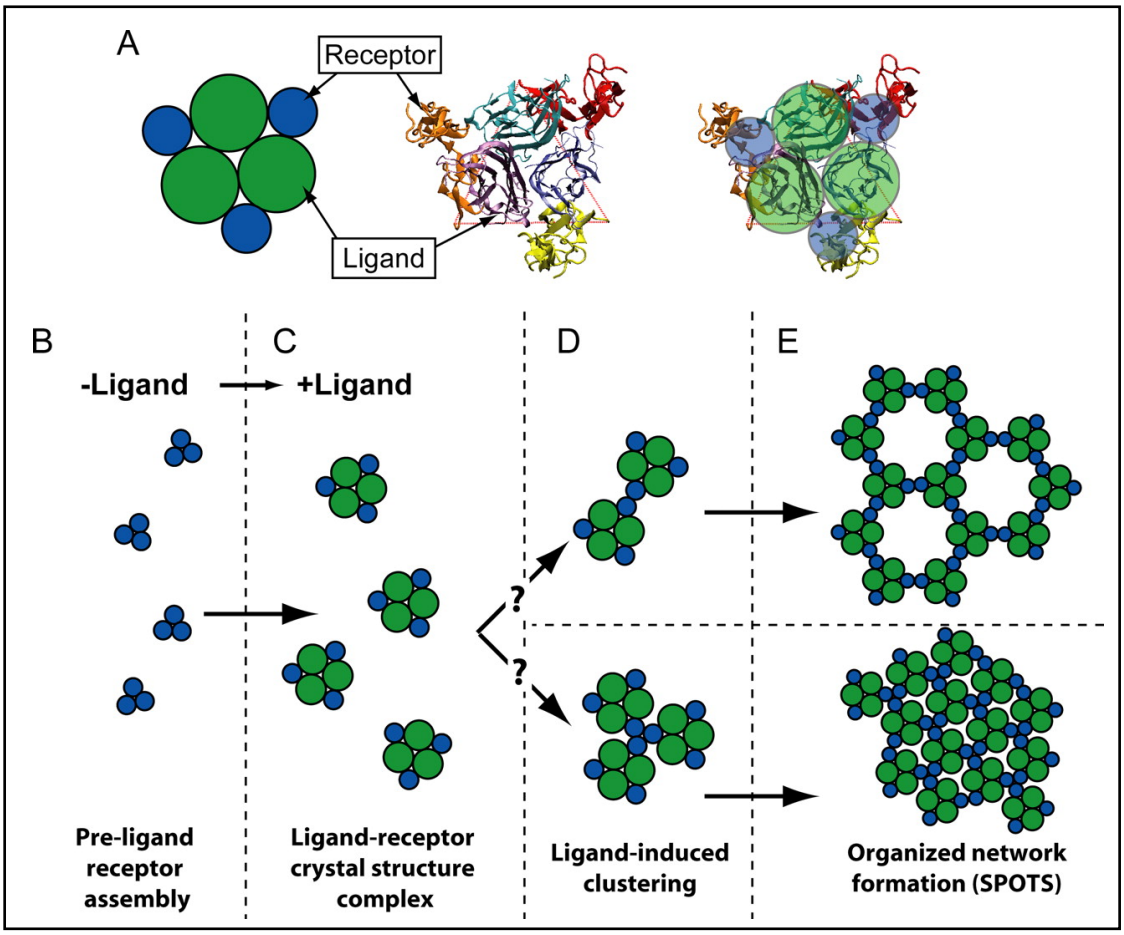

Figure 2. Current model of TRAIL-induced receptor clustering. TRAIL trimers bind preformed receptor trimers to induce receptor clustering with defined topology. From (Valley et al., 2012).

Decoy receptors: DcR1, DcR2 and Osteoprotegerin (OPG)

While DR4 and DR5 can trigger both pro- and non-apoptotic signaling, the decoy receptors mainly function as competitive suppressors of both signaling receptors. DcR2 contains only truncated intracellular domain and DcR1 is a GPI-anchored extracellular protein. Experiments with overexpressed decoy receptors revealed that DcR1 competes with DR4 and DR5 for TRAIL binding, while DcR2 could in addition form complexes with DR5 and thus prevents efficient caspase-8 processing at the DR5-DISC (Clancy et al., 2005; Merino et al., 2006). However, it has been suggested that the decoy receptors may play more than just an inhibitory role. Recently published data showed that DcR2 can activate Akt pathway in a ligand independent manner and DcR1 regulates TRAIL-induced proliferation and migration of pulmonary artery smooth muscle cells (Steinwede et al., 2012).

Expression of DcR1 appears to be specific mainly for lymphocytes and may be responsible for TRAIL resistance of these cells. DcR2 is widely expressed at the level of mRNA but it is usually not expressed at the cell surface (Yagita et al., 2004). Clinical relevance of the decoy receptors was demonstrated in acute myeloblastic leukemia, where expression of both decoy receptors was correlated with resistance to TRAIL-induced apoptosis (Riccioni et al., 2005).

Osteoprotegerin is a TNFR family member that negatively regulates osteoclastogenesis. OPG is a soluble protein, which binds and inhibits RANKL, a ligand for the RANK receptor. RANK is expressed on the surface of osteoclast precursors and triggers NF-κB-dependent osteoclastogenesis upon RANKL binding (Boyce and Xing, 2007). OPG has been also identified *in vitro* as a weak receptor for TRAIL (Emery et al., 1998). Moreover, OPG blocked TRAIL-induced apoptosis in Jurkat cells and vice versa, TRAIL blocked inhibitory function of OPG in the RANKL-RANK pathway (Emery et al., 1998; Zauli et al., 2004). However, *in vivo* relevance of TRAIL-OPG interaction was not confirmed in mouse models lacking mDR5 or in mice treated with TRAIL, both of which displayed normal bone histology (Cretney et al., 2002; Labrinidis et al., 2008).

1.2.3 TRAIL-induced apoptotic signaling: assembly and regulation of the DISC

TRAIL-induced signaling is initiated upon ligation of TRAIL trimers to either DR4 or DR5 receptors and subsequent receptor re-arrangement and clustering leading to the DISC formation. The main components of the DISC are the adaptor protein FADD, pro-caspase-8 and a regulatory molecule cFLIP, resembling catalytically dead pro-caspase-8. These components are routinely identified within the endogenous DISC, but a number of other proteins have been reported to interact with or to have a role in the DISC formation in a cell- or context-specific manner (Shirley et al., 2011).

The first step in formation of the DISC is TRAIL-induced DR4/DR5 rearrangement. This leads to recruitment of the FADD adaptor molecules composed of two similar protein-protein interaction domains - the death domain (DD) and the death effector domain (DED) to the ligated receptors (Kischkel et al., 2000). This binding is mediated through homotypic interaction between FADD-DD and TRAIL receptors DDs and it is essential for further DISC assembly and DR4/DR5-mediated apoptosis (Kuang et al., 2000). The next step is FADD-assisted recruitment of the initiator procaspase-8

and/or 10 and the caspase-8 regulator cFLIP. This recruitment also occurs via homotypic interaction and in this case it is mediated by "dead effector domains" (DEDs, procaspase-8 contains two - DED1 and DED2). Pro-caspase-8 molecules are thus brought into close proximity necessary for conformational changes and proteolytical self-cleavage, which in a time-dependent manner leads to caspase-8 activation (Shi, 2004). Caspase-10 is non-essential for TRAIL-induced apoptosis and its role in this pathway is not well established (Muhlethaler-Mottet et al., 2011; Sprick et al., 2002).

Two splice variants of caspase-8 are cleaved at the DISC, caspase-8a and caspase-8b (55 and 53 kDa). The isoforms are functionally equivalent and differ by one 15 amino acid long exon in caspase-8a. Initial cleavage at the DISC generates p43/p41 fragment attached to the DISC and p10 small subunit of caspase-8. In the second cleavage, which leads to full caspase-8 activation, the p18 subunit is generated. These large and small subunits associate to form $(p18/p10)_2$ tetramers, which are released from the DISC (Lavrik and Krammer, 2012).

The proximity-induced caspase-8 activation in the DISC is modulated by other DED-containing molecule cFLIP(L/S), which have been shown to inhibit caspase-8 activation through competitive interaction with FADD (Sharp et al., 2005; Xiao et al., 2002). Interestingly, cFLIP is expressed in three splice variants – the longest cFLIP-L (56 kDa) resembles pro-caspase-8 and can act at high expression levels as inhibitor of pro-caspase-8 self-processing. However at low expression levels, cFLIP was actually shown to enhance this self-processing of pro-caspase-8. In contrast, two short variants cFLIP-S and-R contain just two DED domains and serve only as rather inhibitors of pro-caspase-8 binding to DISC and its processing. (Ozturk et al., 2012; Sharp et al., 2005).

Recent proteomical analysis and structural modeling of TRAIL- and FasL-induced signaling revealed that the endogenous DISC is a 700kDa complex with FADD:caspase-8 ratio approximately 1:9 and this works also defined a "DED chain model", according to which caspase-8 molecules interact with each other via their DED domains and form chains essential for their activation (Dickens et al., 2012; Schleich et al., 2012).

A number of various posttranslational modifications play a role in the regulation of DISC localization, assembly and stability of its individual components. Palmitoylation of DR4 but not DR5 facilitates efficient oligomerization by targeting the receptor into lipid rafts (Rossin et al., 2009). O-glycosylation of both receptors positively correlates

with TRAIL sensitivity and this finding has possible clinical implications (also see chapter 1.2.5) (Wagner et al., 2007).

At least two phosphorylations directly regulate interaction or activity of some proteins in the DISC. Caspase-8 can be phosphorylated by Src kinase at tyrosine 380 site between p10 and p18 subunits. This phosphorylation inhibits efficient caspase-8 processing and activation at the DISC (Cursi et al., 2006). Phosphorylation of cFLIP, which reduces its recruitment to the DISC, has been observed in hepatocytes upon treatment with bile acid. Decreased cFLIP levels at the DISC led to more efficient caspase-8 processing and thus sensitized hepatocytes to TRAIL-induced apoptosis. (Higuchi et al., 2003). In glioblastoma cells, ubiquitin ligase A20 and RIP1 kinase are constitutively bound to DR5 via homotypic DD interaction. Upon TRAIL binding to DR5, A20 ubiquitinates RIP1 and the RIP1-bound polyubiquitin chains prevent caspase-8 clustering and activation, thus rendering glioblastoma cells resistant to TRAIL (Bellail et al., 2012).

The ubiquitination of various DISC components emerges as an important regulatory mechanism of the early steps in TRAIL-induced pro-apoptotic signaling. Stability of DR4 is compromised by its ubiquitination by MARCH-8 ubiquitin ligase (van de Kooij et al., 2013). Similarly, the essential adapter protein FADD is a subject of degradative ubiquitination mediated by E3 ubiquitin ligase MKRN1. MKRN1 depletion combined with TRAIL treatment significantly enhanced apoptosis and restricted tumor growth in breast cancer xenograft mouse model (Lee et al., 2012). A complex regulation by ubiquitination has been also shown for caspase-8, which undergoes two functionally opposite ubiquitinations during or after its activation at the DISC. During activation, Cullin-3/RBX1 attaches K63-linked ubiquitin chains to DISC-bound procaspase-8 p10 subunits, which are after the first cleavage released from the DISC as p43/p10 dimers and form ubiquitin rich foci in the cytoplasm. The formation of the foci is mediated by p62 protein and is necessary for full caspase activation (Jin et al., 2009). The second ubiquitination occurs immediately after the first one and is mediated by the TRAF2 E3 ubiquitin ligase. This is a K48-linked polyubiquitination that marks activated caspase-8 for rapid proteasomal degradation, therefore serving as “ubiquitin shut-off timer” (Gonzalvez et al., 2012).

Once activated at the DISC, caspase-8 cleaves and activates the effector caspases (caspase-3 and -7), which in type I cells are able to induce full-blown apoptosis. However, in cells with low levels of caspase-8 activation and/or with high levels of

XIAP1 (type II cells) mitochondrial amplification of apoptotic signaling is required for the efficient induction of apoptosis. Activation of MOMP is mediated by caspase-8-processed BH3-only protein Bid (tBid). tBid translocates to the mitochondrial outer membrane and participates in activation of Bax and subsequent MOMP (Gonzalvez and Ashkenazi, 2010) (Figure 4).

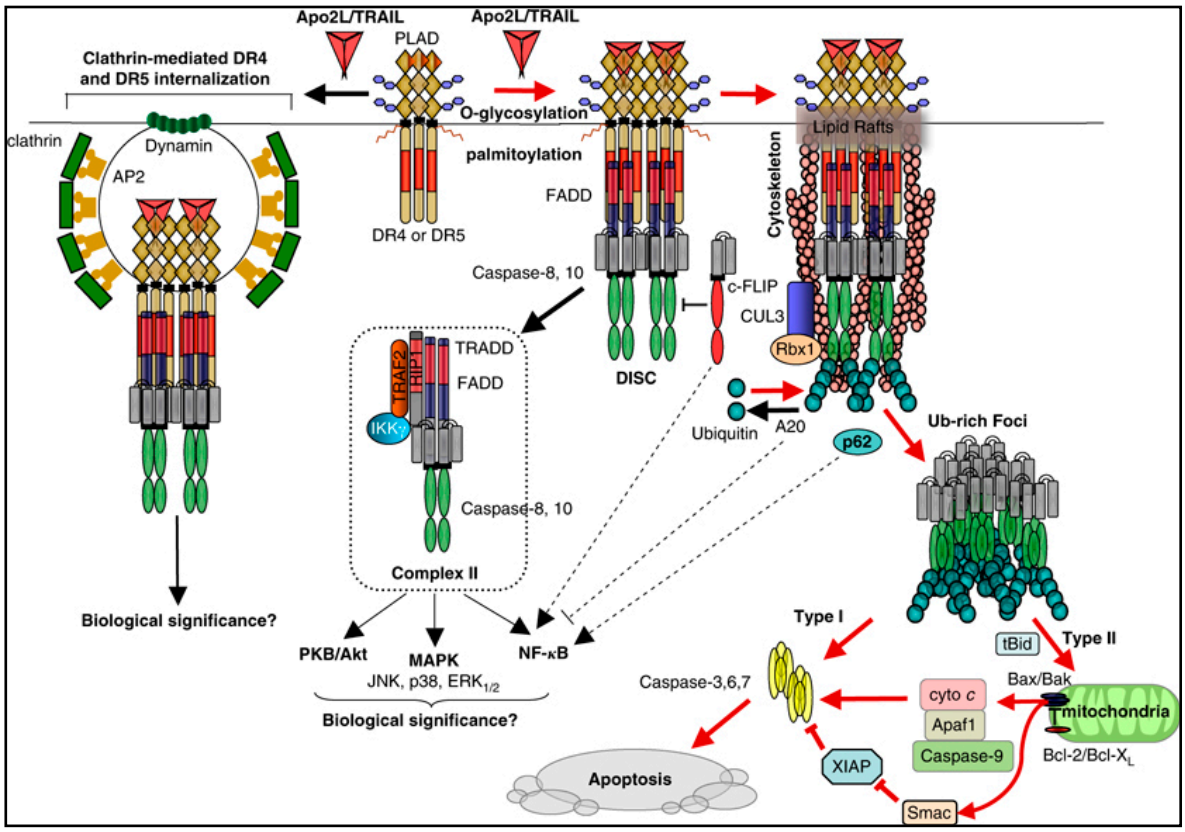

Figure 4. Molecular mechanisms of TRAIL signaling. TRAIL binding induces clustering of DR4/DR5 receptor trimers. The clustering is modulated by palmitoylation and glycosylation of the receptors. The signaling is initiated by recruitment of the FADD adaptor protein to receptors' death domains. FADD serves as the platform for proximity-induced caspase-8 activation, which is negatively regulated by cFLIP. In some cases, efficient caspase-8 activation is mediated by Cullin-3-mediated ubiquitination and p62-mediated translocation into ubiquitin-rich caspase-8 foci. TRAIL-induced apoptotic signaling can proceed either by direct activation of effector caspases-3/-7 by caspase-8 or, in some cell types, the apoptotic signal needs to be amplified by caspase-8-mediated activation of Bid and subsequent MOMP. Apart from apoptotic signaling, TRAIL activates several non-apoptotic pathways via assembly of the secondary cytoplasmic complex composed of TRADD, FADD, RIP1, TRAF2 and IKK proteins. TRAIL-DR4/DR5-receptor complexes are internalized in a clathrin-dependent manner; however, the function of the internalization is not well understood.

1.2.4 Physiological role of TRAIL-induced signaling

The ability of TRAIL to induce apoptosis in numerous cancer cell lines suggested involvement of TRAIL in tumor suppression. This potential anti-tumor role of TRAIL-induced apoptosis was confirmed using SCID mice with induced liver metastasis, in which depletion of TRAIL-expressing liver NK cells increased tumor growth (Takeda et al., 2001). Another evidence for tumor suppressive function of TRAIL comes from $TRAIL^{-/-}$ mice, which developed normally but exhibited increased susceptibility to both spontaneous and experimentally induced tumorigenesis (Cretney et al., 2002). These effects were recapitulated also in mice deficient in mDR5 (Finnberg et al., 2008).

However, on a different genetic background, it was shown that mDR5 knock-out increases lymph node metastasis but not tumorigenesis or tumors size in mice treated with tumor promoter TPA (tetradecanoylphorbol acetate), hinting on the role of TRAIL signaling in restricting metastasis rather than tumor growth itself (Grosse-Wilde et al., 2008). This idea was supported by the study showing that cell detachment-induced cell death (anoikis) is dependent on DR5 in human colorectal carcinoma cell lines (Laguinge et al., 2008).

Experiments with $mDR5^{-/-}$ mice also revealed that TRAIL signaling is important part of antibacterial and antiviral responses in mice. $mDR5^{-/-}$ mice challenged with *Listeria monocytogenes* or *Cytomeglaovirus* exhibited reduced cell death of infected cells and were more resistant to symptoms caused by these infections (Diehl et al., 2004; Zheng et al., 2004).

Two recent studies uncovered a role of TRAIL signaling in lung pathology. The first one showed that TRAIL promotes pulmonary arterial hypertension (PAH) in mice by inducing proliferation and migration of pulmonary artery smooth muscle cells (PASMC) and that PAH symptoms in mice can be suppressed using anti-TRAIL antibodies or TRAIL gene ablation (Hameed et al., 2012). Interestingly, the positive effect of TRAIL on PASMCs was reduced by antibody-mediated blocking of DcR1 receptor, which is lacking an intracellular domain and is considered to play a merely inhibitory role. The second study provides evidence for a beneficial role of TRAIL against pneumonia by showing that *Streptococcus pneumoniae* infected alveolar macrophages are killed by TRAIL-expressing neutrophils (Steinwede et al., 2012).

Taken together, although not much is known about its biological role in other mammals, TRAIL-induced signaling in mice appears to be involved in shaping the immune responses against transformed cells or various infections.

1.2.5 TRAIL-induced signaling and cancer therapy

The ability of TRAIL to selectively kill cancer cells was recognized shortly after its discovery and triggered considerable interest in the pharmacological industry (Ashkenazi et al., 1999; Walczak et al., 1999).

Initially, there were concerns raised about its safety since it was reported that histidine-tagged TRAIL is toxic to human hepatocytes *in vitro* (Jo et al., 2000) and FLAG-tagged version induces strong apoptosis in human brain slices (Nitsch et al., 2000). However, none of these effects were recapitulated when an untagged version of extracellular portion (residues 114−281) of human TRAIL was used and no toxic effects were detected in cynomolgus monkeys administered with this version of TRAIL (Lawrence et al., 2001). This variant of recombinant TRAIL was eventually patented as a potential anticancer therapeutic under trade name dulanermin (US patents US6030945 and US6284236).

To date, TRAIL in combination with various chemotherapeutics reached phase II clinical trials. However, the addition of TRAIL to commonly used drug combinations does not seem to improve chemotherapy outcome, as most recently demonstrated in patients with non-small cell lung carcinoma (NSCLC) (Soria et al., 2011) and it is becoming increasingly clear that most cancers are probably TRAIL-resistant *in vivo*. A recent review extensively discusses the current state of TRAIL-based cancer therapies and suggests that careful selection of patients based on biomarkers predicting TRAIL sensitivity of different cancers may uncover true potential of TRAIL as an anticancer drug (Dimberg et al., 2013). For example, glycosylation of DR4 and DR5 positively correlated with TRAIL sensitivity in numerous melanoma, pancreatic and lung cancer cell lines. Overexpression of specific glycosyltransferases further sensitized target cells to TRAIL, whereas downregulation attenuated their apoptotic response. Moreover, the authors examined expression of these specific glycosyltransferases in a panel of tumor samples and found overexpression in approximately 30% of all cases, thus defining a subset of patients that might benefit from TRAIL based therapies (Wagner et al., 2007).

Another potential biomarker is the presence of mutations causing loss of function of death receptors as demonstrated in head and neck cancer, non-Hodgkin lymphoma, breast cancer or hepatocellular carcinoma (Zhang and Fang, 2004). Moreover, a complete loss of DR4/DR5 was also observed in some cancers, since 8p21-22 chromosome, which encodes all TRAIL receptors except OPG, is often lost in colon, breast, ovarian, hepatocellular or lung cancers. On the other hand, mutations or loss of caspase-8 or FADD are rather rare (Pennarun et al., 2010). Anti-apoptotic molecule cFLIP is another obstacle in potential TRAIL-based therapy. cFLIP recruited to the DISC in high amounts negatively regulates caspase-8 processing. Therefore, cFLIP overexpression may cause TRAIL resistance as shown in colorectal and hepatocellular carcinomas or lymphomas (Safa, 2012).

One way to improve TRAIL based therapies might be co-administration of TRAIL with compounds enhancing sensitivity of cancer cells to TRAIL-mediated killing and intensive search for such compounds is ongoing. To date, a number of compounds have been identified, which enhance TRAIL-mediated killing of cancer cells by various mechanisms including death receptor upregulation or anti-apoptotic proteins such as cFLIP downregulation (Stolfi et al., 2012). One such promising compound appears to be all-trans-retinyl acetate (RAc), which is acting through DR4 and DR5 upregulation together with suppression of decoy receptors. In APC^{min} mice, which bear dominant negative mutation in APC (adenomatous polyposis coli) gene and spontaneously develop colon adenocarcinomas, RAc + TRAIL treatment almost entirely suppressed tumor growth by elimination of premalignant tumors, therefore preventing colon cancer development (Zhang et al., 2010). Another approach, which proved to be efficient in mice model, is triggering apoptosis in tumor-associated endothelial cells, which express high amounts of DR5. FLAG-tagged TRAIL crosslinked with anti-FLAG antibody efficiently disrupted tumor vasculature resulting in reduced tumor growth (Wilson et al., 2012).

