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Role of TRAIL-receptors in apoptosis and nonapoptotic signaling in cancer cells populations undergoing fractional killing

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*ÉCOLE DOCTORALE des Sciences de la Vie
et de la Santé*



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Role of TRAIL-receptors in apoptosis and non-apoptotic signaling in cancer cells populations undergoing fractional killing

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LIST OF ABBREVIATIONS

AHCT - Allogenic hematopoietic cell transplantation
Akt - Protein kinase B (PKB)
allo-BMT - Allogenic bone marrow transplantation
AP2 - Adaptor protein 2
APC - Adenomatous polyposis coli
aSMase - Acid sphingomyelinase
BJ – Normal foreskin fibroblasts
CaCl₂ - Calcium chloride
CAD - Caspase-activated DNase
Cas9 - CRISPR associated protein 9
CD3 - Cluster of differentiation 3
cDNA - Complementary DNA
cFlip - Cellular FLICE-like inhibitory protein
CH₃CO₂K - Potassium acetate
cIAP-1 - Cellular inhibitor of apoptosis 1
cIAP-2 - Cellular inhibitor of apoptosis 2
cIAPs - Cellular inhibitors of apoptosis
CRD - Cysteine rich domains
CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats
CrmA - Cytokine response modifier A
CTSD - Aspartate protease cathepsin D
DAPI - 4',6-diamidino-2-phenylindole
DcR1 – Decoy receptor 1
DcR2 – Decoy receptor 2
DD – Death domain
DED - Death effector domain
DISC – Death inducing signaling complex
DR4 – Death receptor 4
DR5 – Death receptor 5
DTT – Dithiothreitol
ECL - Enhanced chemiluminescence
EDTA - Ethylenediaminetetraacetic acid
EGF - Epidermal growth factor
EGFR - Epidermal growth factor receptor

Erk1/2 - Extracellular signal-regulated kinase 1 and 2
FACS - Fluorescence-activated cell sorting
FADD - FAS-associated death domain protein
FAK - Focal adhesion kinase
FAS – FAS receptor
FASL – FAS ligand
FcγR - Fcγ receptor
FRET - Fluorescence resonance energy transfer
GALNT14 - Polypeptide N-acetylgalactosaminyltransferase 14
GALNT3 - Polypeptide N-Acetylgalactosaminyltransferase 3
GPI - Glycosyl phosphotydylinositol
Grb2 - Growth factor receptor-bound protein 2
GTP - Guanosine triphosphate
GVT - Graft-versus-tumor
HCl - Hydrochloric acid
HCV - Hepatitis C virus
HDAC4 - Histone deacetylase 4
HEK - Human embryonic kidney
HGF - Hepatocyte growth factor
HIV - Human immunodeficiency virus
H-RAS V12 - Activated allele of GTPase H-Ras
hTERT - Human telomerase catalytic subunit
IgG - Immunoglobulin G
IKK – I kappa B kinase
IKK α - IkappaB kinase alpha
IKK β - IkappaB kinase beta
IPTG - Isopropyl β -D-1-thiogalactopyranoside
JAK - Janus kinase
JNK - c-Jun N-terminal kinase
KCl - Potassium chloride
KD – Knock down
KO – Knock out
LB - Lysogeny broth
LUBAC - Linear ubiquitin chain formation complex, linear ubiquitin chain assembly complex
mAb – Monoclonal antibodies
MADD - MAPK-activating death domain containing protein
MAPK - Mitogen-activated protein kinase

MDC - Monodansyl cadaverin
MEK - Mitogen-activated protein kinase kinase
MFI - Mean fluorescent intensities
MLC2 - Myosin Light Chain 2
MLKL - Mixed lineage kinase domain-like protein
MnCl₂ - Manganese (II) chloride
MOMP - Mitochondria outer membrane permeabilization
MOPS - 3-morpholinopropane-1-sulfonic acid
Myc - Proto-oncogene cMyc
Na₂HPO₄ - Disodium phosphate
NaCl - Sodium chloride
NaF - Sodium fluoride
NEMO - NF-kappa-B essential modulator
NK cells - Natural killer cells
NMIIA - Non-muscle myosin IIA
NMIIB - Non-muscle myosin IIB
NSCLC - Non-small cell lung cancer
NTP - Nucleoside triphosphate
P53 - Tumor suppressor protein 53
PAGE - Polyacrylamide gel electrophoresis
PARAs - Pro-apoptotic receptor antagonists
PARP - Poly ADP ribose polymerase
PBS - Phosphate-buffered saline PCR - polymerase chain reaction
PI3 kinase - Phosphoinositide 3-kinase
PIDD - P53-induced protein with a death domain
PIP2 - Phosphatidylinositol 4,5-bisphosphate
PIP3 - Phosphatidylinositol (3,4,5)-trisphosphate
PLAD - Pre-ligand binding assembly domain
RA - Rheumatoid arthritis
RAc - All-trans-retinyl acetate
RANK - Receptor Activator of NF-kappaB
RANKL - Receptor activator of NF-kappaB ligand
Rb protein - Retinoblastoma protein
RbCl₂ - Rubidium chloride
RCE - Raft/caveolar endocytosis
rhTRAIL – Recombinant human TRAIL
RIK3 - Receptor-interacting serine/threonine kinase 3
RIPK1 - Receptor-interacting serine/threonine-protein kinase 1
RSV - Rous sarcoma virus
RT – Reverse transcription

Scr - Tyrosine kinase
SD - Standard deviation
SDS - Sodium dodecyl sulfate
SHARPIN - SHANK Associated RH Domain Interactor
STAT - Signal transducer and activator of transcription
SV40 – Simian virus 40
SV40 ER - Early region of the Simian virus 40 virus
TAB2 - TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein 2
TAB3 - TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein 3
TAK1 - Transforming growth factor beta-activated kinase 1
TBE - Tris/Borate/EDTA
tGVHD - Thymic host versus graft disease
TNF α - Tumor necrosis factor α
TNFR1 - Tumor necrosis factor receptor 1
TNFR2 - Tumor necrosis factor receptor 2
TRADD - Tumor necrosis factor receptor 1 (TNFR1)-associated death domain protein
TRAF1 - TNF Receptor Associated Factor 1
TRAF2 - Tumor necrosis factor receptor-associated factor 2
TRAIL-R1 – Tumor necrosis factor-related apoptosis-inducing ligand receptor 1
TRAIL-R2 – Tumor necrosis factor-related apoptosis-inducing ligand receptor 2
TRAIL-R3 – Tumor necrosis factor-related apoptosis-inducing ligand receptor 3
TRAIL-R4 – Tumor necrosis factor-related apoptosis-inducing ligand receptor 4
TRAIL-Rs - Tumor necrosis factor-related apoptosis-inducing ligand receptors
TRID – TNF receptor internalization domain
Tris (hydroxymethyl) aminomethane - 2-Amino-2-hydroxymethyl-propane-1,3-diol
uPA - urokinase plasminogen activator
uPAR - Urokinase-type plasminogen activator receptor
X-Gal - 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
XIAP - X-linked inhibitor of apoptosis protein TNF α - Tumor necrosis factor alpha

INTRODUCTION

1. CELL HOMEOSTASIS AND CELL DEATH

Cells of an organism can undergo division, proliferation, differentiation and death. These processes balance and ensure a constant number of cells and sustain life of a multicellular organism. Cell death is a crucial mechanism that provides homeostasis, immune system regulation, and protection against viral and microbial pathogens and is an indispensable process during development yielding to the remodeling of organs and tissues. Currently, four major types of cell death are known: apoptosis (intrinsic and extrinsic), necrosis (necroptosis, necrosis and secondary necrosis), autophagy associated cell death (chaperon-mediated autophagy, microautophagy and macroautophagy) and pyroptosis. Notably, different types of cell death have distinct morphological, functional, physiological, immunological consequences and enzymological characteristics that altogether build a very well developed and coordinated system that ensures cell and organism homeostasis.

1.1. Necrosis

Necrosis was discovered as an immunogenic ¹ mechanism of cell death that occurs in an uncontrolled way in response to harsh environmental conditions, such as severe physical damage, hyperthermia and detergent-induced cytolysis ². In that regard, necrotic cell death was shown to be involved in the elimination of unwanted cells, attraction of immune competent cells, regeneration processes, activation-induced cell death of T lymphocytes and different pathophysiological conditions (neurodegeneration, ischemia-reperfusion, etc) ^{3,4}. Importantly, necrotic cell death can also undergo strict regulation provided by a number of proteins. This type of regulated necrosis is called necroptosis. Briefly, necroptosis is induced by the formation of a signaling complex called necrosome in response to death receptors (TNF R1, FAS, DR4 and DR5) stimulation with their specific ligands. Notably, this type of cell death prevails in the absence of caspase activation, suggesting that necroptosis is an alternative cell death pathway ^{5,6}. In that respect, inhibition of death receptor-mediated apoptosis using chemical caspase inhibitors or virally encoded proteins such as CrmA, leads to the association of RIPK1 (Receptor-interacting serine/threonine-protein kinase 1) and RIK3 (Receptor-interacting serine/threonine kinase 3) in a complex called necrosome. Consequently, auto- and transphosphorylation of RIPK1 and RIPK3 result in the oligomerization of MLKL leading to the activation of necroptosis ^{7,8}. The third type of necrotic cell death is called secondary necrosis. In this respect, it was suggested that apoptotic cells that do not undergo phagocytosis trigger secondary necrosis with cytoplasmic swelling, rupture of the plasma membrane, cell disintegration and release of intracellular components ⁹. Overall, the three types of necrosis share a common set of events namely, lysosomal membrane permeabilization, mitochondrial hyperpolarization and plasma membrane permeabilization. Nonetheless, the main difference is that necroptosis and secondary necrosis are characterized by the highly-regulated death receptor-mediated signaling phase followed by the disintegration phase, while necrosis lacks the signaling phase starting immediately from the disintegration phase ².

1.2. Autophagy

Autophagy is a proteolytic catabolic process induced in response to starvation and other stress situations, that leads to the degradation and recycling of cellular components such as proteins, lipids, DNA, RNA molecules and organelles and provides a continuous flow and turnover of building blocks in the cell. Lysosomes are membrane organelles that contain a variety of enzymes which are able to break down proteins, lipids, nucleic acids and carbohydrates, thus playing a central role in the process of degradation. According to the mechanisms by which cellular components are transported to the lysosomes, three types of autophagy are distinguished: chaperon-mediated autophagy, microautophagy and macroautophagy¹⁰. Although the pro-survival and cytoprotective role of autophagy is well defined, autophagic vacuoles are often observed in dying cells. Therefore, it was suggested that autophagy and cell death can act synergistically or inhibitory depending on the cellular context, nature of stimuli and environmental conditions^{4,10-12}.

1.3. Pyroptosis

Pyroptosis is a programmed cell death observed in macrophages, monocytes and dendritic cells infected with microbial pathogens. Therefore, pyroptosis is a part of a host defense system against pathogens. Pyroptosis is a caspase-1-dependent process. Notably, caspase-1 is recruited and activated in the complex called inflammasome. In turn, activated caspase-1 induces digestion of its target proteins (proteins of translation machinery, cytoskeleton proteins, etc.) and pore formation in the plasma membrane leading to cell swelling and lysis associated with the pro-inflammatory response^{13,14}.

1.4. Apoptosis

Apoptosis is a non-immunogenic¹⁵ programmed cell death that is characterized by membrane blebbing, chromatin condensation and formation of apoptotic bodies that undergo engulfment by phagocytes and surrounding cells. Apoptotic cell death plays a crucial role in maintaining homeostasis during embryonic development as well as in adult organisms. Virus or bacteria infected cells as well as cells that go through a malignant transformation undergo apoptosis leading to the elimination of a pathogen niche and preventing tumor development. Apoptosis requires the involvement of two main protein families such as caspases (cysteine aspartate specific proteases) and Bcl-2 family of proteins that can be activated by an intrinsic or extrinsic cell death mechanisms (for details see chapter 2.1, 2.2). Briefly, an intrinsic apoptotic pathway is activated by DNA damage and cytotoxic insults through Bcl2 family members that control mitochondria integrity. In contrast, an extrinsic apoptotic pathway is activated through the stimulation of death receptors: TNFR1, FAS, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) followed by the formation of the death inducing signaling complex (DISC) and activation of caspases. A detailed characterization of the molecular mechanisms of apoptosis will be provided in the following chapters.

Summarizing, different types of cell death ensure distinct mechanisms of intercellular communication, immunological and physiological consequences playing a crucial role in the proper functioning of a multicellular organism.

2. MECHANISMS OF APOPTOTIC CELL DEATH

Cells that are damaged, infected or aged are eliminated by apoptosis. Apoptosis is orchestrated by a group of proteins that belong to the caspase (cysteine aspartate specific proteases) family. Some members of this family act as initiators of apoptosis (caspase 2, 8, 9, 10), while others play an executory function (caspase 3, 6 and 7). Initiator caspases are upstream of executor caspases and lead to the activation of executor caspases that in turn cleave a number of their substrates leading to DNA fragmentation, disassembly of Golgi apparatus, cytoskeleton disruption, chromatin condensation, membrane blebbing and formation of apoptotic bodies.

Caspases are expressed as zymogens which are inactive procaspases that upon stimulation undergo complex activation processes through their cleavage at specific aspartate residues. Importantly, activation of initiator caspases requires their recruitment through DED (death effector domain) to specific signaling platforms such as death inducing signaling complex (DISC) for caspase 8 and 10, apoptosome for caspase 9 and PIDDosome for caspase 2. Consequently, recruitment of initiator caspases to activation platforms leads to their autoproteolytic cleavage and activation. Further, active initiator caspases activate downstream executor caspases through cleavage. There are two main ways of caspase activation: an extrinsic or receptor-mediated pathway and an intrinsic or mitochondria-mediated pathway¹⁶.

2.1 Intrinsic apoptotic pathway

An intrinsic apoptotic pathway occurs in response to internal stimuli, such as DNA damage, transcription/translation inhibition as well as cytotoxic insults and involves mitochondria outer membrane permeabilization (MOMP). The process of MOMP requires proteins that belong to the Bcl-2 family, a protein family involved in the regulation of apoptosis that contains pro- and anti-apoptotic members. Importantly, in normal conditions anti-apoptotic Bcl-2 family members (Bcl-xL, Bcl-2, Mcl1, Bcl-w, A1) prevent mitochondria damage by inhibiting pro-apoptotic members of this family (Bax, Bak, Bok, Bim, Bid, Bik, Puma, Noxa, Bad, Bmf, Hrk)¹⁷. However, upon stress conditions the cell is relieved of the inhibitory effect, thus allowing oligomerization of Bak and Bax and formation of pores in the mitochondria membrane through which cytochrome c is translocated to the cytoplasm. Further, cytochrome c released from mitochondria forms a complex with Apaf-1 (apoptosome) leading to the recruitment and activation of caspase 9. Activated caspase 9 cleaves downstream executor caspases 3, 6, 7, that further cleave their specific substrates leading to cell death. A number of mitochondrial proteins can promote or inhibit apoptosis once released to the cytoplasm. For example, Smac/Diablo binds to XIAP and relieves caspase 9, 3 and 7 from its inhibitory activity¹⁸.

2.2. Extrinsic apoptotic pathway. Type I and type 2 cells

An extrinsic apoptotic pathway is a type of apoptotic cell death that is induced by the stimulation of death receptors of the TNF superfamily (TNFR1, FAS, DR4, DR5) with their specific ligands (TNF α , FASL and TRAIL)¹⁹. Signaling through death receptors can lead to a variety of signaling outcomes, such as proliferation, migration, inflammation and cell death. Triggering of FAS- and TRAIL-induced

apoptosis through an extrinsic pathway requires formation of the death-inducing signaling complex (DISC). Briefly, stimulation of FAS, DR4, and DR5 leads to receptor oligomerization and recruitment of an adaptor molecule FADD followed by the recruitment of initiator caspases 8 or 10, resulting in the assembly of the DISC and initiation of apoptotic signaling. Importantly, once initiator procaspases are activated at the DISC, two outcomes are possible: either the caspase cascade triggered by initiator caspases is sufficient to commit cells to apoptosis (referred as type I cells) or the activation of the intrinsic mitochondrial-apoptotic pathway is required to achieve cell death (type II cells). Contrary to TRAIL and FAS signaling, stimulation of TNFR1 primarily leads to the formation of the pro-survival complex I containing TNFR1, TRADD, TRAF2, RIPK1 and cIAPs, resulting in the activation of pro-inflammatory and pro-survival pathways. Internalization of TNFR1 leads to the formation of the pro-apoptotic complex II comprising of FADD, caspase 8, TRADD or RIPK1 ⁴⁶. A detailed characterization of the molecular mechanisms that regulate and balance different signaling outcomes upon stimulation of death receptors is provided in the following chapters.

3. MOLECULAR MECHANISMS OF TNF FAMILY CYTOKINE SIGNALING

TNF α , FAS and TRAIL signaling systems provide a specific set of mechanisms leading to cell death, survival, migration and pro-inflammatory response depending on the cellular context. Although TNF α , FASL and TRAIL activate a defined set of signaling pathways (apoptotic cell death, pro-inflammatory pathways and pro-survival cascades) and share many similarities in terms of molecular players that orchestrate particular signaling pathways each system has different molecular mechanisms that balance these functionally different signaling outcomes. Therefore, in order to better understand each of those systems, the molecular basis that balance the signaling induced by TNF α , FASL and TRAIL should be discussed in a comprehensive and integrated way.

3.1. Molecular basis of TNF α signaling

3.1.1. Molecular mechanisms of TNF α -induced signaling

TNF receptor 1 stimulation leads to the triggering of two distinct signaling outcomes: survival and death. Soon after TNF α discovery it became obvious that most cell types do not die upon TNF α treatment, and that the primary response to TNF α stimulation is the activation of pro-inflammatory (NF- κ B) and pro-survival pathways (MAPK, Akt, JNK). Interestingly, cells undergo apoptosis if TNF α treatment is performed in combination with protein synthesis inhibitor. These observations revealed three important features of TNF α signaling:

1. TNF α induces expression of pro-survival genes
2. the apoptotic machinery preexists, as it does not require protein synthesis, however apoptosis is not an initial signaling activated by TNF α
3. the activation of pro-survival pathways is a crucial negative regulator of apoptosis induction upon TNF α treatment.

Overall, TNF α -induced NF- κ B-dependent activation of pro-survival genes is sufficient for most cells to survive treatment, even though the apoptotic machinery preexists in the cell. O'Donnell and Ting called this phenomenon «NF- κ B paradox»²⁰. Importantly, numerous studies reported that strict balance between survival and apoptotic pathways activation critically relies on the regulation and functioning of TNF α -induced complex I.

TNF α binds two plasma membrane receptors: TNFR1 and TNFR2^{21,22}. TNF α stimulation of TNFR1 leads to the formation of a membrane-bound TNFR1-containing signaling complex, also known as complex I, resulting in the triggering of pro-inflammatory and pro-survival signaling cascades. Importantly, formation of the complex I is initiated by the recruitment of the adaptor molecule TRADD. TRADD binding to TNFR1 occurs via the death domains. Further, TRADD recruits RIPK1 - Receptor Interacting Protein Kinase 1 (RIPK1) - an essential signaling protein that belongs to the group of threonine/serine protein kinases with distinct non-kinase domains but relatively conservative kinase domains²³. Notably, RIPK1 can also directly interact with TNFR1 and compete for binding with

TRADD²⁴. The ability of RIPK1 to interact with death receptors and adaptor proteins (TRADD and FADD) is determined by the death domains (DD) of both proteins. RIPK1 association with TRADD leads to the recruitment of E3 ubiquitin ligases such as TRAF2, cIAP1, cIAP2 and LUBAC (linear ubiquitin chain formation complex, composed of HOIP), HOIL-1, SHARPIN²⁵. Importantly, it was suggested that TRAF2 mediates recruitment of cIAP1 and cIAP2 followed by the recruitment of LUBAC (linear ubiquitin chain assembly complex)^{26,27}. Overall, these events lead to the assembly of the membrane-bound complex I. Interestingly, shortly after stimulation complex I gets internalized leading to the formation of the pro-apoptotic complex II, composed of FADD and caspase 8, TRADD or RIPK1²⁸ (Fig 1a).

Ubiquitination of RIPK1 plays a pivotal role in the formation and functioning of the complex I and complex II and, thus, in the regulation of TNF α signaling (Fig 1b, c). RIPK1 can exert opposite biological functions- from MAPK and NF- κ B activation to apoptosis and necroptosis depending on its ubiquitination status^{29,30}. In that regard, E3 ubiquitin ligases TRAF2 and cIAP1/2 by being recruited to TNF α -induced signaling complex I, lead to the non-degradative K-63 linkage specific ubiquitination of RIPK1³¹. It has been found that TRAF2 requires TRADD for being recruited to the complex I²⁴ and a lipid mediator sphingosine-1-phosphate in order to function as E3 ligase and attach non-degradative ubiquitin chains to RIPK1³², whereas, cIAPs can act directly. Interestingly, it was suggested that TRAF2 interacts with cIAPs and keeps the level of cIAP1 stable within the complex³³, showing that the presence of TRAF2 is essential for cIAPs to exert E3 ligase function towards RIPK1. Importantly, a single amino acid change (position 377) in RIPK1 leads to the perturbation of NF- κ B and MAPK activation in response to TNF α . Notably, lysine residue in the 377 position of RIPK1 is a major K63-ubiquitin acceptor. These results unravel that RIPK1 ubiquitination is required for the activation of non-apoptotic pathways. Therefore, RIPK1 ubiquitination is considered to be the first checkpoint in TNFR1-mediated death/survival signaling. Ubiquitination of RIPK1 creates a platform for TAB2/TAB3 and NEMO binding^{34,27,35,36} followed by the recruitment of TAK1 and IKK α /IKK β . Further, TAK1 activates IKK α /IKK β by phosphorylation that in turn leads to I κ B α degradation and enables NF- κ B translocation into the nuclei that promotes expression of anti-apoptotic proteins including cFlip, TRAF1, TRAF2, cIAP1, cIAP2, etc. This step of TNF signaling is considered to be a second checkpoint, as apoptotic cell death will be blocked if the expression level of NF- κ B-induced anti-apoptotic proteins is sufficient (Fig 1a).

Importantly, negative regulation of complex I function and positive regulation of death inducing complexes formation is determined by several RIPK1-involved ubiquitin-dependent mechanisms:

1. De-ubiquitination of RIPK1 by CYLD and OTULIN results in the destabilization of complex I, disassociation of TRADD and RIPK1, recruitment of FADD and caspase 8 and transformation of complex I into complex IIb (Fig 1c). In this case apoptosis triggered by complex IIb is usually RIPK1-dependent. Notably, the model proposed by O'Donnell and Ting suggests that NEMO binding to ubiquitinated RIPK1 prevents caspase 8 binding, while RIPK1 de-ubiquitination allows caspase 8 assembly with RIPK1 and triggering of apoptosis²⁰.

2. A20 and CARP-2 convert K63-Ub chains of RIPK1 to K48-Ub chains, while another E3 ligase TRIAD3A directly interacts with K48-Ub linkage and targets RIPK1 to proteosomal degradation. Consequently, most likely this leads to the formation of the complex IIa containing TRADD, FADD and caspase 8 resulting in RIPK1-independent apoptosis^{37, 38} (Fig 1b).

3. De-ubiquitinated RIPK1 can undergo autophosphorylation resulting in the formation of another signaling complex named «rioptosome» containing RIPK1, RIPK3 and MLKL³⁹. This complex leads to the activation of caspase-independent cell death named necroptosis (Fig 1c). Importantly, it was revealed that non-apoptotic RIPK1-mediated cell death can occur due to the direct association of RIPK1 with TNFR1. In that regard, Zheng et al. showed that TRADD is required for TRAF2 recruitment to the complex I as well as for the activation of NF- κ B signaling. Therefore, lack of TRADD in the complex I may prevent TRAF2 recruitment and RIPK1 ubiquitination in complex I resulting in the blocking of survival and apoptotic pathways activation and leading to non-apoptotic cell death²⁴.

Formation of pro-apoptotic or pro-necrotic complexes appear to be cell type-dependent. Interestingly, some cell lines that trigger apoptosis upon TNF α stimulation undergo necroptosis if caspases activity is blocked^{40,41}. In that regard, it was reported that RIPK1 can be cleaved by caspase 8⁴². Therefore, blocking caspase 8 activity may prevent RIPK1 cleavage and enable necroptosis⁴³. This assumption rises a conundrum: on one hand, RIPK1 is required for apoptotic cell death, but on the other hand, this protein undergoes caspase 8-mediated cleavage. Perhaps, RIPK1 dissociated from the complex I supports efficient FADD and caspase 8 recruitment, but once caspase 8 is activated RIPK1 undergoes cleavage. In contrast when TNF α -induced NF- κ B-mediated expression of cFlip is high enough to block caspase 8 cleavage, RIPK1 can undergo autophosphorylation, recruit RIPK3 and MLKL leading to necroptosis. This may serve as a cellular defense mechanism: if one cell death pathway is inhibited, the other takes over.

Importantly, recent data suggests that RIPK1 ubiquitination followed by NF- κ B activation is not the only mechanisms that guides survival and avoids apoptotic signal progression upon TNF α treatment. Dondelinger et al. reported that IKK α /IKK β can prevent RIPK1-dependent apoptosis and necroptosis independently of NF- κ B activation. It has been shown that IKK α /IKK β induce RIPK1 phosphorylation in the complex I and suppress the formation of complex II. Notably, the formation of complex II is prevented in Nec1 (necrostatin 1 – a compound that blocks RIPK1 kinase activity) treated cells highlighting an important role of RIPK1 kinase activity in this process. In that regard, it has been suggested that IKK α /IKK β -induced RIPK1 phosphorylation may negatively regulate RIPK1 kinase activity (autophosphorylation), and, therefore, activation of apoptosis and necroptosis⁴⁴.

Overall, regulation of TNF α signaling largely relies on RIPK1 posttranslational modifications, such as polyubiquitination, autophosphorylation and phosphorylation suggesting a crucial role of RIPK1 in balancing pro-survival and pro-apoptotic signaling activation upon TNF α challenge.

Interestingly, the canonical complex I containing TRADD, RIPK1, TRAF2 and cIAPs is not the only complex triggering pro-survival pathways upon TNF α stimulation. It has been shown that MAPK-activating death domain containing protein (MADD) can interact with TNFR1 and is crucial for the

recruitment of Grb2, Sos1/2 and RAS. Furthermore, this study demonstrated that MADD is essential for Erk1/2 activation but not for the induction of NF- κ B⁴⁵.

Altogether, highly specific regulation of TNF α signaling occurs at initial steps of TNF α signaling and is provided by a number of regulatory proteins that form TNF α -induced signaling complexes and control their functions.