Another possibility of improving TRAIL efficacy against cancer might be utilization of recently designed variants of TRAIL, which are able to selectively target either DR4 or DR5 and which have been shown to be more potent killers than wild- type TRAIL in a cell type-specific manner (Wajant et al., 2011) An alternative way to target death receptors in cancer is application of anti- DR4 or DR5 monoclonal agonistic antibodies, which are in early stages of clinical testing against various cancers (reviewed in (Dimberg et al., 2013)).Recently, a high-throughput screening revealed a small

molecule inducing cell death in cancer cells via DR5 activation. Thus, targeting death receptors with small molecules may be another approach for future death receptor-based cancer therapy (Wang et al., 2013).

In short, although TRAIL or TRAIL-based or –mimicking agents could be well tolerated in patients, their future as potential anti-cancer therapeutics is uncertain and will depend on right combinations of drugs co-administered with them.

1.2.6 TRAIL-induced non-apoptotic pathways

In addition to inducing apoptosis, TRAIL can activate in target cells numerous other signaling pathways including NF-κB pathway, MAP-kinases p38, ERK1/2 and JNK, protein kinase Akt, protein kinase C, RIP1-mediated necroptosis, TAK-1-mediated autophagy and migration (Figure 5).

The role of this "non-apoptotic" signaling is not well characterized and in some cases also controversial. In the following chapters, each of these pathways is shortly described and placed into the context of TRAIL-induced signaling.

1.2.6.1 NF-κB

The NF-κB transcription factors are critical regulators of inflammation, survival, proliferation and apoptosis. Deregulated NF-κB signaling can result into various pathologies including cancer and liver diseases. In humans, the NF-κB transcription factors can be divided into two groups based on structural similarity. Transcription factors c-Rel, RelB and RelA/p65 form so called Rel subfamily and p50 with p52 form NF-κB subfamily. The NF-κB transcription factors are normally held inactive in the cytosol by inhibitory interaction with inhibitor κB proteins (IκBs). Upon activation, which can be mediated by wide spectrum of upstream signals such as interleukins, ligands from the TNF family, growth factors or endotoxins, IκBs are phosphorylated by IKK/NEMO complex. This phospohorylation marks IκBs for ubiquitination and subsequent proteasomal degradation. Once released from the IκB complexes, NF-κB transcription factors translocate into the nucleus where they form dimers to influence transcription of hundreds of genes (Gilmore, 2006).

In the regulation of apoptosis, NF-κB pathway has been shown to induce pro-apoptotic regulators such as c-Myc, p53, FasL, Fas, and also anti-apoptotic proteins such as Bcl-2, IAPs and cFLIP (Shishodia and Aggarwal, 2002).

The NF-κB pathway was together with apoptosis amongst the first recognized TRAIL- induced signaling pathways (Schneider et al., 1997). Similarly as for MAP kinases activation, it was reported that NF-κB induction by TRAIL is dependent on the secondary cytoplasmic complex containing TRADD, FADD, IKK/NEMO, RIP1, TRAF2 and Caspase-8. Depletion of RIP1 or IKK/NEMO, inhibited NF-κB induction by TRAIL (Varfolomeev et al., 2005). However, the molecular mechanism by which is this secondary complex assembled is unknown. Recently, experiments in Jurkat cells suggested that TRAIL-induced NF-κB depends on DR4/DR5 death domains, FADD and Caspase-8 (Grunert et al., 2012). This observation is in contrary with earlier report showing that TRAIL induces NF-κB dependent proliferation in primary childhood leukaemia cells deficient in caspase-8 or functional FADD. Importantly, this work was one of the the first to show that upon dysfunctional extrinsic apoptotic pathway, TRAIL switches from pro-apoptotic molecule into inducer of cell proliferation and survival (Ehrhardt et al., 2003). To date, there are many other reports connecting TRAIL resistance with NF-κB activity in a cell type-dependent manner (reviewed in (Plantivaux et al., 2009)). This makes TRAIL-induced NF-κB a potential obstacle in TRAIL-based therapies.

An interesting observation was recently made in FasL-induced apoptosis, mechanisms of which are very similar to those of TRAIL-induced signaling. The study shows that together with apoptosis, FasL induces NF-κB-dependent cytokine release from apoptotic cells. These cytokines serve as "find-me" signals essential for clearance of apoptotic cells by phagocytes (Cullen et al., 2013). Whether this is also the case in TRAIL-induced apoptosis remains to be investigated.

1.2.6.2 MAP kinase pathways: JNK, p38 and ERK1/2

Mitogen activated protein (MAP) kinases are serine/threonine kinases and form downstream component of typically three-kinase cascade. There are four principal MAPKs groups in mammals – p38, JNK, ERK1/2 and ERK5 and all of them extensively cooperate to control almost all aspects of cell behavior. MAP kinases are rapidly activated by phosphorylation in response to plethora of signals including cytokines

(TNFα, TRAIL), growth factors and various stresses such as UV irradiation, osmotic or heat stress. Once activated, MAPKs phosphorylate and activate a number of diverse substrates including transcription factors from c-jun or c-fos families and other nuclear or cytoplasmic proteins regulating cell proliferation and survival (Chang and Karin, 2001).

As already mentioned, activation of MAP kinases and also NF-κB by TRAIL is dependent on the secondary cytoplasmic complex, which is composed of FADD, caspase-8, RIP1 kinase, TRAF2 and NEMO (Varfolomeev et al., 2005). How is this complex assembled from the primary membrane DISC is unknown.

The MAPK signaling is subject of extremely complex regulation and is highly cell type and stimulus dependent. Since detailed description of MAPK pathways is beyond scope of this work, only a brief description of each pathway is provided with emphasis on cell death regulation.

JNK: c-jun NH2-terminal kinases (JNKs) form group of three genes (JNK1, JNK2, JNK3) encoding ten isoforms that regulate a broad range of cellular processes depending on stimulus and cell type. In pro-apoptotic signaling the activated JNKs can phosphorylate and activate number of cytoplasmic targets such as pro-apoptotic BH3-only proteins Bim and Bad. In the nucleus, major target of JNKs is the c-Jun transcription factor, which in turn transactivates a number of pro-apoptotic genes, including TNFα, Fas, Bak or c-Myc. $JNK^{-/-}$ mouse embryonic fibroblasts fail to release mitochondrial cytochrome c upon various form of cellular stresses and thus are resistant to apoptosis (Dhanasekaran and Reddy, 2008).

TRAIL-induced JNK activation was first observed in HeLa cells and Kym-1 sarcoma cells. While in HeLa cells, TRAIL-induced JNK activation was caspase-8 dependent, TRAIL-treated Kym-1 cells displayed unaffected JNK activation in the presence of caspase inhibitor zVAD-fmk suggesting that TRAIL activates JNK by at least two pathways (Mühlenbeck et al., 1998). Direct contribution of JNK to TRAIL-induced apoptosis was demonstrated in Jurkat cells and human oligodendrocytes where inhibition of JNK significantly reduced TRAIL-induced apoptosis (Herr et al., 1999; Jurewicz et al., 2006).

Recent work analyzed TRAIL-induced JNK activation in more detail and revealed that in colorectal cancer cells, TRAIL activates four JNK1 isoforms – two long isoforms (JNK1α1 and JNK1β1) and two short isoforms (JNK1α2 and JNK1β2). However, stimulation of either DR4 or DR5 by agonistic antibodies induced only

activation of the short isoforms. The study further shows, that while long isoforms can enhance pro-apoptotic signaling, the role of short isoforms JNK1α2 and JNK1β2 is apparently anti-apoptotic. The authors hypothesize that selective DR4 or DR5 ligation leads to formation of receptor complexes with different topology than of those induced by simultaneous ligation of both receptors, which may lead to recruitment of different types or quantities of adaptor proteins needed for selective JNK activation (Mahalingam et al., 2009).

Taken together, based on current *in vitro* and *in vivo* data, TRAIL-induced activation of JNKs seems to play a role in enhancing TRAIL-induced apoptosis.

p38: In general, p38 behaves similarly as JNK, being activated by similar stimuli including DNA damage or pro-inflammatory cytokines. p38 activates other kinases such as Mitogen- and stress-activated protein kinase-1 (MSK1) and MAP kinase-activated protein kinase 2 (MK2) and transcription factors such as p53, CHOP or CREB, which in turn regulate cell cycle progression, inflammation, DNA repair, senescence and apoptosis. Four splice variants exist (p38α, p38β, p38γ p38δ), with p38α being the principal, ubiquitously expressed member. Genetic ablation of p38α in mouse leads to embryonic lethality caused by defective placental angiogenesis. (Zarubin and Han, 2005). The involvement of p38 kinases in apoptosis is highly dependent on the stimulus and cell type. Positive, negative and neutral roles of p38 in apoptotic signaling have been documented. p38 protects early-stage melanoma cells from UV-induced apoptosis by down-regulating Fas receptors and suppression of the NF-κB signaling pathway (Ivanov and Ronai, 2000). Pro-survival effect of p38 has been also documented in prostate cancer cells, where TRAIL-activated p38 up-regulates expression of Mcl-1, thus increasing the apoptotic threshold (Son et al., 2010).

On the other hand, numerous studies have uncovered a pro-apoptotic role of p38. For example, neuronal PC12 cells require p38 for activation of monoamine oxidase, which is a critical mediator of NGF withdrawal-induced apoptosis in these cells (De Zutter and Davis, 2001). Oxidative stress triggers apoptosis via p38 activation in HL-60 leukemia cells and ROS generated by TRAIL treatment in HeLa cells together with p38 contribute to caspase-3 activation (Lee et al., 2002; Zhuang et al., 2000). In DLD-1 cells, even though TRAIL induces p38 pathway, neither inhibition nor overexpression of p38 had any influence on TRAIL-induced apoptosis (Zhang et al., 2004).

ERK1/2: As the other MAP kinases, ERK kinases 1 and 2 represent crucial signaling nodes that transmit signals triggered by numerous inputs including, but not limited to, activated receptor-tyrosine kinases, which activate ERK1/2 through small GTPase Ras. Specificity and output of ERK1/2 signaling network is precisely regulated by subcellular localization of its components and highly depends on cell type and stimulus (Shaul and Seger, 2007).

ERK1 and 2 are approximately 70% homologous and ubiquitously expressed. The function of ERK 2 appears to be more important, since ERK2 knock-out mice die *in utero,* while ERK1-deficient animals are viable, suffering only from compromised thymocyte maturation and memory defects. Both ERK1 and ERK2 also share number of substrates and are usually activated together. The pleiotropic function of ERK1/2 is reflected in a variety of substrates activated or inhibited by ERK1/2-mediated phosphorylation. In the nucleus, the main ERK1/2 substrates such as transcription factors from the Ets family, AP-1, c-myc or p53 are global regulators of cell physiology. The representative cytosolic substrate of ERK1/2 is RSK (ribosomal protein S6 kinase), which can also translocate to the nucleus and activate number of other transcription factors (Shaul and Seger, 2007).

In cell death regulation, activation of ERK1/2 is generally anti-apoptotic. This was first demonstrated in neurons where ERK1/2-activated RSK phosphorylates pro-apoptotic Bcl-2 member Bad on serine-112 causing it to associate with 14-3-3 proteins, leading to Bad inactivation. Also, ERK1/2 phosphorylates CREB transcription factor, which in turn induces expression of a number of anti-apoptotic genes such as *bcl-2* (Bonni et al., 1999).

TRAIL activates ERK1/2 phosphorylation in a number of TRAIL-treated cells (e.g. HeLa) and generally has a suppressive effect on TRAIL-induced apoptosis (Tran et al., 2001). In breast cancer cells, acquired resistance to TRAIL-induced apoptosis was accompanied by constitutive ERK1/2 activation (Lee et al., 2006). In melanoma cells, TRAIL induces rapid ERK1/2 activation, which negatively regulates BAX-dependent MOMP, thus inhibiting apoptosis (Zhang et al., 2003).

The role of TRAIL-mediated ERK1/2 activation has been shown in cardiovascular system. In human endothelial cells, rapid activation of ERK and Akt in response to TRAIL treatment mediates proliferation of these cells. Since TRAIL is also produced by the vascular smooth muscle cells, the role of TRAIL as an angiogenic molecule might be physiologically relevant (Secchiero et al., 2003).

TAK1: MAPKK kinases form a proximal tier of MAP kinase signaling and more than ten MAPKKKs have been described in mammals (Chang and Karin, 2001). TAK1 is a MAPKKK for which relevance to TRAIL signaling has been documented several times. Specifically, TAK1 has been placed upstream of p38, JNK and NF-κB pathways in TRAIL treated HeLa cells. Down-regulation of TAK1 abolished activation of these pathways and increased sensitivity of HeLa cells to TRAIL-induced apoptosis via enhanced activation of caspase-8 (Choo et al., 2006). The mechanism by which TAK1 suppresses apoptosis has been elucidated in keratinocytes and mouse embryonic fibroblasts (MEFs). In MEFs, the TAK1-mediated suppression of apoptosis is transduced via NF-κB-induced activation of cFLIP expression (Lluis et al., 2010). A different mechanism is employed in keratinocytes, where TAK1 prevents ROS accumulation and cIAP degradation induced by TRAIL (Morioka et al., 2009).

An interesting physiological role for TRAIL mediated by TAK1 kinase emerged in non-transformed breast epithelial cells MCF-10A. It has been previously shown that TRAIL-induced autophagy plays a role in lumen formation in MCF-10A *in vitro* acinar morphogenesis model (Dhanasekaran and Reddy, 2008). Recently, it has been shown that TRAIL-activated autophagy in MCF-10A is induced by TAK1-mediated inhibition of mTOR complex 1 (Herrero-Martin et al., 2009).

1.2.6.3 Akt/PKB

With more than a hundred of substrates, Akt kinase is one of the most important regulators of cellular growth, proliferation, survival and metabolism. Akt activation is mediated by dual phosphorylation by PDK1 and mTORC2 and is dependent on PI3K typically activated by receptor tyrosin kinases. Aberrant activation of PI3K/Akt pathway results in hyperproliferation and resistance to apoptosis and is found in numerous cancers (Vivanco and Sawyers, 2002).

Activated PI3K/Akt pathway also renders cancer cells resistant to TRAIL as was shown for the first time in NSCLC cells. NSCLC cells display high PI3K/Akt activity and their treatment with TRAIL fails to activate Bid processing and thus activation of MOMP and apoptosis. TRAIL sensitivity in these cells can be rescued by pharmacological inhibition of PI3K, expression of dominant negative Akt mutant or expression of PTEN phosphatase, a negative regulator of PI3K activity (Kandasamy and

Srivastava, 2002). Other examples of Akt-mediated TRAIL resistance have been demonstrated in several other cancer cell lines including prostate cancer, ovarian cancer and gliomas (Dieterle et al., 2009; Goncharenko-Khaider et al., 2010; Puduvalli et al., 2005).

As already mentioned, direct activation of Akt, together with ERK1/2, by TRAIL enhances proliferation of human endothelial cells (Secchiero et al., 2003). TRAIL activates Akt also in cancers as observed in leukemias and breast cancer cells and Akt inhibition sensitizes these cells to TRAIL induced apoptosis (Xu et al., 2010; Zauli et al., 2005). However, the molecular mechanism by which TRAIL induces PI3K/Akt pathway is unknown. Surprisingly, in a recent work, Akt activation by TRAIL in HeLa cells has been linked to DcR2 receptor, which only possess supposedly non-functional truncated intracellular domain (Lalaoui et al., 2011).

1.2.6.4 Necroptosis

Necrosis has been traditionally considered an uncontrolled catastrophic event characterized by membrane rupture, organelle swelling and metabolic collapse. Recently, however, has been found that even necrotic cell death can be regulated and can be of a physiological relevance. The best known example of regulated necrosis is TNFα-triggered necroptosis. Activation of TNFR1 by TNFα leads to sequential formation of two complexes, second of which (complex II) is able to trigger apoptosis and is composed of adaptors FADD and TRADD, caspase-8 and kinases RIP1 and RIP3. The kinase activity of RIP1 and RIP3 in this complex is blunted by caspase-8-mediated cleavage. However, when the caspase-8 activity is suppressed, the complex-II turns into so-called “necrosome”, which induces necroptosis. The necrosome activity is dependent on RIP1/3 complex and relies on kinase activity of both proteins. Specific RIP1 inhibitor necrostatin-1 prevents TNFR1-induced necroptosis (Vandenabeele et al., 2010).

The first evidence of necrotic-like death induced by TRAIL comes from experiments with murine adenocarcinoma cells, which died after TRAIL treatment but lacked traditional apoptotic markers such as caspase-8 cleavage, tBid generation and phosphatidylserine externalization. In agreement with molecular mechanisms of necroptosis, which were described years later, inhibition of caspase-8 enhanced TRAIL-mediated killing in these cells (Kemp et al., 2003).

Recently it has been shown that HT-29 colorectal cancer cells, which are resistant to TRAIL-induced apoptosis, can be forced to TRAIL-induced necroptosis by low (6.5) extracellular pH and the process is dependent on RIP1/3 complex. This mechanism might be also relevant *in vivo* since tumors often exhibit acidic microenvironment (Jouan-Lanhouet et al., 2012).

1.2.6.5 Migration

Enhanced migration and invasivity allow spreading of cancer cells in the tumor-affected organism. Recent findings indicate that TRAIL is capable of triggering pathways that lead to increased ability of TRAIL-resistant tumor cells to migrate. In non-small cell lung carcinoma (NSCLS) cells, TRAIL treatment led to increased invasivity of the cells via RIP1-mediated activation of Src kinase and transcription factor STAT3 (Azijli et al., 2012). In colorectal cancer cell lines with defective apoptotic signaling, TRAIL enhanced migration capabilities via caspase-8-mediated activation of ROCK1 kinase (Somasekharan et al., 2012). These findings may have therapeutic implications since TRAIL is in clinical trials against both NSCLC and colorectal cancer.

1.2.6.6 Protein kinase C

Protein kinase C (PKC) is a serine/threonine kinase inducible by number of signals including activated G-protein coupled receptors and tyrosine kinase receptors. These signals can result in generation of diacylglycerol, which serves as a platform for PKC activation. Activated PKC is a typical "hub" kinase regulating number of processes such as transcription, membrane remodeling, cell growth and immune responses (Newton, 1995). Activation of various isoforms of PKC is reflected in its either pro- or anti-apoptotic role. In melanoma cells, induction of PKCδ isoform sensitized cells to TRAIL-induced apoptosis, while induction of other isoform (PKCε) increased resistance to TRAIL (Gillespie et al., 2005).

Direct activation of PKC by TRAIL was demonstrated in pancreatic adenocarcinoma cell line PancTuI and colorectal cancer cell line Colo 357. Inhibition of PKC in these cell sensitized them to TRAIL-induced apoptosis. Interestingly, activation of NF-κB pathway, which was also observed in these cell lines upon TRAIL treatment, was dependent on PKC activation (Trauzold et al., 2001). One mechanism by which

PKC inhibits TRAIL signaling is negative regulation of FADD recruitment to the death receptors resulting in inefficient DISC assembly and activation of caspase-8 (Harper et al., 2003).

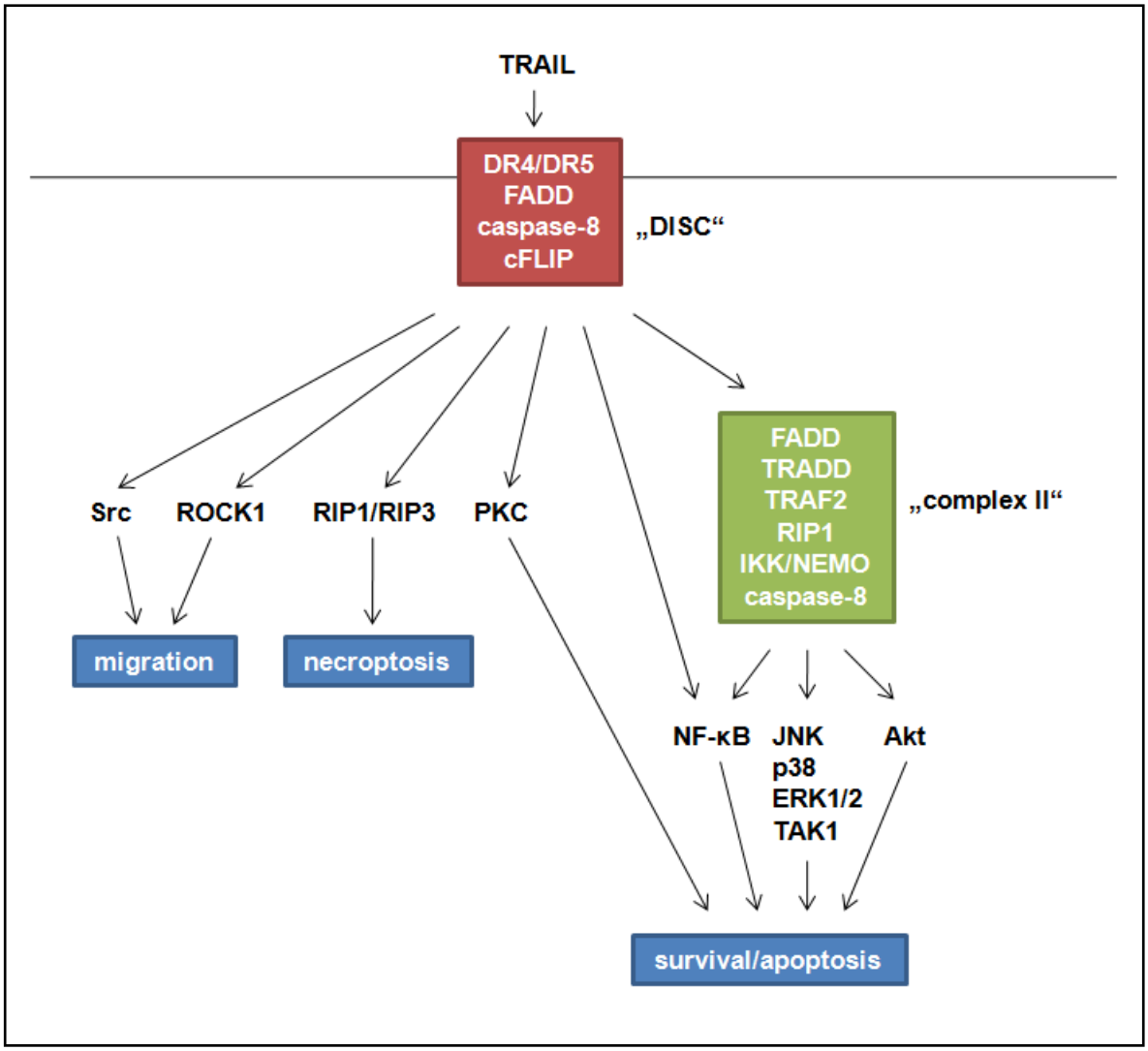

Figure 5. Schematic summary of TRAIL-induced non-apoptotic pathways. TRAIL can activate numerous pathways in a cell type/context specific manner. Depending on pathway, activation proceeds either directly via the DISC or via secondary cytoplasmic complex downstream of the DISC.

1.2.7 Receptor-specific TRAIL-induced signaling

TRAIL initiates signaling via binding to both DR4 and DR5, which are often co-expressed on the surface of cancer cells. However, the relative contribution of either receptor to TRAIL-induced signaling is not well defined. DR4 and DR5 share 58% identity in the extracellular domain and 75% identity in their cytoplasmic death domains (Chaudhary et al., 1997; Walczak et al., 1997). The two receptors also differ in their affinities to TRAIL; DR5 has K_D around 2nM, while DR4 around 70nM (Truneh et al., 2000).

A recent study utilized chimeric receptors to compare the properties of the transmembrane (TM) domains of DR4 and DR5. Using chimeras consisting of the extracellular part derived from TNFR1, transmembrane regions from DR4 or DR5 and intracellular part from Fas, the authors revealed that receptors with the TM region derived from DR5 kill cells more potently than receptors with DR4 TM upon TNFα treatment. Therefore, TM domains of DR4 and DR5 also appear not to be functionally equal (Neumann et al., 2012).

In a study comparing DR4- and DR5-triggered apoptotic signaling, Kelley et al. used phage display approach to select for TRAIL mutants binding exclusively to either DR4 or DR5. Using these receptor-specific mutants, they found that DR5 is a stronger inducer of apoptosis than DR4 in colorectal (Colo205) and breast (MDA-MB-231) cancer cell lines. The authors suggest that both cell lines express equal amounts of DR4 and DR5 but do not provide any evidence for this claim (Kelley et al., 2005). Taken together, based on its high affinity to TRAIL, properties of TM region and biological activity in selected colorectal and breast cancer cell lines, DR5 holds potential to be the dominant proapoptotic TRAIL receptor.

On the other hand, several reports revealed that in many cases the dominant proapototic TRAIL receptor is DR4. This was shown several times in leukemias, where using DR4- and DR5-specific TRAIL mutants revealed that both primary and stable leukemia cells of various origins are killed predominantly via DR4 (MacFarlane et al., 2005a; MacFarlane et al., 2005b; Szegezdi et al., 2011). Dominant function of DR4 was also demonstrated in pancreatic cell lines. These cells were killed strongly by both DR4 and DR5 agonistic antibodies; however, TRAIL was able to induce apoptosis only via DR4 (Lemke et al., 2010). In addition, dominant DR4-mediated apoptotic signaling was demonstrated in colorectal cell line SW948, in which DR4-blocking antibody inhibited

TRAIL- induced apoptosis, whilst blocking of DR5 had no protective effect (van Geelen et al., 2011).

Only a few studies addressed the relative contributions of DR4 and DR5 to non-apoptotic TRAIL-induced pathways. In the pancreatic cancer cells, Lemke et al. showed that similarly as apoptosis, activation of NF-κB is mediated mainly by DR4 (Lemke et al., 2010). In another study, TRAIL treatment induced cell migration of TRAIL-resistant lung cancer cells through activation of several non-apoptotic pathway such as Src, FAK, ERK1/2, Akt kinases and transcription factor STAT3. Notably, all these pathways were dependent on RIP1 and were activated exclusively through DR5 (Azijli et al., 2012).

Receptor-specific regulation has been also suggested for JNK activation in HeLa cells and this study provided the very first evidence of unequal contributions of DR4 and DR5 to TRAIL-induced signaling. Using agonistic receptor-specific antibodies, the authors demonstrated that JNK was activated by DR5 but not DR4 in HeLa cells (Muhlenbeck et al., 2000). An interesting observation was made by comparing the activation of JNK by TRAIL versus agonistic antibodies against DR4 or DR5. TRAIL induced two long and two short isoforms of JNK, whereas antibodies against either DR4 or DR5 were capable of inducing only short JNK isoforms. Moreover, while the long JNK isoforms augmented apoptotic signaling, the short ones displayed anti-apoptotic effect (Mahalingam et al., 2009).

The molecular mechanisms of differential regulation of DR4/DR5 signaling are not well understood. Intuitively, the different levels of both receptors could account for the different signaling strengths. However, no correlation between receptor levels and strength of apoptotic signaling in pancreatic or lymphoid cancer cells has been found (Lemke et al., 2010; MacFarlane et al., 2005a). Another possible explanation of dominant DR4 signaling in several cancer cell lines could be the decoy receptor DcR2, which selectively inhibited DR5 signaling in overexpression experiments (Merino et al., 2006). High levels of DcR2 were indeed detected in pancreatic cancer cell lines, which signal apoptosis via DR4. However, the authors did not examine the involvement of DcR2 in possible inhibition of DR5-mediated signaling (Lemke et al., 2010). The impact of DcR2 on DR5-triggered signaling in lymphoid cancers is also unknown (MacFarlane et al., 2005a; MacFarlane et al., 2005b).

Taken together, it is apparent that DR4 and DR5 signaling is differently regulated in a cell type-specific manner; however, the biochemical basis for the preferential usage of DR4 or DR5 is unknown.

1.2.8 Receptor-specific variants of TRAIL

The first approach that was used to distinguish between DR4 and DR5-mediated signaling was selective stimulation of the two receptors by either DR4 or DR5-specific antibodies (Muhlenbeck et al., 2000). The results of these experiments need to be interpreted with caution, because stimulation of TRAIL receptors with antibodies clearly differs from that induced by natural TRAIL ligand. For example, it has been recently shown in colorectal cancer cell lines that both agonistic DR4 or DR5 antibodies induce apoptosis equally, while TRAIL is able to trigger apoptosis only via DR4 (van Geelen et al., 2011). It is likely that agonistic antibodies induce receptor clustering that is different from that induced by TRAIL.

More recently, TRAIL variants that bind exclusively to either DR4 or DR5 were developed by phage display selection or by computational modeling. Today, there are several selective variants for each receptor (Wajant et al., 2011). The first receptor specific variants were selected using phage display technique. The authors screened a library of TRAIL mutants with substitutions in the receptor-interacting interface for binding to DR4 or DR5 and obtained mutants, which selectively bind to either DR4 or DR5 receptor. In both cases, six amino acid substitutions were necessary for the receptor-specific binding (Kelley et al., 2005).

Another technique that has been successfully employed to design receptor-specific variants of TRAIL is computational modeling with FOLD-X algorithm. FOLD-X allows redesign of proteins in order to change their stability or ligand binding properties. In case of improving the ligand-receptor interaction, FOLD-X takes into account residues at or near the binding interface and substitutes them sequentially for all 20 natural amino acids. For each combination, the global energy of the complex is calculated. In search for DR5 selective TRAIL, the authors modeled the interaction of TRAIL with all receptors except OPG and chose 34 amino acid positions at the receptor binding interface of TRAIL for substitutions. Surprisingly, only a single amino acid in TRAIL was uncovered as a critical determinant of receptor-specificity. In the wild type ligand, aspartate 269 interacts with a specific lysine, which is present in all receptors except DR5. Substituting this aspartate for histidine, an amino acid with opposite charge, diminished the interactions with DR4, DcR1 and DcR2, while the binding to DR5 was even enhanced. Therefore, D269H substitution in TRAIL was able to render a DR5-specific ligand. The authors screened for additional substitutions and found two

additional mutations (E195R or T214R), which slightly enhanced affinity of D269H mutant to DR5 (Van der Sloot et al., 2006).

The DR4-selective variant (designated 4C7) was obtained by the same method as D269H variant. However, the development of DR4 selective variant was complicated by the fact that the structure of TRAIL-DR4 complex is not available. The DR4-selective variant also required more complex rearrangements in the binding site than D269H mutant. Thus, to achieve DR4 selectivity, six substitutions in the TRAIL binding site were required (G131R/R149I/S159R/N199R/K201H/S215D) (Reis et al., 2010).

To date, several additional mutants were prepared by described approaches or their combination (Gasparian et al., 2009; Szegezdi et al., 2011; Tur et al., 2008).

In the experimental part of this thesis, we used 4C7 variant (Reis et al., 2010) and D269H/E195R variant (Van der Sloot et al., 2006) as the DR4 and DR5 selective ligand respectively.

2 Aims of the thesis

The major aim of this thesis was to set-up a system for studying signaling events induced by DR4- and DR5-specific variants of TRAIL in human colorectal cell lines and to perform pilot experiments in the respect to in-detail elucidation of presumed differences in signaling between these two major TRAIL receptors.

The specific aims:

1) Preparation and characterization of a of human recombinant TRAIL ligand modified with a trimerization motif from T4 bacteriophage for enhanced trimer formation and *strep*-tag for affinity purification and protein complex isolation.

2) Preparation of recently developed DR4 or DR5-selective mutants of TRAIL

3) Analysis and quantification of apoptotic signaling initiated by receptor-specific TRAIL variants in selected model colorectal cell lines.

4) Elucidation and comparison of non-apoptotic signaling (p38, JNK, TAK-1, ERK1/2, NF-κB) initiated by receptor-specific TRAIL variants in these model colorectal cell lines,

3 Materials and methods

3.1 Preparation of TRAIL expression vectors

All DNA constructs were prepared following the standard protocols (Sambrook and Russell, 2001) and/or instructions provided by manufacturers of used reagents/kits. Following kits and reagents were used:

Restriction enzymes: NcoI, XhoI, BamHI (Thermo scientific)
Other enzymes: FastAP thermosensitive alkaline phosphatase, T4 DNA ligase (Thermo scientific)
Gel extraction kit: Zymoclean gel DNA recovery kit (Zymo research)
Miniprep kit: Zyppy plasmid miniprep kit (Zymo research)
Bacteria: TOP10 chemically competent *E.coli* (Invitrogen)

cDNA encoding *strep*-tag in-frame with bacteriophage trimerization motif was excised from parental pUC57 plasmid (*strep*-tag and trimerization motif were synthetized by GeneScript) by digestion with NcoI and XhoI and inserted into pBKSII+hTRAIL plasmid, which was digested with the same restriction enzymes. As a result, we obtained bacterial expression vector pBKSII+ with insertion encoding *strep*-tag, T4 bacteriophage trimerization motif and the extracellular portion of human TRAIL (residues 96-281) in one reading frame under control of T7 RNA polymerase promotor sequence (pBKSII+s*trep*-TRItag-hTRAIL).

For the preparation of TRAIL-DR4/DR5 mutants, cDNA encoding DR4 selective TRAIL (variant 4C7 (Reis et al., 2010)) or DR5 selective TRAIL (variant D269H/E195R (Van der Sloot et al., 2006)) was excised by digestion with XhoI and BamHI from parental pUC57 plasmid ordered from Genescript. Insert was then ligated into pBKSII+s*trep*-TRItag-hTRAIL, which was digested by the same enzymes.

Expression vectors for twin-*strep*-TRI-tagged TRAIL variants were prepared as follows. cDNA encoding twin-*strep* tag (two tandem *strep*-tags) was excised by digestion with NcoI and XhoI from parental pUC57 plasmid ordered from Genescript. Insert was then ligated into pBKSII+*strep*-TRItag-hTRAIL, pBKSII+s*trep*-TRItag-DR4-TRAIL or pBKSII+s*trep*-TRItag-DR5-TRAIL, which were digested by the same enzymes. As a

result, we obtained constructs for bacterial expression of following TRAIL variants (Figure 6):

strep-TRI-tagged wild type TRAIL (ST-TRAIL-WT)
twin-*strep*-TRI-tagged wild type TRAIL (TST-TRAIL-WT)
strep-TRI-tagged DR4 selective TRAIL (ST-TRAIL-DR4) (G131R/R149I/S159R/N199R/K201H/S215D)
twin-*strep*-TRI-tagged DR4 selective TRAIL (TST-TRAIL-DR4)
strep-TRI-tagged DR5 selective TRAIL (ST-TRAIL-DR5) (D269H/E195R)
twin-*strep*-TRI-tagged DR4 selective TRAIL (TST-TRAIL-DR5)

All six expression vectors were verified by sequencing at the Centre of DNA sequencing at the Institute of Microbiology of the Czech Academy of Sciences.

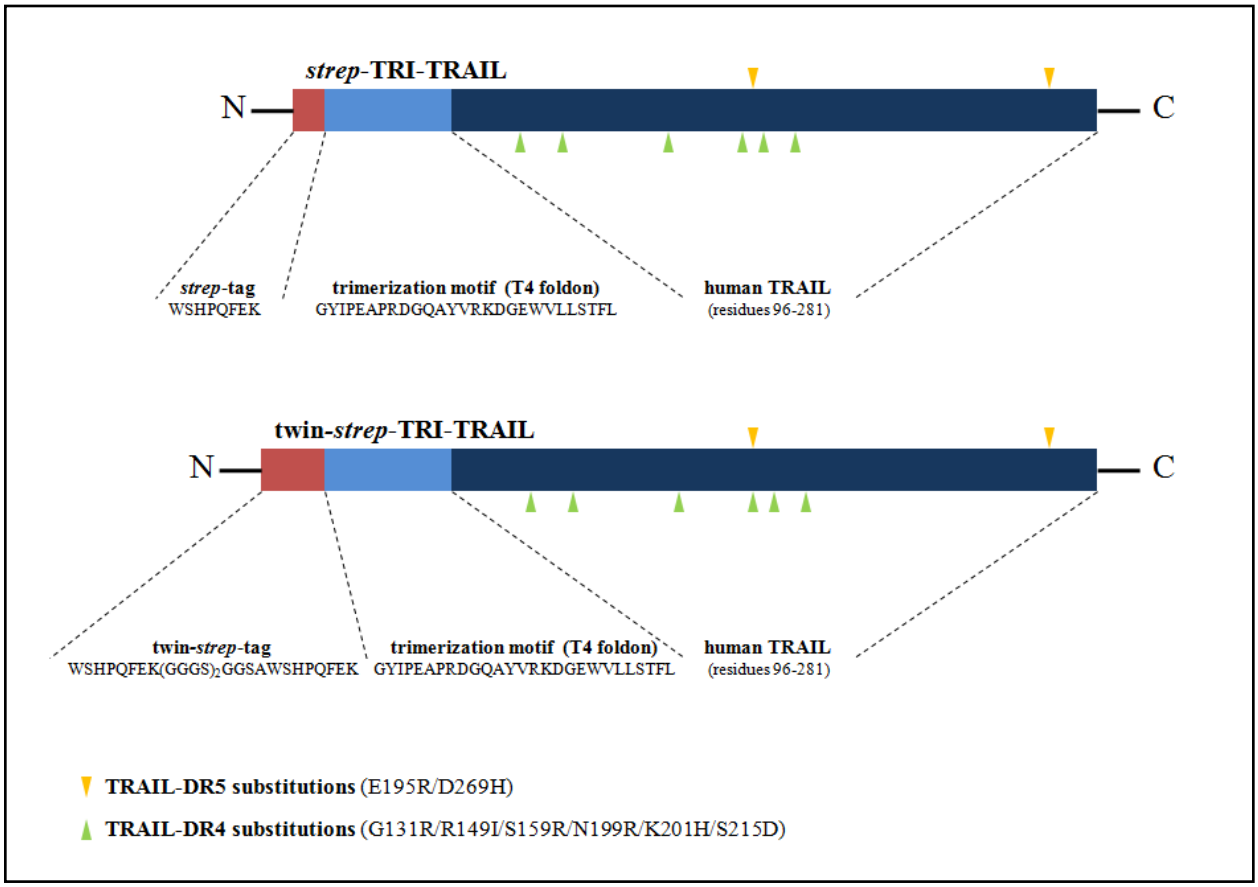

Figure 6. Schematic depiction of (twin)-*strep*-TRI-TRAIL. Amino acid substitutions resulting in DR4 or DR5 selectivity are indicated in green and yellow respectively.

3.2 TRAIL expression and purification

Expression vectors for the respective recombinant TRAIL ligands were transformed into chemically competent *E.coli* cells (BL21-AI™ One Shot® Chemically Competent *E. coli*, Invitrogen). This strain contains chromosomal insertion of T7 RNA polymerase placed under control of araBAD promoter. Therefore, expression of a desired protein from the T7-based expression vector can be induced by adding L-arabinose to the culture in mid-log phase of growth.

The transformation of bacteria with expression vectors for TRAIL and its receptor specific-variants was performed according to the manufacturer's instructions. Briefly, 10ng of plasmid was incubated on ice with 50µl of competent BL21-AI cells for 30 minutes. Cells were then incubated at 42°C for 30 seconds and cooled on ice. Cells were then resuspended in 500 µl of fresh Luria Bertani (LB) medium (Sigma Aldrich), incubated in bacterial shaker at 37°C for 30 minutes, pelleted by centrifugation (16,000x *g*, 30s), resuspended in small volume of LB medium and plated onto LB plates containing ampicilin. The plates were inverted and incubated overnight at 37°C.

To prepare the starting culture for TRAIL expression, a single transformant from previous step was inoculated into 50ml LB media with ampicilin (100ng/ml) and grown overnight in bacterial shaker at 37°C/200rpm. The starting culture was used for inoculation of 0.5l fresh LB medium (1:20 dilution) containing ampicilin (100ng/ml) and the culture was grown with shaking at 37°C/200rpm to reach mid-log phase (OD_{600} = 0.4). TRAIL expression was then induced by adding L-arabinose (Sigma Aldrich) to a final concentration of 0.2% and cells were cultured for additional 5 hours at 25°C/200rpm. The culture was harvested by centrifugation, medium discarded and pellets frozen in -80C.

Purification of s*trep*-tagged TRAIL variants was performed essentially as described in the standard *strep*-tag purification protocol (Schmidt and Skerra, 2007). In brief, TRAIL-containing bacterial pellet from previous step was resuspended in 15ml of TRAIL lysis buffer (20mM HEPES.Na pH 8, 300mM NaCl, 0.1% Tween 20, 5% glycerol, 1mM 2-mercaptoethanol, 1x Roche Complete protease inhibitor), sonicated (5 pulses, 10 seconds each, lysate was cooled on ice between pulses) (Ultrasonic homogenizer, Cole-Parmer). Sonicated lysate was cleared by ultracentrifugation (80,000x *g*, 4°C, 30 min). Affinity purification was performed on a 5 ml Strep-tactin

column (IBA) according to the manufacturer's instructions. Briefly, cleared supernatant was loaded on the column previously equilibrated by washing with 3x5 ml of TRAIL lysis buffer. The column was then washed five times with 0.5 ml Strep-tactin washing buffer (100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA) and *strep*-TRI-/twin-*strep*-TRI-TRAIL was eluted from the column with six times 0.3 ml Strep-tactin elution buffer (Strep-tactin washing buffer supplemented with 2.5mM desthiobiotin) and eluted fractions were collected. Strep-tactin columns eluted with desthiobiotin were regenerated for the next use with five times 5ml of Strep-tactin regeneration buffer (Strep-tactin washing buffer supplemented with 1mM hydroxy-azophenyl-benzoic acid).

5µl of samples from each purification step were taken aside for SDS-PAGE analysis (Figure 7). A nonspecific contaminant co-eluted with all preparations except STS-TRAIL-DR4. In case of TRAIL-DR5 preparations, this contaminant represented less than 50% of total protein content as judged from SDS-PAGE gel (Figure 8). Fractions containing purified TRAIL (typically fractions 3 and 4) were pooled and transferred into TRAIL storage buffer (20mM HEPES.Na, 50mM NaCl, 0.01% Tween 20, 30mM sucrose, 250µM dithiothreitol) using 5ml HiTRAP desalting column (GE Healthcare) connected to ÄKTA FPLC system (GE Healthcare) according to the manufacturer's instructions. Protein concentrations in eluted fractions were measured with Nanodrop at 280nm. Extinction coefficients and molecular weights used for calculations are listed in Table 1. The proteins were stored in aliquots at -80C.