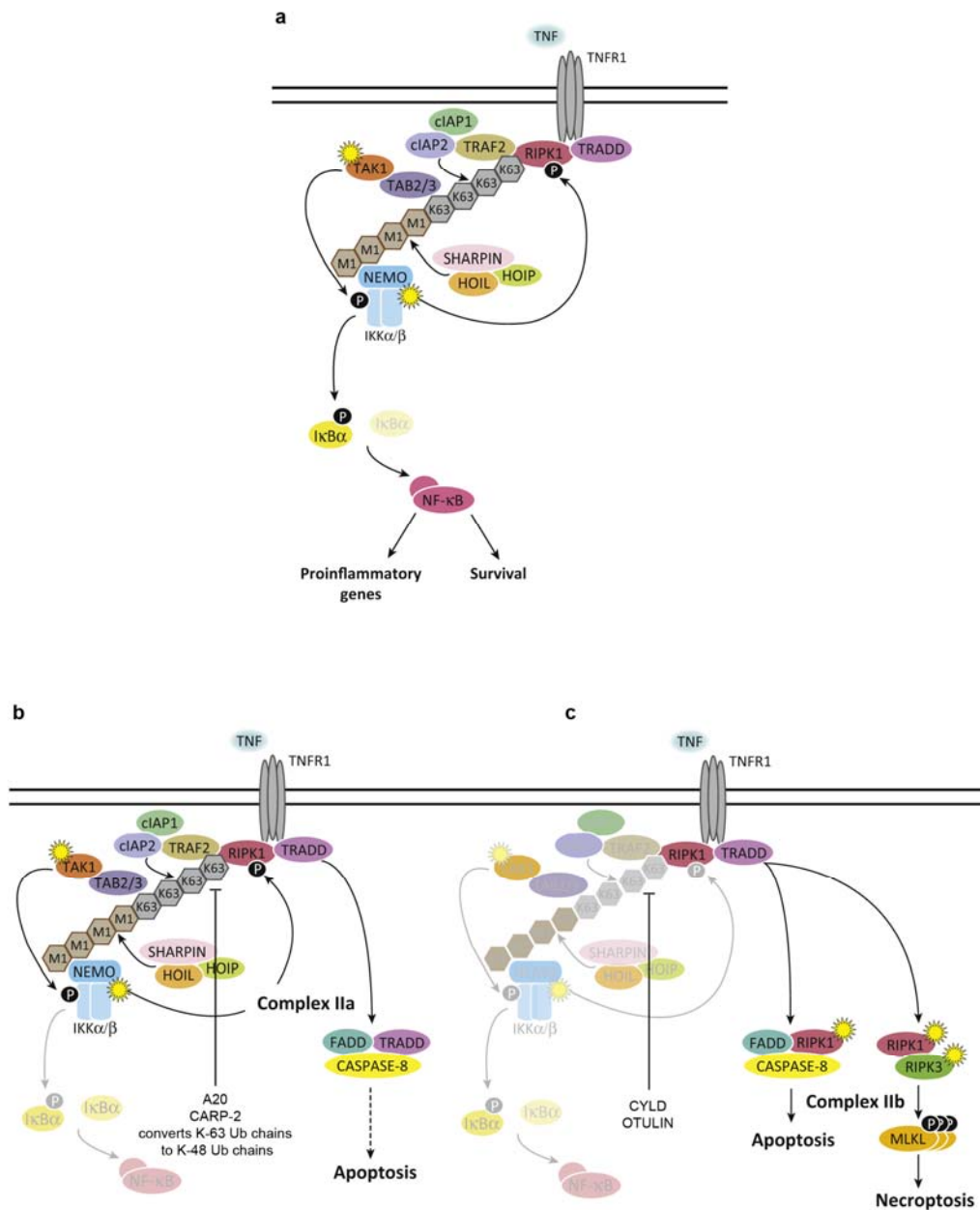


Figure 1. Molecular mechanisms of TNF α signaling. **a** - Formation of the TNF α -induced complex I followed by the triggering of pro-inflammatory and pro-survival response. **b** - Formation of the TNF α -stimulated complex IIa leading to the activation of apoptotic response. **c** - Formation of the TNF α -mediated complex IIb resulting in the induction of apoptosis and/or necroptosis. For details see text. Figure was taken from⁴⁶ and modified.

3.1.2. Historical perspective and current clinical application.

The cytotoxic effect of TNF α was initially demonstrated by William Coley in 1891. He observed that in sarcoma patients who underwent serious bacterial infection some tumors disappeared due to necrotic cell death⁴⁷. However, obvious problems with this approach emerged very soon: in some patients it was very difficult to induce infection, whereas in others infection was lethal. Therefore, due to the unpredictability of this approach, a vaccine containing inactivated bacteria *Streptococcus pyogenes* and *Serratia marcescens* was generated. This product became known as Coley's Toxins and was commercially produced by Parke Davis & Company and widely used for cancer therapy for 30 years since 1899. In the following years W. Coley injected more than 1000 inoperable bone and soft-tissue sarcomas patients with bacterial extracts to activate immune system and induce tumor diminishment^{48,49}. This was one of the first time immunotherapy was applied for cancer. Obviously, molecular determinants and inducers of the effect he had recorded were not known. Only years after Coley's observation, in 1975, Lloyd Old and colleagues identified TNF α in the lipopolysaccharide fraction isolated from bacterial lysates, as an active component of Coley's toxins^{50,51}. However, even though Coley documented many favorable responses for the shrinkage of the malignancy, the follow up of his patients was poorly documented. Moreover, different batches of Coley's Toxins had different activity and the positive response to treatment was not reproducible. Therefore, Coley's work met a lot of criticism⁵². Three years after his discovery Coley received severe criticism published in the *Journal of the American Medical Association (JAMA)*:

«There is no longer much question of the entire failure of the toxin injections, as a cure for sarcomata and malignant growths. During the last six months the alleged remedy has been faithfully tried by many surgeons, but so far not a single well-authenticated case of recovery has been reported»

The following studies of TNF α signaling revealed, that only some tumors responded with the activation of cell death upon stimulation with TNF α . Contrary, more common effect of TNF α treatment was systemic toxicity - lethal inflammatory shock syndrome⁵³. Importantly, this property of TNF α gave rise to another direction of its clinical application, which turned out to be very successful. Currently, TNF α blockers are intensively used in the treatment of rheumatoid arthritis, psoriasis and Crohn's disease^{50,54}. Overall, development and application of TNF α blockers led to the revolution in the treatment of chronic inflammatory diseases.

3.2. TRAIL signaling

3.2.1. TRAIL structure

TRAIL is a type II transmembrane protein. Full length TRAIL consists of a TNF-like C-terminal extracellular receptor-binding domain, a transmembrane helix and a short N-terminal cytoplasmic domain. The extracellular domain of TRAIL can be proteolytically cleaved from the cell surface and form a soluble molecule. Therefore, TRAIL signaling can be initiated via cell to cell contact or by a soluble TRAIL molecule^{55,56}. Membrane bound as well as soluble TRAIL form homotrimers that are non-covalently linked and coordinated by three cysteine residues that bind a central zinc ion which stabilizes TRAIL homotrimers^{57,58}. A crystallographic study showed that TRAIL homotrimers can bind three TRAIL receptors leading to the clustering of TRAIL receptors⁵⁹. Importantly, homotrimeric TRAIL that binds three TRAIL receptors⁵⁹ is much more potent in the activation of cell death as compared to bivalent agonistic TRAIL receptors antibodies that bind only two receptors (crystallographic study)⁶⁰ and need additional crosslinking for an effective apoptotic function⁶¹⁻⁶⁴. Thus, the trimeric structure of TRAIL that induces receptor clustering is essential for an effective apoptotic signaling. Notably, extracellular pH is a critical factor for the conformation of TRAIL molecule. In that regard, Nam and Choi suggested that at physiological pH TRAIL specifically binds to its receptors and induces apoptosis, while at pH less than 4.5 TRAIL undergoes conformational changes leading to its increased affinity to membranes resulting in pores formation, membrane permeabilization and, thus causing nonspecific cytotoxicity⁶⁵. Altogether, structural characteristics of TRAIL molecule play a crucial role in the specificity and efficiency of TRAIL signaling.

3.2.2. TRAIL receptors

TRAIL signaling originates from TRAIL binding to its receptors. TRAIL specifically binds five receptors. The TRAIL receptors – TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 are type 1 transmembrane proteins. The extracellular C-terminal part includes three cysteine rich domains (CRDs). CRDs form a ligand binding site and a pre-ligand assembly domain at the membrane distal region (PLAD)^{66,67}. CRDs are followed by a so-called stalk region and a transmembrane domain. It was suggested that the transmembrane domain plays an important role in the transduction of apoptotic signaling upon TRAIL binding⁶⁸. Two out of five TRAIL receptors - DR4 and DR5- are called death receptors due to the presence of the functional intracellular death domain (DD) and, thus an ability to activate apoptosis upon stimulation. Two others are: decoy receptor 1 (DcR1), which has no death domain, and decoy receptor 2 (DcR2) that has a truncated death domain and, therefore both are incapable of triggering apoptotic cell death. TRAIL can also interact with a soluble receptor – osteoprotegerin, which also binds RANKL, thus preventing its association with the receptor RANK (plays an important role in bone and skeletal homeostasis)⁶⁹.

DR5 is expressed as two functional splicing isoforms referred to DR5 short (DR5-S) and DR5 long (DR5-L). Importantly, both isoforms of DR5 are functionally active and lead to the activation of apoptosis upon stimulation with TRAIL^{70,71}. DR5-S and DR5-L differ by the insertion of 87 bp resulting in the in-frame insertion of 29 amino acids and an inclusion of an intron at the junction between

transmembrane domain and TRAIL binding domain. Notably, DR4 does not contain this insertion and, therefore is more similar to DR5-S. Importantly, DR4 and DR5 levels do not generally show a direct correlation with the sensitivity to TRAIL-induced apoptosis⁷². Furthermore, functional differences between these receptors have not yet been identified. Both of them can trigger apoptosis in different cell lines: DR4 selectively induces apoptosis in chronic lymphocytic leukemia cells⁷³ and in primary lymphoid malignancies⁷⁴, while DR5 often mediates apoptosis in epithelial-derived malignancies^{75,76}.

Contrary to death receptors, DcR1 lacks a death domain and binds to the plasma membrane via GPI (glycosyl phosphotydylinositol)⁷⁷, while DcR2 contains a nonfunctional death domain and is widely expressed on the cell surface of many cell types. It was hypothesized that decoy receptors compete for TRAIL binding with death receptors allowing tumor cells to bypass apoptosis. However, some cells expressing decoy receptors are sensitive to TRAIL⁷⁸⁻⁸¹, and others lacking decoys are resistant⁸¹. Moreover, it has been recently shown that the overexpression of DcR2 correlates with an enhanced level of non-apoptotic signaling activation in a pre-ligand manner⁸²⁻⁸⁴. This indicates that the role of decoy receptors is more complex than originally thought and that DcR2 may not only compete for TRAIL binding with death receptors but may also participate in the activation of non-apoptotic pathways.

Clustering of TRAIL receptors upon exposure to the ligand plays a crucial role in the efficient signaling transduction and may be one of the main determinants of response. Effective clustering is determined by the trimeric structure of TRAIL that provides binding sites for three receptors⁵⁷. Moreover, posttranslational modifications of TRAIL-Rs such as O-glycosylation promote an effective receptor oligomerization⁷⁰. Importantly, receptor oligomerization and clustering promote efficient FADD recruitment, which in turn leads to the recruitment of caspase 8, its effective dimerization and cleavage⁷⁰. In that regard, it was shown that many death receptor agonistic antibodies require crosslinking for their optimal pro-apoptotic activity *in vitro* and *in vivo*^{62-64,85}. Contrary to trimeric TRAIL, death receptor agonistic antibodies can bind only two receptors and this property reduces clustering⁶⁰. Graves et al. recently reported that treatment of several cancer cell lines with a combination of TRAIL and bivalent DR5 agonist antibodies AMG 655 increases their antitumor activity by promoting enhanced clustering. Their model suggests that AMG 655 serves as a bridge between DR5-TRAIL complexes resulting in their stabilization and formation of high ordered receptor structures⁶⁰. The fact that death receptor agonists require an additional crosslinking for efficient receptor clustering may be the main reason for their unsuccessful use in clinical trials. In that regard, it has been shown that TRAIL-R antibodies need additional clustering from leukocyte FcγRs to trigger apoptosis⁶⁴. In humans death receptor agonists have to compete with immunoglobulins for binding to FcγR, while immunodeficient mouse xenograft models usually have very low levels of IgGs (Fig 2a). These findings could potentially explain positive results of death receptor agonists in mouse models and their failure in clinical trials⁶⁴ (Fig 2a). In order to overcome this issue, Gieffers et al. generated a molecule that consists of the Fc part of human IgG1 and two single-chain TRAIL receptor binding domains each of which contains three TRAIL promoter subsequences, thus, creating six receptor binding sites. This molecule shows a significantly higher efficiency in the induction of apoptosis in tumor cell lines and in xenograft mouse models as compared to TRAIL and DR5-agonists⁸⁶ (Fig 2b). Moreover, its activity is independent of cross-linking. Altogether, receptors clustering is definitely an important step in an effective transduction

of apoptotic signaling upon challenge with TRAIL. Notably, the role of receptors clustering in the triggering of non-apoptotic pathways upon TRAIL treatment remains unknown. Furthermore, TRAIL receptors can also form heterocomplexes upon exposure to this cytokine. However, biological significance of this association remains largely unknown.

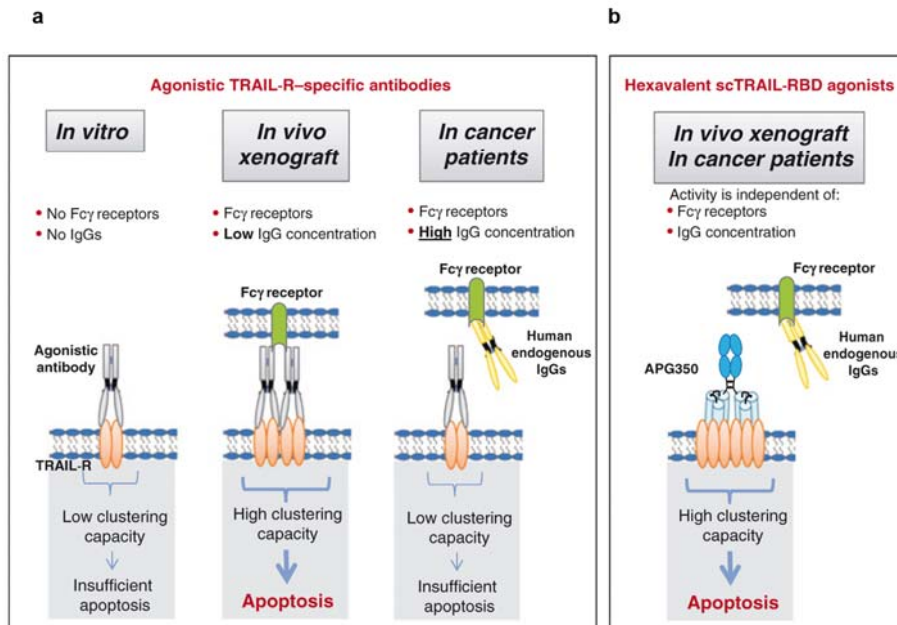


Figure 2. Model of action of agonistic TRAIL receptor antibodies compared with APG350. **a**- Agonistic monoclonal antibodies bind two TRAIL receptors and, therefore, require additional clustering by Fcγ receptors in xenograft models for effective receptor clustering and efficient antitumor activity. Contrary, in humans endogenous IgGs compete for FcγRs and, thus, prevent receptor clustering and diminish pro-apoptotic action of agonistic antibodies. **b** - APG350 bears six TRAIL receptors binding sites and does not require Fcγ receptor-mediated clustering for antitumor activity. For details see text. Figure was taken from ⁸⁶.

3.2.3. TRAIL DISC. Role of FADD, caspase 8 and cFlip in the regulation of the DISC function

TRAIL-R oligomerization and clustering results in the formation of the Death-Inducing Signaling Complex (DISC) (Fig 3a). Receptor clustering is required for the recruitment of the adaptor molecule FADD (FAS associated death domain containing protein) ⁸⁷. FADD is a molecule that contains a death domain (DD) in C-terminus and a death effector domain (DED) in N-terminus. Via its death domain FADD interacts with the intracellular domain of death receptors leading to the formation of a binding platform for signal transduction, therefore, serving as a molecular bridge that links receptors and downstream apoptotic machinery. The death effector domain of FADD molecule allows the recruitment of initiator caspases, such as caspase 8 and 10. Caspase 8 and 10 along with caspase 2 and 9 belong to the initiator caspases class. These caspases have a complex mechanism of activation and require molecular adaptors to be enzymatically activated. Caspase 8 plays a crucial role in apoptotic signal transduction mechanism of death receptors such as FAS, TNFR1 and TRAIL-Rs. Following recruitment

to the DISC caspase 8 undergoes proteolytic activation through several steps. The first cleavage leads to the generation of p12 subunit that is further processed to p10 and p43/p41 subunit. p41/p43 subunits activate each other through trans-catalytic cleavage between DEDs resulting in the generation of p18. p18 and p10 associate into an active caspase 8 heterotetramer and initiate caspases activation and cell death cascade ⁸⁸.

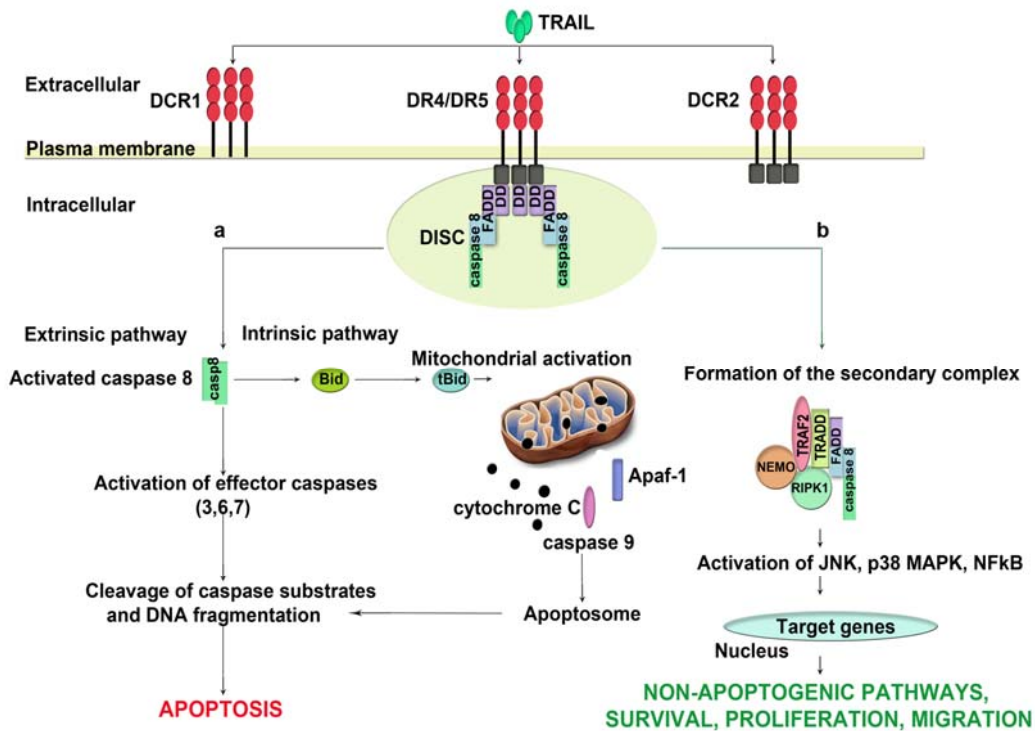


Figure 3. Molecular mechanisms of TRAIL-induced signaling. **a-** TRAIL signaling pathway (adapted from ⁸⁹). TRAIL binds as a homotrimeric molecule to its four receptors. Two decoy receptors (DcR1 and DcR2) either lack or present a truncated intracellular domain, thus do not trigger intracellular signaling upon TRAIL binding. The death receptors 4 and 5 (DR4 and DR5) harbor an intracellular death domain (DD) that, upon TRAIL binding, recruits adaptor protein FADD followed by the recruitment of caspase 8 or 10 forming the death-inducing signaling complex (DISC). Upon DISC formation, initiator caspases 8 and/or 10 get cleaved and activated. Activated initiator caspases lead to the activation of effector caspases 3, 6 and/or 7 directly (extrinsic death pathway) or through the mitochondrial amplification loop (intrinsic death pathway). For details see text. **b-** Under certain conditions, non-apoptotic intracellular signaling can be triggered upon DISC formation, resulting in cell proliferation, migration and survival. For details see text.

The original model of the structure of DISC proposed that the trimeric ligand binds one receptor trimer which in turn leads to the recruitment of three molecules of FADD and three molecules of caspase 8 ⁹⁰. However, this model did not consider the requirement of caspase 8 dimerization for its efficient processing. Further Valley et al. proposed a model of TRAIL receptor clustering in which pre-ligand trimers of DR5 undergo structural reorganization upon TRAIL stimulation, resulting in dimerization of

TRAIL-DR5 trimers⁷¹. Moreover, similar hypothesis were made in FAS system suggesting that three receptors bind three molecules of FADD and three molecules of caspase 8 that are further brought together resulting in the formation of a large organized receptors network^{91,92}. This model could explain how dimerization of caspase 8 is achieved. However, recent studies using mass spectrometry approach revealed that core DISC components such as TRAIL receptor and FADD are present in the native DISC at a ratio 3:1⁹³, suggesting that several receptors might be required for efficient FADD recruitment. Importantly, there is up to several fold more caspase 8 than FADD in the TRAIL DISC⁹³ and FAS DISC⁹⁴. Based on this, a new model was proposed suggesting that caspase 8 is recruited not only to FADD but also interacts with other caspase 8 molecules via DEDs and form chain structures (Fig 4). Indeed, the interaction of caspase 8, caspase10 and cFlip through their DEDs was documented before^{95,96}. Moreover, upon transient overexpression, caspase 8 interacts via DEDs and form fiber structures^{97,98}. By mutating DED2 of caspase 8, Dickens et al. provided a direct experimental proof that caspase 8 is recruited to FADD through DED1 and that the process of caspase 8 chain formation relies on caspase 8 DED1-DED2 interaction⁹³. Notably, DED-mediated assembly of caspase 8 molecules in the DISC is essential for caspase 8 activation and apoptosis. In that regard, Dickens et al. hypothesized that the ability of caspase 8 to form a chain structure provides an important regulatory node that balance apoptotic and non-apoptotic pathways activation upon exposure to TRAIL.

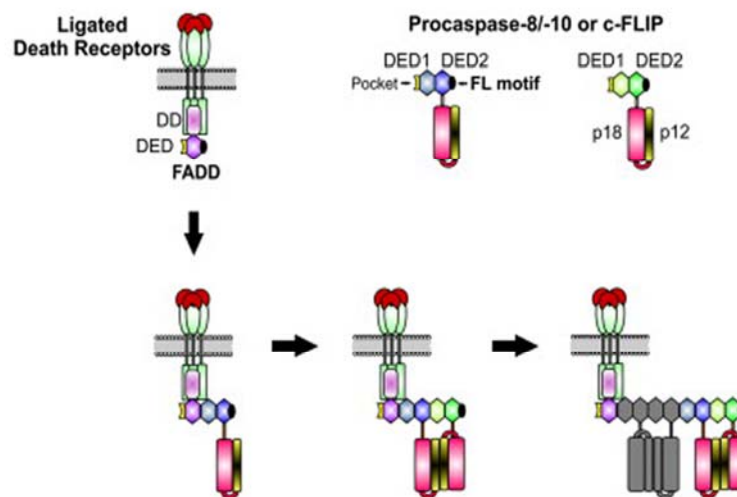


Figure 4. Model of caspase 8 recruitment to the DISC. Death receptors stimulation leads to the recruitment of FADD, which in turn recruits procaspase 8 via DED. Each FADD molecule can recruit only one caspase 8 molecule. Further, each molecule of procaspase 8 associates with another one through DED1- DED2 interaction leading to the formation of chain structures that facilitate caspase 8 activation. For details see text. Figure was taken from⁹³.

Importantly, cFlip is a catalytically inactive caspase 8 homolog that plays a crucial role in the regulation of caspase 8 activity at the DISC. Notably, three cFlip splice isoforms have been identified: cFlipL (long), cFlipS (short) and cFlipR. cFlipL contains two DEDs, however lacks enzymatic activity due to the mutation (cysteine to tyrosine) in the enzymatic domain, while cFlipS (short) consists of two DEDs. Initially, it was believed that cFlip competes with caspase 8 for their binding to the DISC and, therefore inhibits caspase 8 recruitment and activation. However, recent data show that cFlip requires caspase 8 to be recruited to the DISC, suggesting that there is a strict hierarchy in caspase 8/cFlip association with the DISC ⁹⁹. Therefore, this data shows that the inhibitory function of cFlip is not due to the competition with caspase 8 for their binding to the DISC. Furthermore, it was demonstrated that different isoforms of cFlip exhibit differential regulation of caspase 8 activity in the DISC. Notably, cFlipL can activate or inhibit caspase 8 depending on caspase 8: cFlipL ratio ¹⁰⁰⁻¹⁰³. Contrary, cFlip S completely blocks caspase 8 processing by preventing DED-mediated caspase 8 assembly ¹⁰³. Overall, these data suggest that caspase 8 chain formation leads to its effective activation and, thus is an important regulatory node in the TRAIL signaling cascade.

In that regard, several mutations in caspase 8 gene were identified in numerous gastric, hepatocellular ^{104,105} and head and neck squamous cell carcinomas ¹⁰⁶. Some missense mutations detected in advanced gastric carcinomas lead to the change in amino acids of DEDs without affecting other domains. Importantly, expression of these mutants significantly decreases cell death, however, does not prevent interaction with FADD ¹⁰⁴. Even though, DED mutants can interact with FADD, formation of caspase 8 chains might be perturbed, thus blocking its activity. Furthermore, expression of caspase 8 gene bearing frameshift mutation leading to the termination of amino acid synthesis in the p10 protease subunit results in the blocking of caspase 8 activity, however, does not prevent association with FADD ¹⁰⁵. This might enable activation of inappropriate alternative cell survival signaling pathways where the catalytic activity of caspase 8 is not required ¹⁰². This hypothesis is supported by recent research of Ando et al. who reported that the catalytically inactive mutant of caspase 8 failed to induce apoptosis, but enhanced NF- κ B activation relative to wild type ¹⁰⁶.