TRAIL variant	Extinction coefficient ($cm^{-1} M^{-1}$)	Molecular weight (g/mol)
strep-tagged wild type (**ST-TRAIL-WT**)	38240	27770
twin-*strep*-tagged wild type (**TST-TRAIL-WT**)	45210	28846
strep-tagged DR4 selective (**ST-TRAIL-DR4**)	38240	27974
twin-*strep*-tagged DR4 selective (**TST-TRAIL-DR4**)	45210	28816
strep-tagged DR5 selective (**ST-TRAIL-DR5**)	38240	27819
twin-*strep*-tagged DR4 selective (**TST-TRAIL-DR5**)	45210	28895

Table 1. Extinction coefficients and molecular weights used for calculations of TRAIL variants concentrations. Values were determined using Peptide property calculator (http://www.basic.northwestern.edu/biotools/proteincalc.html)

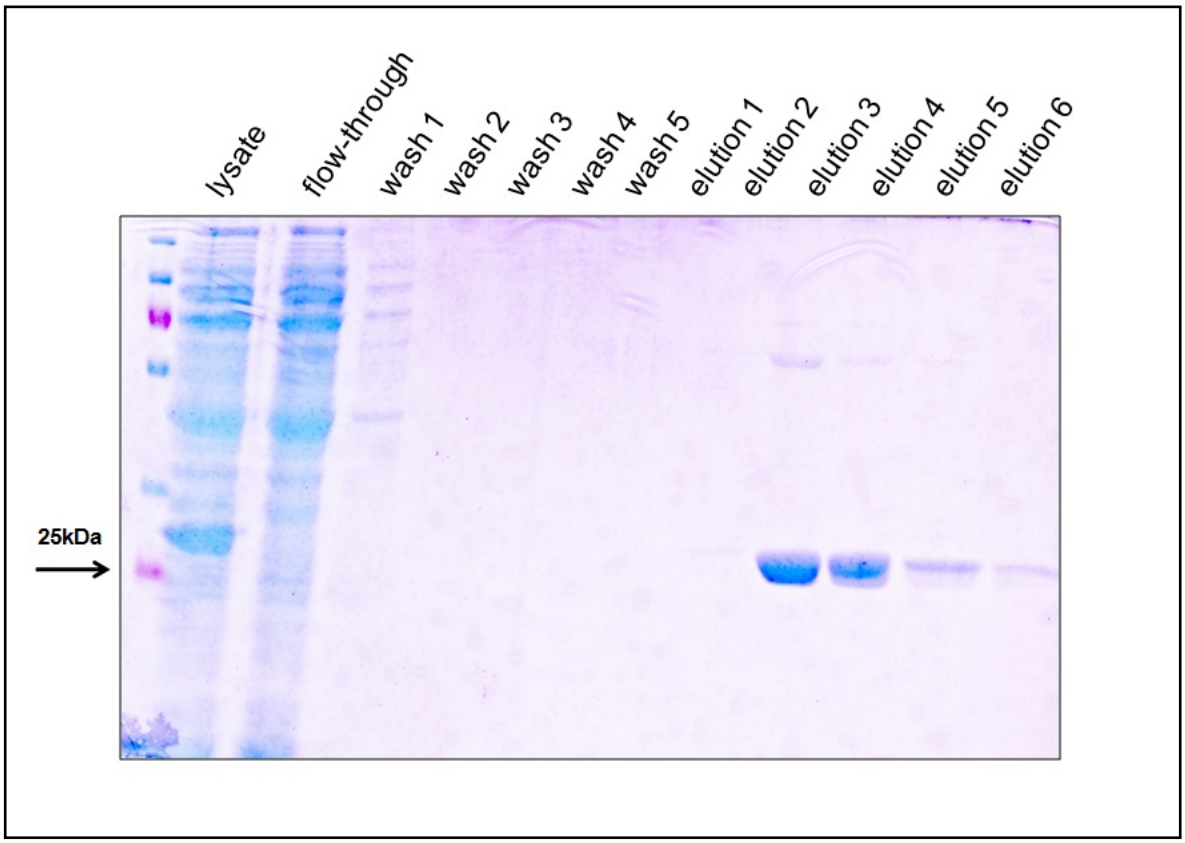

Figure 7. Purification of ST-TRAIL-WT on Strep-tactin sepharose column. 5 µl of fraction from each step was resolved on a 12% polyacrylamide gel. The gel was stained by coomassie brilliant blue.

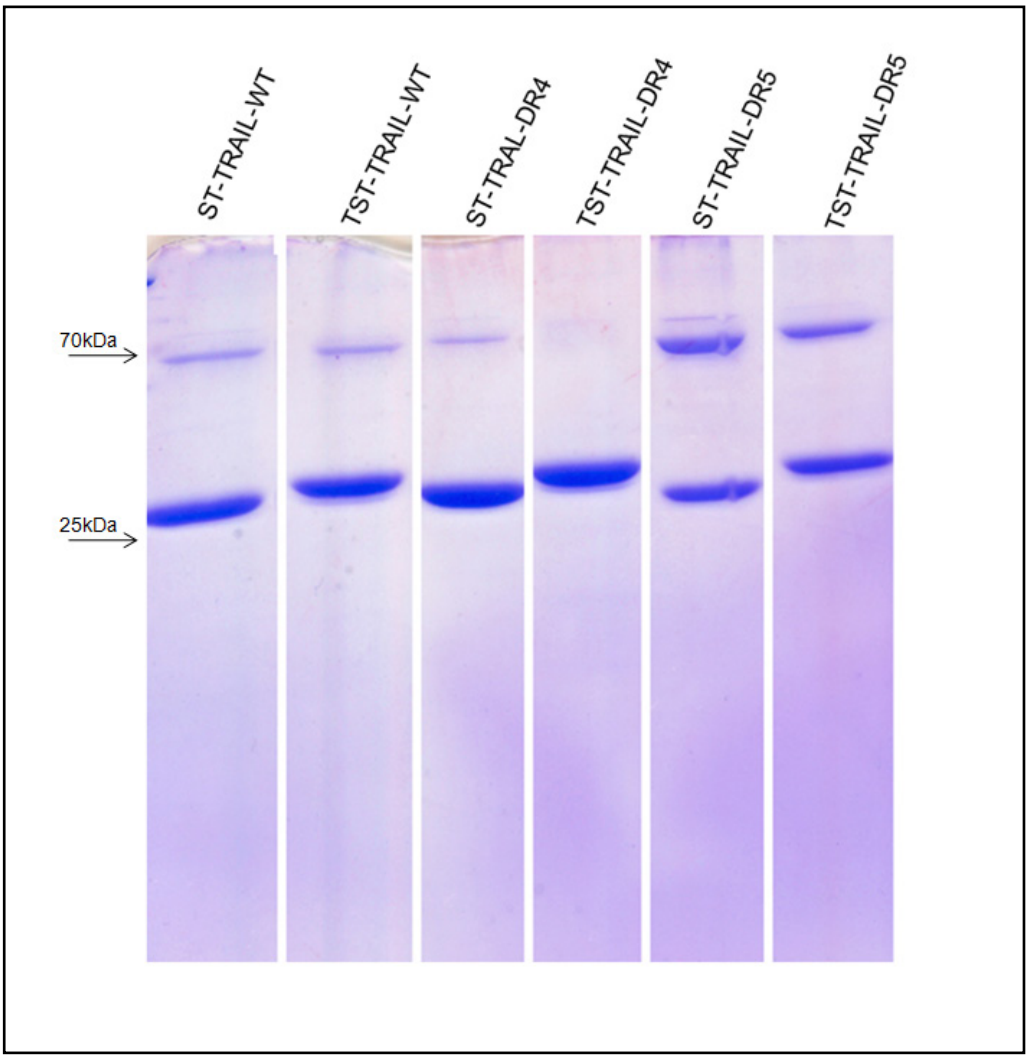

Figure 8. Purity of all TRAIL variants used in this work. 1µg of each TRAIL preparation was resolved on a 12% polyacrylamide gel. The gel was stained by coomassie brilliant blue. All preparations contained approximately 70kDa non-specific contaminant.

3.3 Coomassie briliant blue staining

Samples were resolved by SDS-PAGE and the gels were incubated for 1 hour in Commassie brilliant blue staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid). The gels were then transferred into Coomassie destaining solution (40% methanol, 10% acetic acid) and incubated until background of the gel was destained.

3.4 Size exclusion chromatography

100µg of ST-TRAIL-WT was loaded on Superdex 200 HR 10/30 column (GE healthcare) connected to ÄKTA FPLC system (GE Healthcare) equilibrated with PBS. The molecular weight of ST-TRAIL was calculated comparing ST-TRAIL elution profile to the elution profile of Gel filtration standard (BioRad). Data were analyzed in Unicorn software (GE healthcare) and plot was generated in Excel (Microsoft).

3.5 Mammalian cell culture

Cell lines were maintained at low confluency in RPMI (DLD-1, Jurkat, Ramos) or DMEM (HT-29, SW620) supplemented with 10% FBS and antibiotics. Cells were grown in 5% CO_2 at 37°C.

DLD-1, HT-29 and SW620 are colorectal adenocarcinoma cells, Jurkat are acute T-cell leukemia cells and Ramos are Burkitt's lymphoma cells. All cell lines were obtained from American Type Culture Collection (ATCC).

3.6 Detection and quantification of apoptosis

Suspension cell lines (Ramos, Jurkat) were cultivated in 96-well plates and adherent cell lines (DLD-1, HT-29) were seeded into 24-well plates 24 hours prior treatment. Distinct concentrations of TRAIL or its mutants were added to these cell cultures and cells were treated for indicated times in 5% CO_2 at 37°C. After the treatment, adherent cells were trypsinized, harvested by centrifugation at (200x *g,* 4°C, 3 min), and washed twice with ice cold PBS. The percentage of apoptotic cells was analyzed by Annexin V assay, in which fluorophore-conjugated Annexin V protein added to samples specifically binds to phosphatidylserine externalized by apoptotic cells. The Annexin V assay was performed as following: each sample was once washed with 50µl of Annexin V-binding buffer (Apronex), pelleted by centrifugation and again resuspended in 100µl Annexin V-binding buffer containing 1ul of Annexin V-FITC (Apronex). Cells were incubated for 20 minutes in dark on ice. To stain dead/necrotic cells, Hoechst 33258, a fluorescent dye, which is excluded by healthy cells but enters dead cells, was added into samples to concentration 4 µg/ml directly before measurement. The samples were analyzed by flow-cytometry (LSR-II, BD Biosciences).

Data were analyzed and histograms were generated using FlowJo software (Tree Star). Graphs including statistical analysis were generated in Excel 2007 (Microsoft).

3.7 Cell surface staining of TRAIL receptors

Cells were grown in 24-well plate to reach ~80% confluency, trypsinized and harvested by centrifugation (5810R centrifuge, 200 x g, 4°C, 3 min). 1-2 x 10^5 of cells were used per sample. Cells were resuspended in 20µl of ice cold PBS with 0.2% gelatine and 0.1% $NaNO_3$ (PBS-GA) with respective primary antibodies (5µg/ml) and incubated on ice for 30 minutes. After incubation, cells were washed twice with ice cold PBS-GA and pelleted by centrifugation. Pellets were resuspended in 20µl of secondary antibodies (goat anti-mouse conjugated with phycoerythrin, Jackson) diluted in PBS-GA (1:100) and incubated on ice for 30 minutes. After incubation, cells were washed twice with 200µl of ice cold PBS-GA. Cells were pelleted by centrifugation and resuspended in 90µl of PBS-GA. Finally, 10µl of Hoechst 33258 was added to the final concentration of 4µg/ml to stain dead cells, which were later excluded from analysis. The entire procedure was carried out on ice. Samples were analyzed by flow cytometry (LSRII, BD Biosciences). Data were analyzed and histograms were generated in FlowJo software (Tree Star). Samples stained only with the secondary antibody were used as negative controls.

primary antibodies used :

DR4 DR-4-02 (Exbio)
DR5 DR-5-01-1 (Exbio)
DcR1 HS301 (Enzo Life Sciences)
DcR2 HS402 - (Enzo Life Sciences)

3.8 TRAIL treatment of cancer cells and western blotting

Cells were seeded on 6cm plates and grown to approximately 80% confluency. TRAIL (wild type or its receptors-specific variants) was added to cells to the specified final concentration and cells were incubated in 5% CO_2 at 37°C for indicated time periods. After the treatment, plates were transferred onto ice, washed twice with ice cold

PBS to remove medium, scraped in 100µl of triton-x lysis buffer (1% Triton X-100, 25mM Tris-HCl pH 7.4, 150 mM NaCl, 10mM NaF, 1mM EDTA, 1mM Na_3VO_4) and transferred into eppendorf tubes. Lysates were vortexed, incubated on ice for 30 minutes and cell lysates were cleared by centrifugation at 16,000 x g, 4°C, 30 min. Protein concentration in cleared lysates was determined by BCA method (Pierce) according to the manufacturer's instructions. Lysates were mixed 1:1 with 2x SDS sample buffer (100mM Tris-HCl pH 6.8, 4% SDS, 12% glycerol, 0.2M dithiothreitol, 0.01% bromophenol blue) and denatured for 5 minutes at 95°C in a heating block. Equal amounts of proteins (40µg/well) were then resolved by SDS-PAGE on 12% acrylamide gels. Proteins were transferred from the gels to PVDF membrane (BioTrace PVDF, Pall Life Sciences) using BioRadTrans-Blot SD semi-dry (20V/20min). The membrane was then blocked in PBS/Tween containing 5% non-fat milk for 1 hour at room temperature. After blocking, the membrane was incubated overnight at 4°C with appropriate primary antibody (usually diluted to 1:1000 in PBS/Tween containing 1% non-fat milk, 0.1% sodium azide to the final concentration 1-2 µg/ml), washed 3x10 minutes with PBS/TWEEN and incubated with HRP-conjugated secondary antibody diluted to 1:1000 in PBS/Tween containing 1% non-fat milk at room temperature for 1 hour. Stained proteins were detected using SuperSignal West Femto Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions.

List of antibodies used for western blotting:

NF-κB p65 (H-286) (Santa Cruz, K1609)
phospho-NF-κB p65 (Cell Signaling, 3033)
p38 (Cell Signaling, 9212)
Phospho-p38 (Thr180/Tyr182) (D3F9) (Cell Signaling, 4511S)
Akt (Cell Signaling, 9272)
phospho-Akt (Cell Signaling, 9271)
p44/42 MAPK (Erk1/2) (137F5) (Cell Signaling, 4695)
phospho- p44/42 MAPK (Erk1/2) (Cell Signaling, 4370)
IκBα (Cell Signaling, 2682)
phospho-IκBα (14D4) (Cell Signaling, 2859)
SAPK/JNK (Cell Signaling, 9252)
phospho-SAPK/JNK(T183/Y185) (Cell Signaling, 9255S)
TAK1 (Cell Signaling, 4505)
phospho-TAK1 (Thr184/187) 90C7 (Cell Signaling, 4508)
DR4 (ABCAM, ab8414)
DR5 (Sigma, D3938)

FADD (BD Biosciences, 610400)
FLIP (NF-6) (Enzo, ALX-804-428)
caspase-8 (BD Pharmingen, 551243)
α-tubulin (TU-01) (Exbio, 11-250-C025)

3.9 Affinity purification of the DISC

For each time point, cells were grown in one 150mm petri dish to reach 80% confluency. Cells were cooled on ice, washed once with ice cold PBS and incubated on ice in the medium containing 1µg/ml of twin-*strep*-TRAIL-WT, twin-*strep*-TRAIL-DR4 or twin-*strep*-TRAIL-DR5 for 20 minutes. This step allows TRAIL to saturate receptors, but DISC formation is attenuated due to the low temperature. After 20 minutes, medium containing TRAIL was changed for the fresh warm medium and cells were transferred to 37°C to allow DISC formation and processing. Cells were harvested at indicated time points as follows. Cells were washed twice with ice cold PBS, scraped in approximately 10ml of ice cold PBS, transferred to 15ml tubes and pelleted by centrifugation. Pellets were lysed in appropriate volume (depending on pellet size, approximately 500 µl) of DISC-lysis buffer (20mM Tris-HCl pH7,5; 150mM NaCl; 1% Tritone-X; 10% glycerol; 1mM EDTA; 1mM Sodium orthovanadate; 10mM Sodium fluoride; 1x Roche Complete protease inhibitor), incubated on ice for 30 minutes and cleared by centrifugation (16,000 x *g*, 4°C, 30 min). Total protein concentrations in cleared lysates were determined using BCA method (Pierce) and equal volumes of equally concentrated lysates were rotated with 20µl of Strep-tactin Sepharose beads (IBA) over night at 4°C. Prior incubation with lysates, beads were equilibrated in DISC lysis buffer. As a loading control, 50µl of total lysates were taken aside for further SDS analysis („load“). After the incubation beads were washed five times as follows: beads were pelleted by centrifugation (speed time), lysates were discarded, beads were resuspended in 1ml DISC-lysis buffer, incubated at 4°C on a rotator for 5 minutes and pelleted again. After the last washing step, beads were resuspended in 80µl 2xSDS sample buffer per 2mg of total protein in the starting lysate and protein complexes were eluted by incubating at 95°C for 5 minutes. Boiled samples were filtered through 200µl filter tips to remove beads. Samples (20µl) were resolved by SDS-PAGE on 12% gels and western blotting was performed as described in previous chapter.

4 Results

4.1 ST-TRAIL purification and characterization

Several variants of the human recombinant TRAIL were or are used in TRAIL-related research such as histidine tagged, leucine zipper tagged or untagged TRAIL (Pennarun et al., 2010). We decided to prepare a novel variant designated *strep*-TRI-TRAIL (ST-TRAIL) with enhanced stability and functionality. ST-TRAIL is composed of human recombinant TRAIL (residues 96-281), fused with trimerization motif from T4 bacteriphage (T4 „foldon“) and s*trep*-tag epitope (Figure 6).

Strep-tag is a short peptide tag suitable for one-step purification of proteins on Strep-tactin matrices. The s*trep*-tag:Strep-tactin interaction is highly specific and proteins can be isolated in a single step to high purities. The proteins are eluted from Strep-tactin under physiological conditions using desthiobiotin, which competitively binds to Strep-tactin. This system is therefore suitable for purifications of functional proteins (Schmidt and Skerra, 2007). *Strep*-tag could be also with an advantage used for isolation and characterization of protein complexes. However, for this purposes, it is recommended to use double *strep*-tag (twin-*strep*-tag) for its stronger interaction with Strep-tactin Sepharose. Twin-s*trep*-tag allows isolation of highly pure complexes, which were shown to be suitable even for mass spectrometry analysis (Junttila et al., 2005). Therefore, mainly for the isolation of TRAIL DISC complexes we prepared also double *strep*-tagged TRAIL variants (twin-*strep*-TRI-TRAIL). To enhance trimer formation of our ligand, we used T4 foldon, which has been successfully employed to stabilize FasL trimers (see https://www.iba-lifesciences.com/details/product/226.html). T4 foldon is a short peptide sequence derived from fibritin of T4 bacteriophage (Figure 6).

ST-TRAIL was produced in *E.coli* and purified by affinity chromatography on Strep-tactin column (IBA) followed by FPLC desalting (ÄKTA FPLC, GE healthcare) as described in Methods section. Purity of ST-TRAIL was more than 90% judging from coomassie-stained SDS PAGE (Figure 7). Yields were approximately 3mg per 1 liter of bacterial culture. The analysis of ST-TRAIL by gel filtration on Superdex 200 HR showed that it elutes at apparent molecular weight 68 kDa, confirming its preferential formation of stable trimers (Figure 9).

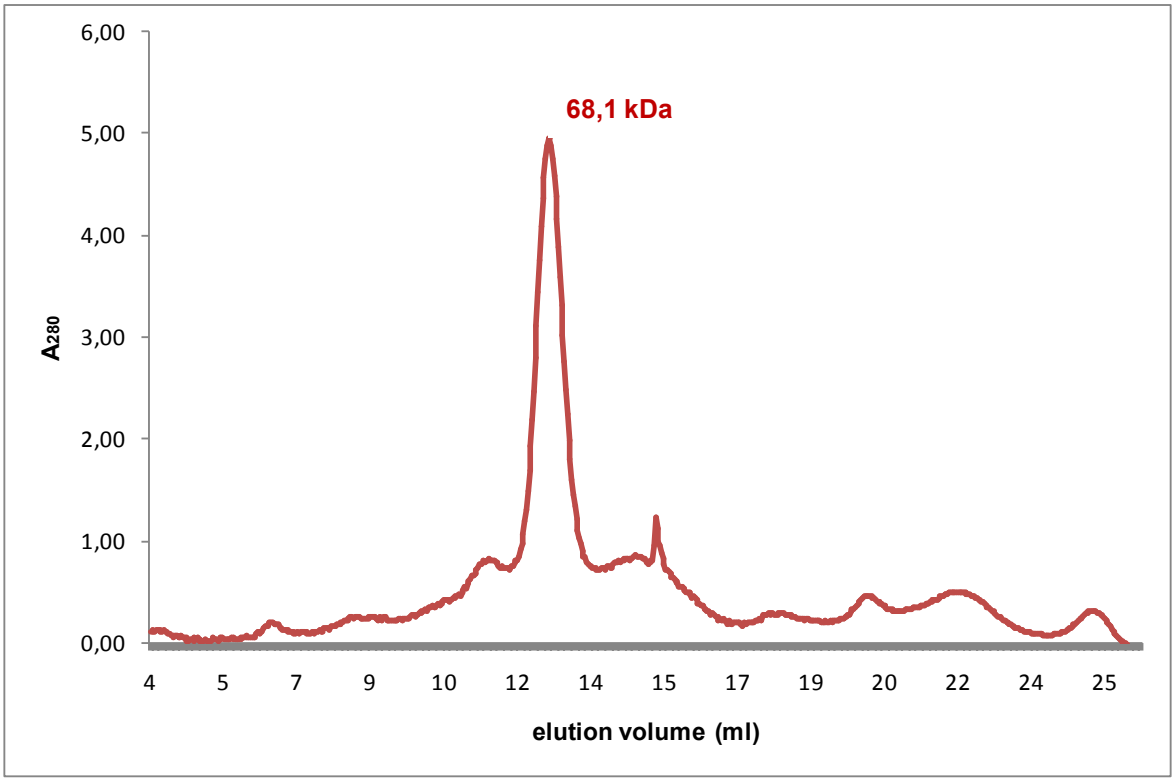

Figure 9. ST-TRAIL forms trimers under physiological conditions. 100µg of ST-TRAIL preparation was separated by size-exclusion chromatography on Superdex 200 column previously equilibrated with BioRad gel filtration standards. The major peak corresponds to ST-TRAIL trimers.

To test the biological activity of ST-TRAIL, we treated TRAIL-sensitive Jurkat cells with increasing concentrations of ST-TRAIL in parallel with traditionally used His-TRAIL. The treated cells were stained by Annexin V-FITC and analyzed by flow cytometry. We found that ST-TRAIL kills Jurkat cell more efficiently than His-TRAIL (Figure 10), possibly due to formation of more stable trimers.