An additional level of DISC regulation at the level of caspase 8 is added by posttranslational modifications of caspase 8. Two recent studies identified polyubiquitination as a mechanism of positive and negative regulation of apoptosis upon TRAIL treatment at the level of caspase 8. Jin et al. found that culling 3 - based E3 ligase is associated with the DISC upon TRAIL treatment and leads to caspase 8 polyubiquitination followed by the recruitment of ubiquitin binding protein p62 that promotes caspase 8 aggregation and processing ¹⁰⁷. In contrast, another ubiquitin ligase - TRAF2, was shown to be recruited to the DISC, where it exerts an anti-apoptotic function through K48 linkage specific polyubiquitination of p18 targeting it to proteosomal degradation ¹⁰⁸. Moreover, caspase 8 can undergo phosphorylation that modulates its functionality. Caspase 8 phosphorylation by Erk1/2 ¹⁰⁹, focal adhesion kinase and proto-oncogene tyrosine protein kinase (SCR) ^{110,111} prevents caspase 8 cleavage at the DISC.

Importantly, signaling through TRAIL receptors can occur independently of the canonical DISC ¹¹²⁻¹¹⁵. In that respect, it was shown that endogenously expressed TRAIL can induce DR5-mediated cancer cell

invasion independently of FADD and TRAIL receptor death domains; however it requires the membrane proximal domain of DR5 for Rac1 recruitment and Akt activation¹¹⁵. These results point to a very critical feature of TRAIL signaling: cells that lack functional DISC components including the adaptor molecule FADD and/or functional receptor death domain, can respond to TRAIL treatment by the activation of signaling pathways that lead to tumor progression, invasion and metastasis. Therefore, death receptors by itself can serve as a signaling bifurcation point depending on the recruitment of certain adaptor and signaling molecules.

3.2.4. TRAIL-induced non-apoptotic complex/es. RIPK1 and TRAF2 as a modulators of response to TRAIL

Importantly, besides being able to trigger apoptotic cell death, TRAIL can also induce activation of pro-inflammatory and pro-survival pathways¹¹⁶. The mechanisms of TRAIL-induced caspase activation and apoptosis are well defined, however the molecular basis regulating activation of survival pathways is not clear and often controversial. Initially, it was reported that TRADD and RIPK1, known to participate in the triggering of pro-survival cascades in TNF α signaling, are recruited to DR4 and DR5. Importantly, these findings were obtained in overexpression models, and were not reproducible under endogenous conditions^{117,118}. Later Varfolomeev et al. hypothesized that in terms of pro-apoptotic and pro-survival complexes formation, TRAIL and TNF α signaling are similar; however with an opposite sequence of events: first TRAIL induces the formation of the pro-apoptotic receptor-associated DISC, followed by the assembly of a cytoplasmic secondary complex resulting in survival pathways initiation¹¹⁹. Indeed, TRAIL receptor immunoprecipitation did not lead to the detection of RIPK1, TRAF2 or NEMO in TRAIL treated HT1080 cells. In contrast, secondary IP of RIPK1 from DISC – immunodepleted lysates revealed FADD and caspase 8 as well as TRAF2 and NEMO being co-immunoprecipitated with RIPK1 (Fig 3b)¹¹⁹. These results suggested that indeed TRAIL can induce the formation of the primary receptor-associated DISC and a secondary intracellular non-apoptotic signaling platform. To support the role of the secondary complex in the activation of pro-survival pathways, it was shown that the knock down of either RIPK1, FADD or caspase 8 prevents I κ B α phosphorylation, whereas cFlip and TRAF2 depletion results in an increased level of I κ B α phosphorylation^{119,120}. Interestingly, inhibition of caspase 8 activity abrogated JNK and p38MAPK activation, delayed I κ B α phosphorylation and attenuated RIPK1/FADD association.

On the other hand, further studies revealed that RIPK1 is recruited to the DISC upon TRAIL treatment in resistant to TRAIL-induced cell death NSCLC cells¹¹². Moreover, a recent study of Gonzalez et al. provided evidences for TRAF2 co-immunoprecipitation with DR4 and DR5¹⁰⁸. Furthermore, it has been suggested that Akt-phosphorylated MADD interacts with DR4 and DR5 in a TRAIL-independent manner and prevents FADD binding to the DISC suggesting its anti-apoptotic role in the regulation of TRAIL signaling¹²¹. These findings suggest that anti-apoptotic proteins such as RIPK1, MADD and TRAF2 can be recruited to TRAIL receptors. Overall, these data highlight that the mechanisms of secondary complex formation and composition are still poorly understood and may be cell type dependent. From the mechanistic point of view, it was reported that TRAIL-induced RIPK1 and cFlip association with TRAIL receptors determines DISC localization within non-lipid raft compartments of

the plasma membrane and plays a crucial role in the regulation of death versus survival pathways activation. In that respect, Song et al. reported that TRAIL-stimulated DISC of TRAIL sensitive NSCLC cells is formed in both raft and non-raft fractions of the plasma membrane, whereas DISC assembly in TRAIL resistant NSCLC cells occurs exclusively in the non-raft fraction. Furthermore, it was shown that the DISC of TRAIL resistant NSCLC cells contains RIPK1 and cFlip. RIPK1 and cFlip knock down lead to the redistribution of the DISC to the lipid raft fraction, caspase 8 cleavage, activation of apoptosis and disruption of Erk1/2 and NF- κ B triggering in resistant NSCLC cells ¹²². This data provides evidence that subcellular compartmentalization of TRAIL-induced complexes may play a crucial role in the activation of apoptotic and survival pathways.

Altogether, the rising number of evidence shows that anti-apoptotic proteins such as RIPK1, TRAF2 and MADD can be recruited to the DISC and lead to the blocking of apoptosis and/or activation of pro-survival pathways. This data supports that diversification of TRAIL signaling occurs at the level of the DISC. However, the molecular mechanisms that modulate TRAIL signaling bifurcation remain largely elusive (see chapter 4).

3.2.5. Functional role of endogenous TRAIL

TRAIL and its receptors are expressed in cells from the spleen, lung, liver, prostate, cerebellum, colon, kidney, spine, thyroid, tonsil, innate and adaptive immune system cells ^{81,123}. Importantly, expression of TRAIL and TRAIL receptors on the surface of natural killers, macrophages, neutrophils, dendritic cells, T and B lymphocytes plays an essential role in the regulation of immune cells functions such as immunosuppressive, immunoregulatory, pro-viral and antiviral, homeostatic and immune surveillance ^{124,125}. Among those functions, TRAIL-induced apoptosis is essential for the proper functioning of the immune system ¹²⁶. Indeed, TRAIL-induced activation of apoptosis is crucial for the removal of auto reactive T and B cells, killing of infected cells, immune surveillance against tumors and metastasis ¹²³.

TRAIL in the regulation of immune system functioning

An important role of TRAIL in response to various viral infections has been demonstrated. For example, TRAIL-expressing NK cells eliminate encephalomyocarditis-infected cells ¹²⁷. On the other hand, CD8 T cells that express FASL and TRAIL induce apoptosis in influenza virus infected cells and HCV infected hepatocytes, while sparing normal liver cells ¹²³. Importantly, the role of TRAIL in the antiviral function of immune system largely depends on the nature of viral infection. Contrary to the antiviral role of TRAIL in encephalomyocarditis and influenza virus infected cells, TRAIL signaling can also support pathogenesis of the disease in HIV infected patients. Briefly, persistent upregulation of IFN α expression during HIV infection leads to the increased expression of TRAIL and TRAIL receptors on pDCs cells (type I interferon producing cells – IkpDC3), CD4 T cells and memory B cells (Fig 5). Consequently, upregulation of TRAIL and TRAIL-R expression results in the triggering of apoptosis by infected as well as non-infected CD4 T cells, leading to the development of immunodeficiency syndrome during HIV infection ¹²⁸⁻¹³⁰. This example of TRAIL signaling in the immune system demonstrates that TRAIL not only plays a role in antiviral response, but also in the pathogenesis of some virus-induced diseases.

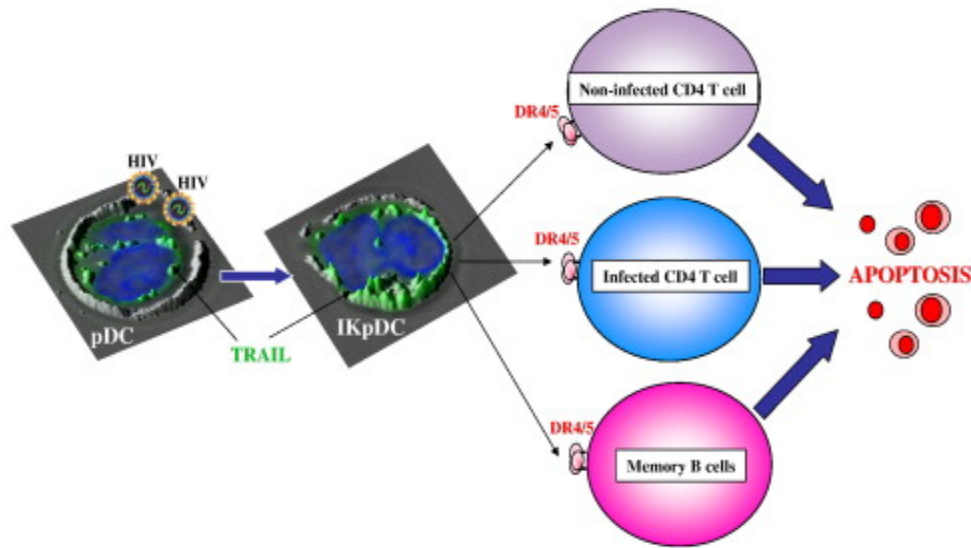


Figure 5. Role of TRAIL in the progression of HIV infection. For details see text. Figure was taken from ¹³⁰.

Importantly, the role of TRAIL in immune homeostasis has been unraveled. In that regard, it was shown that CD8 T cells acquire distinct genetic programs by being primed in the presence or absence of CD4 T helper lymphocytes. Briefly, CD8 T cells primed in the presence of CD4 T helper lymphocytes (CD8 “helped” cells) acquire the ability for an autonomous secondary expansion upon re-stimulation with an antigen - the key feature of immune memory. Contrary, CD8 T cells primed in the absence of CD4 helper T cells (CD8 “helpless” cells) do not have the capacity to undergo a second round of expansion upon re-stimulation with an antigen. Moreover, CD8 “helpless” cells undergo cell death upon second round of stimulation. Interestingly, CD8 “helpless” cells are characterized by an increased level of TRAIL and FAS expression upon re-stimulation, whereas Bcl-2, Bcl-xL and cFlip (anti-apoptotic proteins) are selectively upregulated in CD8 “helped” cells. Blocking TRAIL receptors in CD8 “helpless” cells leads to the ability of these cells to undergo secondary expansion upon re-stimulation with an antigen. Furthermore, TRAIL secreted by CD8 “helpless” cells inhibits secondary expansion of CD8 “helped” cells. These results show an important role of TRAIL signaling in the regulation of CD8 T cells secondary expansion upon re-stimulation. Overall, these data, suggest that endogenous TRAIL exhibits a crucial role in the regulation of immune system and also in the maintenance of immune homeostasis (Fig 6) ^{131,132}.

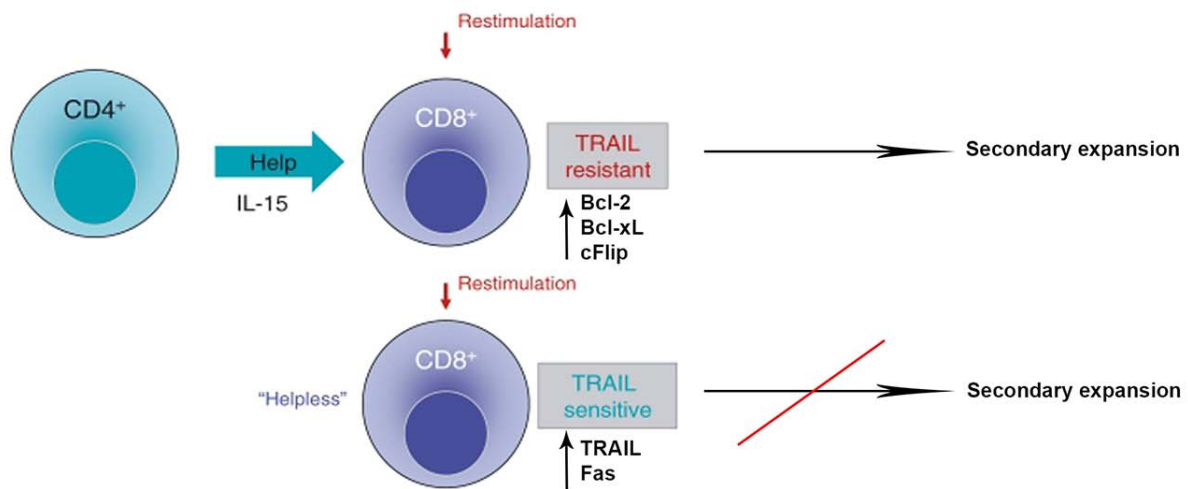


Figure 6. Role of TRAIL in T-cells homeostasis. For details see text. Figure was taken from ¹²³ and modified.

TRAIL and autoimmune disease

Several studies indicate that TRAIL can also contribute to the control of autoimmune diseases, including rheumatoid arthritis (RA) ¹³²⁻¹³⁵. This disease is characterized by a hyperplasia of the synovium that consists of fibroblast- and macrophage-like synoviocytes. Consequently, expanding synoviocyte invasion leads to the irreversible destruction of the bone and cartilage. Importantly, immune cells (macrophages, lymphocytes, dendritic cells and neutrophils) that infiltrate synovium create an inflammatory environment, therefore promoting hyperplasia of synoviocytes. Notably, TRAIL was shown to down-regulate the autoimmune response in RA by inducing apoptosis in cells that populate the synovium (Fig 7) ^{125,136-138}. In that regard, Song et al. demonstrated that blocking endogenous TRAIL with the soluble TRAIL receptor (DR5) prevents TRAIL-induced apoptosis in lymphocytes and leads to an enhanced arthritic inflammation in the mouse collagen-induced arthritis model ¹²⁵. However, some studies revealed that only a part of fibroblast-like synoviocytes die upon exposure to TRAIL, while those cells that survive activate a proliferative response by the triggering of Erk1/2, p38MAPK and Akt pathways ¹³⁹⁻¹⁴¹. Altogether, the role of TRAIL in the suppression or progression of rheumatoid arthritis remains elusive and further studies are necessary in order to evaluate the actual role of endogenous TRAIL in rheumatoid arthritis.

On the other hand, TRAIL was also shown to be involved in the etiology of type one and type two diabetes ¹⁴². *In vivo* studies suggested that TRAIL might protect against diabetes by inducing apoptosis in T cells and /or supporting survival of beta-cells of the pancreas in type one diabetes ¹⁴³⁻¹⁴⁵, as well as by preventing fat mass accumulation and exerting an anti-inflammatory function in type two diabetes ^{146,147}. Nevertheless, the precise molecular mechanisms of TRAIL protective action against diabetes are poorly understood and need further studies.

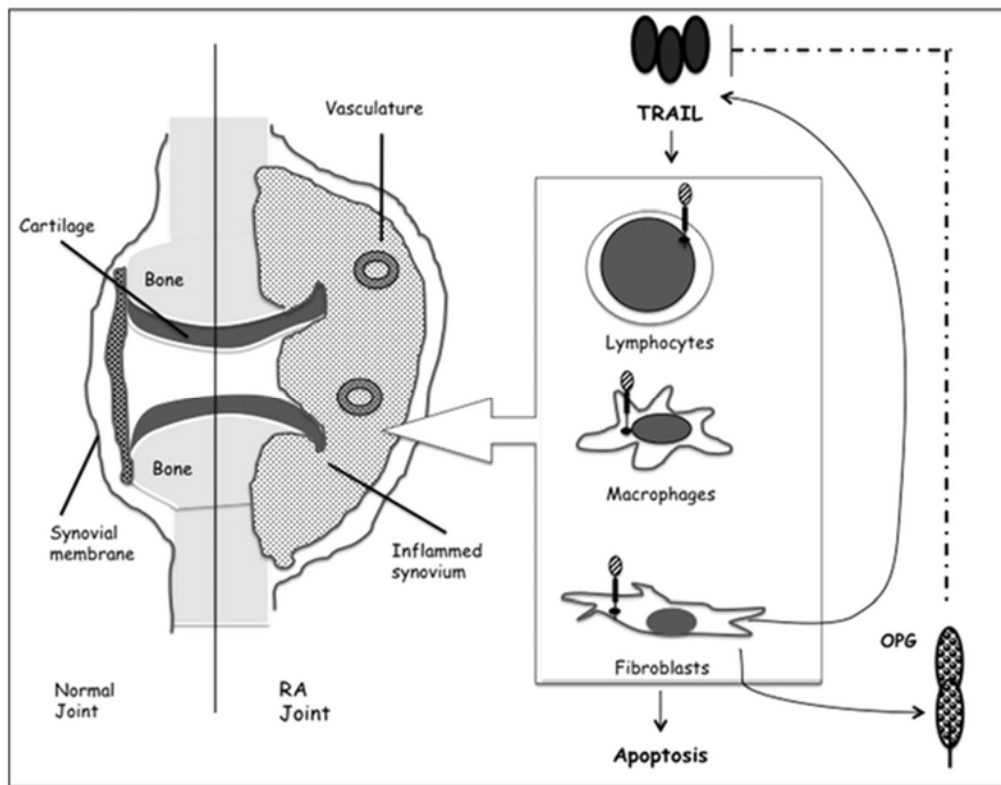


Figure 7. Role of TRAIL in rheumatoid arthritis. TRAIL can induce apoptosis in synovial cells. Moreover, synovial fibroblasts besides triggering TRAIL-induced apoptosis, constitutively express and synthesize TRAIL. Furthermore, they synthesize OPG that plays a role of receptor antagonist and inhibits TRAIL-mediated apoptosis. For details see text. Figure was taken from ¹⁴⁸.

TRAIL in host versus graft disease

It was shown that TRAIL plays a critical role in the thymic host versus graft disease (tGVHD) caused by an allogeneic bone marrow transplantation (allo-BMT). Briefly, tGVHD is the most common complication after allogeneic bone marrow transplantation initiated by host-reactive donor T cells leading to the damage of the architecture and composition of the thymic microenvironment. However, the molecular mechanisms regulating an interaction of donor alloreactive T cells and host thymic tissue remain poorly defined. Interestingly, Na et al. suggested that donor alloactivated T cells that rapidly infiltrate thymus can express TRAIL leading to the induction of cytolytic pathways in host thymic cells resulting in the damage of thymus cytoarchitecture ¹⁴⁹. Therefore, improved T cell depletion strategies and methods that allow blocking of TRAIL signaling can help to prevent tGVHD and improve thymopoiesis after an allo-BMT ¹⁴⁹. However, the authors of another study reported that TRAIL does not have any significant impact on the development of host-versus-graft disease ¹⁵⁰. Therefore, the actual role of TRAIL in the progression of tGVHD is poorly understood and remains controversial.

TRAIL and cancer

Immune surveillance against tumors is mediated by the adaptive and the innate immune system. Natural killer (NK) cells present a component of the innate immune system that provides natural protection against the development of primary tumors^{151,152}. The adaptive immune system is presented by CD8 positive T cells. Importantly, a number of reports indicate that endogenous TRAIL plays a crucial role in NK and T cell-mediated immune surveillance against tumors in mammals^{85,131,153-157}. In that regard, it was shown that TRAIL expressed by liver natural killer cells plays an anti-metastatic function in several different cancer models¹⁵³. Briefly, administration of anti-TRAIL mAb enhanced liver metastasis of L929 and LB27.4 cells. Importantly, anti-TRAIL mAb administration in NK cell – depleted mice did not significantly enhance metastasis suggesting that TRAIL is involved in the NK cell-mediated suppression of L929 and LB27.4 liver metastasis¹⁵³. Moreover, it was reported that TRAIL deficient mice are predisposed to the development of spontaneous hematological malignancies¹⁵⁷. Overall, these results provided a clear evidence for the physiological role of TRAIL in innate immune system-mediated tumor suppression.

Importantly, it was also reported that TRAIL is involved in T cell-mediated tumor immunity in graft-versus-tumor scenario (GVT) after an allogenic hematopoietic cell transplantation (AHCT)¹⁵⁰. AHCT is a therapeutic procedure used in a variety of hematopoietic malignancies. Schmaltz et al. reported that mice from two different models of GVHD and GVT that were recipients of donor TRAIL^{-/-} T cells had significantly decreased survival (mortality was caused by tumor) as compared to those mice receiving wild type T cells. Therefore, this study provides evidences that increasing expression of TRAIL on donor T cells may enhance the GVT effect after AHCT. Notably, another evidence of TRAIL involvement in the adaptive immune response against tumors was provided by Takeda et al. It was shown that the administration of anti-DR5 monoclonal antibodies results in the rejection of primary tumors which leads to the development of T-cell specific immunity and resistance to the second challenge with tumor⁸⁵.

Therefore, endogenously expressed TRAIL is an important effector molecule for immune surveillance and has promise for the immunotherapy of tumors. These observations gave rise to research lines aimed to evaluate the potential of exogenously administrated TRAIL in anti-cancer therapy.

3.2.6 Potential therapeutic advantages of TRAIL

TRAIL was discovered as a ligand that selectively induced apoptosis in tumor cells while sparing normal cells^{158,159}. Moreover, TRAIL did not lead to the systemic shock syndrome, observed with TNF α , and did not cause cytotoxic effects seen with FasL¹⁶⁰. Therefore, TRAIL has drawn particular attention as a candidate that could be used for systemic cancer treatment without being toxic.

Normal cell response to TRAIL

Initial *in vitro* studies raised concerns regarding cytotoxicity of TRAIL towards normal hepatocytes, prostate, brain and endothelial cells. In the late 1990s, in order to facilitate the production of TRAIL, rhTRAIL was tagged at its amino-terminal end using His- and Flag-tag. *In vitro* studies using tagged rhTRAIL revealed cytotoxicity of the ligand against normal cells such as isolated human astrocytes, hepatocytes, human brain cells, endothelial and prostate cells bringing up a problem regarding the toxicity of systemic TRAIL administration in humans^{80,161-166}. However, further studies utilizing non-tagged TRAIL had shown negligible cytotoxic effects of TRAIL in normal cells, and suggested that previous observations could have been resulted from the usage of tagged TRAIL^{167,168}. In that regard, using circular dichroism approach, it was suggested that His-tagged TRAIL had less ordered structure, formed aggregates at 37°C and was relatively unstable in solution compared to untagged TRAIL¹⁶⁷. Furthermore, it was reported that while different versions of tagged TRAIL display toxic effect on primary human hepatocytes, the same compounds exert moderate cytotoxic effect on hepatic explants obtained from the same patients¹⁶⁹. Liver is a complex organ containing several cell types that can modulate hepatocytes response to TRAIL. Therefore, taking into account this report, primary human hepatocytes may not be a suitable model to study the hepatotoxicity of TRAIL. Moreover, Volkmann et al. reported that hepatic explants obtained from patients suffering from hepatitis C and steatosis were sensitive to TRAIL-induced toxicity suggesting that the clinical application of TRAIL should be restricted to patients that do not suffer from inflammatory liver diseases.

Importantly, resistance of the majority of normal cells to TRAIL-induced apoptosis does not mean that TRAIL cannot trigger any other pathways in this cellular context. In that regard, it was reported that TRAIL activates pro-survival and pro-migratory pathways in human endothelial cells^{165,170,171} and vascular smooth muscle cells¹⁷². These results could argue for the possible role of TRAIL in the endothelial homeostasis and angiogenesis. Therefore, further safe clinical administration of TRAIL requires critical evaluation of all possible adverse effects associated with the application of TRAIL, including cytotoxicity and non-apoptotic signaling activation in normal cells.

Cancer cell response to TRAIL

According to their response to TRAIL, cancer cells can be grouped into three classes; (i) the entire population commits to apoptosis or (ii) is inherently resistant to cell death induced by TRAIL and (iii) only a fraction of the cell population dies while another survives the treatment¹⁷³ (referred to as “fractional killing”¹⁷⁴). Importantly, in those populations of cancer cells that display fractional killing in response to TRAIL, cells that survive treatment may «sense» TRAIL and undergo activation of survival, migratory and pro-inflammatory pathways such as Erk1/2, p38MAPK, NF-κB and Akt^{173,175,176}. Therefore, TRAIL treatment applied to tumors that respond by fractional killing can result in an increased proliferation of TRAIL survivors, enhanced migration and metastasis. Thus, this phenomenon requires further studies in order to determine the molecular mechanisms balancing TRAIL-induced apoptotic and non-apoptotic signaling activation.

The heterogeneity in response to TRAIL within the same population can arise due to several different factors, mechanisms and cell states, among them:

1. Genetic differences between cells within the population. This type of heterogeneity within the population occurs when a cell acquires a stable heritable genetic alteration and gives rise to the clone that responds differently to the stimuli as compared to the rest of the population ¹⁷⁷;
2. Epigenetic differences. This type of heterogeneity occurs when a small subpopulation of cells acquire altered chromatin state that can change a transcriptional profile of these cells, thus, influencing cellular response to a particular stimuli ¹⁷⁸;
3. Different phases of the cell cycle. Levels of proteins playing a key role in the regulation of apoptosis are highly variable during the cell cycle. Therefore, the response of cancer cells to TRAIL within the same population can be dramatically different (apoptosis or survival activation, different time from exposure to ligand to cell death, etc.) depending on the cell cycle phase at the moment of treatment. In that regard, it has been shown that mitotic cells display higher sensitivity to TRAIL than those in G1 and S phase due to the decreased basal level of pErk1/2 in mitotic cells ¹⁰⁹.
4. Natural stochastic fluctuations in protein levels. In this case two newborn sister cells are very similar in protein levels, as they inherited it from the parental cell. However, after several cell division due to the performance of protein biosynthesis machinery each sister cell accumulates differences in protein levels, therefore, becoming as similar with its sister cell as with any other cell randomly chosen from the population ¹⁷⁵. Briefly, P. Sorger's team using live microscopy and mathematical modeling of cellular processes involved in the apoptotic cascade in response to TRAIL suggested that death and survival signaling are activated within the same cell of the population. However, the final outcome (death or survival) strongly relies on the natural cell-to-cell variability in the level and concentration of pro- and anti-apoptotic proteins before exposure to TRAIL ^{175,179}. They have shown that two sister cells behave the same in response to TRAIL ('time-to-death') up to 50h after «birth» (approximately two cell divisions), however later they behave as two randomly chosen cells from the population. Importantly, the variability in time-to-death arises from pre-MOMP processes, such as TRAIL binding to its receptors, formation of the DISC and activation of effector caspases (caspase 8 and 10) suggesting that early events of TRAIL signaling play a crucial role in TRAIL signaling regulation. Overall this data shows that the non-genetic cell-to-cell variability causes two genetically identical cells to respond differently to the same stimuli due to its acquisition of a unique proteome short time after «birth» and gives rise to two distinct phenotypes. Interestingly, when cell fate of two sister cells was analyzed, mostly both of them died or both of them survived without correlation to time-to-death.
5. Receptors internalization, cytoskeletal rearrangements, formation of functionally different TRAIL-induced complexes and their localization in different cellular and plasma membrane compartments, post translational modifications of TRAIL-DISC members. Detailed characterization of mentioned above factors in the regulation of TRAIL signaling is provided in chapter 3 and 4.

TRAIL-induced cancer cell resistance

An increasing number of evidences suggests that treatment of drug-sensitive cancer cell populations with anticancer agents results not only in the death of the majority of the population but also in the generation of a small subpopulation of drug resistant cells. Importantly, often resistance established in response to drug has a reversible nature, meaning that when drug treatment is discontinued cells revert to the initial response suggesting that acquired resistance to anticancer drugs does not require a stable genetic mutation. This phenomenon was called “re-treatment response”^{180,181}. In that regard, it was observed that some patients with non-small lung carcinoma that initially respond well to the treatment with EGFR tyrosine kinase inhibitors later show therapy failure. Interestingly, efficient response to EGFR tyrosine kinase inhibitors is observed again after “drug holiday”^{180,181}. This and other results¹⁸² indicate an establishment of a reversible “drug tolerant state” upon exposure to anticancer drugs.

Importantly, the phenomenon of reversible resistance is also observed in a number of TRAIL treated cancer cell populations. Indeed, we¹⁷³ and others¹⁷⁴ have shown that cancer cells surviving the first TRAIL treatment may acquire resistance to TRAIL-induced apoptosis upon continues exposures. However, when TRAIL treatment is discontinued cells revert to the initial response to this ligand, suggesting that TRAIL-induced resistance is not due to the selection of inherently resistant clones, but that survival is induced and maintained in TRAIL-dependent manner^{176,183}. Importantly, cells can enter the state of reversible resistance even in the presence of caspases inhibitors, suggesting that the establishment of resistance is not only based on cell-to-cell variability, but is also ligand-induced and ligand-maintained¹⁷⁶. In that respect, we unraveled that TRAIL induces non-apoptotic pathway activation at early time points in response to the ligand. This data suggests that activation of TRAIL-induced apoptotic and non-apoptotic pathways may occur within the initial population. Therefore, it is possible that the establishment of TRAIL resistance is based on (i) the stochastic cell-to-cell variability that determines initial cellular response to TRAIL and (ii) TRAIL-induced activation of pro-survival pathways that further allows the establishment and maintenance of resistance to this cytokine. When TRAIL treatment is discontinued and, thus the trigger that ensures survival is removed, cells revert to the initial response after several cell cycles. A detailed discussion of TRAIL-induced fractional killing and the reversible resistance phenomenon is provided in chapters: “Results and Discussion” and “Discussion”.

Altogether, further studies are required to unravel molecular players that modulate TRAIL signaling and balance apoptotic and pro-survival pathways activation occurred within isogenic cell populations. These results will provide insights for evaluation of whether combined therapies that target components promoting the activation of survival signaling would allow the use of TRAIL against tumors that respond in fractional killing, without the risk of generating tumor progression and resistance.

4. NATURE OF SIGNALING BIFURCATION (APOPTOSIS VERSUS SURVIVAL) IN RESPONSE TO TRAIL, TNF α AND FASL

4.1. Functional role of receptor internalization in signaling for death or survival

Endocytosis - is a process of internalization of membrane-associated proteins through the formation of membrane bound vesicles. This process plays a crucial role in the nutrition uptake, growth factor signaling, etc. Clathrin-mediated endocytosis is one of the best characterized pathways of endocytosis. The endocytosis machinery contains an adaptor protein 2 (AP2) that provides a link between clathrin and membrane proteins that undergo trafficking. Plasma membrane invagination of clathrin coated pits requires further GTP (guanosine triphosphate) hydrolysis of dynamin leading to the release of endocytic transport vesicles from the plasma membrane. After uncoating and fusion with early endosomes the fate of vesicles and their cargo can vary from lysosomal degradation to recycling and trafficking back to the plasma membrane. Notably, endocytosis can also occur in a clathrin-independent manner¹⁸⁴⁻¹⁸⁷. For example, raft/caveolar endocytosis (RCE) is one form of the clathrin-independent endocytosis. This type of endocytosis is based on the invagination of the caveolae and cholesterol/sphingolipid - enriched plasma membrane domains (lipid rafts)¹⁸⁸. Furthermore, several dynamin-independent endocytosis pathways have been also discovered^{189,190}. Importantly, after stimulation and clustering many plasma membrane receptors undergo internalization that is usually associated with the down regulation of signaling transduction, as it leads to the degradation of receptors. However, it has been reported that internalized receptors are still active and that signaling continues along the endocytic pathways¹⁹¹⁻¹⁹⁴. Moreover, receptors internalization can support formation of signaling complexes and intensify the signal¹⁹⁵.

An important role of receptor localization within different subcellular compartments in cell fate decisions has been reasonably demonstrated in TNF α and FAS signaling and to significantly lesser extend in TRAIL-induced cascades. A number of early studies showed that TNF α -induced apoptosis is internalization-dependent¹⁹⁶⁻¹⁹⁸, whereas the formation of complex I and the activation of pro-survival signaling occurs at the plasma membrane and is independent of internalization¹⁹⁸. Contrary, Woo et al. reported that TNFR1 internalization is required for the activation of MAPK and Akt pathway as well as for the production of reactive oxygen species, but not for NF- κ B signaling in non-phagocytic cells¹⁹⁹. Importantly and contrary to the previous statement that complex II lacks TNFR1 (see chapter 3.1.1)^{28,200}, it was suggested that TNFR1 is internalized while being in complex with TRADD, FADD and caspase 8^{201,202}. The discrepancy between findings of different groups can be due to distinct immunoprecipitation conditions. Deletion of the TNFR1 internalization domain (TRID) blocks FADD, TRADD and caspase 8 recruitment and almost entirely inhibit apoptosis, suggesting that TNFR1 internalization is crucial for the recruitment of pro-apoptotic components. In contrast, blocking of internalization did not affect RIPK1 and TRAF2 recruitment to TNFR1, but changed its binding kinetics from transient recruitment to a long-term durable binding. Overall, these observations suggest that TNFR1 internalization serves as a specific platform for the recruitment of DISC components and transduction of apoptotic signaling²⁰³. Thus, the endosomal compartment should be considered as an important component of TNF α -induced signaling. In that regard, it has been reported that endo-

lysosomal enzyme A-SMase activated by stimulated TNFR1²⁰⁴⁻²⁰⁷, FAS²⁰⁸⁻²¹⁰ and TRAIL^{211,212} plays a role in transmitting apoptotic signaling through the generation of ceramide (pro-apoptotic lipid second messengers)²¹³⁻²¹⁵ that activates its downstream target - aspartate protease cathepsin D (CTSD). Briefly, specific binding of ceramid results in an autocatalytic activation of CTSD, which in turn results in mitochondrial apoptotic pathway through Bid cleavage, followed by caspases activation²⁰⁷ (Fig 8).

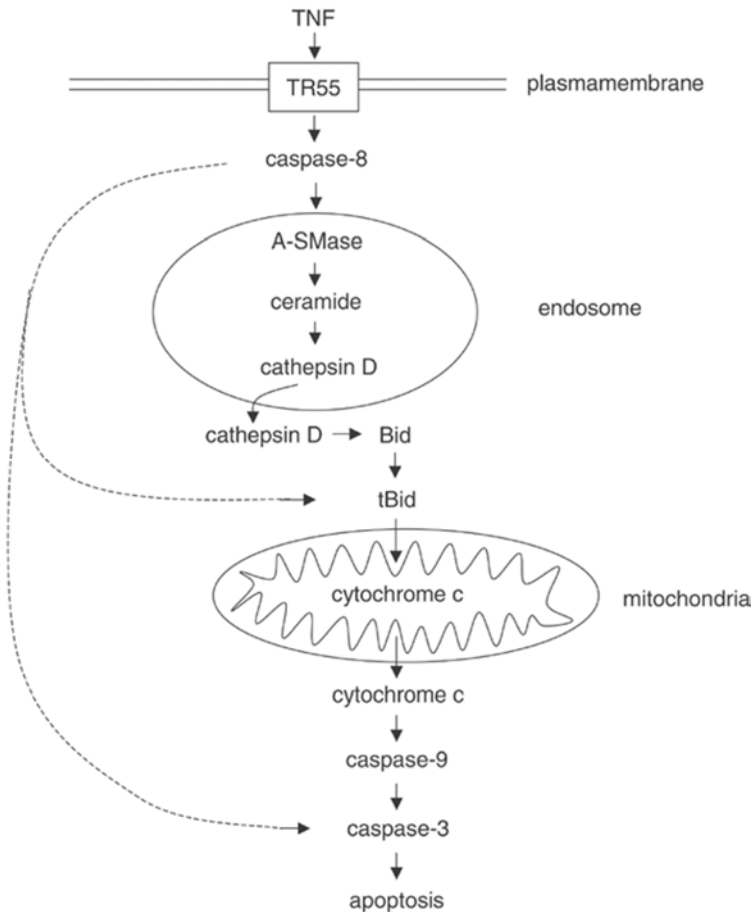


Figure 8. Role of CTSD in TNF α -induced apoptosis. For details see text. Figure was taken from²⁰⁷.

Furthermore, the role of internalization in apoptosis of type I cells has been also confirmed for FAS signaling^{216,217}. In brief, isolation of plasma membrane and endosomes membrane fractions followed by immunoprecipitation of FAS ligand from these fractions revealed that FAS treatment leads to the redistribution of FAS receptors into the endosomes compartment. Moreover, FADD and caspase 8 associated with FAS receptors were detected exclusively in the endosomes membrane fraction. This result suggests that FASL-induced DISC is formed in endosomes. Furthermore, using different approaches, such as modulation of PIP2 (Phosphatidylinositol 4,5-bisphosphate minor phospholipid component of the plasma membrane, that have been shown to regulate clathrin-mediated endocytosis) levels and knock down of members of the endocytic machinery (AP2 and clathrin heavy chain) it has been reported that FAS internalization is indeed a crucial event for apoptosis. Contrary, blocking

receptor internalization did not prevent NF- κ B and Erk1/2 activation upon exposure to FAS ligand ²¹⁷. Overall, subcellular localization of TNF α - and FAS-induced signaling complexes appears to play a crucial role in the internalization-dependent apoptosis and internalization-independent non-apoptotic/pro-survival pathways.

Nonetheless, it was suggested that internalization negatively regulates TRAIL-induced apoptosis in breast cancer cell lines, most likely through internalization-dependent DR4 cleavage ²¹⁸. Interestingly, TRAIL induces caspase-mediated cleavage of AP2, clathrin heavy chain and dynamin in cell lines sensitive to TRAIL-induced apoptosis (Hela, BJAB, Colo205), while this effect was not observed in resistant cell lines (HCT8). Moreover, blocking endocytosis by incubating cells at 4°C or utilizing a thermo-sensitive dynamin mutant showed a significant increase in caspases activation upon TRAIL treatment suggesting a negative role of internalization in the triggering of apoptosis. The role of TRAIL-Rs internalization in the activation of pro-survival pathways was not addressed in this study and remains an open question in the field ²¹⁹. On the other hand, Akazawa et al. obtained an opposite result using Huh - 7 (malignant liver cell lines) cells stressing the importance of DR5 internalization for its trafficking to lysosomes followed by the release of lysosomal proteases that contribute to efficient apoptosis ⁷⁵. Given that Akazawa et al. reproduced the data of Austin et al. using Hela cells, it was suggested that the role of TRAIL-Rs internalization in apoptotic signaling activation is more likely to be cell type dependent rather than a general rule for all cell types. Akazawa et al. proposed that the role of TRAIL-Rs internalization depends on whether cells belong to «type L cells» (lysosomal cytotoxic signaling) that requires lysosomal proteases for apoptotic signaling (Huh-7 cells) or «type D cells» (direct signaling), which is independent of lysosomal permeabilization (HeLa cells) ⁷⁵.

Overall, these results reveal an important role of death receptor internalization in the spatial and temporal regulation of different pathways by trafficking signaling complexes to a particular cellular compartments in order to access certain molecules required for efficient signaling transduction.

4.2. Functional role of lipid rafts localization of death receptors in the triggering of death or survival

Lipid rafts are micro domains of the plasma membrane enriched in sphingolipids and cholesterol. These plasma membrane compartments represent detergent resistant membrane domains, obtained upon lysis by mild nonionic detergents at low temperatures. Lipid rafts have dynamic structure and are characterized by ever changing content of lipids and proteins. It has been reported that these plasma membrane micro domains provide specific signaling platforms for the activation of various signaling pathways by bringing receptors in close proximity with different signaling molecules, favor specific protein-protein interaction and lead to the activation of signaling pathways ^{189,220}. Thus, lipid rafts play an important role in signal transduction.

Interestingly, it was found that lipid rafts play a distinct role in the regulation of TNF α and FAS signaling. TNFR1 is translocated to lipid raft within seconds after stimulation resulting in TRADD, RIPK1 and TRAF2 recruitment followed by NF- κ B activation. Importantly, lipid rafts disruption by cholesterol depletion prevents phosphorylation of I κ B α and leads to the induction of apoptosis ²²¹.

Contrary, other findings suggest that TNFR1 translocation to lipid rafts supports Erk1/2 activation, but not NF- κ B²²². Altogether, this data shows that lipid rafts play an important role in TNF α -induced pro-survival signaling activation. Contrary, the role of lipid rafts in FAS signaling is more likely to be cell type dependent^{223,224}. In that respect, lipid raft disruption in type I cells (thymocytes, peripheral T cells, etc) inhibits apoptosis, whereas, it does not affect cell death in type II cells (hepatocytes) upon exposure to FAS ligand. Notably, FAS receptor posttranslational modifications such as palmitoylation and S-glutathionylation may play a crucial role in the redistribution of FAS receptors into lipid rafts and sensitization to apoptosis^{225,226}. Even though lipid rafts play functionally different roles in TNF α and FAS signaling, one should consider that survival activation upon TNF α stimulation and apoptosis induction upon exposure to FAS is a primary receptor-associated response to the stimuli. This can suggest that the role of lipid raft is to increase the efficiency of signaling through receptor-associated complexes.

On the other hand, it was shown that the translocation of death receptors into lipid rafts upon TRAIL treatment leads to the initiation of apoptosis in TRAIL-susceptible NSCLC cells^{122,227}. Contrary, RIPK1 and cFlip mediated assembly of the TRAIL-DISC in non-raft compartments of the plasma membrane, leads to the activation of NF- κ B and Erk1/2 in NSCLC cells resistant to TRAIL-induced apoptosis¹²². Additional evidence suggests that non-receptor tyrosine kinase Ack1 can associate with DR4 and DR5 and leads to the translocation of death receptors into lipid raft domains upon TRAIL treatment. This redistribution of DR4 and DR5 was shown to play an important role in caspase 8 recruitment and apoptosis²²⁸. Similarly to FAS signaling, palmitoylation appears to play a role in receptor translocation to lipid raft domains and in the triggering of apoptotic signaling²²⁹. Given that lipid rafts support receptor clustering and oligomerization, data obtained for TRAIL signaling might suggest that the activation of apoptotic or survival pathways upon TRAIL challenge may require different level of death receptor clustering. Furthermore, localization of TRAIL-induced complexes in raft or non-raft micro domains might lead to a certain availability of the downstream pro- and anti-apoptotic components, thus supporting one or the other signaling outcome^{230,231}.

4.3. Role of cytoskeleton in cell fate decisions

The cytoskeleton is a dynamic structure that consists of filamentous polymers (actin filaments, microtubules and intermediate filaments) and regulatory proteins that are in constant flux. The role of the cytoskeleton is grouped and subdivided into three main functions: (i) spatial compartmentalization of cellular components, (ii) transport of intracellular cargo, (iii) generation of forces that provide and support cellular movement, adhesion, migration, changes in shape and enable cells to resist deformation and mechanical stress. Architecture, stabilization, growth, polymerization and depolymerization of cytoskeletal polymers are controlled by several classes of regulatory proteins that respond to external and internal stimuli and lead to cytoskeleton rearrangements. Actin filaments, microtubules and intermediate filaments are different in several characteristics, such as - assembly dynamics, flexibility, polarity and type of molecular motors. Members of kinesin and dynein families serve as molecular motors of microtubules, while myosin proteins are molecular motors of actin filaments. Contrary to

microtubules and actin filaments intermediate filaments are not polarized and cannot provide directional movement of molecular motors²³²⁻²³⁴.

Interestingly, the actomyosin cytoskeleton plays a role in the formation and maintenance of lipid raft domains that were shown to play an important regulatory function in TNF α , FAS and TRAIL signaling. Initially, it was reported that lipid raft domains are associated with cytoskeleton proteins, such as actin, NMIIa and MLC2 and lipid raft associated protein - caveolin-1. Moreover, these data were supported by imaging studies suggesting an association of lipid raft domains with the subcortical cytoskeleton^{235,236}. Importantly, using FRET approach it was found that the cytoskeleton plays a crucial role in lipid ordering events through its interaction with the plasma membrane^{237,238}. A number of proteins were suggested to be molecular bridges connecting plasma membrane and the cytoskeleton, among them - members of ezrin family, such as ezrin, radixin, moesin and merlin²³⁹⁻²⁴¹. Additionally, phosphoinositides are factors that were shown to be crucial in cytoskeleton and plasma membrane interaction²⁴². One of the existing cytoskeleton-mediated lipid ordering models proposes that actin interacting with the plasma membrane through phosphoinositides creates compressive forces on the above lying plasma membrane. Compressive forces are supported by the tension impaired by myosin II activity. Altogether, it leads to a certain lipid ordering and to the formation and maintenance of lipid raft domains that are optimal in size and time to be biologically functional²⁴³.

Importantly, the role of the cytoskeleton in the formation of lipid rafts suggests that the cytoskeleton supports a concentration of specific proteins and lipids in specific plasma membrane domains for biological functions. Therefore, the cytoskeleton provides an important regulation of protein functions resulting in enhancing or attenuation of signaling pathways. Such cytoskeleton-mediated regulation of protein function was reported for the member of Src family of non-receptor protein tyrosine kinases, Lck²⁴⁴. Briefly, Lck is expressed in T-cells and is known to participate in T-cell neoplasms. In that respect, efficient regulation of Lck function is important in order to avoid T-cell neoplasms. Notably, it was suggested that Lck function is inhibited by its association with lipid rafts domains. This localization excludes Lck from its activator CD45 (receptor-linked protein tyrosine phosphatase) and, thus prevents Lck from CD45-induced dephosphorylation and activation. Contrary, cytoskeleton disruption by depolymerization of actin filaments using Lat B or inhibiting NMII activity with blebbistatin as well as neomycin-induced PIP2 sequestering results in CD45 and Lck interaction, Lck dephosphorylation and activation²⁴². Lck regulation provides an example of cytoskeleton-dependent lipid raft-mediated regulation of the protein function.

Importantly, an involvement of the cytoskeleton in the regulation of death receptor signaling has been demonstrated. Sensitivity of T cells to FASL-induced apoptosis is dependent on receptor clustering in lipid raft domains of the plasma membrane²⁴⁵ that is mediated by the actin cytoskeleton^{216,241,245} through association with ezrin²⁴¹. Furthermore, it was demonstrated that actin inhibitor Ltn A diminished recruitment of FADD, decreased FAS receptor clustering and prevented FAS receptor internalization²¹⁶. Interestingly, adhesion, a process regulated and mediated by the cytoskeleton, plays the role of a positive regulator in FAS-mediated apoptosis by modulating protein redistribution. Adhesion of hematopoietic cancer cells to fibronectin leads to the block of caspase 8 activation due to

the translocation of the membrane-associated cFlip pool to the cytosolic fraction, its recruitment to the DISC and abolishing apoptotic response²⁴⁶. Furthermore, the role of the cytoskeleton in the regulation of TRAIL signaling has also been suggested. Briefly, E-cadherin-mediated coupling of TRAIL receptors to the actin cytoskeleton plays a role in efficient DR4/DR5 clustering resulting in increased caspase 8 recruitment to the DISC and its activation²⁴⁷.

Therefore, the cytoskeleton plays a crucial role in receptor clustering, translocation to lipid raft domains and internalization, thus providing one more essential mechanism of TNF receptor family signaling regulation that guides cells towards life or death.

5. TRAIL IN CLINICAL TRIALS

Numerous studies using exogenously administrated TRAIL provided evidences that exogenous TRAIL induces apoptosis in cancer cells *in vitro* and *in vivo* while causing negligible effect on normal cells. Therefore, the heightened interest to TRAIL as potential therapeutic agent against cancer led to the development of several pro-apoptotic receptor antagonists (PARAs) including recombinant human ligand (rhTRAIL) targeting both TRAIL receptors²⁴⁸; agonistic monoclonal antibodies targeting DR4 (mapatumumab) or DR5 (lexatumumab)^{18,249-254}, TRAIL receptor specific peptides^{89,166} and TRAIL-receptor selective mutants^{74,255,256}. Despite our limited knowledge regarding the mechanisms of tumor selective action of TRAIL and molecular basis of TRAIL-induced non-apoptotic pathways activation, a number of clinical trials have been launched in order to evaluate the potential of PARAs for the treatment of cancer¹⁶⁶. Clinical trials are based on several pharmacological strategies, including adenoviral delivery of TRAIL coding sequence to cancer cells and administration of pro-apoptotic receptor antagonists²⁵⁷. PARAs have been intensively tested in preclinical and clinical trials as a monotherapy and in combination with other drugs (irinotecan, camptothecin, bortezomib, cisplatin, carboplatin, 5-fluorouracin, carboplatin, paclitaxel, doxorubicin, gemcitabine, sorafenib).

Phase I clinical trials of mapatumumab demonstrated well tolerability, and the maximum tolerated dose has not been reached yet. The most frequent adverse effects caused by the administration of mapatumumab were fatigue, fever, myalgia, hypotension, nausea, and pyrexia^{251,258,259}. However, in phase II clinical trials no significant clinical activity of mapatumumab administrated as a single agent for NSCLC²⁵⁴, colorectal cancer²⁵³, relapsed or refractory non-Hodgkin's lymphoma²⁶⁰ treatment was observed. Administration of mapatumumab in combination with gemcitabine and cisplatin²⁶¹ or with carboplatin and paclitaxel²⁵⁹ in patients with advanced solid tumors led to partial response in 20% of the patients and to stable disease in 45% of the patients. In contrast, recent phase II clinical trial did not demonstrate any improvement in disease history from adding mapatumumab to carboplatin and paclitaxel in patients with non-small cell lung carcinoma²⁶². Altogether, clinical trials have shown that the best outcome of mapatumumab treatment is stable disease and only in some cases positive response. Similar results were obtained for lexatumumab. Lexatumumab is well tolerated and can be safely administrated. However, the best outcome obtained due to the treatment is stable disease - in 20-30% of the patients involved in the study^{250,252,263}. Clinical efficiency has been also tested for DR5-targeting Apomab and AMG-655. Both of them are safe and well tolerated. Similar to lexatumumab and mapatumumab clinical trials, treatment of colorectal and granulosa cancer cell ovarian cancer patients with Apomab yielded to 9% of partial response and 52% of stable disease among patients. AMG-655, applying for NSCLC patients led to 5% of partial response and 48% of stable disease²⁶⁴.

Overall, low toxicity and good tolerability further argues for PARAs investigation in anticancer therapy. However, despite expected results, based on *in vitro* and *in vivo* studies, clinical trials indicate that stable disease is the best clinical outcome of PARAs or rhTRAIL application for the majority of patients. Moreover, results of clinical trials also indicate that a number of human tumors are resistant to TRAIL monotherapy. Notably, most clinical trials have recruited people with advanced and refractory tumors, therefore studying the efficiency of TRAIL treatment in less advanced stages of cancer should be taken into consideration. In that regard, it was shown that TRAIL treatment in combination with all-trans-

retinyl acetate (RAc) efficiently induces apoptosis in adenomatous polyposis coli (APC)-deficient premalignant tumor cells. Importantly, APC deficiency leads to the repression of cFlip expression, while RAc induces upregulation of death receptors and suppression of decoy receptors expression leading to the sensitization to TRAIL-induced apoptosis²⁶⁵. Overall, this study demonstrates TRAIL-based approach that allows efficient and selective elimination of premalignant tumor cells. This finding supports further efforts in establishing TRAIL-based therapies in combination with other anticancer drugs that can sensitize cancer cells to TRAIL-induced apoptosis via modulation of TRAIL signaling.

Undoubtedly, humanized antibodies against TRAIL receptors and recombinant human TRAIL are well established as therapeutic agents and give promises in clinical trials. However, both strategies have some disadvantages. Recombinant human TRAIL binds to all TRAIL receptors, death and decoys, thus reducing its apoptogenic efficiency. Contrary, humanized antibodies against TRAIL receptors specifically bind to a certain death receptor (DR4 or DR5), however the capacity of mAb to cluster TRAIL receptors is lower as compared to rhTRAIL. Moreover, another issues that arise from utilizing rhTRAIL and mAb are differences in their functionality, purity and stability from batch to batch. Besides that the price of synthesis and storage of these products is high. Thus, specific multivalent DR5 selective synthetic peptides that activate TRAIL signaling were developed. In that regard, our group reported that TRAIL DR5/^{mim} exerts selective anti-tumor activity *in vivo* as a single agent⁸⁹.

Importantly, one of the biggest issues in TRAIL based therapy is the potential of this ligand to activate pro-survival, proliferation and migration pathways in cancer cells. Initially, pro-survival response of tumor cells to TRAIL treatment was mentioned *in vitro* in populations of cancer cells resistant to TRAIL-induced apoptosis. Given that many tumors are resistant to TRAIL-induced apoptosis and can undergo activation of pro-survival pathways upon TRAIL treatment it is important to identify patients that will profit from TRAIL therapy. Therefore, significant number of studies aiming to identify molecular markers of sensitivity to TRAIL has been performed. In that regard, it was found that sensitivity to TRAIL positively correlates with the expression of uPA in the stepwise transformation models of epithelial and mesenchymal origin (for details see “Results and Discussion” and “Publications”)¹⁸³ and the expression of O-glycosylation enzymes, such as GALNT14 in non-small cell lung carcinoma, pancreatic cancer and melanoma cell lines, GALNT3 in colorectal cancer⁷⁰.

On the other hand, there are many cancer cell lines and tumors that undergo fractional killing upon TRAIL treatment, meaning that a part of the population dies whereas the other one survives TRAIL treatment and undergoes activation of non-apoptotic/pro-survival pathways^{174,175,183}. This phenomenon of TRAIL signaling bifurcation is poorly understood at the molecular level. Our team identified PLAU (uPA) as a tumor specific factor that regulates fractional killing and fractional survival of transformed cells by affecting DISC composition (for details see “Results and Discussion” and “Publication 1”). These results suggest that bifurcation of TRAIL signaling towards triggering of pro-apoptotic and pro-survival pathways occurs at early steps of response to TRAIL¹⁸³. Thus, there is an urgent need in unraveling the regulatory network that modulates apoptogenic and pro-survival action of TRAIL which will provide basis for the development of TRAIL-based cancer therapies that aim to avoid TRAIL-mediated tumor progression.