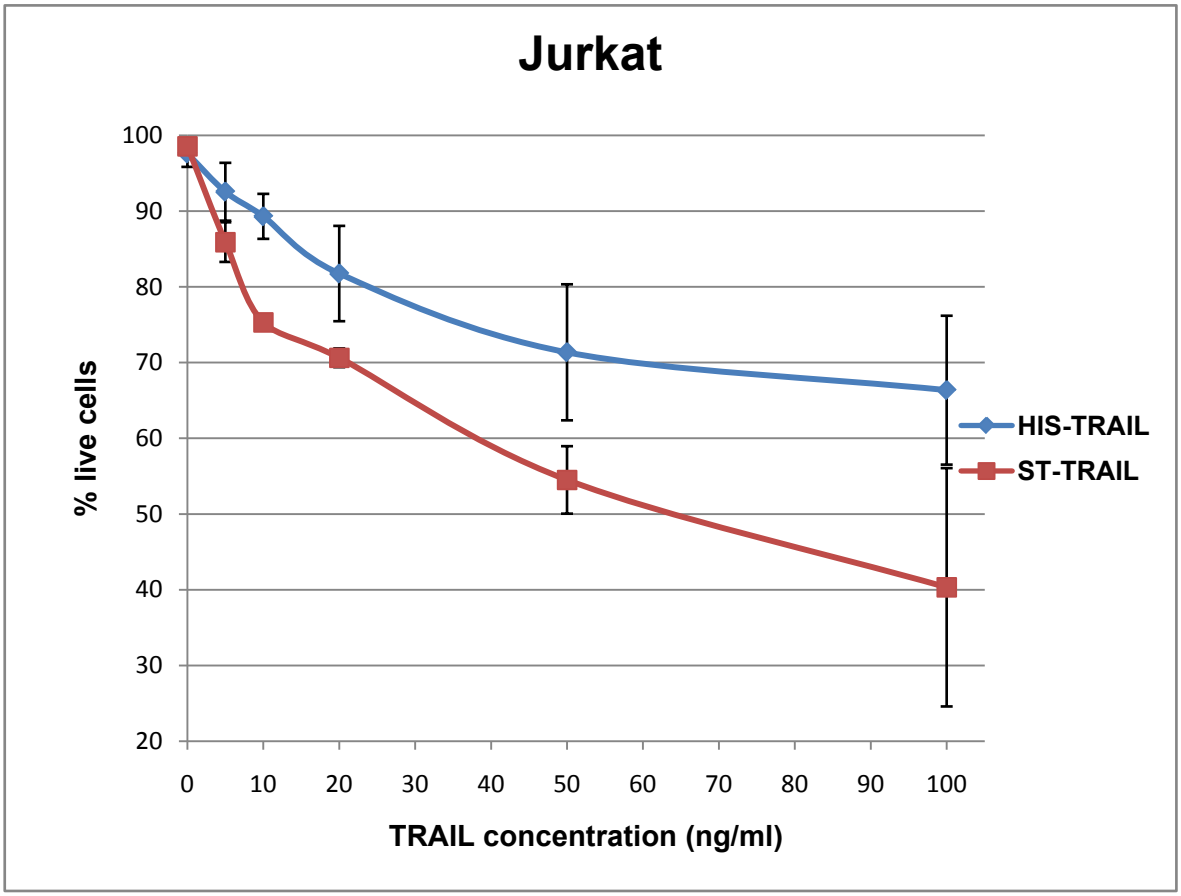

Figure 10. Induction of apoptosis in Jurkat cells by ST-TRAIL and His-TRAIL. The cells were incubated for 3 hours with 5, 10, 20, 50 or 100 ng/ml of ST-TRAIL or His-TRAIL. Cells were then stained by Annexin V-FITC and analyzed by flow cytometry. The results are average of three independent experiments. The error bars indicate averages ± SDs.

4.2 Preparation of DR4- and DR5-specific mutants of TRAIL

To obtain receptor-specific variants of ST-TRAIL, we modified our original ST-TRAIL constructs by introducing published substitutions into receptor binding domain of ST-TRAIL. These modifications are G131R/R149I/S159R/N199R/K201H/S215D for DR4-selective mutant (Reis et al., 2010) and D269H/E195R for DR5-selective mutant (Van der Sloot et al., 2006). Here we refer to these variants as to **TRAIL-DR4** and **TRAIL-DR5 (**Figure 6). Both proteins were expressed and purified by the same procedure as wild type ST-TRAIL. However, the yields were up to 10 times lower and in the case of DR5-selective variant, a non-specific contaminant co-eluted with our protein. We made several attempts to improve its purity such as using different buffers or further purification by gel filtration (Superdex 200 HR, data not shown). Unfortunately, we were not successful and since TRAIL-DR5 was functional, we decided to continue in

experiments with this impure preparation, taking in account this contamination in calculating working concentrations of DR5-specific TRAIL (due to approx. 50% contamination as judged from gel electrophoresis (see Figure 8), we used twice as much DR5-specific ligand than WT or DR4-specific one).

To verify the selectivity of TRAIL-DR4 and TRAIL-DR5 we induced apoptosis with these ligands in Jurkat and Ramos cell lines. Jurkat cells (human T-cell leukemia model) are known to signal apoptosis exclusively through DR5 and no detectable levels of DR4 are present on the surface of these cells. Ramos cells (human Burkitt lymphoma cells) on the other hand, signal through DR4 and no DR5 is present (MacFarlane et al., 2005b). Thus, these two cell lines serve as perfect models for testing the receptor-selectivity of TRAIL-DR4 and TRAIL-DR5. As a result (Figure 11), we show that Jurkat cells are killed by both TRAIL-WT and TRAIL-DR5 but not by DR4 and the situation is opposite in Ramos cells, which are killed by TRAIL-DR4 and TRAIL-WT but not by TRAIL-DR4. Therefore, we concluded that both mutants are functional and selective, tools for the induction of TRAIL-mediated, receptor-specific apoptosis/signaling.

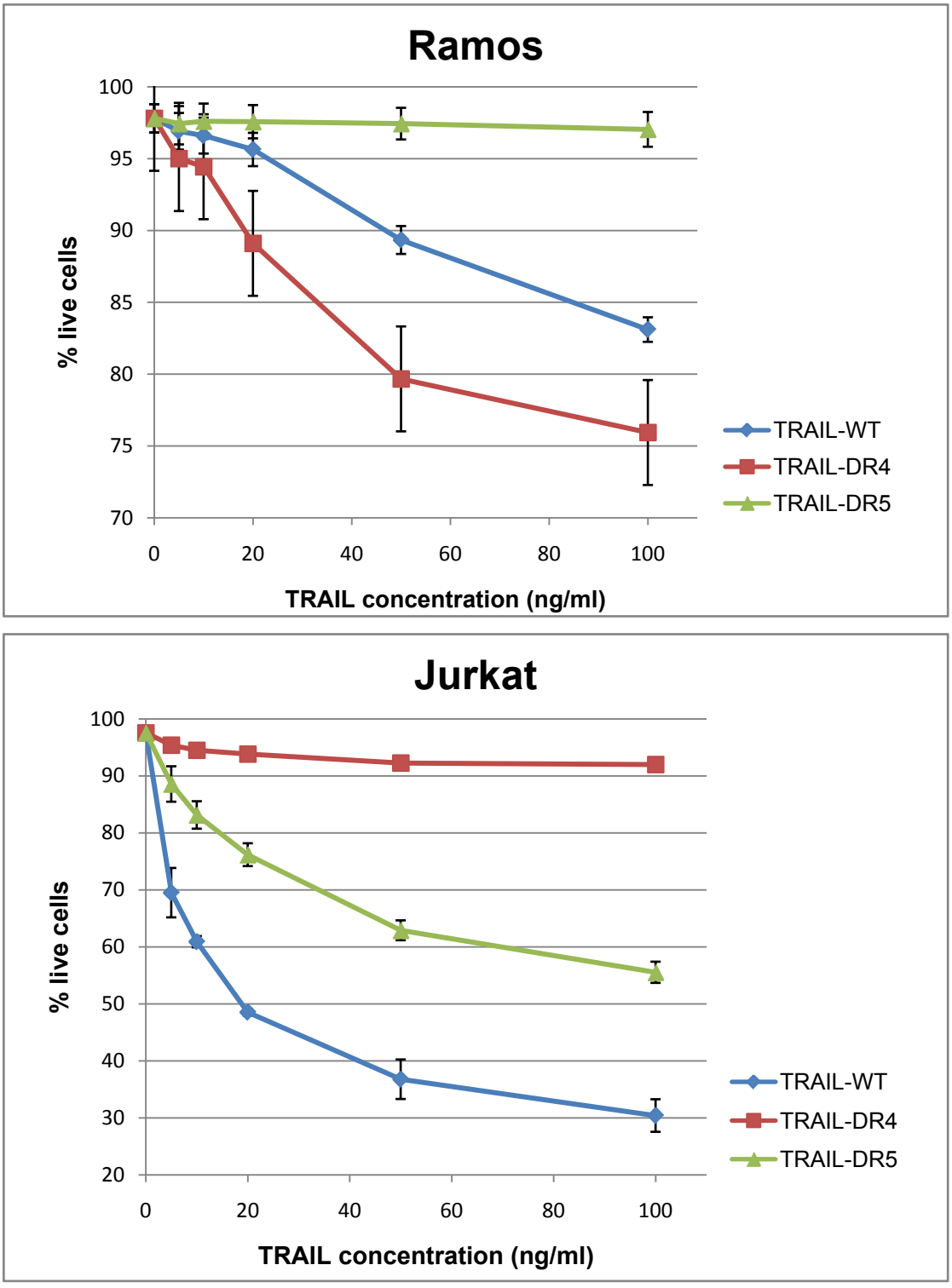

Figure 11. Induction of apoptosis in Jurkat and Ramos cells with wild type TRAIL and DR4/DR5 selective variants. The cells were incubated for 5 hours with 5, 10, 20, 50 or 100 ng/ml of TRAIL variants. Cells were stained by Annexin V-FITC and analyzed by flow cytometry. The results are average of three independent experiments. The error bars indicate ± SD.

4.3 Determination of TRAIL receptors expression and TRAIL-sensitivity in colorectal cell lines DLD-1, HT-29 and SW620

In selected model colorectal cancer cell lines, we first analyzed and quantified expression of TRAIL receptors. Cells were stained with respective antibodies and analyzed by flow cytometry (Figure 12). We show that all cell lines express both DR4 and DR5 at their cell surface, while decoy receptors were limitedly detected only in SW620 cell line.

To evaluate sensitivity of the model colorectal cancer cells to TRAIL-induced apoptosis, we treated tested cell lines with TRAIL-WT (100ng/ml, 5 hours) (Figure 13). In agreement with the published data, HT-29 and SW620 were resistant, while DLD-1 cell line was sensitive to TRAIL-induced apoptosis.

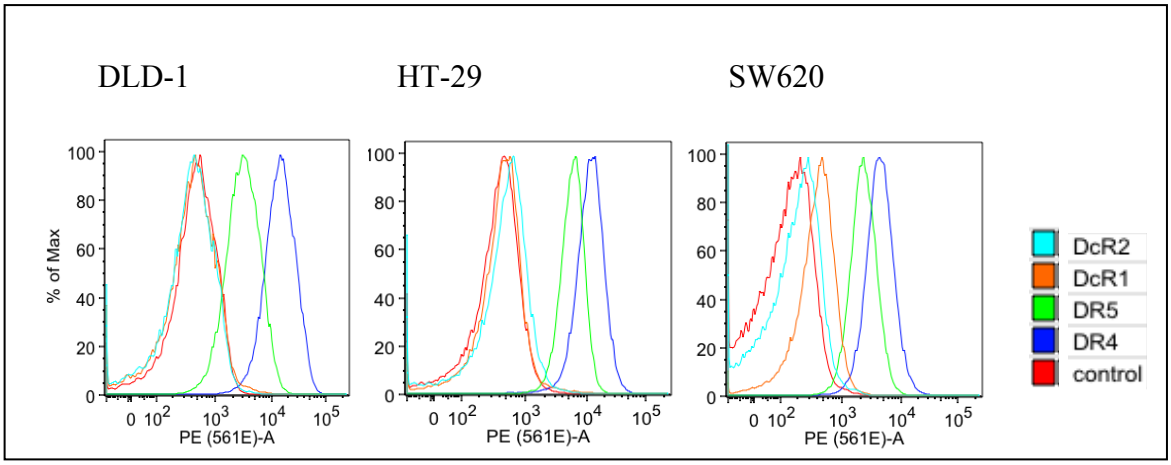

Figure 12. TRAIL receptors profiling of DLD-1, HT-29 and SW620 cells. Cells were stained with primary antibodies against DR4, DR5, DcR1 or DcR2 and subsequently with secondary antibody conjugated with phycoerythrin. Control samples were stained with secondary antibody only. The results are representatives of three independent experiments.

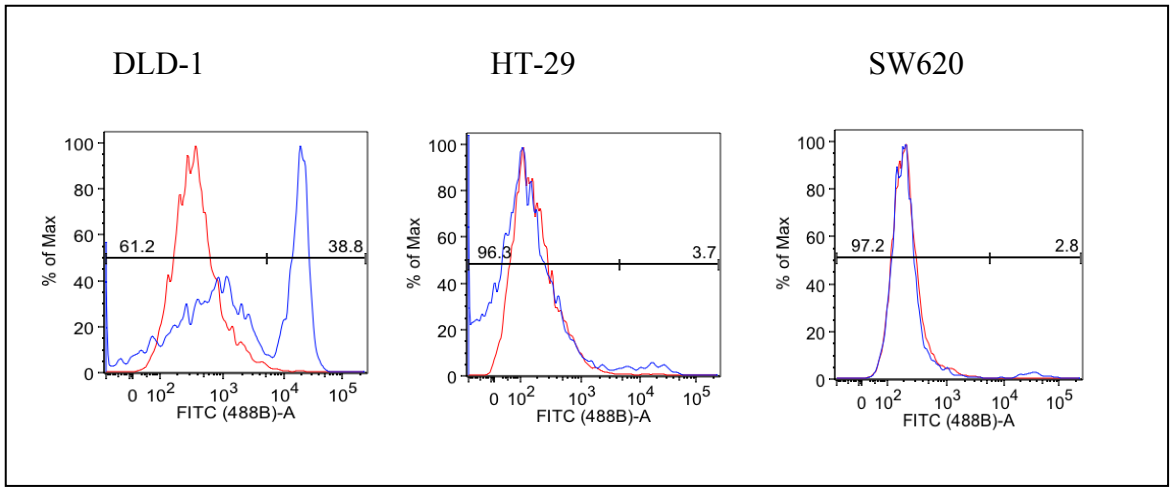

Figure 13. Induction of apoptosis in DLD-1, HT-29 and SW620 colorectal cancer cell with TRAIL-WT. Cell were treated with 100ng/ml ST-TRAIL-WT for 5 hours, stained with Annexin V-FITC and analyzed by flow cytometry. Untreated cells were used as controls (red line), TRAIL-treated cells are represented as a blue line. We found that DLD-1 cells are sensitive, while HT-29 and SW620 are resistant to TRAIL-induced apoptosis. The results are representatives of three independent experiments.

4.4 Receptor-specific induction of apoptosis in DLD-1 cells

To determine relative contributions of DR4 and DR5 to TRAIL-induced apoptosis in TRAIL-sensitive DLD-1 cell line, we treated these cells in parallel with TRAIL-WT, TRAIL-DR4 or TRAIL-DR5 and analyzed them for apoptosis by Annexin- V-FITC staining and flow cytometry. We found that the most potent mediator of TRAIL-induced apoptosis in DLD-1 cells is DR4-specific ligand, which induced apoptosis in these cells three fold more efficiently than its DR5-specific counterpart (Figure 14).

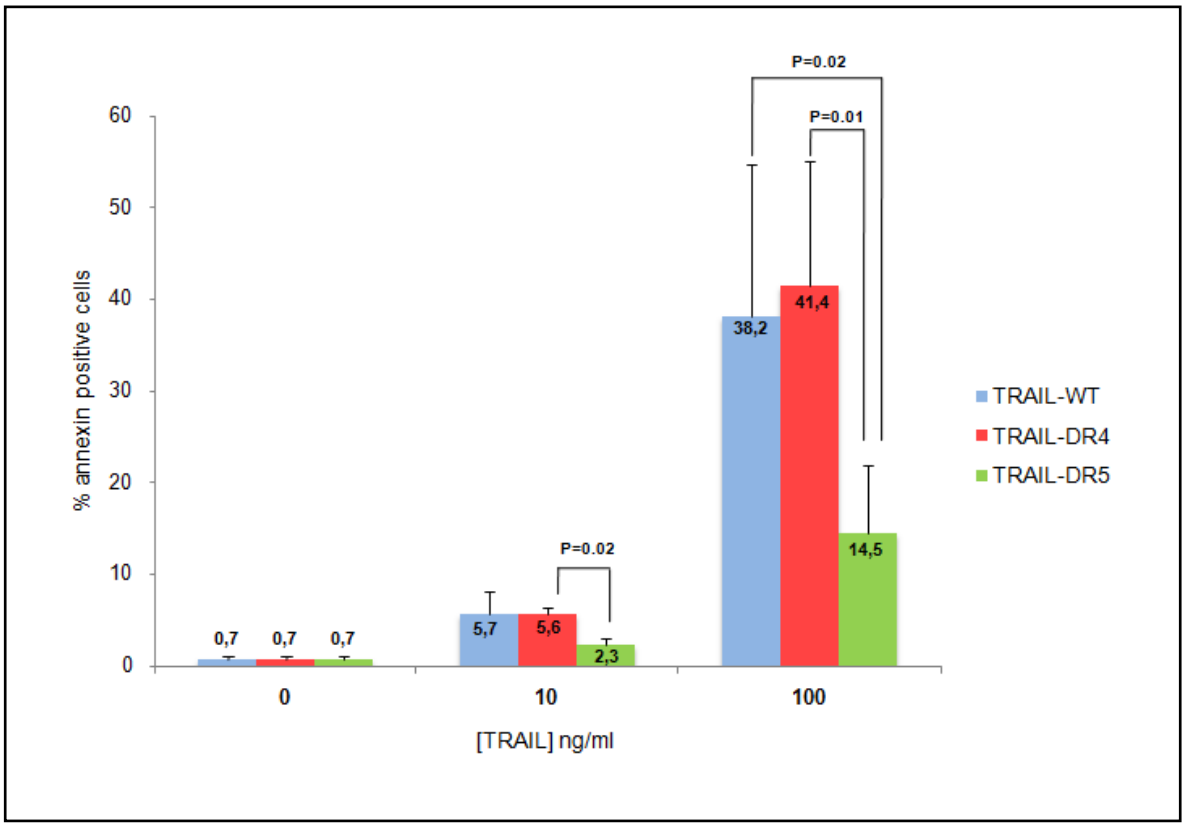

Figure 14. Receptor specific induction of apoptosis in DLD-1 cell line. Cells were treated with 10 or 100 ng/ml of TRAIL-WT, TRAIL-DR4 or TRAIL-DR5 for 5 hours. Untreated cells were used as controls. Phosphatidylserine externalization was analyzed by Annexin-V-FITC staining and cells were quantified by flow cytometry. Results are average of three independent experiments. Error bars indicate ±SD. Statistical significance was calculated using Student's t-test.

4.5 Analysis of the TRAIL DISC formation in DLD-1 and HT-29 cells

To examine the role of receptor-specific TRAIL ligands in the formation and activation of the DISC in the model colorectal cancer cell lines, we performed affinity purification of the DISC complexes via twin-*strep*-TRI-TRAIL variants and analyzed the pulled-down complexes by western blotting (Figure 15).

In DLD-1 cells, we in the agreement with the analyses of receptor-specific induction of apoptosis (Figure 14) observed that DR4-selective ligand induced potent FADD recruitment to the receptor, which was also reflected in higher amounts of the cleaved caspase-8 within the DISC (Figure 15, lane 4.). DR4-specific ligand also apparently induced even more effective DISC formation and attracted more FADD and caspase-8 than the TRAIL-WT (Figure 15, compare lanes 5-8 with lanes 1-4). In

contrast, DR5-specific ligand induced only very poor DISC formation and processing of caspase-8, despite efficient binding to and precipitation of the (Figure 15, lanes 9-12). All three TRAILs (WT, DR- and DR5-specific) also attracted RIP1 kinase into the DISC complex and again TRAIL-WT and DR4-specific mutant did it more efficiently. However, due to very low levels of cFLIP expression in DLD-1 cells we observed only low association of cFLIP with the DISC. Strangely, we also noticed in DR5-specific DISC precipitates stained with anti-cFLIP antibody presence of a approx. 70 kDa band that decreased with the time of treatment (Figure 15, lanes 9-12).

Even though that HT-29 cells are resistant to TRAIL-induced apoptosis, we followed DISC formation and caspase-8 processing also in these cells (Figure 16). To our surprise, we similarly as for DLD-1 cells noticed that only wild type TRAIL and the DR4-specific mutant not only bound to and precipitated appropriate TRAIL receptors, but also triggered efficient DISC formation documented by significantly increased levels of the adapter protein FADD, caspase-8 and by caspase-8 processing (Figure 16, lanes 1-8). Despite HT-29 cells express higher levels of DR5 than DLD-1 cells and DR5-specific ligand efficiently precipitated DR5 receptor, also in these cells DR5-specific ligand was unable to trigger comparable DISC formation as the wild type or DR4-specific ligand (Figure 16, lanes 9-12). In contrast to the DISC precipitates from DLD-1 cells, TRAIL-triggered DISCs in HT-29 cells contained high levels of cFLIP, especially its partly processed p43 fragment. These high levels of cFLIP (also noticed in DR5-specific DISC precipitates) could be behind the resistance of these cells to TRAIL-induced apoptosis.

Similarly as in DLD-1 cells, we also noticed the presence of RIP1 kinase in especially TRAIL-WT and DR4-specific DISC precipitates (Fig. 16, lower panel). This finding was somehow surprising as the RIP1 kinase, which is important for TRAIL-induced non-apoptotic signaling, has not been routinely identified in the membrane DISCs (Cao et al., 2011).