Overall, these data suggest that TRAIL treatment combined with other approaches, such as: other anticancer drugs and/or identifying molecular markers of sensitivity/resistance to TRAIL should be prioritized in clinical strategy involving TRAIL.

AIMS OF THE THESIS

The overall aim of the thesis was to contribute to the understanding of the modulation of TRAIL-induced fractional killing in clonal populations of cancer cells. To shed the light on this issue our sub-goals included:

1. To analyze the role of individual TRAIL receptors in the activation of pro- and anti-apoptotic pathways in response to TRAIL
2. To study the formation, composition and localization of TRAIL-induced pro- and anti-apoptotic signaling platforms
3. To unravel the role of DISC and non-apoptotic signaling complex components in the activation of different TRAIL-stimulated pathways

EXPERIMENTAL MODEL. STEPWISE TRANSFORMATION SYSTEMS

Cancer is a genetic disease that develops from almost every tissue through the stepwise accumulation of genetic mutations²⁶⁶. Human cancer cell lines derived from human tumor specimens are extensively used in order to identify molecules and pathways involved in malignant transformation as well as for preclinical testing of potential therapeutic anti-cancer compounds. Although, using human cancer cell lines led to a significant progress in understanding cancer cell biology, these experimental models have numerous properties that limit their practical use. In that regard, human-derived cancer cell lines bear an unknown number of genetic mutations that significantly complicates studying the contribution of different signaling pathways into the process of transformation. In this respect, it was shown that the re-expression of tumor suppressor gene PTEN (Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase) in PTEN deficient human cancer cell lines did not re-establish some functions of PTEN, such as apoptosis and cell cycle arrest²⁶⁷. This observation suggests that there are some other mechanisms that regulate PTEN pathway in these cancer cell lines. Moreover, patient-derived cancer cell lines represent only a subset of late stage tumors, often derived from metastatic lesions and do not represent different stages of tumors²⁶⁶. Furthermore, often it is difficult to generate stable cell line using tumor explants, thus available cancer cell lines do not represent most types of human cancer²⁶⁸. Additionally, sometimes using the same cell lines in different laboratories gives unreliable data. This could arise from the fact that human tumors are often characterized by the intra-tumor genetic heterogeneity. Therefore, numerous passaging of cell lines derived from human tumors can lead to the selection of faster growing clones that can eventually lead to the generation of sublines²⁶⁸.

Therefore, given that natural processes of cancerogenesis are highly complex, variable to each type of tumorigenic stimuli and specific to different cell types, an alternative approach to study human cancer was developed. This approach is based on the introduction of defined genetic elements into normal human cells to generate experimental models of tumorigenesis *in vitro*. However, one of the major obstacles in the *in vitro* propagation of primary human cell cultures is a limited life span of the cells. This mechanism can serve as a barrier to the malignant transformation *in vitro*. Therefore, studying the molecular mechanisms that regulate the life span and in particular the role of telomeres in this process led to a significant progress in understanding the basis of malignant transformation. Briefly, telomeres are regions of repetitive sequence at the end of chromosomes, that are synthesized by the ribonucleoprotein – reverse transcriptase polymerase, that consists of a telomerase RNA component (TERC) and a protein component – a telomere reverse transcriptase (TERT)²⁶⁹. RNA component serves as a template for telomere synthesis that involves catalytic subunit of TERT. Notably, normal cells are characterized by the shortening of telomeres during lifespan, while immortalized and cancer cells maintain the stable length of telomeres. In that respect, the expression of telomerase is low and transient in normal cells²⁷⁰⁻²⁷³, whereas telomerase is upregulated in most human cancer cells. Furthermore, the overexpression of human TERT stabilizes the length of telomeres and facilitates immortalization²⁷⁴, while the expression of catalytically inactive TERT in cancer cells results in the shortening of telomeres and apoptosis^{275,276}, suggesting that TERT plays an important role during tumorigenesis.

In agreement with these observations, initial attempts led to the generation of two types of transformed human cell lines based on normal human cells. In these models, normal primary human cells (human embryonic kidney - HEK, foreskin fibroblasts - BJ) are transformed in a stepwise manner by the introduction of the catalytic subunit of telomerase (hTERT), the early region of the SV40 virus (SV40 ER) and the activated allele of H-RAS (H-RAS-V12)^{277,278}. Ectopic expression of the catalytic subunit of telomerase (hTERT) in normal primary human epithelial (HEK) and fibroblast (BJ) cells provides telomerase activity and results in telomeres elongation and stabilization leading to the immortalization of the cells. Furthermore, the early region of the simian virus (SV40ER) inactivates tumor suppressors Rb and p53, enables cell cycle progression and prevents cells from undergoing senescence. An oncogenic allele of H-RAS (H-RAS V12) provides cells with continuous growth signal and makes them independent from external growth signals. It was shown that cells expressing hTERT, SV40-ER and H-RAS V12 injected into nude mice lead to the efficient tumor growth. Importantly, hTERT expression by its own does not lead to the tumorigenesis of human cells, but is crucial for oncogenesis as it allows “pre-cancerous cells” to proliferate beyond the normal number of cell cycles and then to cooperate with oncogenic mutations leading to malignant transformation. Overall, this approach led to the successful transformation of a wide variety of primary human cells (Fig 9).

Combinations of genes				AIG	Tumors	Human cell types	Refs
hTERT	LT	ST	RAS	+	+	Fibroblasts Embryonic kidney epithelial cells Mammary epithelial cells Airway epithelial cells Glial cells Endothelial cells Mesothelial cells	[40,42–46,67]
hTERT	HPV E6 and E7	ST	RAS	+	+	Fibroblasts	[41,42]
hTERT	cyclin D1, CDK4 ^{R24C} and p53DD ^b	ST	RAS	+	+	Embryonic kidney epithelial cells	[41]
hTERT	^c cyclin D1–CDK4 ^{R24C} and p53 ^{-/-}	ST	RAS	+	+	Fibroblasts	[47]
hTERT	p16 ^{INK4a} -deficient		RAS or MYC	+	+ ^f	Fibroblasts	[48]
Telomerase ^d	E1A and MDM2		RAS	+	+	Fibroblasts	[55]
Telomerase ^d	CDK4		RAS	+	+	Keratinocytes	[49]
hTERT	LT	B56γ–shRNA ^g	RAS	+	+	Embryonic kidney epithelial cells	[52]
hTERT	LT	PtdIns3K	RAS or MYC	+	+ ^g	Mammary epithelial cells	[67]
hTERT	LT	MYC	RAS	+	+	Embryonic kidney epithelial cells	[66]

^aAbbreviations: AIG, anchorage-independent growth in soft agar; HPV, human papillomavirus; hTERT, human telomere reverse transcriptase; LT, simian virus 40 (SV40) early region large T antigen; PtdIns3K, phosphatidylinositol-3-kinase; ST, SV40 early region small t antigen.

^bCyclin D1–CDK4^{R24C}, co-expression of cyclin D1 and the R24C mutant form of CDK4 to phenocopy loss of p16 function. p53DD is a dominantly interfering mutant allele of p53 that inactivates p53.

^cCyclin D1–CDK4^{R24C} is a fusion protein of Cyclin D1 and CDK4^{R24C}. p53^{-/-} cells were created by targeting p53 by homologous recombination.

^dTumors derived these cells express telomerase, although hTERT was not introduced into the cells *in vitro*.

^eB56γ–shRNA is a short-hairpin RNA (shRNA) that is targeted to the PP2A subunit, B56γ.

^fCo-expression of RAS and MYC enabled tumor formation.

^gExpression of LT, hTERT, PtdIns3K and RAS enabled tumor formation.

Figure 9. Transformation of primary human cells by the introduction of defined genetic elements. Taken was taken from ²⁶⁶.

Overall, stepwise transformation systems represent a valuable experimental models as these cellular systems display several hallmarks of tumorigenesis and allow the comparison between cancer cells and their normal progenitors, which is difficult to achieve with cancer cell lines derived from primary human tumors. Moreover, contrary to cancer cells derived from primary human tumors, the genetic alterations that lead to the malignant transformation is well defined in stepwise transformation systems, providing

a possibility to study hTERT-, SV40-ER- and H-RAS-V12-mediated mechanisms involved in transformation - associated processes.

An important feature of stepwise transformation systems for our studies is that the sensitivity to TRAIL-induced apoptosis is acquired along the transformation process. In this respect, we and others observed that normal cells of epithelial (human embryonic kidney; HEK) and mesenchymal (foreskin fibroblasts; BJ) origins are resistant to TRAIL-induced cell death^{183,279}. Pre-transformed, immortalized cells expressing hTERT and SV40ER from both systems (BJE and HA1E cells) undergo weak induction of apoptosis upon TRAIL treatment. Importantly, hTERT, SV40ER and H-RAS-V12 expressing cells display high sensitivity to TRAIL-induced death^{173,279,280}. In that regard, it was proposed that sensitization of transformed cells to TRAIL-induced apoptosis compared to their normal counterparts is due to constitutively active RAS that acts through Raf1/MEK/Erk1/2 pathway and results in the phosphorylation and stabilization of cMyc (transcription factor)^{279,280}. Further, cMyc leads to the overexpression of death receptors and enhanced apoptosis^{279,280}. Indeed, the role of MYC in the regulation of the sensitivity to TRAIL has been also reported for other transformed cells²⁸¹. In this respect, it was shown that cFlip appears to be a direct target of cMyc. In this case, cMyc overexpression leads to the downregulation of cFlip expression, enhanced caspase 8 cleavage and sensitization of cancer cells to TRAIL-induced apoptosis. Notably, large-scale analysis of genomic targets of the human cMyc gene revealed that caspase 8 and caspase 9 can also be cMyc target genes²⁸². Indeed, exogenous expression of cMyc in BJ cells leads to the upregulation of caspase 8 expression²⁸⁰. Additionally, ectopic expression of cMyc in normal fibroblasts sensitizes them to various apoptotic stimuli, including FAS and TNF α ²⁸³. Altogether, RAS-dependent stabilization of cMyc can indeed be required for the sensitization of transformed cells from stepwise transformation models to TRAIL-induced apoptosis. Overall, transformed cells undergo apoptosis upon TRAIL treatment while normal cells are resistant to TRAIL-induced death suggesting that stepwise tumorigenesis models exhibit the basics of tumor-selective apoptogenic action of TRAIL. Thus, these cellular systems serve as valuable tools for studying the molecular basis that underlie normal cells resistance to TRAIL-induced apoptosis as well as to understand mechanisms that control cancer cell sensitivity to TRAIL-mediated death.

Interestingly, we found that transformed cells from the stepwise transformation systems display fractional killing when exposed to TRAIL, meaning that only part of the clonal cell population dies, while the other part of the population survives TRAIL treatment and continues to proliferate¹⁸³. Furthermore, cells surviving first treatment remains resistance to the following exposures to TRAIL. However, when TRAIL treatment is discontinued cells revert to the initial response after several cell cycles. The reversible nature of resistance suggests that it is not due to the selection of inherently resistant clones within the initial population, but that its establishment and maintenance is TRAIL-mediated. Importantly, fractional killing and establishment of reversible resistance in response to TRAIL were also reported for cancer cell lines (MCF10A)¹⁷⁴, suggesting that observed phenomenon is not restricted to transformed cells from the stepwise transformation system.

RESULTS AND DISCUSSION

1. Characterization of the stepwise tumorigenesis system as a model to study the molecular mechanisms underlying modulation of TRAIL-induced apoptosis versus survival pathways activation

Considering that TRAIL signaling starts from DISC formation, we hypothesized that events regulating resistance to TRAIL-induced apoptosis in normal cells and apoptosis versus survival response in transformed cells take place at this level. In that regard, it has been reported that the differential response of normal and transformed cells to TRAIL-induced apoptosis mainly occurs due to the different expression levels of DISC components in normal and transformed cells and/or due to the imbalance of TRAIL receptors expression at the cell surface of normal and cancer cells²⁸⁴. Therefore, we first analyzed the surface expression level of TRAIL receptors in normal versus transformed cells by direct surface TRAIL-Rs immunolabeling and flow cytometry analysis of the mean fluorescent intensities (MFI) (Fig 10a, 10b). Importantly, we did not observe any differences in DR4, DR5 and DcR2 expression levels between normal and transformed cells (Fig 10c). Interestingly, DcR1 is expressed at the cell surface of BJ cells, but is not present at the cell surface of BJELR cells (Fig 10a-c). Further, we set to identify the functional role of TRAIL-Rs in BJ and BJELR cells. Down-regulation of DR5 levels by siRNA-based approach resulted in a complete block of cell death upon TRAIL treatment in both cell lines, whereas DcR2 depletion enhanced apoptosis upon treatment in BJ and BJELR cells (Fig 10d, 10e). These results go in line with their previously reported “death” (DR5) and “decoy” (DcR2) nature. Surprisingly, knock down of DR4 did not result in blocking of apoptosis or sensitization to TRAIL-induced cell death (Fig 10d, 10e). Notably, DcR1 knock down did not significantly affect sensitivity to TRAIL neither in normal nor in transformed cells, suggesting that DcR1 has no major effect on the resistance to TRAIL-induced apoptosis in normal cells, even though it is expressed at the cellular surface (Fig 10a-e). These data provided evidences that the difference in TRAIL signaling in normal and transformed cells does not rely on an altered expression of TRAIL receptors at the cell surface or their distinct functional role in TRAIL signaling. However, it does not exclude the possibility of less (normal cells) and more (transformed cells) efficient clustering of TRAIL receptors, an important event for efficient TRAIL signaling upon stimulation with the ligand. Importantly, when grown *in vitro* normal cells are larger (confluency reached at 1×10^6 cells/6010mm²) than transformed cells (confluency reached at 5×10^6 cells/6010mm²). Therefore, equal levels of TRAIL-R expression at the surface of normal versus transformed cells does not necessarily mean the same spatial distribution (number of TRAIL receptors per surface area and distance between two receptors, for instance) and mobility within the plasma membrane. Thus, the efficiency of TRAIL-induced clustering can be different in normal and transformed cells.

Given that normal and transformed cells express TRAIL receptors at the cell surface, thus having potential to form a DISC, we analyzed the composition of the death inducing signaling complex formed upon TRAIL treatment in BJ and BJELR cells. Given that DR5 is the main apoptotic receptor in our experimental model, DISC was immunoprecipitated using anti-DR5 antibodies. Indeed, DISC immunoprecipitation (IP) experiments showed that TRAIL can induce DISC formation in both BJ and

BJELR cell lines (Fig 10f). DR4, DR5 and DcR2 formed heterocomplexes upon TRAIL treatment, which led to the recruitment of FADD, caspase 8 and cFlip (Fig 10f). As mentioned before DcR2 protects cells from apoptosis (Fig 10d, 10e). Interestingly, even though DR4 is recruited to the DISC, it is not required for apoptotic signaling (Fig 10d, 10e). Importantly, FADD and caspase 8 knock down blocked apoptotic cell death in BJ and BJELR cells suggesting that both proteins are required for apoptotic cell death (Fig 10h, i). Contrary, cFlip depletion sensitized both cell lines to TRAIL-induced cell death evidencing that cFlip acts as a negative regulator of TRAIL-stimulated apoptosis in BJ and BJELR cells (Fig 10h, i).

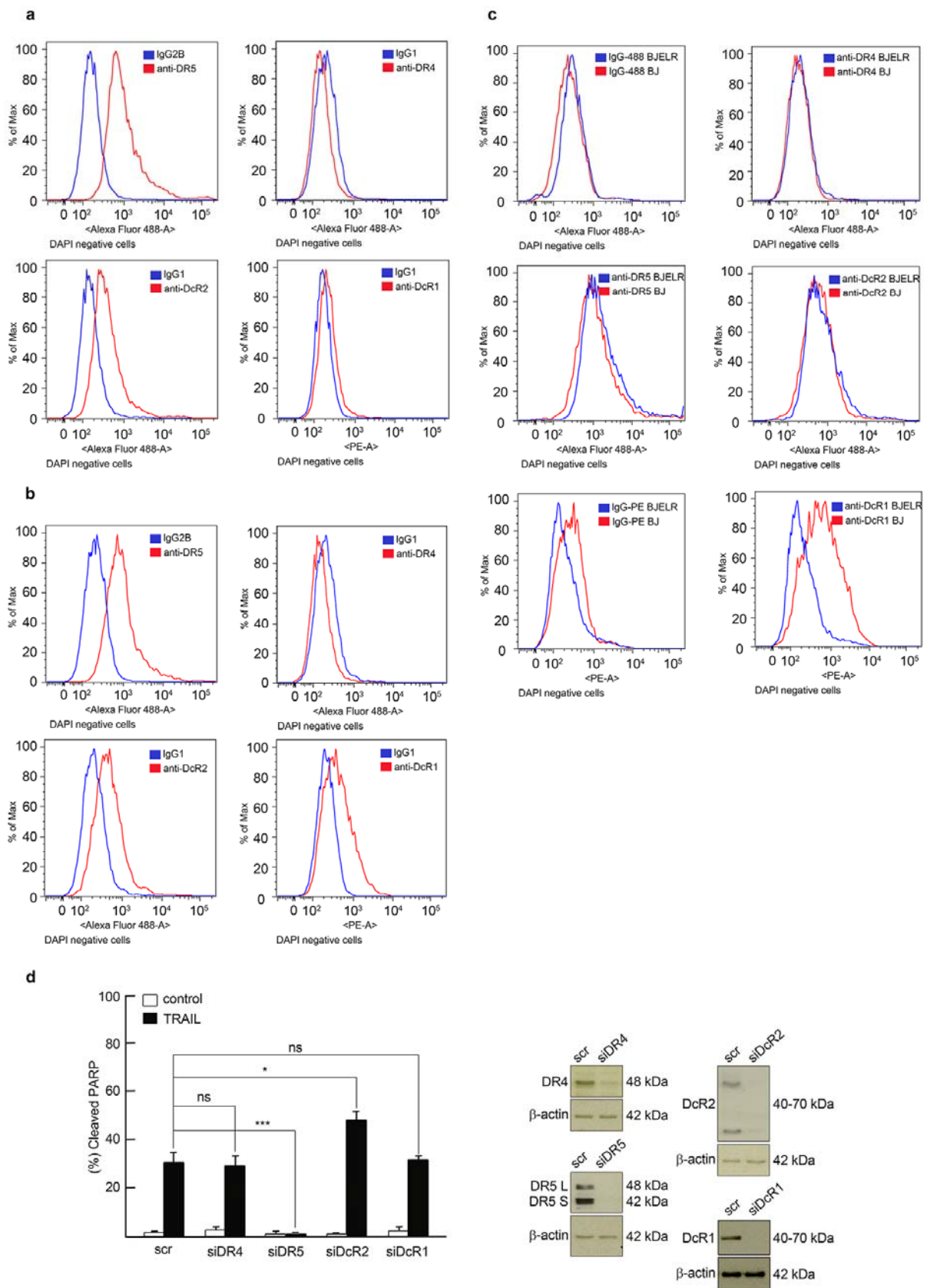
Although BJ and BJELR cells form a DISC, decreased recruitment of DR4, FADD and caspase 8 cleavage upon TRAIL treatment was detected in BJ cells (Fig 10f). Moreover, increased co-immunoprecipitation of DR5 with cFlip and DcR2 was observed in normal cells as compared to transformed cells (Fig 10f). In that regard, we observed that total expression levels of cFlip and DcR2 proteins (anti-apoptotic proteins in the system) are higher in BJ cells, whereas caspase 8 (pro-apoptotic protein in the system) is less expressed in BJ cells as compared to BJELR cells (Fig 10g). Notably, even though the basal expression level of DcR2 is higher and co-immunoprecipitation with DR5 is increased in normal cells, the surface expression level of DcR2 in normal and transformed cells is not different. Moreover, we did not observe any difference in the total expression level of FADD in BJ versus BJELR cells (Fig 10g); however, the recruitment of FADD to the DISC is lower in normal cells as compared to transformed cells (Fig 10f). Thus, decreased caspase 8 recruitment to the DISC of normal cells may be due to the lower recruitment of FADD, and not because of reduced caspase 8 expression in BJ cells as compared to BJELR cells. This suggests that differences in DISC composition observed in normal and transformed cells may not be due to the differential expression of DISC components and that additional DISC-modulating mechanisms exist to support resistance of normal cells and sensitivity of transformed cells to TRAIL-induced apoptosis.

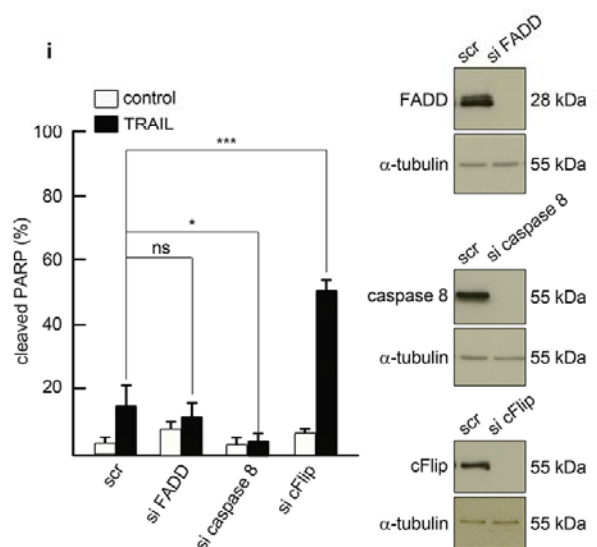
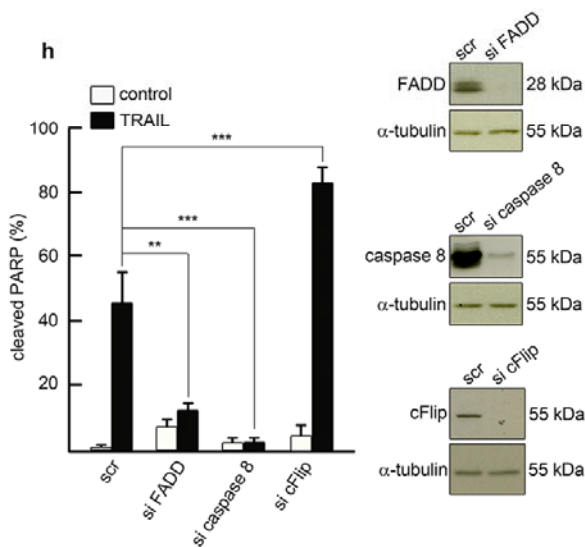
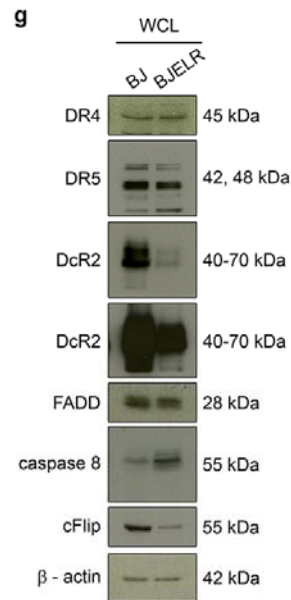
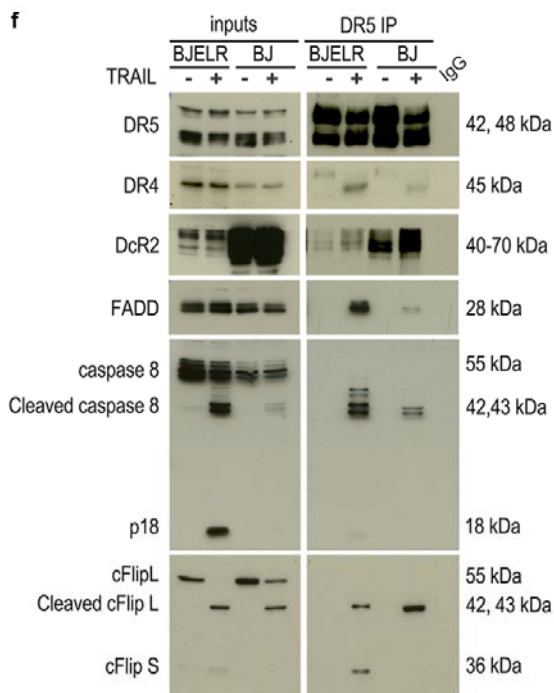
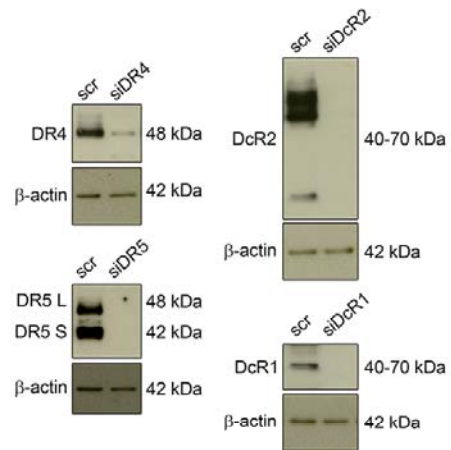
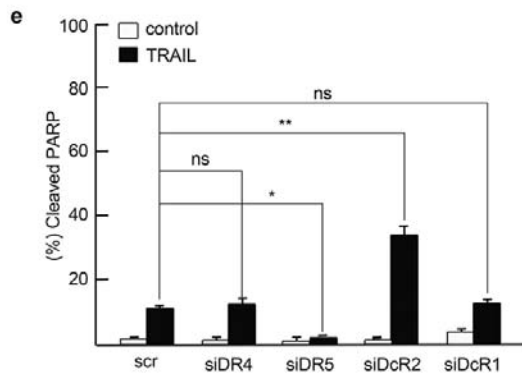
In those lines, using liquid chromatography-supported mass spectrometry (LC-MS/MS) our lab found that non-muscle myosins class II (NMIIA and NMIIB) as well as actin are co-immunoprecipitating partners of the DISC in BJ and BJELR cells (Sup fig 1a) (Dr. C. Schulz and Dr. V. Pavet, unpublished data). Interestingly, Western blot analysis revealed that the association of NMIIA, NMIIB, MLC2 and actin with the DISC of normal cells is significantly higher as compared to transformed cells (Sup fig 1a, p.86). These results showed a direct correlation between the level of NMII-DISC co-immunoprecipitation and the resistance to TRAIL-induced apoptosis in normal cells. In contrast, transformed cells that are sensitive to ligand-mediated death showed reduced NMII-DISC co-IP. Based on these results, it was hypothesized that the actomyosin cytoskeleton can modulate response to TRAIL by maintenance of resistance to TRAIL-mediated apoptosis in normal cells. Notably, the conformation of NMII plays a crucial role in its interaction with other proteins. Briefly, non-muscle myosins class II (NMII) consists of two heavy chains, two essential light chains (ELC) and two regulatory light chains, referred to as myosin light chain 2 (MLC2). Phosphorylation of MLC2 by different kinases is essential for NMII to display an active conformation that in turn enables NMII-protein interactions²⁸⁵. To study the impact of NMII on TRAIL signaling two complementary approaches aiming to modify NMII structure and possibly prevent its association with the TRAIL receptors were proposed: (i) treatment

with the non-competitive inhibitor blebbistatin, known to inactivate and disassemble NMIs, (ii) treatment with ML7, a specific inhibitor of MLCK, a key kinase that phosphorylates MLC2. We found that Blebbistatin and ML7 treatment prior to TRAIL challenge led to an increased level of PARP cleavage in both cell lines, showing that NMIs modulate TRAIL-induced apoptosis (Sup fig 1b, c, p.86). Furthermore, we found that Blebbistatin and ML7 treatment enhanced caspase 8 cleavage upon TRAIL challenge in BJ and BJELR cells suggesting that NMIs regulate TRAIL signaling at the level of the DISC (Sup fig 1b, c, p. 86). Overall, these data support our initial hypothesis that NMII-DISC association may at least partially be involved in the maintenance of normal cells resistance to TRAIL-induced cell death by modifying DISC composition. In this respect, we hypothesize that NMIs may determine a specific subcellular localization of the DISC that does not support efficient DISC formation either by affecting receptor clustering, hampering FADD and caspase 8 recruitment or compartmentalizing activated caspase 8 far from its cytoplasmic targets. In that regard, it was recently reported that E-cadherin-mediated coupling of TRAIL receptors to the actin cytoskeleton plays an important role in efficient DR4/DR5 clustering resulting in enhanced caspase 8 recruitment to the DISC and its activation²⁴⁷. Furthermore, ezrin-mediated association of FAS receptors with the actin cytoskeleton supports receptor clustering in lipid raft domains of the plasma membrane and determines T-cells sensitivity to apoptosis²⁴¹. Overall, further studies are required to unravel precise molecular mechanisms by which NMIs can support normal cells resistance to TRAIL-stimulated apoptosis.

Figure 10. Characterization of the (BJ) stepwise transformation model. a - Surface levels of TRAIL-Rs in BJELR cells. **b -** Surface levels of TRAIL-Rs in BJ cells. **c -** Comparative analysis of surface expression levels of TRAIL-Rs in BJ versus BJELR cells. In **a, b and c** surface levels of Death Receptor 5 (“anti-DR5”), Death Receptor 4 (“anti-DR4”), Decoy Receptor 1 (“anti-DcR1”) and Decoy Receptor 2 (“anti-DcR2”) were analyzed by flow cytometry. Isotypic IgG1 or IgG2B labeling was used as a control for background fluorescence. **d and e -** Impact of TRAIL-Rs knock down on BJELR (**d**) and BJ (**e**) sensitivity to TRAIL. BJ and BJELR cells were transfected either with siRNAs targeting *DR4* (“DR4 KD”), *DR5* (“DR4 KD”), *DcR1* (“DcR1 KD”) or *DcR2* (“DcR2 KD”) mRNA or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection cell populations were either left untreated (“control”) or challenged with 1µg/ml TRAIL (“TRAIL”) during 1h and the percentage of cells with the positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. Efficiency of DR4, DR5, DcR1 and DcR2 knock down at 48 h post-transfection was analyzed by Western blot. α - Tubulin, loading control. **f –** DISC composition in BJ and BJELR cells. BJ and BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Immunoprecipitation of Death Receptor 5 (DR5-IP) was performed and co-immunoprecipitation of DISC components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as control of non-specific binding. **g –** Total protein expression levels of DISC components in normal and transformed cells. **h and i -** Impact of FADD, caspase 8 and cFlip knock down on BJELR (**h**) and BJ (**i**) sensitivity to TRAIL. BJ and BJELR cells were transfected either with siRNAs targeting *FADD* (“si FADD”), *caspase 8* (“si caspase 8”) or *cFlip* (“si cFlip”) mRNA or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection cell populations were either left untreated (“control”) or challenged with 1µg/ml TRAIL (“TRAIL”) during 1h and the percentage of cells with the positive labeling for cleaved PARP was analyzed by flow cytometry. Histograms represent the mean +/- Standard Deviation (SD) from at least five independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05.

Figure 10





2. Normal cells activate non-apoptotic pathways upon TRAIL treatment

Importantly, numerous studies have shown that besides induction of apoptosis TRAIL can also trigger non-apoptotic/pro-survival pathways (Akt, Erk1/2, p38MAPK, JNK, NF- κ B). Notably, TRAIL-induced activation of non-apoptotic signaling has been reported to take place in both cancer and normal cells. Indeed, it was reported that TRAIL leads to the activation of Akt, Erk1/2 and NF- κ B in human endothelial cells, suggesting that TRAIL can exert proangiogenic effect^{165,170,171}. Additionally, TRAIL can also stimulate proliferation and migration in vascular smooth muscle cells in Erk1/2-dependent manner¹⁷¹. Therefore, we set to identify whether normal cells from the stepwise tumorigenesis system activate non-apoptotic pathways upon exposure to TRAIL while being resistant to TRAIL-induced apoptosis. Importantly, we observed that normal BJ cells activate non-apoptotic signaling such as Akt, p38MAPK, NF- κ B and ERK1/2 upon TRAIL treatment (Fig 11a). Notably, TRAIL-induced activation of pro-survival pathways in normal cells can serve as a mechanism of normal cells resistance to TRAIL-induced cell death. In that regard, the activation of Akt, p38MAPK, NF- κ B and Erk1/2 can lead to the expression of non-apoptotic proteins such as cFlip, cIAPs, DcR2 and TNF α that can block the progression of apoptotic signaling³⁶ (Fig 17c,d). On the other hand, triggering of non-apoptotic pathways upon TRAIL treatment can be a consequence of the inability of normal cells to induce cell death. In that regard, it was shown that blocking caspase 8 activity can potentiate activation of non-apoptotic pathways, particularly NF- κ B. Thus, inability of caspase 8 to undergo cleavage can shift the cellular response from pro-apoptotic to pro-survival upon TRAIL treatment. Overall, even though the majority of normal cell types are resistant to TRAIL-induced apoptosis, normal cells can activate non-apoptotic pathways upon TRAIL challenge. Notably, the molecular mechanisms underlying triggering of non-apoptotic pathways in this cellular context is unknown.

Given our previous results suggesting that NMIIIs may support normal cells resistance to TRAIL-induced apoptosis we set to analyze whether NMIIIs can participate in the activation of non-apoptotic pathways. In that regard, our results show a direct correlation between the level of NMII-DISC association and the triggering of non-apoptotic signaling pathways upon TRAIL treatment in normal cells. Moreover, blebbistatin and ML7 treatment leads to the sensitization of normal cells to TRAIL-induced cell death (Sup fig 1b, p.86). Therefore, we hypothesized that NMIIIs can support the formation of the DISC whose composition will favor the triggering of pro-survival pathways upon exposure to TRAIL. To study this we treated BJ cells with blebbistatin and ML7 inhibitor followed by TRAIL challenge and analyzed phosphorylation levels of Erk1/2 and Akt. Interestingly, our preliminary data showed a significant decrease in the level of TRAIL-induced Erk1/2 and Akt phosphorylation when Blebbistatin or ML7 treatment was applied prior to TRAIL challenge (Sup fig 1d, p.86). These results suggest that NMIIIs may play an important role in the activation of non-apoptotic pathways in response to TRAIL.

3. TRAIL induces activation of non-apoptotic and pro-survival pathways in transformed cells and leads to the establishment of reversible resistance

According to their response to TRAIL, cancer cells can be grouped into three classes; (i) the entire population commits to apoptosis or (ii) is inherently resistant to cell death induced by TRAIL and (iii) only a fraction of the cell population dies while another survives the treatment and may even proliferate¹⁸³ (referred to as “fractional killing”¹⁷⁴). Notably, an increasing number of evidence suggests that treatment of drug sensitive cancer cell populations with anticancer agents results not only in the death of majority of the population but also generates a small subpopulation of drug resistant cells. Importantly, often resistance established in response to a drug has a reversible nature, meaning that when drug treatment is discontinued cells revert to the initial response suggesting that acquired resistance to anticancer drugs does not require a stable genetic mutation. This phenomenon was called “re-treatment response”¹⁷⁸. In that regard, we found that transformed cells (BJELR, HA1ER) from stepwise transformation systems undergo fractional killing when exposed to TRAIL (Fig 11b, c). Moreover, those cells surviving a first cytokine stimulation acquire resistance to TRAIL-induced apoptosis upon further exposures¹⁸³. However, when TRAIL treatment is discontinued cells revert to the initial response to ligand. The reversible nature of resistance suggests that TRAIL-induced resistance in BJELR cells is not due to the selection of inherently resistant clones, but that its establishment and maintenance is TRAIL-dependent¹⁸³.

Interestingly, it was previously demonstrated that pre-existing differences in expression levels and /or activities of apoptotic proteins among cells of clonal populations determine the “chance” of TRAIL-stimulated cell death and the time required for its execution¹⁷⁵. For instance, it was suggested that the ratio of caspase 3:XIAP that depends on the cell-to-cell variability plays a crucial role in death versus survival decision upon TRAIL treatment¹⁷⁹. Although preexisting differences might play a pivotal role in the initial response to ligand there is evidence suggesting that reversible resistance is a state that is TRAIL-induced and TRAIL-maintained. In that regard, it has been reported that the entire population of cells enter resistant state, if cell death is blocked using caspases inhibitor¹⁷⁴. This suggests that based on cell-to-cell variability TRAIL induces not only apoptotic cell death in the subpopulation of cells but also activates pro-survival pathways that might participate in the establishment and maintenance of resistance to repetitive ligand treatments. Importantly, activation of pro- and anti-apoptotic pathways may occur in the same cell but the time, intensity and extent to which one or the other signaling is activated will be determined by the differences in protein expression levels. Indeed, numerous studies reported that TRAIL can induce activation of non-apoptotic pathways such as Akt, p38MAPK, NF- κ B, Erk1/2 and JNK that can support cancer cells survival, proliferation and migration²⁸⁶. Based on this, it is possible to hypothesize that death and survival signaling are competing upon TRAIL treatment and based on stochastic differences in protein levels¹⁷⁵ (that determine time and probability of TRAIL-induced signaling) one pathway will eventually overcome the other one and lead to cell death or survival. Permanent TRAIL treatment further maintains TRAIL survivors under a selective pressure and leads to the constant activation of non-apoptotic pathways that in turn helps cells to survive as long as the treatment is administrated. However, when TRAIL treatment is discontinued cells start accumulating

distinct levels of proteins leading to the re-establishment of cell-to-cell variability. Therefore, new exposure to the ligand leads to the fractional killing response.

Importantly, we found that Akt, NF- κ B, p38MAPK and Erk1/2 signaling is activated at early time points of response to TRAIL suggesting that triggering of non-apoptotic pathways occur within the initial population (Fig 11d). Notably, TRAIL-induced activation of signaling pathways such as Akt, NF- κ B, p38MAPK, JNK and Erk1/2 can contribute to both outcomes: apoptotic or survival depending on cellular context. For instance, it was shown that the MAPK pathway mediates an increased caspase 8 recruitment to the DISC upon TRAIL treatment in RAS-transformed HEK cells suggesting a pro-apoptotic function of MAPK pathway in this cellular context²⁷⁹. Therefore, using specific inhibitors we analyzed the functional role of TRAIL-induced Erk1/2, Akt, p38MAPK, NF- κ B activation in BJELR cells. We found that blocking Erk1/2 phosphorylation by using MEK1/2 inhibitor (U0126) significantly lowered cellular threshold to TRAIL-induced apoptosis in BJELR cells (Fig 11e, 11f). Moreover, blocking Akt and p38MAPK by using PI3 kinase inhibitor (LY294002) and p38MAP kinase inhibitor (PD169316) augmented apoptotic response to TRAIL. Contrary, inhibition of NF- κ B signaling using IKK inhibitor - Bay 11-7082 did not modify apoptotic response to this cytokine. These data indicate that Erk1/2, Akt and p38MAPK cascades support survival upon exposure to TRAIL in our experimental model, while NF- κ B does not play a main pro-survival function in BJELR cells.

We observed that pre-incubation of BJELR cells with pan-caspase inhibitor zVAD.fmk results in a complete block of cell death following TRAIL challenge (Fig 11g). In contrast, phosphorylation of Erk1/2, Akt, p38MAPK and I κ B α is independent of caspase activity (Fig 11h). These results indicate that TRAIL induces a dual signaling in our experimental model including the activation of caspase-dependent cell death, as well as the triggering of non-apoptotic and pro-survival signaling cascades independently of the protease activity of initiator and executor caspases. Notably, we detected significantly higher levels of I κ B α phosphorylation upon TRAIL challenge when caspase 8 activity is blocked as compared to controls (Fig 11h). Therefore, we hypothesized that decisions that balance apoptotic and non-apoptotic/pro-survival pathways activation and thus regulating fractional killing, are made at early steps of TRAIL signaling such as DISC formation.

In that regard, several mechanisms for TRAIL signaling regulation at the level of the DISC have been previously reported. Interestingly, it was found that the sensitivity to TRAIL positively correlates with the expression of O-glycosylation enzymes, such as GALNT14 in non-small cell lung cancer, pancreatic cancer and melanoma cell lines as well as GALNT3 in colorectal cancer⁷⁰. It was found that GALNT14 induces DR4 and DR5 O-glycosylation and supports receptor clustering which in turn results in efficient caspase 8 recruitment and cleavage. On the other hand, two recent studies identified that polyubiquitination reveals a mechanism of positive and negative regulation of apoptotic signaling at the level of caspase 8 recruited to the DISC upon TRAIL treatment. In that regard, it was reported that culling 3 - based E3 ligase is associated with the DISC upon TRAIL treatment and leads to caspase 8 polyubiquitination followed by the recruitment of ubiquitin binding protein p62 that promotes caspase 8 aggregation and processing¹⁰⁷. Contrary, another ubiquitin ligase - TRAF2, was shown to be recruited to the DISC and exert anti-apoptotic function through K48 linkage specific polyubiquitination of p18

targeting it to proteosomal degradation¹⁰⁸. Moreover, caspase 8 can undergo phosphorylation that modulates its functionality. It was reported that caspase 8 phosphorylation by Erk1/2¹⁰⁹, focal adhesion kinase or proto-oncogene tyrosine protein kinase (Scr)^{110,111} prevents caspase 8 cleavage at the DISC.

In those lines, we identified PLAU (uPA) as a tumor-specific factor that regulates fractional killing of transformed cells by modifying DISC composition¹⁸³ (see publication Pavet et al, (2014) in the section “Publication 1”). Briefly, extracellular protease urokinase-type plasminogen activator (uPA; also known as urokinase) and its receptor uPAR play an important role in the proteolysis of the extracellular matrix. Moreover, numerous studies have shown that besides its function in the regulation of proteolysis uPAR can promote cell survival, proliferation, invasion and migration mainly acting through MAPK, Akt, FAK, STAT and JAK pathways²⁸⁷. Importantly, we reported that uPA depletion in BJELR cells leads to TRAIL-induced caspase 8 cleavage at earlier time points of exposure to TRAIL as compared to control cells, suggesting that uPA affects the kinetics of TRAIL-mediated apoptosis (Fig 12a). These results indicate that uPA modulates TRAIL signaling at the level of the DISC. In that regard, it was reported that uPAR can physically interact with a number of receptors, among them several integrins, fMLP receptor FPRL1 and EGF receptor. Moreover, it has been shown that in most cases uPAR changes the ligand-dependent signaling of these receptors, and leads to the formation of altered signaling complexes^{288,289}. Therefore, we analyzed the impact of uPA depletion on DISC composition. Indeed, we observed that TRAIL treatment results in decreased DR5 and DCR2 co-immunoprecipitation and increased DR5 and FADD association in uPA knock down cells as compared to control (Fig 12b). However, neither uPA nor its receptor- uPAR were identified in the DISC suggesting that uPA/uPAR’s interaction with the DISC may be less stable than the association of other DISC components or uPA indirectly modulates DISC composition. Taking into account our previous results regarding the pro-survival role of Erk1/2 and Akt in BJELR cells and considering the role of uPA/uPAR system in the modulation of those signaling pathways, we set to analyze whether uPA modulates pErk1/2 and pAkt level in our experimental system. In this context we found that knocking down uPA leads to a decreased basal level of pErk1/2, which may result in lowering the cellular threshold to TRAIL-induced apoptosis in uPA depleted BJELR cells (Fig 12c)¹⁸³ (paper attached). Therefore, uPA regulates TRAIL signaling by two different mechanisms: (i) by modulation pErk1/2 level that supports cancer cell survival, and (ii) by altering DISC composition, which leads to the formation of the less efficient apoptotic DISC. Importantly, these results reinforce our hypothesis that initial events of TRAIL signaling and regulation of DISC composition play a crucial role in setting a threshold for TRAIL-induced activation of apoptotic versus survival signaling and balancing fractional killing in transformed cells.

Taken together, our data support that stepwise transformation models are valuable systems to (ii) unravel the molecular mechanisms that maintain resistance in normal cells to TRAIL-induced cell death, (iii) discover the molecular basis that balance fractional killing in tumor cells upon exposure to TRAIL, (iv) study the nature and features of TRAIL-induced resistance in cancer cells. In my thesis work we focused on studying the molecular mechanisms of TRAIL-induced fractional killing in cancer cells. In that regard, particular efforts were made to uncover the role of individual TRAIL receptors and initial

TRAIL-induced molecular events (formation of signaling complexes) in the regulation of fractional killing.

Figure 11. TRAIL induces fractional killing in transformed cells. **a** - Phosphorylation levels of Erk1/2, Akt, p38MAPK and regulator of NF- κ B signaling (I κ B α) in normal cells upon TRAIL treatment. BJ cells were either left untreated (“0”) or treated with TRAIL (1 μ g/ml) for 15min, 30min, 1h, 3h, 6h or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of at least five independent biological replicates. **b**- Cleavage of caspase 8 observed in the population of BJELR cells upon TRAIL treatment. Cell death in untreated BJELR cells (control) and after 6 h of treatment with 1 μ g/ml of TRAIL (TRAIL) is displayed. Apoptosis was analyzed by flow cytometry as the percentage of cleaved caspase 8 positive cells. Histogram represents the mean \pm Standard Deviation (SD) from at least three independent biological replicates. **c**- Cleavage of PARP observed in the population of BJELR cells upon TRAIL treatment. Cell death in untreated BJELR cells (control) and after 6 h of treatment with 1 μ g/ml of TRAIL (TRAIL) is displayed. Apoptosis was analyzed by flow cytometry as the percentage of cleaved PARP positive cells. Histogram represents the mean \pm Standard Deviation (SD) from at least three independent biological replicates. **d**- Phosphorylation levels of Erk1/2, Akt, p38MAPK and a regulator of NF- κ B signaling (I κ B α) in transformed cells upon TRAIL treatment. BJELR cells were either left untreated (“0”) or treated with TRAIL (1 μ g/ml) for 1, 2, 3, 4, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of at least five independent biological replicates. **e**- Requirement of Erk1/2, Akt and p38MAPK and NF- κ B signaling for cell survival upon TRAIL challenge. BJELR cells were either treated for 1 h with 20 μ M U0126 MEK1/2 inhibitor, 50 μ M LY294002 PI3 kinase inhibitor, 25 μ M PD169316 p38 MAP kinase inhibitor, 40 μ M Bay 11-7082 NF- κ B inhibitor or vehicle (DMSO). Apoptosis in untreated samples (control) and upon 5h of treatment with 1 μ g/ml of TRAIL (TRAIL) was analyzed by flow cytometry as the percentage of cells with positive labeling for cleaved PARP. Histogram represents the mean \pm Standard Deviation (SD) from at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P* value < 0.0005, ***P* value < 0.005, **P* value < 0.05. **f**- Impact of U0126 MEK1/2, LY294002 PI3K, PD169316 p38MAPK and Bay 11-7082 NF- κ B inhibitors on the phosphorylation level of Erk1/2, Akt, p38MAPK and I κ B α . Western blot assays of total Erk1/2, Akt, p38MAPK, I κ B α and phosphorylated Erk1/2 (pErk1/2; Thr202/Tyr204), Akt (Thr 308), Akt (Ser 473), p38MAPK (Thr180/Tyr182), I κ B α Ser32/36) in BJELR cells treated with 20 μ M U0126 MEK1/2, 50 μ M LY294002 PI3K, 25 μ M PD169316 p38MAPK, 40 μ M Bay 11-7082 NF- κ B inhibitors or vehicle (DMSO) during 1 or 5 h. α -Tubulin, loading control. **g**- Role of caspases activity in the triggering of apoptotic signaling. BJELR cells were either pretreated with 20 μ M pan-caspase inhibitor (“zVAD”) or vehicle (“DMSO”) for 1h and then either left untreated (“control”) or treated with TRAIL (1 μ g/ml) for 6h (“TRAIL”). Apoptosis was analyzed by flow cytometry as the percentage of cleaved PARP positive cells. Histogram represents the mean \pm Standard Deviation (SD) from at least three independent biological replicates. **h**- Role of caspases activity in the triggering of non-apoptotic signaling. BJELR cells were either left untreated (“mock”), pretreated with 20 μ M pan-caspase inhibitor (“zVAD”) or vehicle (“DMSO”) for 1h and then either left untreated (“0”) or treated with TRAIL (1 μ g/ml) for 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.

Figure 11

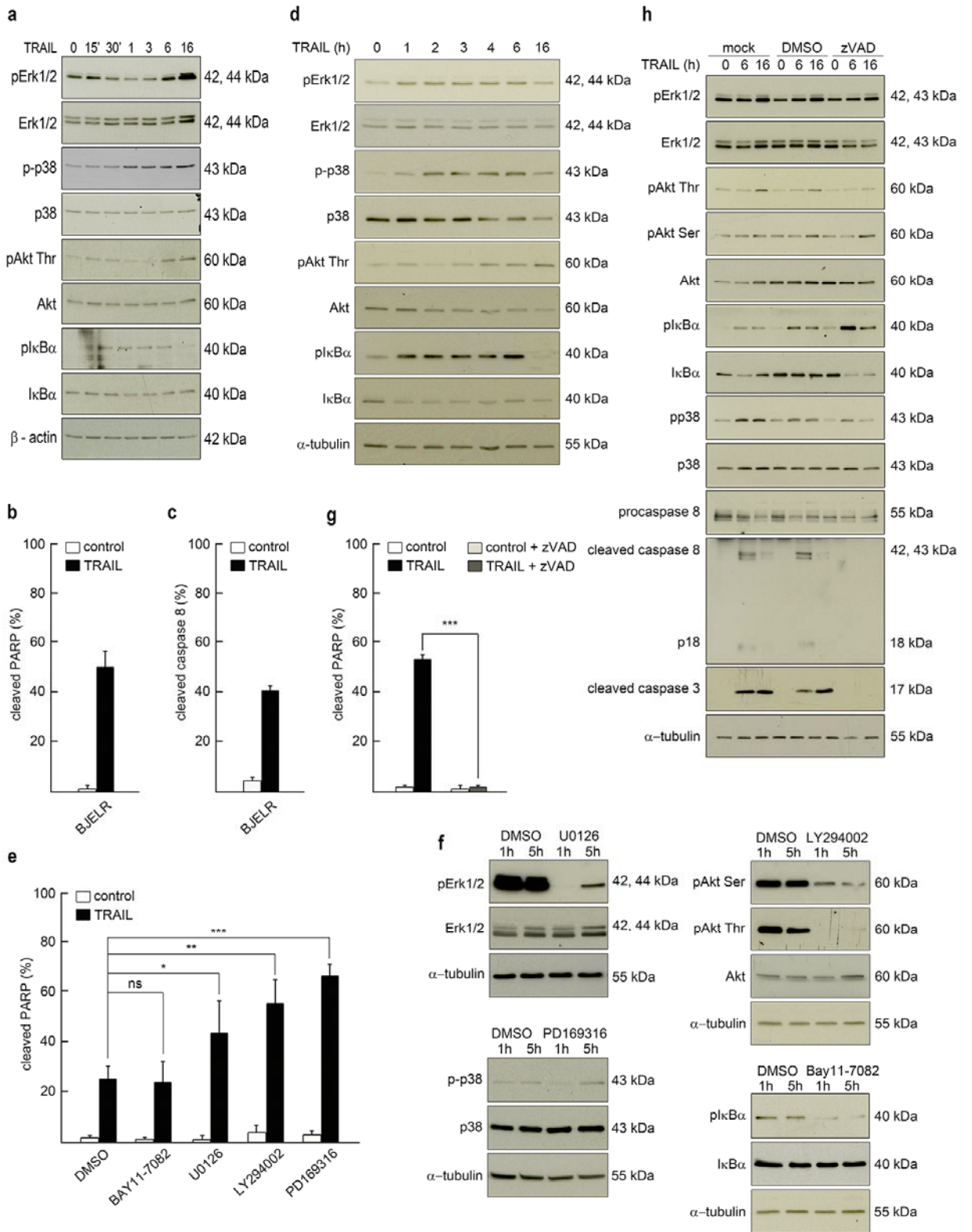


Figure 12

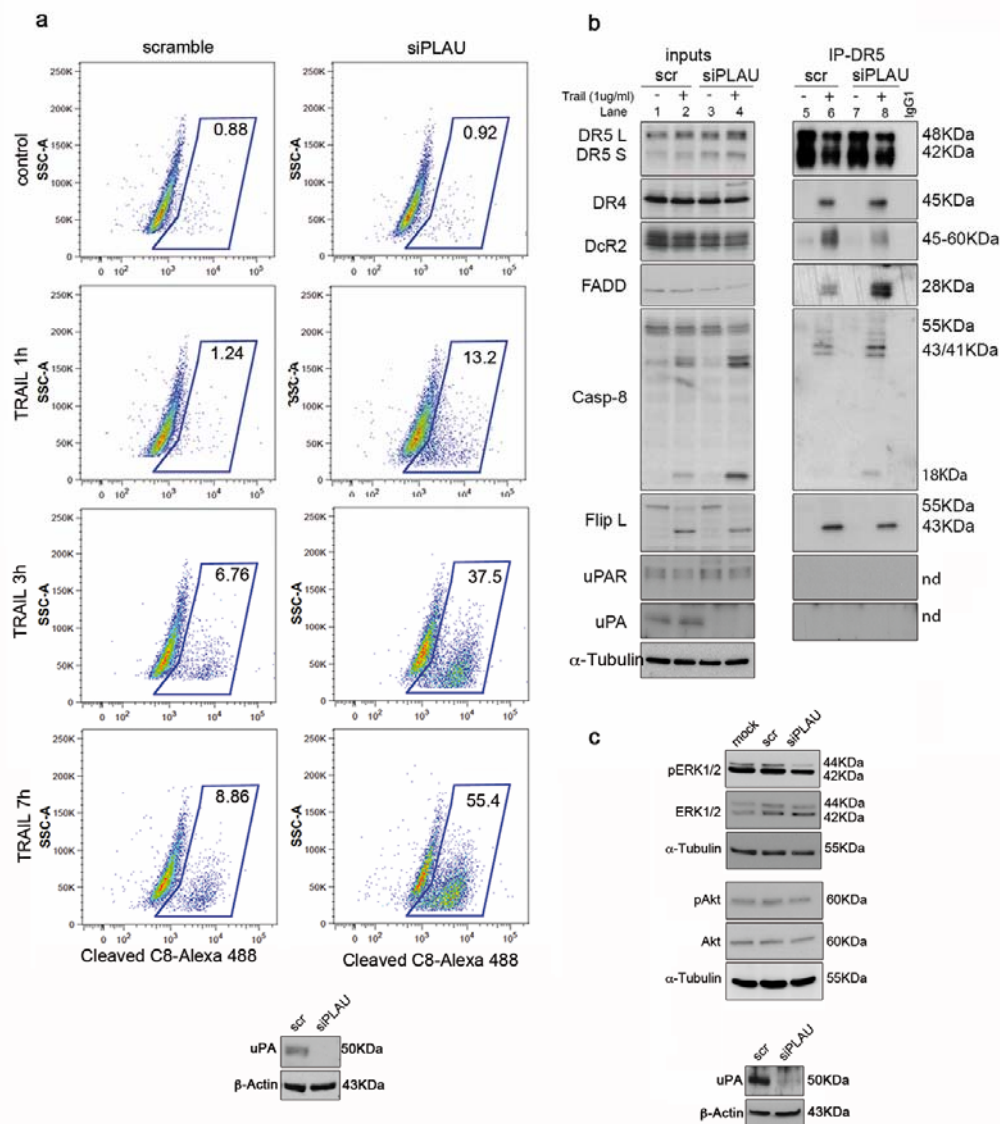


Figure 12. Role of uPA in the regulation of TRAIL-induced apoptosis. **a**-Time course analysis of procaspase-8 cleavage. BJELR cells were transfected either with pooled siRNAs targeting *PLAU* mRNA (“siPLAU”) or non-targeting scramble siRNAs (“scramble”). Forty-eight hours post-transfection cell populations were either left untreated or challenged with 1 μ g/ml TRAIL during 1, 3 or 7 h and the percentage of cells displaying cleaved caspase-8 (“C8”) was determined by flow cytometry. Images from one representative experiment out of three independent replicates are shown. Percentage of cleaved C8-positive cells is indicated. **b**- DISC composition in uPA depleted cells. BJELR cells were transfected either with pooled siRNAs targeting *PLAU* mRNA (“siPLAU”) or non-targeting scramble siRNAs (“scr”) and 48 h post-transfection cell populations were either left untreated or challenged with TRAIL (1 μ g/ml) during 30 min. Immunoprecipitation of Death Receptor 5 (IP-DR5) was performed and co-immunoprecipitation of cognate DISC components, uPA and uPAR was analyzed by Western blot. Immunoprecipitation using isotopic IgG1 (“IgG1”) was used as a background control. **c**- Phosphorylation status of Erk1/2 and Akt upon uPA knock down. BJELR cells were left non-transfected (“mock”) or transfected either with pooled siRNAs targeting *PLAU* mRNA (“siPLAU”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection, total and phosphorylated protein levels of Erk1/2 (Thr202/Tyr204) and Akt (Ser 473) were analyzed by Western blot. α -Tubulin, loading control.