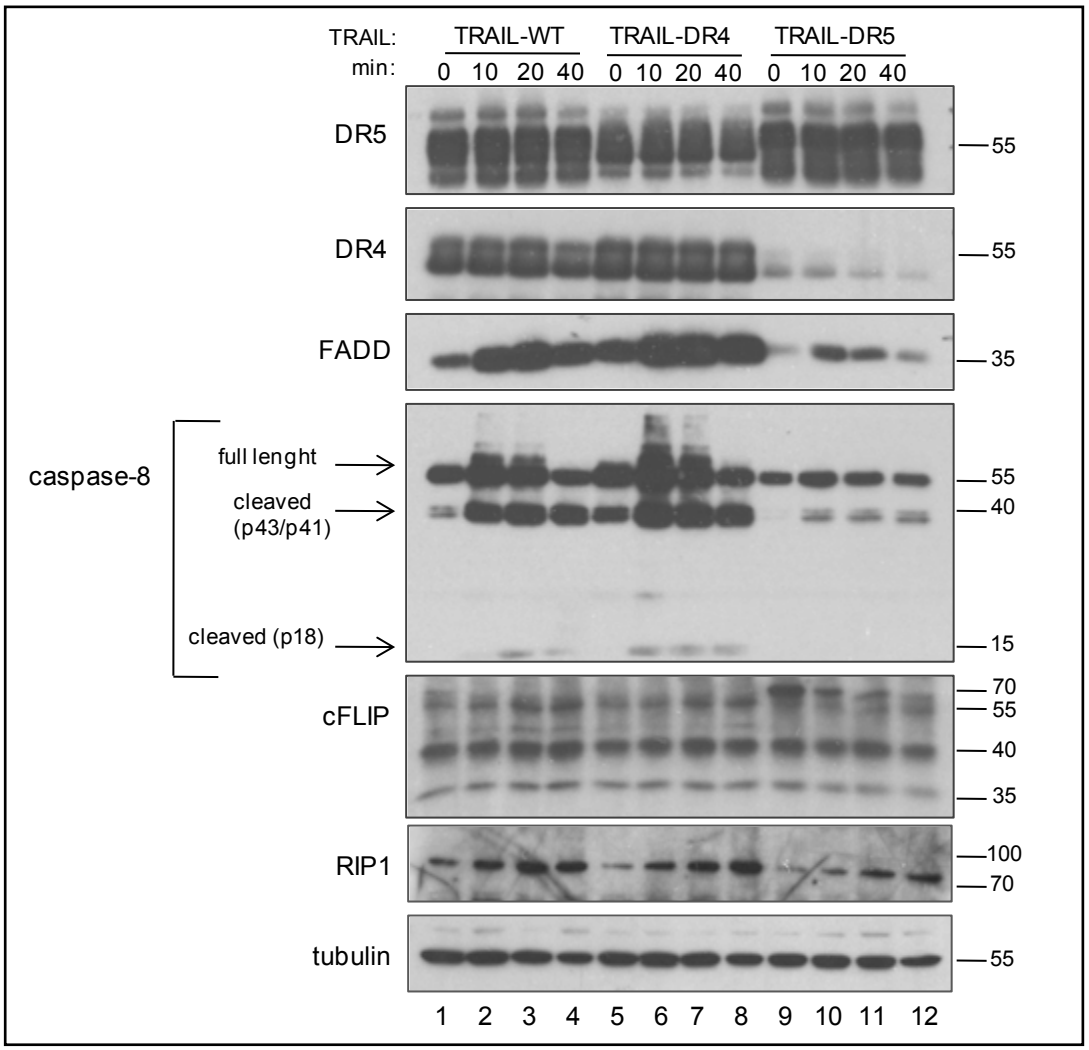

Figure 15. DISC analysis in DLD-1 cells. DLD-1 cells were treated with 1μg/ml of TRAIL-WT, TRAIL-DR4 and TRAIL-DR5 for indicated time points and affinity purification of the DISC complexes was performed as described in Methods section. Samples were analyzed by western blotting with antibodies against core DISC components. Bands stained with DR5-specific antibody in DR4-specific DISC precipitates are likely originating from a cross-reactivity of this anti-DR5 antibody with DR4 (these bands are also distinguishable in TRAIL-WT precipitates stained with anti-DR5 antibody). With anti-tubulin antibody were stained the cell lysates used for DISC precipitations.

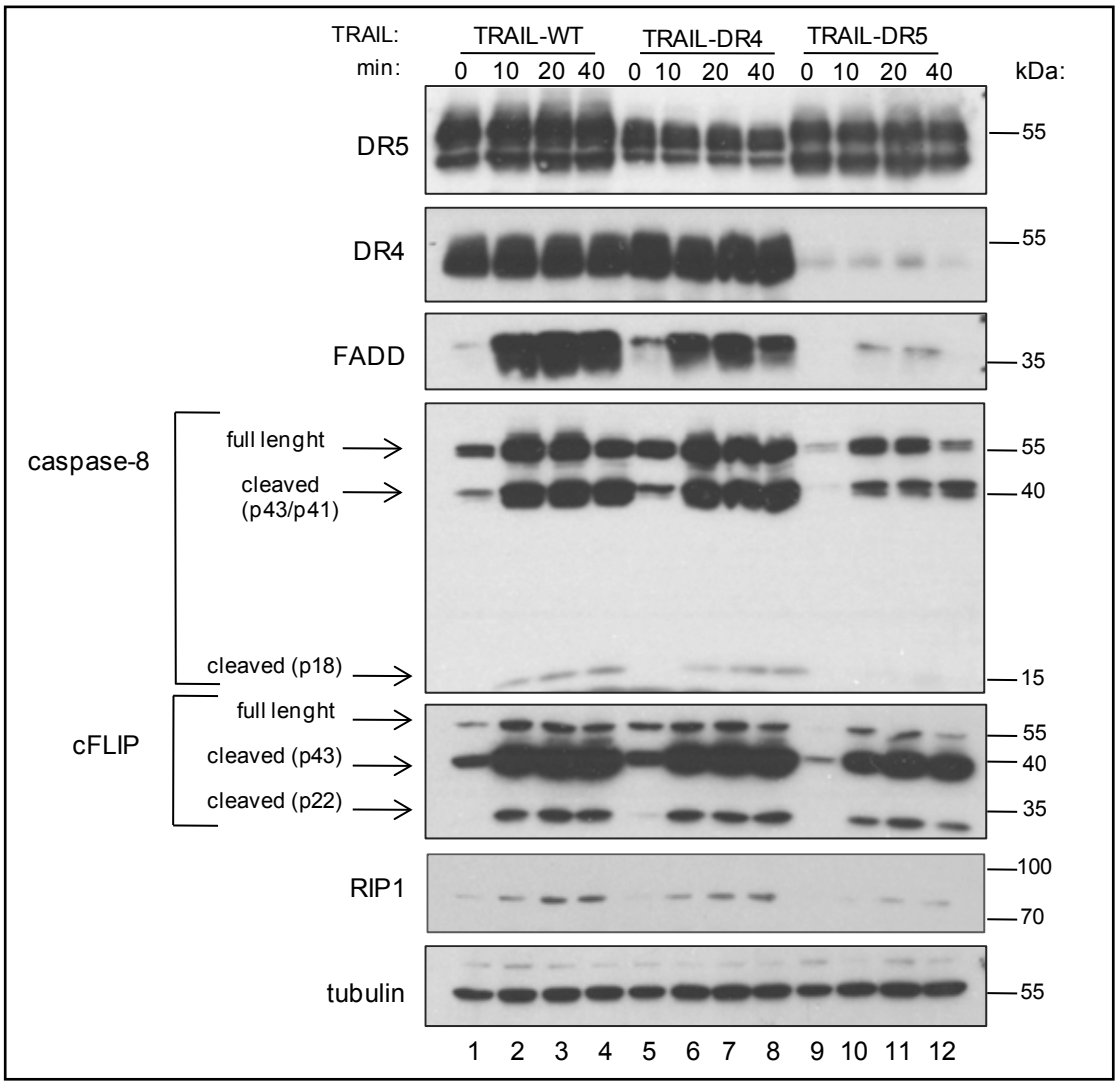

Figure 16. DISC analysis in HT-29 cells. DLD-1 cells were treated with 1µg/ml of twin-*strep*-TRI-tagged TRAIL-WT, TRAIL-DR4 and TRAIL-DR5 for indicated time points and affinity purification of the DISC complexes was performed as described in Methods section. Samples were analyzed by western blotting with antibodies against core DISC components. For anti-DR5-specific antibody-stained band in DR4 precipitates see Fig. 15 legend.

4.6 Mapping the non-apoptotic signaling pathways induced by TRAIL and its receptor-specific variants in colorectal cancer cells

The non-apoptotic or non-apoptotic signaling triggered by TRAIL or its receptor-specific variants was analyzed in addition to DLD-1 and HT-29 also in another TRAIL-resistant colorectal cancer cell line SW620. We used phospho-specific antibodies against proteins in these signaling pathways that are known to be phosphorylated upon the TRAIL treatment and mapped activation of p38, JNK, ERK1/2, TAK1 and NF-κB pathways in these cells. To activate TRAIL-induced signaling, we treated cells in parallel with 100ng/ml of TRAIL-WT, TRAIL-DR4 or TRAIL-DR5 for time periods ranging from 15 to 180 minutes and analyzed the samples by western blotting. Analyzing the data obtained from two independent experiments in all three tested cells lines and presented in Figures 17-19 we can summarize that:

1) Despite the huge differences in the pro-apoptotic signaling between DR4- and DR5-specific ligands in both DLD-1 and HT-29 cells, majority of the non-apoptotic signaling is in these cells activated similarly by all three ligands.

2) In some cells, one of the non-apoptotic signaling pathways is not activated at all (TAK1 kinase in all cell lines, JNK kinases in HT-29 and SW620) or active even without TRAIL treatment (ERK kinases in HT-29 or Akt kinase in SW620 cells).

3) In some cells we noticed differences between DR4- and DR5-activated signaling (see below).

Activation of the individual non-apoptotic signaling pathways in the treated cells:

JNKs: JNK1/2 kinases are expressed in all three cell lines but their TRAIL-mediated phosphorylation was triggered only in DLD-1 at later time points (60 min after the TRAIL treatment and later) and similarly as apoptotic signaling mainly in WT- or DR4-treated cells (Figs. 17-19, 1st panels).

p38 MAPK: p38 kinase was expressed and activated (phosphorylated) by TRAIL-triggered signaling in all three tested cells lines and in contrast to JNK kinases by all three ligands to similar extent (Figsures 17-19, 2nd panels). However, we also observed significant differences in the cell-specific activation of p38 kinase among the tested cell lines. While in DLD-1 and SW620 was phosphorylation of p38 rather late event, its

TRAIL-triggered phosphorylation in HT-29 cells occurred very early (already at the first analyzed 15 min time point – compare Figures 17, 19 with Figure 18).

ERK1/2: ERK1/2 kinases are also expressed in all three tested cell lines and activated by all three ligands (Figures 17-19, 3rd panels). DLD-1 cells contain relatively low levels of these kinases and both ERK1 and ERK2 are activated as early as 15 min after adding TRAIL (Figure 17, 3rd panels). However, TRAIL-resistant SW620 and even more notably HT-29 cells both express high levels of these kinases and especially ERK2 is in these cells constitutively phosphorylated. Nevertheless, all three ligands can indistinguishably enhance their phosphorylation, though with a different kinetics. HT-29 cells in this aspect resemble DLD-1 cells and TRAIL-induced phosphorylation of both kinases is detectable already 15 min after starting their treatment with TRAIL. However, TRAIL-mediated activation of ERK in SW620 cells is delayed and mainly ERK2 kinase becomes detectably phosphorylated between 30-60 min of the treatment (compare 3rd panels in Figures 18 and 19).

Akt: This kinase is significantly activated/phosphorylated even in untreated SW620 cells and TRAIL-treatment of these cells has only very limited effect on its further phosphorylation. Basal phosphorylation of Akt kinase in other two cell lines is significantly lower and their treatment with TRAIL-WT or its receptor-specific variants leads to its enhanced phosphorylation (Figures 17,18, 4th panels). Even though in both cell lines all three tested TRAIL ligands induce phosphorylation of Akt, the phosphorylation signature differs between these two cell lines. While TRAIL treatment of DLD-1 cells leads to time-dependent, gradual increase of Akt phosphorylation, TRAIL-treated HT-29 cells display rapid phosphorylation of Akt as early as at 15 min time point and then over time gradual decrease of phosphorylated Akt (most evident in cells treated with DR4-specific TRAIL, compare 4th panels in Figures 17 and 18).

NF-κB: For the analysis of TRAIL-induced NF-κB signaling in colorectal carcinoma cells, we employed both analysis of IκB phosphorylation and also the later phosphorylation of the p65 subunit. In DLD-1 and SW620 cells TRAIL-WT and its receptor-specific variants induce activation of NF-κB signaling pathway with similar efficacy and kinetic (Figs 17, 19, 5th panels). In contrary, in HT-29 we observed a significant difference between DR4- and DR-specific activation of NF-κB signaling

pathway. DR5-specific ligand triggered in comparison to either DR4-specific variant or TRAIL-WT earlier and more pronounced activation of NF-κB signaling (Figure 18, 5th panel).

TAK1: Though TAK1 kinase was as a protein expressed in all three tested colorectal cancer cell lines, TRAIL treatment of neither of them led to TAK1 phosphorylation (Figures 17-19, 6th panels).

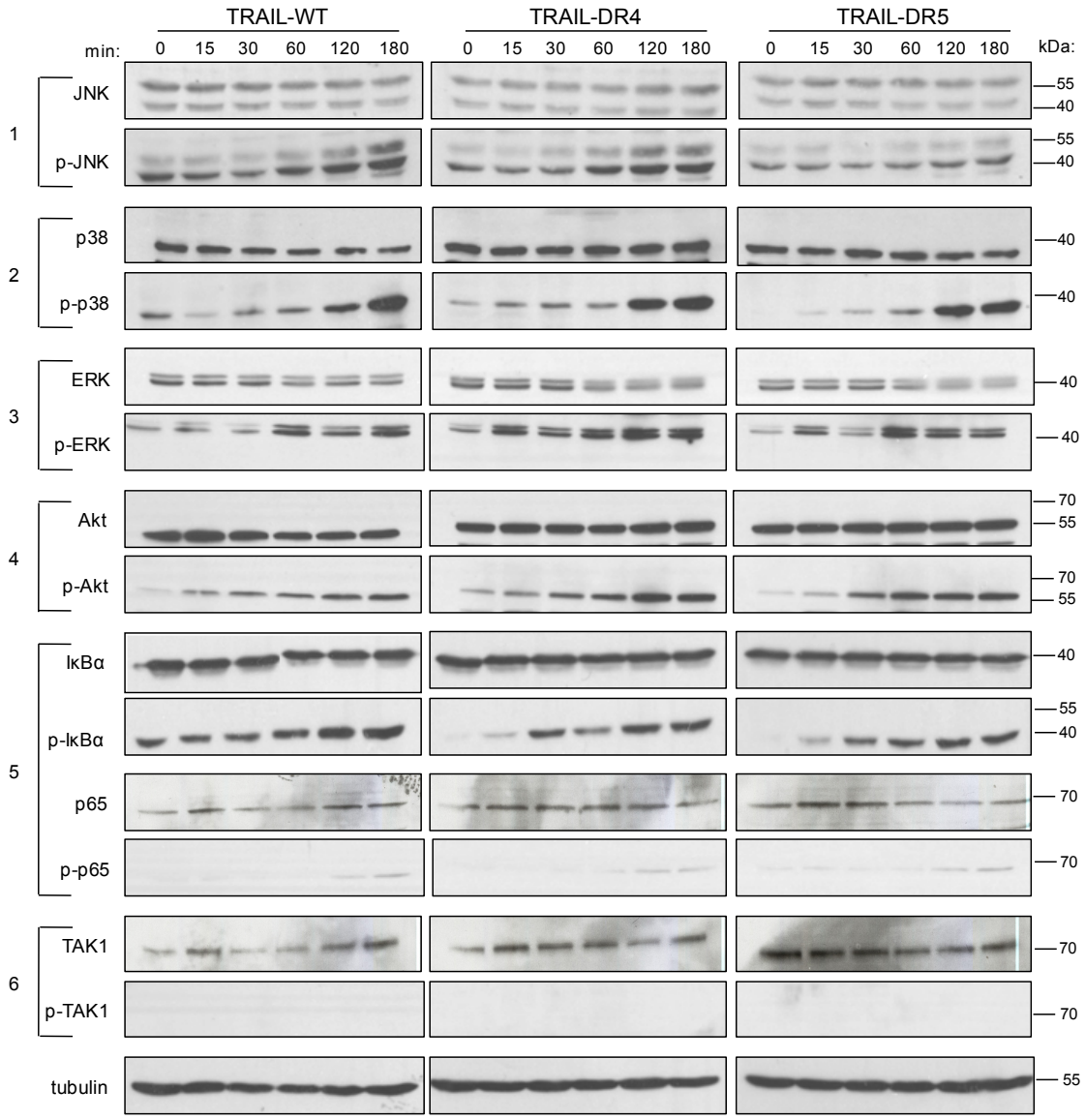

Figure 17. Receptor-specific induction of non-apoptotic TRAIL signaling in DLD-1 cells. Cells were treated with 100ng/ml of TRAIL-WT, TRAIL-DR4 or TRAIL-DR5 for indicated time periods. Activation of TRAIL-induced pathways was analyzed by western blotting with indicated antibodies. Representative western blots from two independent experiments are shown. Tubulin was used as a loading control.

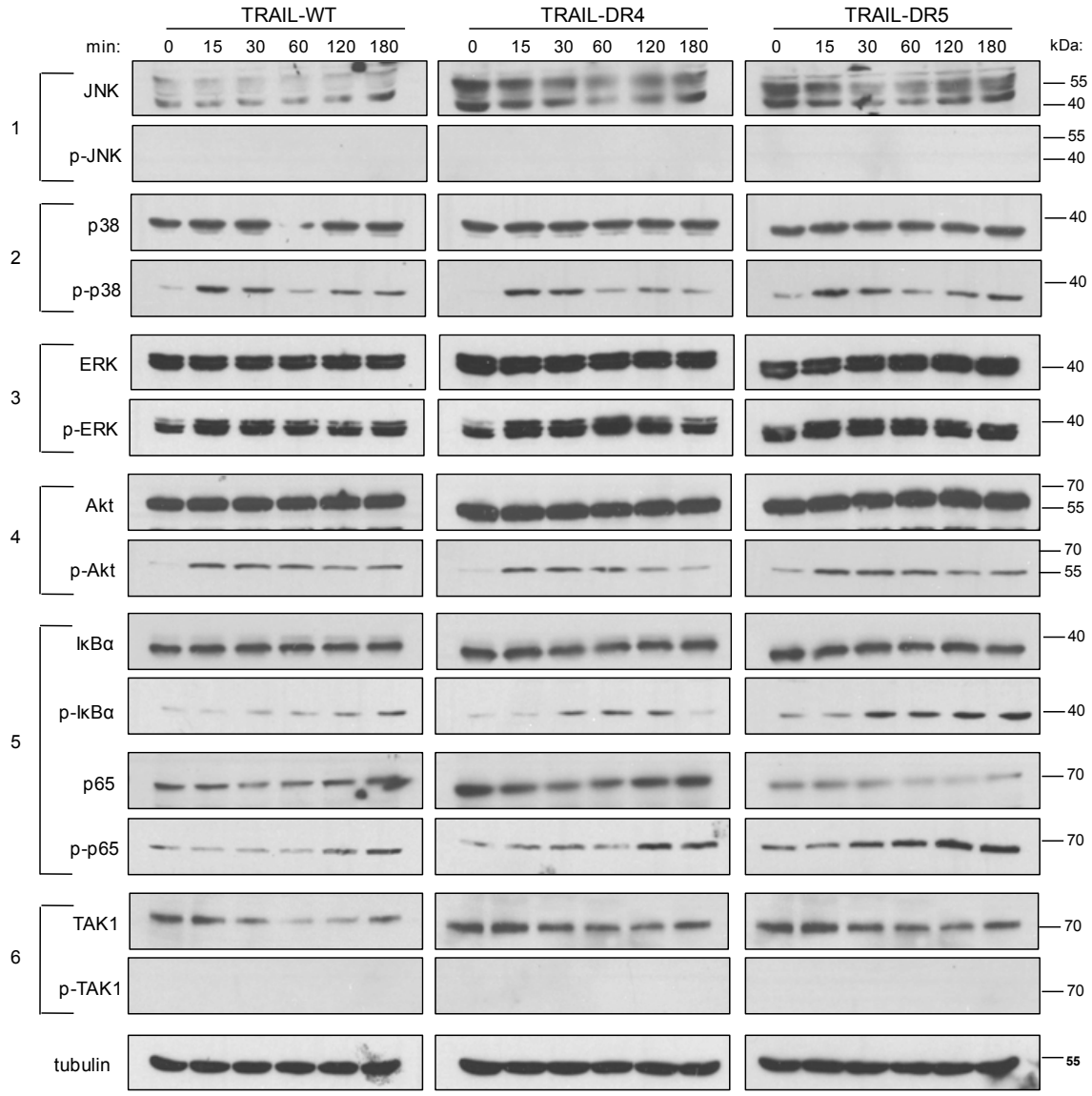

Figure 18. Receptor-specific induction of non-apoptotic TRAIL signaling in HT-29 cells. Cells were treated with 100ng/ml of TRAIL-WT, TRAIL-DR4 or TRAIL-DR5 for indicated time periods. Activation of TRAIL-induced pathways was analyzed by western blotting with indicated antibodies. Representative western blots from two independent experiments are shown. Tubulin was used as a loading control.

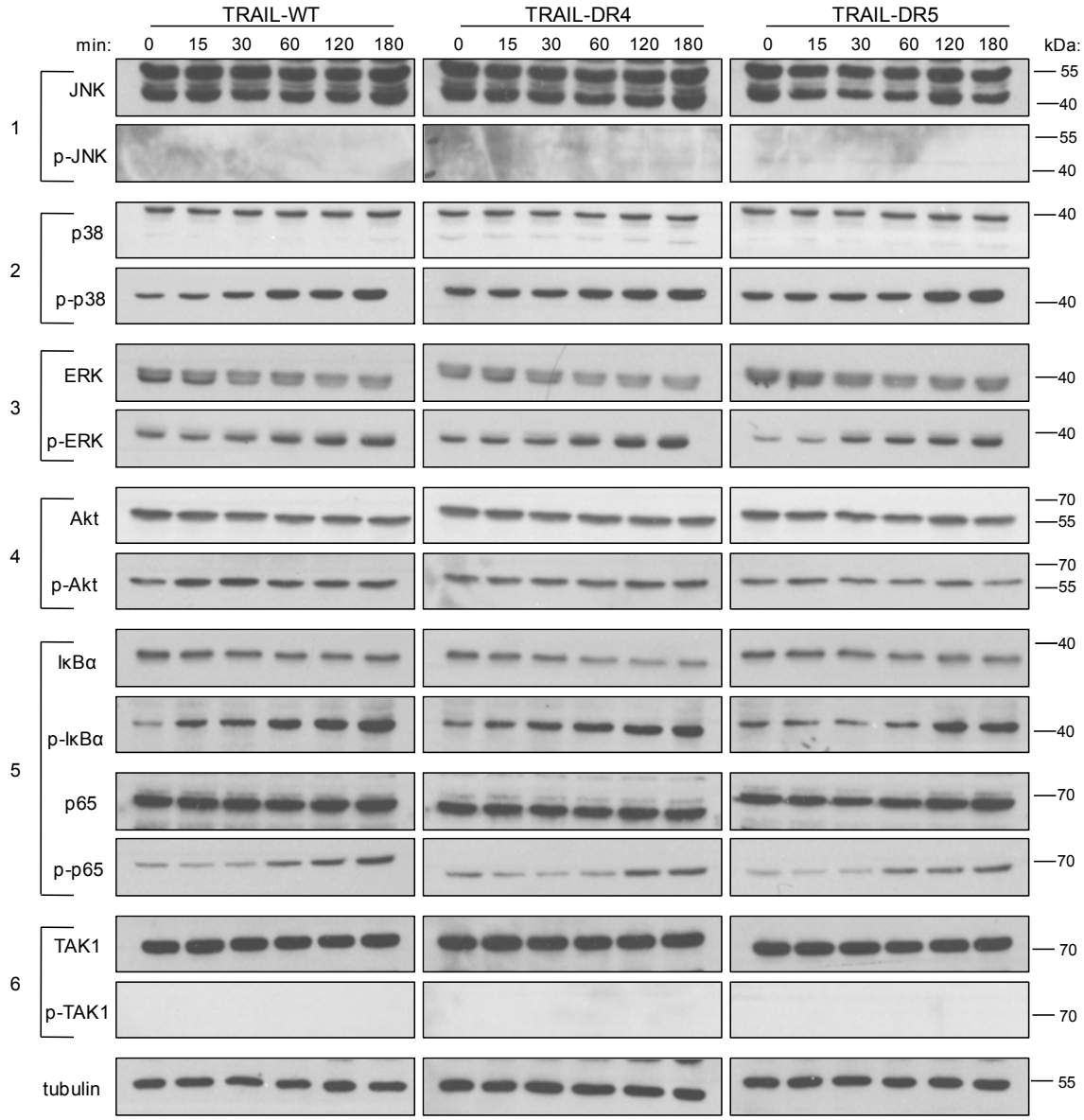

Figure 19. Receptor-specific induction of non-apoptotic TRAIL signaling in SW620 cells. Cells were treated with 100ng/ml of TRAIL-WT, TRAIL-DR4 or TRAIL-DR5 for indicated time periods. Activation of TRAIL-induced pathways was analyzed by western blotting with indicated antibodies. Representative western blots from two independent experiments are shown. Tubulin was used as a loading control.

5 Discussion

Many aspects of TRAIL-induced apoptotic and non-apoptotic signaling in human cells are still unknown or poorly investigated. In this work we aimed to target one of these poorly investigated aspects, and analyzed whether and how differs apoptotic and non-apoptotic signaling from the individually-activated TRAIL receptors DR4 and DR5. To address this aim, we used receptor-specific variants of the recombinant ligand and as initial model cells, we chose several well-known human colorectal cancer cell lines.

We prepared a novel version of human recombinant TRAIL fused with trimerization motif from T4 bacteriophage and *strep*-tag or twin-strep-tag. These recombinant ligands were produced in *E.coli* and *strep*-tag allowed their one-step purification from the bacterial lysate. In its biological activity, ST-TRAIL surpassed histidine-tagged TRAIL perhaps due to enhanced trimer formation documented also by its gel filtration analysis. Into this novel recombinant TRAIL, we introduced mutations, which turned it into DR4- or DR5-specific variants. As a DR4-selective ligand, we used 4C7 variant (Gasparian et al., 2009) containing substitutions (G131R/R149I/S159R/ N199R/K201H/ S215D). As a DR5-selective one, we used variant D269H/E195R (Van der Sloot et al., 2006). Though TRAIL-WT and the DR4-specific variant were produced and purified to approx. 90-95% homogeneity, we encountered a problem with the expression and purification of the DR5-specific ligand. The yield of this recombinant ligand was about 5-fold lower than the other two ligands and it co-purified with an unidentified *E. coli* protein of an apparent molecular weight approx. 70 kDa (likely a heat shock protein). Though the purity of this DR5-specific ligand was about 60%, it was active and thus we decided to use it in our receptor-specific experiments. So far our attempts to purify it further failed, but we are well aware of this problem and aim to use combinations of ion-exchange and hydrophobic chromatography to achieve its purity comparable with the DR4-specific ligand.

To date only a few reports touching analysis of receptor-specific apoptotic signaling in cancer cell lines were published (see chapter 1.2.7) and no systematic comparison of DR4 versus DR5-induced non-apoptotic signaling in cancer cells has been published. In this Thesis, we compared activation of pro-apoptotic and non-apoptotic (activation of JNK, p38, ERK, Akt and TAK1 kinases and NF-kB pathways) in three colorectal cell lines; one sensitive (DLD-1) and two resistant (HT-29 and SW620) to TRAIL-induced apoptosis.

In sensitive DLD-1 cells, we found out that TRAIL-induced apoptotic signaling preferentially proceeds via DR4 receptor and that the pro-apoptotic contribution of DR5 is rather low. This observation is in agreement with previous report, which showed that colorectal cell line SW948 is also predominantly killed through DR4-mediated apoptotic signaling (van Geelen et al., 2011). However, another colorectal cell line, Colo-205 was shown to signal mainly via DR5 (Kelley et al., 2005).

One of the reasons of markedly higher sensitivity of DLD-1cells to DR4-induced apoptosis could be relatively higher expression of the DR4 receptor in these cells. Indeed data from our and others laboratories (Cho et al., 2012) data show that DR4 expression is higher in DLD-1 and also in other colorectal cell lines (Ziauddin et al., 2010). Thus, predominant DR4 signaling in DLD-1 may be simply caused by higher DR4 levels expressed. However, it has been previously shown that no correlation between DR4/DR5 expression levels and apoptotic sensitivity exists in leukemias and pancreatic cancers that despite high levels of DR5 trigger DR4-mediated apoptosis (Lemke et al., 2010; MacFarlane et al., 2005a).

In addition, our analysis of the DISC composition suggests that DR5-DISC contains sufficient amount of DR5 receptors, which are, however, unable to efficiently recruit adaptor FADD, a crucial component of TRAIL-induced apoptotic pathway. Therefore, reduced ability of DR5 to recruit FADD and subsequent FADD-dependent casase-8 recruitment appear to be the main reasons for predominant DR4-activated apoptotic signaling in DLD-1 cells.

Similarly, in TRAIL-resistant HT-29 cells the DISC formation and also caspase-8 processing was by far more efficient, when TRAIL-induced signaling was activated by the DR4-specific ligand. This difference between DR4- and DR5-specific ligand-triggered DISC formations was even more pronounced (significantly less FADD and caspase-8 in DR-assembled DISCs) in HT-29 cells. An exception was cFLIP, which has been associated with DR5-activated DISCs at relatively high levels. This finding was somehow surprising and possible explanation of it could be a) higher affinity of cFLIP to FADD and b) similarly as for caspase-8, formation of DED1-DED2-mediated clustering of cFLIP molecules (Dickens et al., 2012; Schleich et al., 2012).

It has been shown that DcR2 is able to selectively inhibit DR5-mediated DISC formation in overexpression experiments (Merino et al., 2006). To examine whether DcR2 may account for decreased DR5-DISC formation, we measured DcR2 levels by flow cytometry. We did not detect expression of DcR2 neither in DLD-1 cells nor in any

other cell line tested. Thus, DcR2 expression is probably not the cause of the selective DR4 signaling in DLD-1 and HT-29 cells. Other potential reasons for the weak FADD recruitment to DR5 receptor might be presence of mutation(s) in the intracellular part of the receptor or selective recruitment of negative regulator/s of FADD to DR5. We have not addressed this possibility yet.

Interestingly, we identified RIP1 kinase in both DLD-1 and HT-29 DISCs. The role of RIP1 in the DISC is controversial. RIP1 has not been routinely identified within endogenous DISCs in TRAIL-treated cells (Kischkel et al., 2000; Varfolomeev et al., 2005), but is an essential component of the secondary signaling complex downstream of the DISC, which is responsible for activation of several non-apoptotic TRAIL-induced pathways (Varfolomeev et al., 2005). In contrast, other study claimed that RIP1 was identified in the endogenous DISC (Lin et al., 2000). It has been also suggested that RIP1 is recruited to the DISC in a TRADD dependent manner (Cao et al., 2011). However, we did not detect TRADD in HT-29 or DLD-1 DISC (data not shown), suggesting other mechanism of RIP1 recruitment in our system. The role of RIP1 at the level of the DISC remains unresolved.

Recently, a ubiquitin ligase cullin-3 was identified as a DISC component in lung cancer cell lines. Cullin-3-mediated polyubiquitination of caspase-8 was determined to be critical for TRAIL-induced apoptosis in these cells (Jin et al., 2009). Though this effect appears to be cell type restricted, as cullin-3 has not been identified in the DISC of hematopoietic cell lines (Dickens et al., 2012). However, we did not detect any associated cullin-3 isolated DISCs from TRAIL-treated DLD-1 or HT-29 cells (data not shown).

Taken together, our data and published work suggest that in several colorectal cancer cell lines, TRAIL exerts its apoptotic activity mainly via DR4. Dysfunctional DR5 signaling might be caused by insufficient recruitment of the FADD adaptor protein to the DR5-DISC. Moreover, published literature suggests that DR4 is the main apoptotic receptor also in pancreatic cancers and leukemias (Lemke et al., 2010; MacFarlane et al., 2005a).

In addition to apoptotic signaling, we also analyzed the ability of DR4- and DR5-specific ligands to trigger JNK, p38, ERK, Akt and NF-κB pathways in colorectal cancer cell lines DLD-1, HT-29 and SW620. Reflecting significantly more efficient activation of pro-apoptotic signaling in DLD-1 and HT-29 cells by the DR4-specific

ligand, we expected that also the non-apoptotic signaling will be in these cells more pronouncedly activated by the DR4-specific variant. Surprisingly, we found that in all cells tested, both receptors were mostly quite similar in their capabilities of triggering all of these pathways. Therefore, it appears that even small amounts of core DISC components recruited to ligated DR5 are able to trigger full activation of the non-apoptotic pathways. This assumption is supported in a study showing that in several pancreatic cancer cell lines, NF-κB was equally activated by varying TRAIL concentrations (10ng/ml and 100 ng/ml) (Lemke et al., 2010). Alternatively, activation of these pathways may be dependent on yet unknown components of the DISC equally distributed to DR4 and DR5. It has been shown that TRAIL-induced MAP kinases and NF-κB are dependent on secondary complex downstream of the DISC composed of FADD, TRAF2, TRADD, IKK/NEMO, caspase-8 and RIP1. Despite decreased DR5-DISC assembly, it is possible that this secondary complex is more efficiently formed upon stimulation of either receptor.

Although in most cases both receptors were able to activate TRAIL-induced non-apoptotic signaling to similar extent, we did reveal several significant differences in receptor-specific triggering of some of these pathways in analyzed colorectal cancer cells.

In DLD-1 cells, JNK appears to be activated predominantly via DR4. It has been shown that TRAIL-induced JNK activation can be both caspase-dependent and caspase-independent (Muhlenbeck et al., 2000). We hypothesize that in our setting, JNK phosphorylation was caspase-8-dependent, as it was triggered only via DR4 (and TRAIL-WT), which, as we found, is a principal inducer of apoptosis in these cells. It is likely that rather than a specific target in TRAIL signaling, JNK was probably activated as a response to stress caused by progressing apoptosis (its phosphorylation was detectable only in later time points upon DR4 treatment, when substantial portion of cells had likely underwent apoptosis). In agreement with this, we did not detected JNK activation in any of the TRAIL-resistant cancer cell lines. To clarify the issue, the effect of caspases inhibition on JNK activation should be examined and vice versa, to show whether is there a specific effect of JNK in apoptosis, specific inhibition of JNK should be performed. The finding that JNK is inducible by TRAIL in DLD-1 cells is in contrary with earlier report, which did not reveal any phosphorylation of JNK in DLD-1 upon TRAIL treatment (Zhang et al., 2004). We have, however, used higher concentrations of TRAIL and longer treatment times.

In HT-29 cell line, we observed reproducible differences in the receptor-specific activation of NF-κB signaling. Paradoxically, we found that DR5 is the strongest NF-κB activator, as documented by more rapid and efficient phosphorylation of IκBα and the NF-κB p65 subunit. There is a controversy in the field concerning the mechanism of TRAIL induced NF-κB pathway. In Jurkat cells, the activation of NF-κB by TRAIL was shown to be dependent on FADD recruited to DR4/DR5 death domains (Grunert et al., 2012). This was probably not the case in our system, as the activation of NF-κB signaling was more strongly mediated via DR5 receptor, which poorly performed inthe recruitment of FADD and caspase-8 to the DISC. The receptor-specific induction of NF-κB was previously observed in pancreatic cancer cells, where DR4, a principal mediator of TRAIL killing in these cells, but not DR5, induced NF-κB activation (Lemke et al., 2010).

One mechanism of IκBα and MAP kinases activation by TRAIL is formation of the secondary complex downstream of the DISC (Varfolomeev et al., 2005), but how is the formation of this complex regulated is unknown. It is therefore possible that DR5 stimulation in HT-29 leads to more efficient assembly of this secondary complex. This hypothesis is supported by the fact that p38 and Akt were also activated more strongly via DR5, although the difference is not as pronounced as in the case of IκBα and p65 phosphorylation. Alternatively, as TRAIL-induced NF-κB activation likely depends on the interaction of p43 fragment of cFLIP with TRAF2 (Kataoka and Tschopp, 2004), relatively strong association of cFLIP-p43 with the DR5 DISC and very inefficient recruitment and activation of caspase-8 could be behind stronger, DR5-mediated activation of NF-κB signaling in these cells. Another possible explanation of stronger DR5-mediated NF-κB activation may be selective recruitment of unknown factors to the DR5-DISC, which may then mediate NF-κB activation. Indeed, it was published that in primary leukaemia cells, TRAIL induces NF-κB by yet unknown mechanism, independently of caspase-8 and FADD (Ehrhardt et al., 2003).

Taken together, this study provides the first systematic insight into DR4/DR5-specific TRAIL signaling in colorectal cell lines and uncovers potential interesting and useful differences in TRAIL receptor-specific signaling in these cells.

6 Conclusions

1) We prepared and characterized a novel version of recombinant human TRAIL modified with trimerization motif from T4 bacteriophage for enhanced trimer formation and *strep*-tag (or double *strep*-tag) for affinity purification and protein complex isolation (ST-TRAIL). ST-TRAIL can be isolated in one step from bacterial lysate, displays stable trimer formation and higher activity than histidine-tagged version of TRAIL.

2) We prepared DR4- and DR5-specific variants of ST-TRAIL by introducing published mutations into their receptor-binding domains. Both selective variants (designated TRAIL-DR4 and TRAIL-DR5) were purified by the same protocol as the wild type ligand. TRAIL-DR5 preparation contains impurity, which we failed to remove so far. Nevertheless, our test confirmed that both proteins are active and selective.

3) We quantified apoptotic signaling initiated by receptor-specific TRAIL variants in sensitive DLD-1 colorectal cell line. Our data suggest that principal mediator of TRAIL-induced apoptosis in these cells is DR4.

4) We examined composition of the DISC in DLD-1 and HT-29 cell lines. We revealed that in both cells, the DISC is efficiently formed only when DR4 is activated, confirming our previous observation that DR4 is the main mediator of TRAIL-induced apoptosis in DLD-1 cells. In addition, we found that cullin3, which is essential for caspase-8 activation in some cell types, is not present in DLD-1 or HT-29 DISCs. On the other hand, RIP1 kinase, which is not a core component of the DISC was present in both DLD-1 and HT-29 DISC isolations.

5) We examined TRAIL-induced activation of JNK, p38, ERK1/2, TAK1 and NF-κB in DLD-1, HT-29 and SW620 cells. We found that both DR4 and DR5 are in most cases very similar in their ability to trigger these pathways. However, we observed that NF-κB is triggered mainly via DR5 in HT-29 cells and JNK mainly via DR4 in DLD-1 cells. No signifant differences were detected in SW620 cells.

7 References

Allen, J.E., and W.S. El-Deiry. 2012. Regulation of the human TRAIL gene. *Cancer Biol Ther*. 13:1143-1151.

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*. 104:155-162.

Azijli, K., S. Yuvaraj, M.P. Peppelenbosch, T. Wurdinger, H. Dekker, J. Joore, E. van Dijk, W.J. Quax, G.J. Peters, S. de Jong, and F.A. Kruyt. 2012. Kinome profiling of non-apoptotic TRAIL signaling reveals RIP1-Src-STAT3-dependent invasion in resistant non-small cell lung cancer cells. *J Cell Sci*. 125:4651-4661.

Bellail, A.C., J.J. Olson, X. Yang, Z.J. Chen, and C. Hao. 2012. A20 ubiquitin ligase-mediated polyubiquitination of RIP1 inhibits caspase-8 cleavage and TRAIL-induced apoptosis in glioblastoma. *Cancer Discov*. 2:140-155.

Bonni, A., A. Brunet, A.E. West, S.R. Datta, M.A. Takasu, and M.E. Greenberg. 1999. Cell Survival Promoted by the Ras-MAPK Signaling Pathway by Transcription-Dependent and -Independent Mechanisms. *Science*. 286:1358-1362.

Boyce, B.F., and L. Xing. 2007. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res Ther*. 9 Suppl 1:S1.

Bridgham, J.T., J.A. Wilder, H. Hollocher, and A.L. Johnson. 2003. All in the family: evolutionary and functional relationships among death receptors. *Cell Death Differ*. 10:19-25.

Cao, X., Y.L. Pobezinskaya, M.J. Morgan, and Z.G. Liu. 2011. The role of TRADD in TRAIL-induced apoptosis and signaling. *FASEB J*. 25:1353-1358.

Clancy, L., K. Mruk, K. Archer, M. Woelfel, J. Mongkolsapaya, G. Screaton, M.J. Lenardo, and F.K. Chan. 2005. Preligand assembly domain-mediated ligand-independent association between TRAIL receptor 4 (TR4) and TR2 regulates TRAIL-induced apoptosis. *Proc Natl Acad Sci U S A*. 102:18099-18104.

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*. 168:1356-1361.

Cullen, S.P., C.M. Henry, C.J. Kearney, S.E. Logue, M. Feoktistova, G.A. Tynan, E.C. Lavelle, M. Leverkus, and S.J. Martin. 2013. Fas/CD95-Induced Chemokines Can Serve as "Find-Me" Signals for Apoptotic Cells. *Mol Cell*. 49:1034-1048.

Cursi, S., A. Rufini, V. Stagni, I. Condo, V. Matafora, A. Bachi, A.P. Bonifazi, L. Coppola, G. Superti-Furga, R. Testi, and D. Barila. 2006. Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *Embo J*. 25:1895-1905.

De Zutter, G.S., and R.J. Davis. 2001. Pro-apoptotic gene expression mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Proc Natl Acad Sci U S A*. 98:6168-6173.

Dhanasekaran, D.N., and E.P. Reddy. 2008. JNK signaling in apoptosis. *Oncogene*. 27:6245-6251.

Dickens, L.S., R.S. Boyd, R. Jukes-Jones, M.A. Hughes, G.L. Robinson, L. Fairall, J.W. Schwabe, K. Cain, and M. Macfarlane. 2012. A death effector domain chain DISC model reveals a crucial role for caspase-8 chain assembly in mediating apoptotic cell death. *Mol Cell*. 47:291-305.

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*. 21:877-889.

Dieterle, A., R. Orth, M. Daubrawa, A. Grotemeier, S. Alers, S. Ullrich, R. Lammers, S. Wesselborg, and B. Stork. 2009. The Akt inhibitor triciribine sensitizes prostate carcinoma cells to TRAIL-induced apoptosis. *Int J Cancer*. 125:932-941.

Dimberg, L.Y., C.K. Anderson, R. Camidge, K. Behbakht, A. Thorburn, and H.L. Ford. 2013. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. *Oncogene*. 32:1341-1350.

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*. 22:3842-3852.

Eimon, P.M., E. Kratz, E. Varfolomeev, S.G. Hymowitz, H. Stern, J. Zha, and A. Ashkenazi. 2006. Delineation of the cell-extrinsic apoptosis pathway in the zebrafish. *Cell Death Differ*. 13:1619-1630.

Elmore, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 35:495-516.

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*. 273:14363-14367.

Finnberg, N., A.J. Klein-Szanto, and W.S. El-Deiry. 2008. TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. *J Clin Invest*. 118:111-123.

Fridman, A., I. Pak, B.D. Butts, M. Hoek, D.W. Nicholson, and H. Mehmet. 2013. MerCASBA: an updated and refined database of caspase substrates. *Apoptosis*. 18:369-371.

Gasparian, M.E., B.V. Chernyak, D.A. Dolgikh, A.V. Yagolovich, E.N. Popova, A.M. Sycheva, S.A. Moshkovskii, and M.P. Kirpichnikov. 2009. Generation of new TRAIL mutants DR5-A and DR5-B with improved selectivity to death receptor 5. *Apoptosis*. 14:778-787.

Gillespie, S., X.D. Zhang, and P. Hersey. 2005. Variable expression of protein kinase Cε in human melanoma cells regulates sensitivity to TRAIL-induced apoptosis. *Molecular Cancer Therapeutics*. 4:668-676.

Gilmore, T.D. 2006. Introduction to NF-[kappa]B: players, pathways, perspectives. *Oncogene*. 25:6680-6684.

Goncharenko-Khaider, N., D. Lane, I. Matte, C. Rancourt, and A. Piche. 2010. The inhibition of Bid expression by Akt leads to resistance to TRAIL-induced apoptosis in ovarian cancer cells. *Oncogene*. 29:5523-5536.

Gonzalvez, F., and A. Ashkenazi. 2010. New insights into apoptosis signaling by Apo2L/TRAIL. *Oncogene*. 29:4752-4765.

Gonzalvez, F., D. Lawrence, B. Yang, S. Yee, R. Pitti, S. Marsters, V.C. Pham, J.P. Stephan, J. Lill, and A. Ashkenazi. 2012. TRAF2 Sets a Threshold for Extrinsic Apoptosis by Tagging Caspase-8 with a Ubiquitin Shutoff Timer. *Mol Cell*. 48:888-899.

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*. 118:100-110.

Grunert, M., K. Gottschalk, J. Kapahnke, S. Gundisch, A. Kieser, and I. Jeremias. 2012. The adaptor protein FADD and the initiator caspase-8 mediate activation of NF-kappaB by TRAIL. *Cell Death Dis*. 3:e414.

Hameed, A.G., N.D. Arnold, J. Chamberlain, J.A. Pickworth, C. Paiva, S. Dawson, S. Cross, L. Long, L. Zhao, N.W. Morrell, D.C. Crossman, C.M. Newman, D.G. Kiely, S.E. Francis, and A. Lawrie. 2012. Inhibition of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) reverses experimental pulmonary hypertension. *J Exp Med*. 209:1919-1935.

Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-674.

Harper, N., M.A. Hughes, S.N. Farrow, G.M. Cohen, and M. MacFarlane. 2003. Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. *J Biol Chem*. 278:44338-44347.

Herr, I., D. Wilhelm, E. Meyer, I. Jeremias, P. Angel, and K.M. Debatin. 1999. JNK/SAPK activity contributes to TRAIL-induced apoptosis. *Cell Death Differ*. 6:130-135.