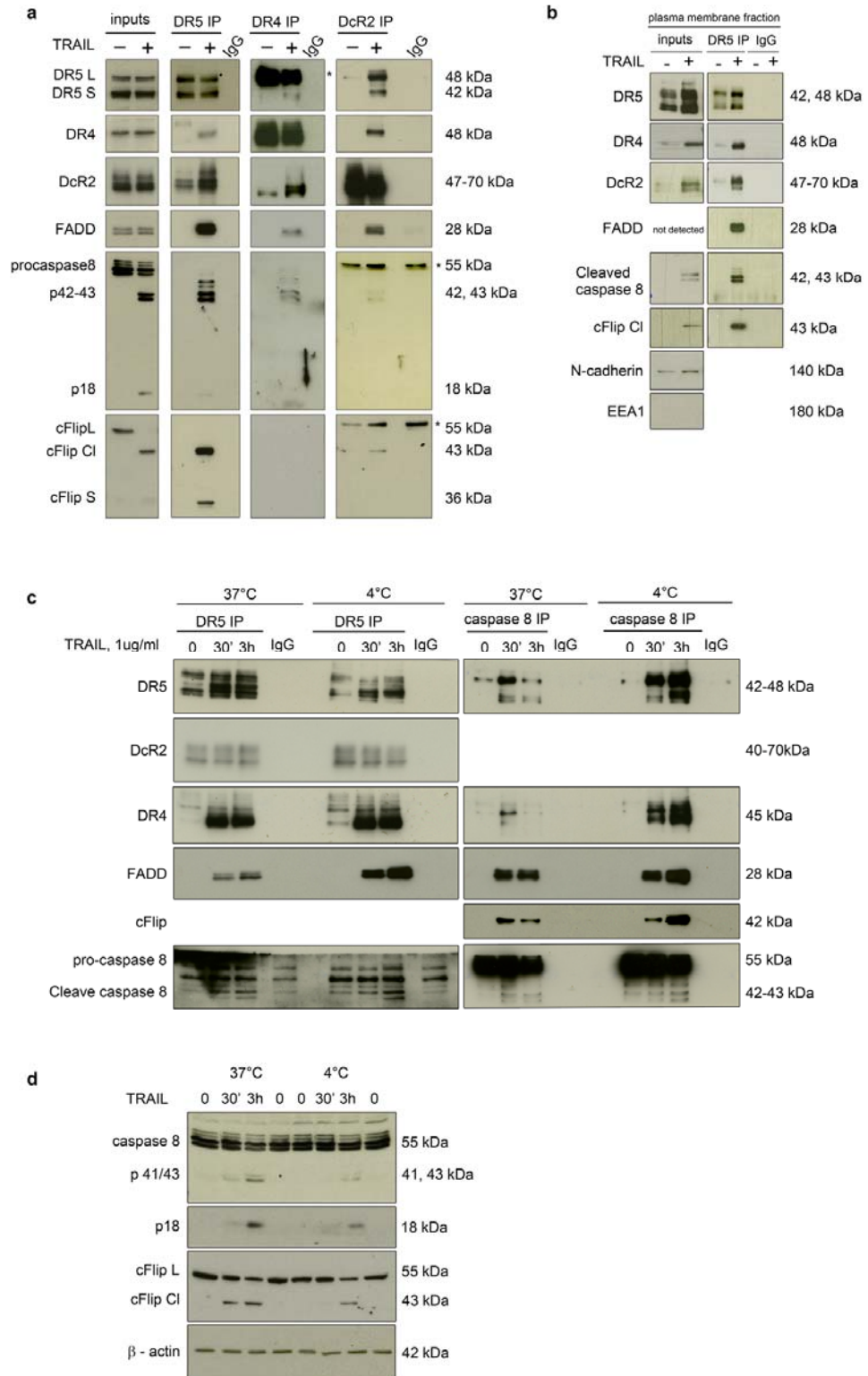
4. Pro-apoptotic and pro-survival platforms are assembled in response to TRAIL

Taking into account our above-described results we hypothesized that the phenomenon of fractional killing largely relies on the initial events of TRAIL signaling. Therefore, to shed light on the molecular basis that balance fractional killing in clonal populations of cancer cells, we analyzed which are the signaling platforms that are formed in response to TRAIL in our cellular model. Initially, using flow cytometry assays we found that DR4, DR5 and DcR2 are expressed at the cell surface of BJELR cells (Fig 10a). Importantly, previous reports showed that different TRAIL receptors can participate in the activation of different pathways from apoptotic to pro-survival suggesting that TRAIL receptors can serve as a bifurcation point for the induction of functionally different signaling outcomes^{84,112-115,290}. Based on this data we set to determine whether different TRAIL-Rs form distinct complexes or ligand-induced clustering leads to the formation of DR4/DR5/DcR2 heterocomplexes. To shed light on this matter we performed immunoprecipitation of DR4, DR5 and DcR2 followed by the identification of previously reported DISC components by Western blot. We found that DR4, DR5 and DcR2 are associated in heterocomplexes upon TRAIL treatment, which in turn results in the recruitment of FADD, cFlip and caspase 8 (Fig 13a). Given that, it has been reported that the assembly of the pro-apoptotic DISC upon FAS stimulation occurs in the early endosomes^{216,217}, and that the formation of the apoptotic complex II upon TNF α treatment also takes place in the cytosol after internalization of complex I²⁸, we set to analyze the subcellular localization of the TRAIL-induced DISC. Contrary to Fas and TNF α signaling, extraction of the plasma membrane fraction followed by DR5 immunoprecipitation revealed that TRAIL-induced DISC is formed at the plasma membrane in our experimental system (Fig 13b). To study how plasma membrane localization of the DISC affects its composition we performed DR5 immunoprecipitation from cells treated with TRAIL at 4°C in order to block internalization. Interestingly, we detected significantly higher FADD association with DR5 after 3h of TRAIL treatment at 4°C as compared to 37°C (Fig 13c). Moreover, caspase 8 immunoprecipitation indicated dramatically higher caspase 8 association with DR5, DR4, FADD and cFlip after 3h of TRAIL treatment at 4°C as compared to 37°C (Fig 13c). These results suggest that plasma membrane localization of the DISC supports recruitment of its components and/or blocking of internalization increases stability of the complex. However, even though association of DISC members was significantly higher at 4°C as compared to 37°C, caspase 8 and cFlip cleavage was decreased at 4°C (Fig 13d). This might indicate that even though a DISC is formed at the plasma membrane, it requires internalization for efficient signaling transduction (for the recruitment of additional members, availability of substrates, etc). In that regard, it was proposed that the role of TRAIL-R internalization in apoptosis depends on whether cells belong to «type L cells» (lysosomal cytotoxic signaling) that requires lysosomal proteases for apoptotic signaling or «type D cells» (direct signaling), that is independent of lysosomal permeabilization⁷⁵. However, given that biochemical reactions including caspase 8 and cFlip cleavage may be slowed down at low temperatures, the effect of 4°C on the efficiency of apoptotic cell death may be indirect and independent of the blocking of internalization. Other strategies (e.g. monodansyl cadaverin (MDC) treatment, clathrin heavy chain depletion and dynamin mutants) have to be used in order to define the requirement of internalization for the transduction of apoptotic signaling in our experimental model.

Overall, our results show that TRAIL induces DISC formation at the plasma membrane and suggest that plasma membrane localization supports recruitment of its members.

Figure 13. TRAIL-induced DISC is formed at the plasma membrane. **a** – DISC composition in BJELR cells. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Immunoprecipitation of Death Receptor 5 (DR5-IP), Death Receptor 4 (DR4-IP), and Decoy Receptor 2 (DcR2-IP) was performed and co-immunoprecipitation of DISC components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as a background control. *The issue concerning the cross reactivity of antibodies that were used for immunoprecipitation experiments is described in the “Materials and Methods: Immunoprecipitation” section. **b** – DISC assembly in BJELR cells takes place at the plasma membrane. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Further plasma membrane fraction was extracted from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of Death Receptor 5 (DR5-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of DISC components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. **c** – Impact of internalization on the DISC formation and/or its stability. BJELR cells were either left at 37°C or placed at 4°C for 15 min. Further, BJELR cells kept at 37°C and 4°C were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 (“30”) min or 3h (“3h”). Immunoprecipitation of DR5 (“DR5 IP”) and caspase 8 (“caspase 8 IP”) was performed and co-immunoprecipitation of DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as a background control. Image corresponds to one representative experiment out of three independent biological replicates. **d** – Impact of internalization on caspase 8 and cFlip cleavage. BJELR cells were either left at 37°C or placed at 4°C for 15 min. Further, BJELR cells kept at 37°C and 4°C were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 (“30”) min or 3h (“3h”). Cells were collected for Western blot. Total and cleaved levels of caspase 8 and cFlip were analyzed by Western blot. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.

Figure 13



On the other hand, the triggering of non-apoptotic signaling in response to TRAIL has been ascribed to the formation of a “secondary complex” composed by FADD, Caspase 8 as well as RIPK1, TRAF2, TRADD and NEMO ¹¹⁹. Although TRAIL-induced assembly of such secondary complex has been reported for cancer cells, the molecular mechanisms leading to its formation remain elusive. It has been demonstrated that following the formation of the primary complex that leads to kinases activation, TNFR1 stimulation leads to the assembly of a secondary pro-apoptotic complex ²⁸. Based on this Varfolomeev et al. hypothesized that TRAIL may act in a similar way but with an opposite sequence of events: first it induces formation of the apoptotic DISC and then a secondary complex that activates kinases ¹¹⁹. In that regard, it has been reported that DISC immunoprecipitation from TRAIL treated HT1080 cell line ¹¹⁹ (display fractional killing upon TRAIL treatment) did not lead to the detection of RIPK1, TRAF2 or NEMO. Contrary, secondary IP of RIPK1 from DISC – immunodepleted lysates revealed FADD and caspase 8 as well as TRAF2 and NEMO being co-immunoprecipitated with RIPK1 ¹¹⁹. Therefore, these results suggested that TRAIL may induce the formation of the primary receptor-associated DISC and the secondary intracellular non-apoptotic signaling platform. Importantly, later Song et al. provided evidences that RIPK1 can be recruited to the receptor-associated DISC of TRAIL resistant NSCLC – A549 cell line ¹¹². These controversial results could be explained by differences in response to TRAIL. Indeed, HT1080 cells undergo fractional killing upon exposure to TRAIL, while A-549 cells are fully resistant to ligand-induced apoptosis. Moreover, recently it has been shown that TRAF2 is assembled with the DISC upon exposure to TRAIL, where it leads to caspase 8 ubiquitination and its proteosomal degradation ¹⁰⁸. Overall, these results suggest that TRAIL has the potential to form not only apoptotic DISC but also non-apoptotic signaling platforms that may lead to the activation of non-apoptotic/pro-survival signaling; however, their composition and mechanisms of formation may be cell type-dependent.

Taking into account these reports and the fact that TRAIL induces non-apoptotic pathways activation in our experimental model, we set to identify whether the formation of the non-apoptotic signaling platform takes place also in BJELR cells. For that, we performed DR5 immunoprecipitation from the whole cell lysate followed by the detection of RIPK1 and TRAF2 as well as other DISC components by Western blot. Our results showed that both RIPK1 and TRAF2 are recruited to the DISC upon TRAIL treatment (Fig 14a). Moreover, TRAF2 immunoprecipitation led to the detection of TRAIL-induced DR5/TRAF2 association (Fig 14b). These data goes in line with previous reports indicating that RIPK1 and TRAF2 are recruited to TRAIL-Rs upon TRAIL treatment and exert non-apoptotic function. Importantly, it is well documented that TNF α -induced complex I and complex II are temporary and spatially separated, as they are formed at the plasma membrane and in the cytosol, respectively ²⁸. Taking this into account and given that the apoptotic DISC is formed at the plasma membrane we evaluated whether the non-apoptotic signaling platform is also formed in the same cellular compartment. For that we extracted plasma membrane fraction followed by DR5 and RIPK1 immunoprecipitation. Notably, DR5 immunoprecipitation revealed an association of DR5 with RIPK1 as well as with other DISC components (FADD, caspase 8, DR4, DcR2, cFlip) upon TRAIL treatment (Fig 14c). On the other hand, RIPK1 immunoprecipitation from the plasma membrane indicated TRAIL-induced binding of RIPK1 to DR5, DcR2, TRAF2 and cFlip (Fig 14d, 17c-WT). Interestingly, neither FADD nor caspase 8 were detected in RIPK1 IP suggesting that RIPK1 and TRAF2 might associate with DR5 independently of

FADD and caspase 8. In that regard, it was previously reported that RIPK1 can directly interact with death receptors through death domains²⁴. It was also suggested that TRAF2 can be recruited directly to TNFR2, independently of the death domain. Moreover, it was shown that TRAF2 can interact with cFlip (p43), but not with cFlipS, cFlip (233-480), cFlip (233-376) or Flip (p12)²⁹¹. Further experiments, in particular caspase 8 and FADD immunoprecipitation from the plasma membrane fraction are required in order to confirm that caspase 8 and FADD are indeed not recruited to the non-apoptotic complex at the plasma membrane (see “Ongoing work and perspectives”). Overall, our initial observations suggest that TRAIL may induce formation of two different plasma membrane located DR5-associated complexes: (i) the DISC, which includes TRAIL-Rs, FADD, caspase 8 and cFlip (Fig 14g (b)); and (ii) the non-apoptotic signaling platform that consists of DR5, DcR2, RIPK1, TRAF2 and cFlip, but does not contain FADD and caspase 8 (Fig 14g (a)). Notably, immunoprecipitation experiments targeting caspase 8 from the whole cell lysate revealed that RIPK1, TRAF2, FADD as well as TRAIL receptors co-immunoprecipitate with this caspase upon TRAIL challenge (Fig 14e). Based on this data we hypothesized that the non-apoptotic signaling complex may undergo internalization which in turn leads to the recruitment of caspase 8 through its interaction with cFlip via DED (death effector domain) of both proteins (Fig 14g (c)). On the other hand, during internalization RIPK1/TRAF2/cFlip can potentially dissociate from TRAIL receptors, which in turn may lead to the association of RIPK1/TRAF2/cFlip with FADD (Fig 14g (d)) resulting in the recruitment of caspase 8 via DED of FADD molecule. Thus, caspase 8 immunoprecipitation from the whole cell lysate leads to the pull down of caspase 8 from two different complexes, leading to the identification of components that form the DISC on one hand and the non-apoptotic signaling platform/s on the other (Fig 14g (e)).

Interestingly, our preliminary data showed that TRAIL treatment of BJELR cells at 4°C completely prevented I κ B α phosphorylation, suggesting that even though TRAIL-induced non-apoptotic signaling platform is formed at the plasma membrane, the triggering of non-apoptotic pathways may require internalization (Fig 14f). This may support our hypothesis that the non-apoptotic signaling platform undergoes internalization, recruits caspase 8 in the cytosolic compartment and triggers non-apoptotic pathways. However, given that enzymatic reactions can be slowed down at 4°C, an observed effect may not be directly related to the block of internalization. Therefore, further experiments using alternative approaches to prevent internalization are required in order to characterize the role of internalization in the triggering of I κ B α as well as in the induction of other non-apoptotic pathways (Erk1/2, Akt, p38MAPK) (see “Ongoing work and perspectives”).

Altogether, our results suggest that TRAIL-induced formation of both pro-apoptotic and pro-survival platforms in populations of BJELR cells primarily occurs at the plasma membrane. Further internalization might be required for the recruitment of additional components and transduction of TRAIL-induced apoptotic and/or non-apoptotic signaling.

Figure 14

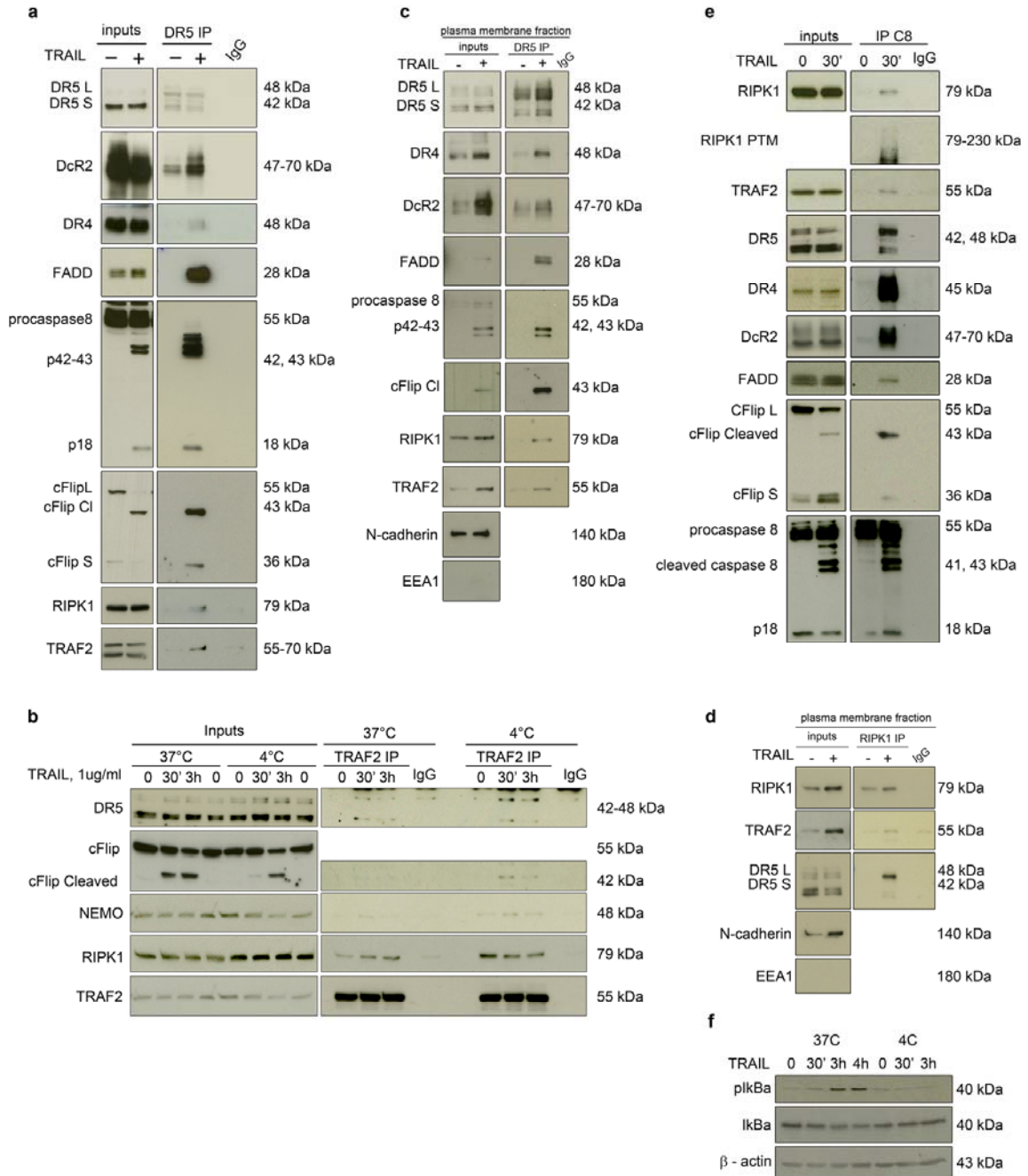


Figure 14. TRAIL induces formation of the non-apoptotic signaling platform. **a-** Co-immunoprecipitation of RIPK1 and TRAF2 with DR5 upon TRAIL treatment. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 (“+”) min. Immunoprecipitation of DR5 (DR5 IP) was performed and co-immunoprecipitation of DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. Image corresponds to one representative experiment out of three independent biological replicates. **b-** Co-immunoprecipitation of DR5 and RIPK1 with TRAF2 upon TRAIL treatment. BJELR cells were either left at 37°C or placed at 4°C for 15 min. Further, BJELR cells kept at 37°C and 4°C were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 (“30”) min or 3h (“3h”). Immunoprecipitation of TRAF2 (“TRAF2 IP”) was performed and co-immunoprecipitation of DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. Image corresponds to one representative experiment out of three independent biological replicates. **c** – RIPK1 and TRAF2 are recruited to the DISC at the plasma membrane. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Further plasma membrane fraction was extracted from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of Death Receptor 5 (DR5-I) was performed from the plasma membrane fraction and co-immunoprecipitation of DISC and secondary complex components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as a background control. **d** – TRAIL-induced non-apoptotic signaling complex is formed at the plasma membrane. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Further plasma membrane fraction was separated from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of RIPK1 (RIPK1-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of DISC and secondary complex components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. **e-** TRAIL-induced co-immunoprecipitation of caspase 8 with RIPK1 and TRAF2. BJELR cells were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 (“30”) min. Immunoprecipitation of caspase 8 (“c8 IP”) was performed and co-immunoprecipitation of the DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as a background control. **f-** Impact of the internalization on the TRAIL-induced phosphorylation of IκBα. BJELR cells were either left at 37°C or placed at 4°C for 15 min. Further, BJELR cells kept at 37°C and 4°C were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 (“30”) min or 3h (“3h”). Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of IκBα (Ser32/36) were analyzed. β- Actin, loading control. Image corresponds to one representative experiment out of at least three independent biological replicates.

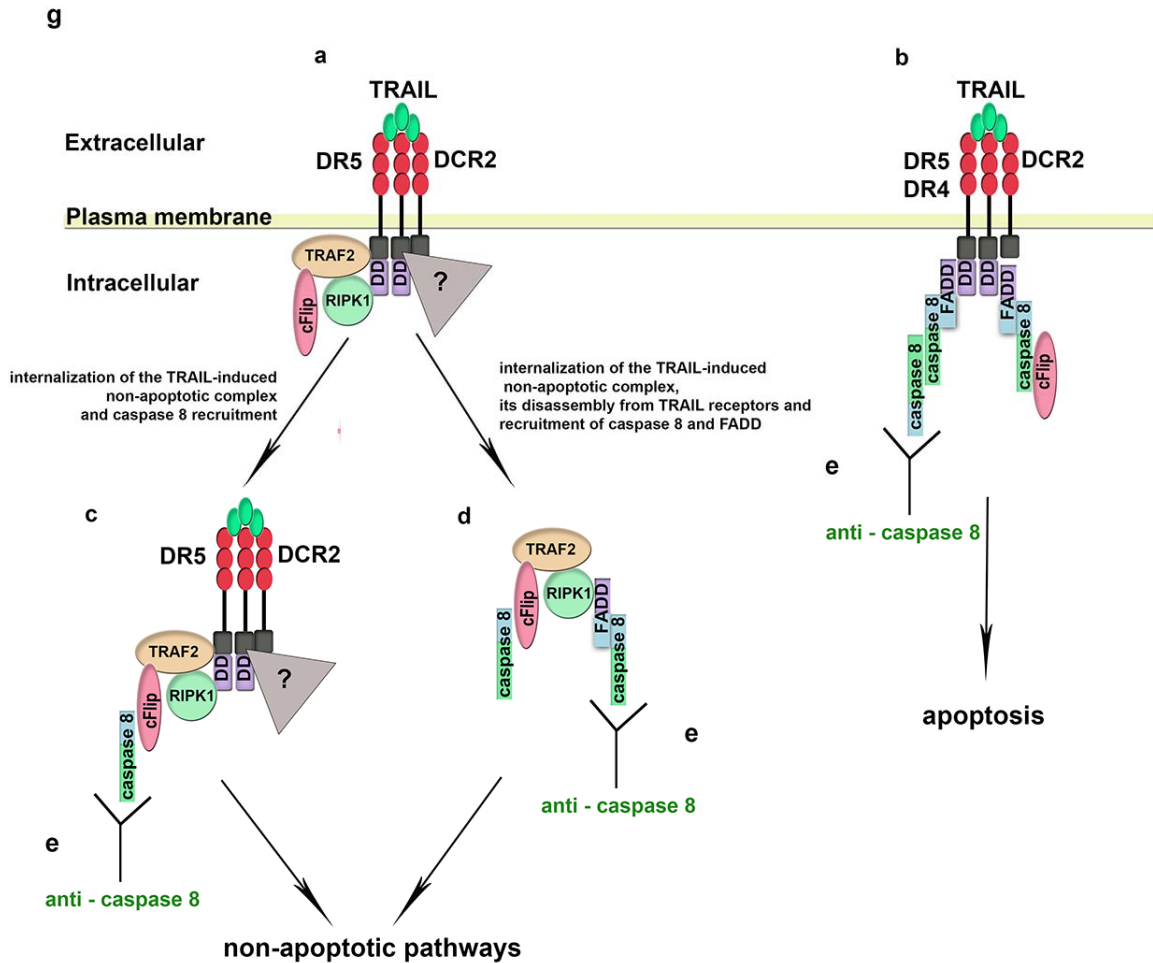


Figure 14 continued. TRAIL induces formation of the non-apoptotic signaling platform- A model depicting the localization, composition and possible mechanisms of formation of the DISC and TRAIL-induced non-apoptotic signaling complex/es. **(a)** The non-apoptotic signaling platform comprising of DR5, DcR2, RIPK1, TRAF2 and cFlip is formed at the plasma membrane upon TRAIL treatment. **(b)** Assembly of the TRAIL-induced DISC formed by DR5, DR4, DcR2, FADD, caspase 8 and cFlip takes place at the plasma membrane upon exposure to ligand. **(c)** Non-apoptotic signaling complex may undergo internalization, which in turn leads to the recruitment of caspase 8 possibly through its interaction with cFlip via DED (death effector domain) of both proteins. **(d)** During internalization RIPK1/TRAF2/cFlip can potentially dissociate from TRAIL receptors, which in turn may lead to the association of RIPK1/TRAF2/cFlip with FADD followed by caspase 8 recruitment. **(e)** Caspase 8 immunoprecipitation leads to the pull down of caspase 8 from two or more different complexes, leading to the identification of components that form a DISC on one hand and non-apoptotic signaling platform/s on the other hand.