Herrero-Martin, G., M. Hoyer-Hansen, C. Garcia-Garcia, C. Fumarola, T. Farkas, A. Lopez-Rivas, and M. Jaattela. 2009. TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells. *Embo J*. 28:677-685.

Higuchi, H., J.H. Yoon, A. Grambihler, N. Werneburg, S.F. Bronk, and G.J. Gores. 2003. Bile acids stimulate cFLIP phosphorylation enhancing TRAIL-mediated apoptosis. *J Biol Chem*. 278:454-461.

Holland, P.M. 2011. Targeting Apo2L/TRAIL receptors by soluble Apo2L/TRAIL. *Cancer Lett*.

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*. 4:563-571.

Chahrazade, K., and H. Walczak. 2011. Dual Philosophy in Death Receptor Signalling. *The Open Cell Signaling Journal*. 3:27-34.

Chang, L., and M. Karin. 2001. Mammalian MAP kinase signalling cascades. *Nature*. 410:37-40.

Chaudhary, P.M., M. Eby, A. Jasmin, A. Bookwalter, J. Murray, and L. Hood. 1997. Death Receptor 5, a New Member of the TNFR Family, and DR4 Induce FADD-Dependent Apoptosis and Activate the NF-ºB Pathway. *Immunity*. 7:821-830.

Cho, M.H., E.J. Lee, M. Son, J.H. Lee, D. Yoo, J.W. Kim, S.W. Park, J.S. Shin, and J. Cheon. 2012. A magnetic switch for the control of cell death signalling in in vitro and in vivo systems. *Nat Mater*. 11:1038-1043.

Choo, M.K., N. Kawasaki, P. Singhirunnusorn, K. Koizumi, S. Sato, S. Akira, I. Saiki, and H. Sakurai. 2006. Blockade of transforming growth factor-beta-activated kinase 1 activity enhances TRAIL-induced apoptosis through activation of a caspase cascade. *Mol Cancer Ther*. 5:2970-2976.

Ivanov, V.N., and Z. Ronai. 2000. p38 protects human melanoma cells from UV-induced apoptosis through down-regulation of NF-kappaB activity and Fas expression. *Oncogene*. 19:3003-3012.

Jin, Z., Y. Li, R. Pitti, D. Lawrence, V.C. Pham, J.R. Lill, and A. Ashkenazi. 2009. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell*. 137:721-735.

Jo, M., T.-H. Kim, D.-W. Seol, J.E. Esplen, K. Dorko, T.R. Billiar, and S.C. Strom. 2000. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med*. 6:564-567.

Jouan-Lanhouet, S., M.I. Arshad, C. Piquet-Pellorce, C. Martin-Chouly, G. Le Moigne-Muller, F. Van Herreweghe, N. Takahashi, O. Sergent, D. Lagadic-Gossmann, P. Vandenabeele, M. Samson, and M.T. Dimanche-Boitrel. 2012. TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. *Cell Death Differ*. 19:2003-2014.

Junttila, M.R., S. Saarinen, T. Schmidt, J. Kast, and J. Westermarck. 2005. Single-step Strep-tag purification for the isolation and identification of protein complexes from mammalian cells. *Proteomics*. 5:1199-1203.

Jurewicz, A., M. Matysiak, S. Andrzejak, and K. Selmaj. 2006. TRAIL-induced death of human adult oligodendrocytes is mediated by JNK pathway. *Glia*. 53:158-166.

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*. 62:4929-4937.

Kataoka, T., and J. Tschopp. 2004. N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappaB signaling pathway. *Mol Cell Biol*. 24:2627-2636.

Kelley, R.F., K. Totpal, S.H. Lindstrom, M. Mathieu, K. Billeci, L. DeForge, R. Pai, S.G. Hymowitz, and A. Ashkenazi. 2005. Receptor-selective mutants of apoptosis-inducing ligand

2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution. *J Biol Chem*. 280:2205-2212.

Kemp, T.J., J.S. Kim, S.A. Crist, and T.S. Griffith. 2003. Induction of necrotic tumor cell death by TRAIL/Apo-2L. *Apoptosis*. 8:587-599.

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*. 26:239-257.

Kischkel, F.C., D.A. Lawrence, A. Chuntharapai, P. Schow, K.J. Kim, and A. Ashkenazi. 2000. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity*. 12:611-620.

Kuang, A.A., G.E. Diehl, J. Zhang, and A. Winoto. 2000. FADD is required for DR4- and DR5-mediated apoptosis: lack of trail-induced apoptosis in FADD-deficient mouse embryonic fibroblasts. *J Biol Chem*. 275:25065-25068.

Kwon, D., J.H. Yoon, S.-Y. Shin, T.-H. Jang, H.-G. Kim, I. So, J.-H. Jeon, and H.H. Park. 2012. A comprehensive manually curated protein–protein interaction database for the Death Domain superfamily. *Nucleic Acids Research*. 40:D331-D336.

Labrinidis, A., V. Liapis, M. Thai le, G.J. Atkins, C. Vincent, S. Hay, N.A. Sims, A.C. Zannettino, D.M. Findlay, and A. Evdokiou. 2008. Does Apo2L/TRAIL play any physiologic role in osteoclastogenesis? *Blood*. 111:5411-5412; autor reply 5413.

Laguinge, L.M., R.N. Samara, W. Wang, W.S. El-Deiry, G. Corner, L. Augenlicht, L. Mishra, and J.M. Jessup. 2008. DR5 receptor mediates anoikis in human colorectal carcinoma cell lines. *Cancer Res*. 68:909-917.

Lalaoui, N., A. Morle, D. Merino, G. Jacquemin, E. Iessi, A. Morizot, S. Shirley, B. Robert, E. Solary, C. Garrido, and O. Micheau. 2011. TRAIL-R4 promotes tumor growth and resistance to apoptosis in cervical carcinoma HeLa cells through AKT. *PLoS One*. 6:e19679.

Lavrik, I.N., and P.H. Krammer. 2012. Regulation of CD95/Fas signaling at the DISC. *Cell Death Differ*. 19:36-41.

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*. 7:383-385.

Lee, E.W., J.H. Kim, Y.H. Ahn, J. Seo, A. Ko, M. Jeong, S.J. Kim, J.Y. Ro, K.M. Park, H.W. Lee, E.J. Park, K.H. Chun, and J. Song. 2012. Ubiquitination and degradation of the FADD adaptor protein regulate death receptor-mediated apoptosis and necroptosis. *Nat Commun*. 3:978.

Lee, M.W., S.C. Park, Y.G. Yang, S.O. Yim, H.S. Chae, J.H. Bach, H.J. Lee, K.Y. Kim, W.B. Lee, and S.S. Kim. 2002. The involvement of reactive oxygen species (ROS) and p38 mitogen-activated protein (MAP) kinase in TRAIL/Apo2L-induced apoptosis. *FEBS Lett*. 512:313-318.

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*. 351:1024-1030.

Lemke, J., A. Noack, D. Adam, V. Tchikov, U. Bertsch, C. Roder, S. Schutze, H. Wajant, H. Kalthoff, and A. Trauzold. 2010. TRAIL signaling is mediated by DR4 in pancreatic tumor cells despite the expression of functional DR5. *J Mol Med (Berl)*. 88:729-740.

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*. 20:6638-6645.

Lluis, J.M., U. Nachbur, W.D. Cook, I.E. Gentle, D. Moujalled, M. Moulin, W.W. Wong, N. Khan, D. Chau, B.A. Callus, J.E. Vince, J. Silke, and D.L. Vaux. 2010. TAK1 is required for survival

of mouse fibroblasts treated with TRAIL, and does so by NF-kappaB dependent induction of cFLIPL. *PLoS One*. 5:e8620.

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

MacFarlane, M., S.L. Kohlhaas, M.J. Sutcliffe, M.J.S. Dyer, and G.M. Cohen. 2005b. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res*. 65:11265-11270.

Mahalingam, D., M. Keane, G. Pirianov, H. Mehmet, A. Samali, and E. Szegezdi. 2009. Differential activation of JNK1 isoforms by TRAIL receptors modulate apoptosis of colon cancer cell lines. *Br J Cancer*. 100:1415-1424.

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*. 26:7046-7055.

Morioka, S., E. Omori, T. Kajino, R. Kajino-Sakamoto, K. Matsumoto, and J. Ninomiya-Tsuji. 2009. TAK1 kinase determines TRAIL sensitivity by modulating reactive oxygen species and cIAP. *Oncogene*. 28:2257-2265.

Mühlenbeck, F., E. Haas, R. Schwenzer, G. Schubert, M. Grell, C. Smith, P. Scheurich, and H. Wajant. 1998. TRAIL/Apo2L Activates c-Jun NH2-terminal Kinase (JNK) via Caspase-dependent and Caspase-independent Pathways. *J Biol Chem*. 273:33091-33098.

Muhlenbeck, F., P. Schneider, J.L. Bodmer, R. Schwenzer, A. Hauser, G. Schubert, P. Scheurich, D. Moosmayer, J. Tschopp, and H. Wajant. 2000. The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. *J Biol Chem*. 275:32208-32213.

Muhlethaler-Mottet, A., M. Flahaut, K.B. Bourloud, K. Nardou, A. Coulon, J. Liberman, M. Thome, and N. Gross. 2011. Individual caspase-10 isoforms play distinct and opposing roles in the initiation of death receptor-mediated tumour cell apoptosis. *Cell Death and Dis*. 2:e125.

Neumann, S., T. Bidon, M. Branschadel, A. Krippner-Heidenreich, P. Scheurich, and M. Doszczak. 2012. The transmembrane domains of TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 co-regulate apoptotic signaling capacity. *PLoS One*. 7:e42526.

Newton, A.C. 1995. Protein Kinase C: Structure, Function, and Regulation. *J Biol Chem*. 270:28495-28498.

Nitsch, R., I. Bechmann, R.A. Deisz, D. Haas, T.N. Lehmann, U. Wendling, and F. Zipp. 2000. Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). *Lancet*. 356:827-828.

Ozturk, S., K. Schleich, and I.N. Lavrik. 2012. Cellular FLICE-like inhibitory proteins (c-FLIPs): fine-tuners of life and death decisions. *Exp Cell Res*. 318:1324-1331.

Pan, G., K. O'Rourke, A.M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, and V.M. Dixit. 1997. The Receptor for the Cytotoxic Ligand TRAIL. *Science*. 276:111-113.

Pennarun, B., A. Meijer, E.G.E. de Vries, J.H. Kleibeuker, F. Kruyt, and S. de Jong. 2010. Playing the DISC: Turning on TRAIL death receptor-mediated apoptosis in cancer. *Bba-Rev Cancer*. 1805:123-140.

Plantivaux, A., E. Szegezdi, A. Samali, and L. Egan. 2009. Is there a role for nuclear factor kappaB in tumor necrosis factor-related apoptosis-inducing ligand resistance? *Annals of the New York Academy of Sciences*. 1171:38-49.

Puduvalli, V.K., D. Sampath, J.M. Bruner, J. Nangia, R. Xu, and A.P. Kyritsis. 2005. TRAIL-induced apoptosis in gliomas is enhanced by Akt-inhibition and is independent of JNK activation. *Apoptosis*. 10:233-243.

Reis, C.R., A.M. van der Sloot, A. Natoni, E. Szegezdi, R. Setroikromo, M. Meijer, K. Sjollema, F. Stricher, R.H. Cool, A. Samali, L. Serrano, and W.J. Quax. 2010. Rapid and efficient

cancer cell killing mediated by high-affinity death receptor homotrimerizing TRAIL variants. *Cell Death and Dis*. 1:e83.

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*. 90:612-624.

Rossin, A., M. Derouet, F. Abdel-sater, and A.-O. Hueber. 2009. Palmitoylation of the TRAIL receptor DR4 confers an efficient TRAIL-induced cell death signalling. *Biochem J*. 419:185-192.

Safa, A.R. 2012. c-FLIP, a master anti-apoptotic regulator. *Exp Oncol*. 34:176-184.

Sambrook, J., and D.W. Russell. 2001. Molecular cloning: a laboratory manual. CSHL press.

Secchiero, P., A. Gonelli, E. Carnevale, D. Milani, A. Pandolfi, D. Zella, and G. Zauli. 2003. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. *Circulation*. 107:2250-2256.

Sharp, D.A., D.A. Lawrence, and A. Ashkenazi. 2005. Selective knockdown of the long variant of cellular FLICE inhibitory protein augments death receptor-mediated caspase-8 activation and apoptosis. *J Biol Chem*. 280:19401-19409.

Shaul, Y.D., and R. Seger. 2007. The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1773:1213-1226.

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*. 277:818-821.

Shi, Y. 2004. Caspase Activation: Revisiting the Induced Proximity Model. *Cell*. 117:855-858.

Shirley, S., A. Morizot, and O. Micheau. 2011. Regulating TRAIL receptor-induced cell death at the membrane : a deadly discussion. *Recent Pat Anticancer Drug Discov*. 6:311-323.

Shishodia, S., and B.B. Aggarwal. 2002. Nuclear factor-kappaB activation: a question of life or death. *J Biochem Mol Biol*. 35:28-40.

Schleich, K., U. Warnken, N. Fricker, S. Ozturk, P. Richter, K. Kammerer, M. Schnolzer, P.H. Krammer, and I.N. Lavrik. 2012. Stoichiometry of the CD95 death-inducing signaling complex: experimental and modeling evidence for a death effector domain chain model. *Mol Cell*. 47:306-319.

Schmidt, T.G., and A. Skerra. 2007. The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nat Protoc*. 2:1528-1535.

Schneider, P., M. Thome, K. Burns, J.L. Bodmer, K. Hofmann, T. Kataoka, N. Holler, and J. Tschopp. 1997. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity*. 7:831-836.

Somasekharan, S.P., M. Koc, A. Morizot, O. Micheau, P.H. Sorensen, O. Gaide, L. Andera, and J.C. Martinou. 2012. TRAIL promotes membrane blebbing, detachment and migration of cells displaying a dysfunctional intrinsic pathway of apoptosis. *Apoptosis*.

Son, J.K., S. Varadarajan, and S.B. Bratton. 2010. TRAIL-activated stress kinases suppress apoptosis through transcriptional upregulation of MCL-1. *Cell Death Differ*. 17:1288-1301.

Soria, J.-C., Z. Márk, P. Zatloukal, B. Szima, I. Albert, E. Juhász, J.-L. Pujol, J. Kozielski, N. Baker, D. Smethurst, Y.-j. Hei, A. Ashkenazi, H. Stern, L. Amler, Y. Pan, and F. Blackhall. 2011. Randomized Phase II Study of Dulanermin in Combination With Paclitaxel, Carboplatin, and Bevacizumab in Advanced Non–Small-Cell Lung Cancer. *J Clin Oncol*.

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*. 21:4520-4530.

Steinwede, K., S. Henken, J. Bohling, R. Maus, B. Ueberberg, C. Brumshagen, E.L. Brincks, T.S. Griffith, T. Welte, and U.A. Maus. 2012. TNF-related apoptosis-inducing ligand (TRAIL) exerts therapeutic efficacy for the treatment of pneumococcal pneumonia in mice. *J Exp Med*. 209:1937-1952.

Stolfi, C., F. Pallone, and G. Monteleone. 2012. Molecular Targets of TRAIL-Sensitizing Agents in Colorectal Cancer. *Int J Mol Sci*. 13:7886-7901.

Szegezdi, E., C.R. Reis, A.M.v.d. Sloot, A. Natoni, A. O'Reilly, J. Reeve, R.H. Cool, M. O'Dwyer, S. Knapper, L. Serrano, W.J. Quax, and A. Samali. 2011. Targeting AML through DR4 with a novel variant of rhTRAIL. *Journal of Cellular and Molecular Medicine*. 15:2216-2231.

Tait, S.W.G., and D.R. Green. 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol*. 11:621-632.

Takeda, K., Y. Hayakawa, M.J. Smyth, N. Kayagaki, N. Yamaguchi, S. Kakuta, Y. Iwakura, H. Yagita, and K. Okumura. 2001. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med*. 7:94-100.

Tran, S.E.F., T.H. Holmström, M. Ahonen, V.-M. Kähäri, and J.E. Eriksson. 2001. MAPK/ERK Overrides the Apoptotic Signaling from Fas, TNF, and TRAIL Receptors. *J Biol Chem*. 276:16484-16490.

Trauzold, A., H. Wermann, A. Arlt, S. Schutze, H. Schafer, S. Oestern, C. Roder, H. Ungefroren, E. Lampe, M. Heinrich, H. Walczak, and H. Kalthoff. 2001. CD95 and TRAIL receptor-mediated activation of protein kinase C and NF-kappaB contributes to apoptosis resistance in ductal pancreatic adenocarcinoma cells. *Oncogene*. 20:4258-4269.

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*. 275:23319-23325.

Tur, V., A.M. van der Sloot, C.R. Reis, E. Szegezdi, R.H. Cool, A. Samali, L. Serrano, and W.J. Quax. 2008. DR4-selective tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) variants obtained by structure-based design. *J Biol Chem*. 283:20560-20568.

Valley, C.C., A.K. Lewis, D.J. Mudaliar, J.D. Perlmutter, A.R. Braun, C.B. Karim, D.D. Thomas, J.R. Brody, and J.N. Sachs. 2012. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces death receptor 5 networks that are highly organized. *J Biol Chem*. 287:21265-21278.

van de Kooij, B., I. Verbrugge, E. de Vries, M. Gijsen, V. Montserrat, C. Maas, J. Neefjes, and J. Borst. 2013. Ubiquitination by the membrane-associated RING-CH-8 (MARCH-8) ligase controls steady-state cell surface expression of the death receptor TRAIL-R1. *J Biol Chem*.

Van der Sloot, A.M., V. Tur, E. Szegezdi, M.M. Mullally, R.H. Cool, A. Samali, L. Serrano, and W.J. Quax. 2006. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *P Natl Acad Sci USA*. 103:8634-8639.

van Geelen, C.M., B. Pennarun, P.T. Le, E.G. de Vries, and S. de Jong. 2011. Modulation of TRAIL resistance in colon carcinoma cells: different contributions of DR4 and DR5. *BMC Cancer*. 11:39.

Vandenabeele, P., L. Galluzzi, T. Vanden Berghe, and G. Kroemer. 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol*. 11:700-714.

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*. 280:40599-40608.

Vivanco, I., and C.L. Sawyers. 2002. The phosphatidylinositol 3-Kinase-AKT pathway in human cancer. *Nat Rev Cancer*. 2:489-501.

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*. 13:1070-1077.

Wajant, H., J. Gerspach, and K. Pfizenmaier. 2011. Engineering death receptor ligands for cancer therapy. *Cancer Lett*.

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*. 16:5386-5397.

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*. 5:157-163.

Wang, G., X. Wang, H. Yu, S. Wei, N. Williams, D.L. Holmes, R. Halfmann, J. Naidoo, L. Wang, L. Li, S. Chen, P. Harran, X. Lei, and X. Wang. 2013. Small-molecule activation of the TRAIL receptor DR5 in human cancer cells. *Nat Chem Biol*. 9:84-89.

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*. 3:673-682.

Wilson, N.S., A. Yang, B. Yang, S. Couto, H. Stern, A. Gogineni, R. Pitti, S. Marsters, R.M. Weimer, M. Singh, and A. Ashkenazi. 2012. Proapoptotic activation of death receptor 5 on tumor endothelial cells disrupts the vasculature and reduces tumor growth. *Cancer Cell*. 22:80-90.

Xiao, C., B.F. Yang, N. Asadi, F. Beguinot, and C. Hao. 2002. Tumor necrosis factor-related apoptosis-inducing ligand-induced death-inducing signaling complex and its modulation by c-FLIP and PED/PEA-15 in glioma cells. *J Biol Chem*. 277:25020-25025.

Xu, G., and Y. Shi. 2007. Apoptosis signaling pathways and lymphocyte homeostasis. *Cell Res*. 17:759-771.

Xu, J., J.Y. Zhou, W.Z. Wei, and G.S. Wu. 2010. Activation of the Akt survival pathway contributes to TRAIL resistance in cancer cells. *PLoS One*. 5:e10226.

Yagita, H., K. Takeda, Y. Hayakawa, M.J. Smyth, and K. Okumura. 2004. TRAIL and its receptors as targets for cancer therapy. *Cancer Science*. 95:777-783.

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.

Zarubin, T., and J. Han. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Res*. 15:11-18.

Zauli, G., E. Rimondi, V. Nicolin, E. Melloni, C. Celeghini, and P. Secchiero. 2004. TNF-related apoptosis-inducing ligand (TRAIL) blocks osteoclastic differentiation induced by RANKL plus M-CSF. *Blood*. 104:2044-2050.

Zauli, G., S. Sancilio, A. Cataldi, N. Sabatini, D. Bosco, and R. Di Pietro. 2005. PI-3K/Akt and NF-κB/IκBα pathways are activated in Jurkat T cells in response to TRAIL treatment. *Journal of Cellular Physiology*. 202:900-911.

Zhang, L., and B. Fang. 2004. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther*. 12:228-237.

Zhang, L., X. Ren, E. Alt, X. Bai, S. Huang, Z. Xu, P.M. Lynch, M.P. Moyer, X.F. Wen, and X. Wu. 2010. Chemoprevention of colorectal cancer by targeting APC-deficient cells for apoptosis. *Nature*. 464:1058-1061.

Zhang, L., H. Zhu, J.J. Davis, D. Jacob, S. Wu, F. Teraishi, A. Gutierrez, Y. Wang, and B. Fang. 2004. Lack of p38 MAP kinase activation in TRAIL-resistant cells is not related to the resistance to TRAIL-mediated cell death. *Cancer Biol Ther*. 3:296-301.

Zhang, X.D., J.M. Borrow, X.Y. Zhang, T. Nguyen, and P. Hersey. 2003. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. *Oncogene*. 22:2869-2881.

Zheng, S.J., J. Jiang, H. Shen, and Y.H. Chen. 2004. Reduced apoptosis and ameliorated listeriosis in TRAIL-null mice. *J Immunol*. 173:5652-5658.

Zhuang, S., J.T. Demirs, and I.E. Kochevar. 2000. p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J Biol Chem*. 275:25939-25948.

Ziauddin, M.F., Z.S. Guo, M.E. O'Malley, F. Austin, P.J. Popovic, M.A. Kavanagh, J. Li, M. Sathaiah, P. Thirunavukarasu, B. Fang, Y.J. Lee, and D.L. Bartlett. 2010. TRAIL gene-armed oncolytic poxvirus and oxaliplatin can work synergistically against colorectal cancer. *Gene Ther*. 17:550-559.