5. Role of TRAIL-Rs and TRAIL-Rs binding proteins in the triggering of apoptotic and non-apoptotic pathways

Our above-described results show that TRAIL induces the activation of apoptotic as well as non-apoptotic signaling pathways in BJELR cells. Furthermore, our preliminary data suggest that TRAIL may induce the formation of two different plasma membrane-located DR5-associated complexes: (i) the DISC, which includes TRAIL-Rs, FADD, caspase 8 and Flip (Fig 14g (b)); and (ii) the non-apoptotic signaling platform that consists of DR5 and DcR2, RIPK1, TRAF2 and cFlip, but lacks FADD and caspase 8 (Fig 14g (a)). Following our initial hypothesis that the bifurcation of TRAIL-induced signaling for the activation of apoptotic and non-apoptotic pathways relies on the initial events of TRAIL signaling we studied the role of the aforementioned proteins in the triggering of both apoptotic and non-apoptotic cascades. For that, we used siRNA-mediated approach to knock down TRAIL receptors as well as FADD, caspase 8, cFlip, RIPK1 and TRAF2. The impact of the knock down of each protein on the sensitivity to TRAIL-induced apoptosis was analyzed by measuring the percentage of cells displaying positive labeling for cleaved PARP by flow cytometry. Importantly, siRNA-mediated depletion of DR5 resulted in a complete block of cell death upon TRAIL treatment, whereas downregulation of DcR2 enhanced the apoptotic response upon exposure to this ligand. These results go in line with their reported “death” (DR5) and “decoy” (DcR2) nature (Fig 10d). Surprisingly, knock down of DR4 resulted neither in the block of apoptosis nor in the sensitization to TRAIL-induced cell death. Thus, although DR4 is recruited to the DISC it is not fully required for apoptotic and pro-survival signaling activation (Fig 10e). Further, we analyzed the role of FADD and caspase 8 in the triggering of apoptotic pathway in our cellular model. We observed that cell death is blocked in the absence of FADD or caspase 8 suggesting that FADD is the main adaptor molecule, while caspase 8 is the main initiator caspase in our experimental system (Fig 10h). Importantly, knock down of cFlip decreases the cellular threshold to TRAIL-induced cell death, indicating an anti-apoptotic role of cFlip in BJELR cells (Figure 10h).

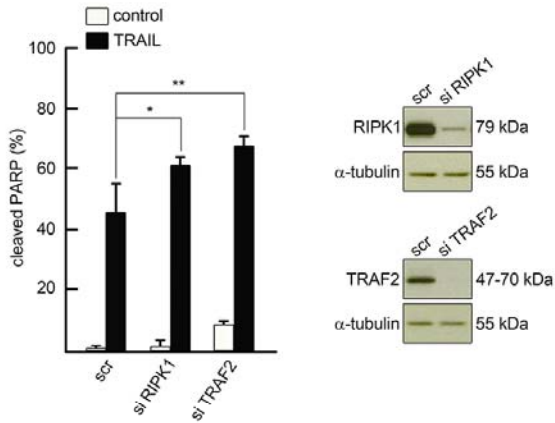
Notably, siRNA-mediated depletion of RIPK1 and TRAF2 led to an increased cell death upon TRAIL treatment, suggesting a pro-survival function of these two proteins in our system (Fig 15a). In that respect, it was reported that TRAF2, due to its E3 ligase activity, leads to K63 linkage specific ubiquitination of RIPK1, which in turn enables RIPK1-mediated activation of NF- κ B upon TNF α treatment³¹. In contrast, deubiquitination of RIPK1 switches its function to pro-apoptotic³⁰. To identify whether cell death observed in TRAF2 knock down cells is due to a change in the function of RIPK1 from anti- to pro-apoptotic we performed double knock down of RIPK1 and TRAF2. We did not detect any significant difference between the percentage of TRAIL-induced cell death observed in TRAF2 knock down as compared to RIPK1/TRAF2 double knock down, suggesting that cell death observed in TRAF2 knock down is not due to the switch in RIPK1 function in the absence of TRAF2, but occurs due to the anti-apoptotic function of TRAF2 itself (Fig 15b). In that regard, it was recently reported that TRAF2 can induce K48 linkage specific ubiquitination of caspase 8 targeting it to proteosomal degradation¹⁰⁸. On the other hand, it was found that cullin 3 - based E3 ligase is associated with the DISC upon TRAIL treatment and leads to caspase 8 polyubiquitination followed by the recruitment of ubiquitin binding protein p62 that promotes caspase 8 aggregation and processing¹⁰⁷. Importantly, TRAIL receptors can also undergo posttranslational modifications that have a major impact on their pro-

apoptotic function^{70,229}. Overall, these results suggest that posttranslational modifications of DISC members may play a crucial role in the regulation of TRAIL signaling. Therefore, given that TRAF2 and RIPK1 are recruited to DR5-containing complex/es and taking into account their possible role in posttranslational modifications we analyzed whether the recruitment of TRAF2 and RIPK1 to the DISC can modify the function of TRAIL receptors. To shed light on this issue we set to study whether cell death observed in RIPK1 and TRAF2 knockdown is still DR5-dependent or is due to the acquisition of a pro-apoptotic role by DR4 in the absence of RIPK1 and/or TRAF2. For that we performed double knock down of RIPK1/DR5 and TRAF2/DR5 and analyzed cell death rate upon TRAIL treatment. Double knock down of RIPK1/DR5 and TRAF2/DR5 led to a complete block of cell death, suggesting that the non-apoptotic function of RIPK1 and TRAF2 is not due to their direct impact (posttranslational modifications) on the function of TRAIL receptors (Fig 15c). Finally, treatment of RIPK1, TRAF2 and RIPK1/TRAF2 knock down cells with pan caspase inhibitor zVAD.fmk resulted in the block of death upon TRAIL treatment, suggesting that cell death observed in these conditions is still caspase-mediated (Fig 15b).

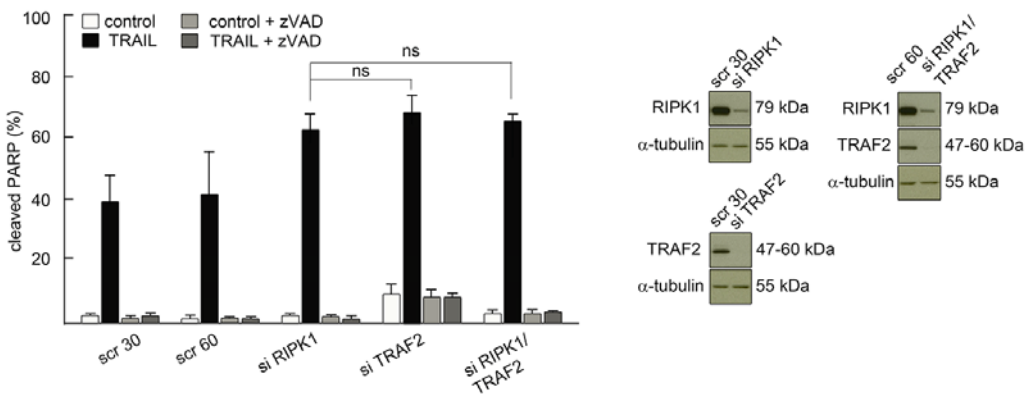
Figure 15. RIPK1 and TRAF2 play a pro-survival role in BJELR cells. **a-** Impact of RIPK1 and TRAF2 knock down on BJELR sensitivity to TRAIL. BJELR cells were transfected either with siRNAs targeting *RIPK1* (“si RIPK1”) and *TRAF2* (“si TRAF2”) mRNA or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection cell populations were either left untreated (“control”) or challenged with 1µg/ml TRAIL (“TRAIL”) during 1h and the percentage of cells with positive labeling for cleaved PARP was analyzed by flow cytometry. Histograms represent the mean +/- Standard Deviation (SD) from at least five independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P* value< 0.0005, ***P* value<0.005, **P* value<0.05. **b-** Impact of TRAF2 on the RIPK1 function. BJELR cells were transfected either with siRNAs targeting *RIPK1* (“si RIPK1”), *TRAF2* (“si TRAF2”), *RIPK1/TRAF2* (“si RIPK1/TRAF2”) mRNA or non-targeting scramble siRNAs (“scr 30” and “scr 60”). Forty-eight hours post-transfection cell populations were either left untreated or pretreated with 20µM zVAD for 1h and then cell populations were either left untreated (“control” and “control+zVAD”) or challenged with 1µg/ml TRAIL (TRAIL) during 1h (“TRAIL” and “TRAIL+zVAD”) and the percentage of cells with positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least five independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P* value< 0.0005, ***P* value<0.005, **P* value<0.05. Efficiency of RIPK1, TRAF2 and RIPK1/TRAF2 knock down at 48 h post-transfection was analyzed by Western blot. α - Tubulin, loading control. **c-** Impact of RIPK1 and TRAF2 on the functional role of TRAIL receptors. BJELR cells were transfected either with siRNAs targeting *RIPK1* (“si RIPK1”), *TRAF2* (“si TRAF2”), *DR5* (“si DR5”), *DR5/TRAF2* (“si DR5/TRAF2”), *DR5/RIPK1* (“si DR5/RIPK1”) mRNA or non-targeting scramble siRNAs (“scr 30” and “scr 60”). Forty-eight hours post-transfection cell populations were either left untreated (“control”) or challenged with 1µg/ml TRAIL (TRAIL) during 1h (“TRAIL”) and the percentage of cells with positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least five independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P* value< 0.0005, ***P* value<0.005, **P* value<0.05. Efficiency of RIPK1, TRAF2, DR5, DR5/TRAF2 and DR5/RIPK1 knock down at 48 h post-transfection was analyzed by Western blot. α - Tubulin, loading control.

Figure 15

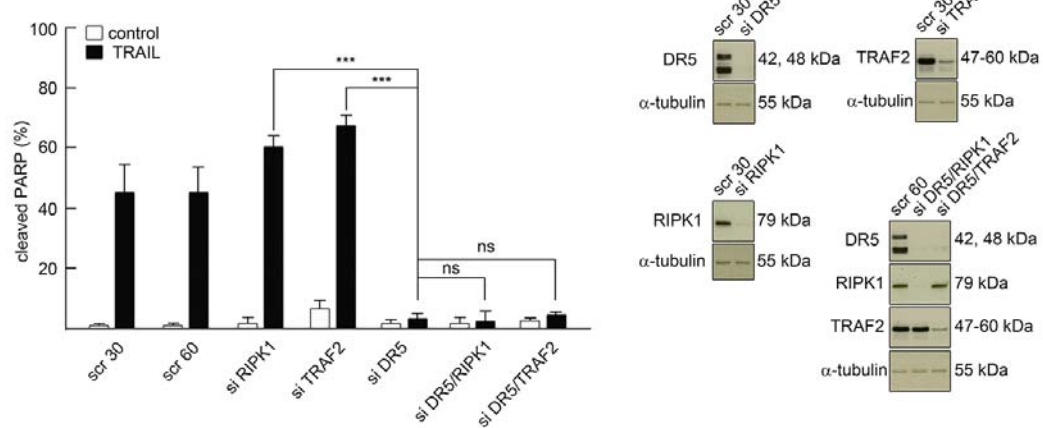
a



b



c



To evaluate the involvement of DISC components in the activation of non-apoptotic cascades, each DISC member was knocked down by siRNA-mediated approaches, cells were further exposed to TRAIL and phosphorylation levels of Erk1/2, p38MAPK, Akt and I κ B α were estimated by Western blot. We observed that FADD was required for p38MAPK and I κ B α phosphorylation upon TRAIL challenge (Fig 16a). Moreover, TRAIL-induced Erk1/2 phosphorylation was delayed upon FADD knock down (Fig 16b). Interestingly, Akt phosphorylation in response to TRAIL took place independently of FADD (Fig 16a). Overall, our results show that FADD participates in the triggering of TRAIL-induced non-apoptotic and pro-survival pathways, while being indispensable for apoptotic signaling. In line with our findings, Varfolomeev et al. also reported that FADD depletion leads to the block of I κ B α activation and reduces the phosphorylation level of p38MAPK¹¹⁹ in response to TRAIL. Importantly, it has been previously reported that a FADD mutant lacking the death domain inhibits mitogen-induced proliferation of mature T cells, suggesting that the function of FADD is not restricted to being pro-apoptotic^{292,293}.

Interestingly, our results showed that caspase 8 is required for TRAIL-induced I κ B α phosphorylation as well as p38MAPK and Erk1/2 activation, while it is not required for Akt phosphorylation (Fig 16c, d). In that respect, it was found that caspase 8 is indispensable for lymphocytes proliferation^{294,295}. These results indicate that the role of caspase 8 is not restricted to pro-apoptotic. Briefly, deficiency of caspase 8 in human and murine T cells diminishes their capacity to proliferate and leads to the development of immunodeficiency syndrome^{294,295}. Moreover, it was previously reported that the presence of caspase 8 in TRAIL-induced secondary complex is required for the activation of p38MAPK, JNK and I κ B α ¹¹⁹. These data support our finding of a role of caspase 8 in non-apoptotic and pro-survival pathways, while also being indispensable for TRAIL-induced apoptosis. Apparently, these two opposite functions of caspase 8 require precise control and regulation mechanisms. In that regard, it was shown that active caspase 8 is localized in different cellular compartments of T cells that undergo activation with proliferation (CD3) or apoptotic (FAS) stimuli²³⁰. In brief, CD3 stimulation leads to the redistribution of a small portion of active caspase 8 to lipid raft domains of the plasma membrane, while FAS treatment results in the profound activation of caspase 8 exclusively in the cytosolic fraction²³⁰. Therefore, spatial localization of caspase 8 may strongly affect its functional role, which can be achieved due to an altered access to different caspase 8 substrates. Thus, in line with our previous results suggesting that caspase 8 is recruited to the DISC as well as may participate in the formation of TRAIL-induced non-apoptotic signaling complex, we hypothesized that the role of caspase 8 can vary depending on its localization in one or the other complex, that in turn can be located in different subcellular/plasma membrane compartments. This hypothesis may be supported by the fact that DISC-associated caspase 8 is detected in the plasma membrane fraction, while our preliminary data suggest that caspase 8 association with RIPK1 does not take place at the plasma membrane. In contrast, caspase 8 and RIPK1 assembly is detected when immunoprecipitation is performed from the whole cell lysate. Overall, these results suggest that interaction of caspase 8 with two functionally different signaling complexes may take place in different cellular compartments; this distinct compartmentalization could regulate caspase 8 function within these complexes. Given that FADD is required for the activation of the same non-apoptotic pathways as caspase 8, and is an adaptor molecule required for caspase 8 recruitment to the DISC, it is possible that FADD function in the activation of apoptotic vs non-apoptotic signaling relies on the same

mechanisms that control caspase 8 function.

We have shown that while apoptotic signaling requires caspase 8 cleavage, the triggering of non-apoptotic pathways is independent of caspase 8 cleavage (Fig 11g, h). Interestingly, as already mentioned before, blocking of caspase cleavage enhanced I κ B α phosphorylation. Based on this we set to analyze whether caspases inhibition also supports the formation of non-apoptotic signaling platforms. Our preliminary data suggest that inhibition of caspases cleavage increased caspase 8 - RIPK1 association upon TRAIL treatment (Fig 16e), suggesting that inhibition of caspase activity supports the formation of signaling platform/s that balance the system towards the activation of non-apoptotic pathways. In that respect, it was reported that caspase 8 can induce RIPK1 cleavage⁴². Notably, we also observed RIPK1 cleavage upon TRAIL treatment in BJELR cells (Fig 16f). Therefore, blocking caspase 8 can prevent RIPK1 cleavage supporting the formation of non-apoptotic signaling platform/s and the activation of pro-survival and non-apoptotic pathways.

Further, we analyzed the impact of cFlip on the phosphorylation of kinases upon TRAIL treatment. Our initial results show that cFlip is required for the activation of p38MAPK and Erk1/2 in response to TRAIL (Fig 16g). Importantly, it was reported that different cFlip isoforms (cFlipS and cFlipL) exert a differential impact on the function of the DISC and consequently on the modulation of TRAIL signaling⁹⁹⁻¹⁰². Briefly, cFlipL can activate or inhibit caspase 8 depending on caspase 8: cFlipL ratio at the DISC⁹⁹⁻¹⁰². In contrast, cFlipS completely blocks caspase 8 processing by preventing DED-mediated caspase 8 assembly⁹⁹. Moreover, it was reported that cFlipL, but not cFlipS, is required for NF- κ B activation^{291,296}. Given that siRNA used in our experiments targets both: short and long isoform, we cannot discriminate whether both of them or only one of the isoforms is needed for the phenotype that we observe in cFlip knock down cells. Importantly, zVAD treatment that prevents not only caspase 8 but also cFlip cleavage (Fig 16e) does not block the activation of p38MAPK and Erk1/2, suggesting that cleavage of cFlipL is not necessary for the triggering of above mentioned kinases (Fig 11h).

Notably, RIPK1 depletion abrogated TRAIL-induced activation of I κ B α , p38MAPK, but not Erk1/2 and Akt (Fig 16h). Our results go in line with previous reports suggesting that RIPK1 is required for the activation of p38MAPK and NF- κ B^{119,120}. Interestingly, we did not observe any significant effect of TRAF2 knock down on the activation of Erk1/2, p38MAPK or Akt upon TRAIL challenge (Fig 16h). Moreover, TRAF2 depletion did not prevent TRAIL-induced I κ B α phosphorylation (Fig 16h). In that regard, the role of TRAF2 in the activation of NF- κ B upon TNF α treatment is well characterized³⁰. It has been found that TRAF2 is required for cIAPs to induce K63 linkage specific ubiquitination of RIPK1, an event necessary for NF- κ B activation³⁰. Varfolomeev et al. also showed that TRAF2 is not necessary for I κ B α phosphorylation upon TRAIL treatment¹¹⁹. Therefore, there might be additional mechanisms different from those existing in TNF α signaling that enables RIPK1-mediated I κ B α phosphorylation. Importantly, even though TRAF2 did not exert any significant effect on the activation of non-apoptotic pathways, TRAF2 depletion increased the level of caspase 8 cleavage, suggesting that TRAF2 modulates TRAIL signaling at the level of the DISC. In that regard, it was recently reported that TRAF2 can be recruited to the DISC upon TRAIL treatment and induce K48 linkage specific ubiquitination of caspase 8 guiding it to proteosomal degradation¹⁰⁷. Therefore, sensitization to TRAIL-induced apoptosis in TRAF2 knock down cells might be due to its effect on caspase 8 ubiquitination and

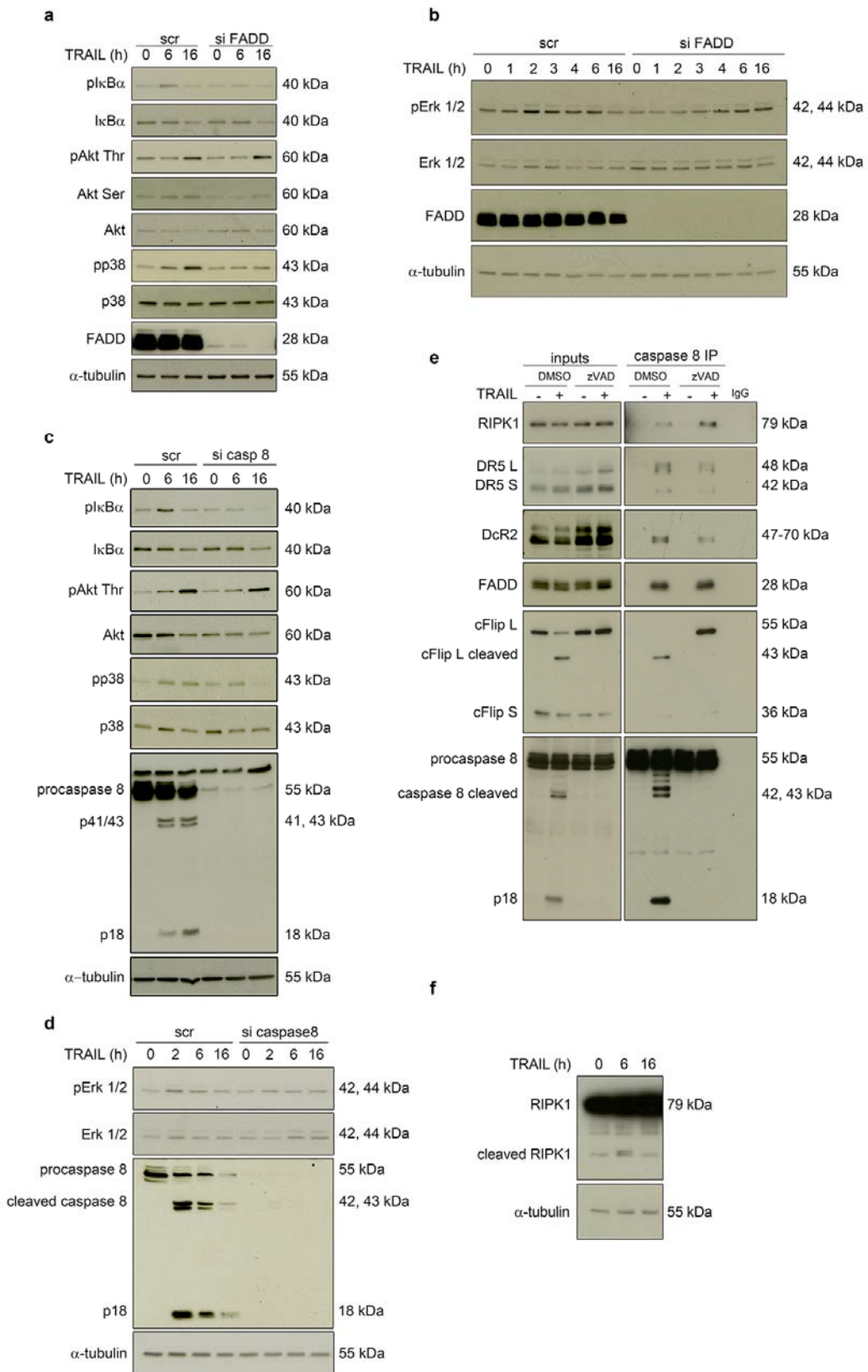
degradation in our model.

To summarize, our results show that TRAF2 depletion does not affect the activation of either non-apoptotic cascades. Contrary, RIPK1, FADD and caspase 8 are required for I κ B α and p38MAPK phosphorylation upon TRAIL challenge. TRAIL-induced Erk1/2 phosphorylation is not affected by RIPK1 downregulation, whereas the activation of this signaling cascade is delayed upon FADD knock down and abolished under conditions of caspase 8 depletion. Our preliminary data support that cFlip is also required for Erk1/2 activation as well as p38MAPK phosphorylation. Interestingly, Akt phosphorylation in response to TRAIL takes place independently of FADD, caspase 8, cFlip, RIPK1 and TRAF2. Indeed, it was recently shown that the activation of Akt upon TRAIL treatment occurs independently of FADD and the TRAIL receptor death domain, but requires the membrane-proximal domain for Akt induction¹¹⁵. These results point to a very critical feature of TRAIL signaling - cells that lack functional DISC components including the adaptor molecule FADD and/or functional receptor death domain can respond to TRAIL treatment by the activation of pathways that lead to tumor progression, invasion and metastasis. Therefore, death receptors by themselves can serve as a signaling bifurcation point in the activation of death or survival pathways depending on the recruitment of certain adaptor and signaling molecules.

Overall, our results indicate that the activation of non-apoptotic cascades, such as Erk1/2, p38MAPK, and NF- κ B relies on canonical DISC and secondary complex components, whereas activation of Akt is independent of these proteins and apparently involves additional yet unidentified TRAIL-induced signaling platforms. Our results suggest that TRAIL may lead to the formation of several complexes, with different composition, triggering distinct signaling from apoptosis to Erk1/2, p38MAPK, NF- κ B and Akt pathways.

Figure 16. Role of FADD, caspase 8, cFlip, RIPK1 and TRAF2 in the triggering of non-apoptotic pathways. **a-** Impact of FADD knock down on the triggering of Akt, p38MAPK and regulator of NF- κ B signaling (I κ B α). BJELR cells were transfected either with siRNAs targeting *FADD* mRNA (“si FADD”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1 μ g/ml) during 6h or 16h. Apoptotic cells were discarded by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates. **b-** Impact of FADD knock down on the phosphorylation level of Erk1/2. BJELR cells were transfected either with siRNAs targeting *FADD* mRNA (“si FADD”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1 μ g/ml) during 1, 2, 3, 4, 6h or 16h. Apoptotic cells were discarded by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates. **c-** Impact of caspase 8 knock down on the induction of Akt, p38MAPK and regulator of NF- κ B signaling (I κ B α). BJELR cells were transfected either with siRNAs targeting *caspase 8* mRNA (“si Casp8”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1 μ g/ml) during 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent replicates.

Figure 16



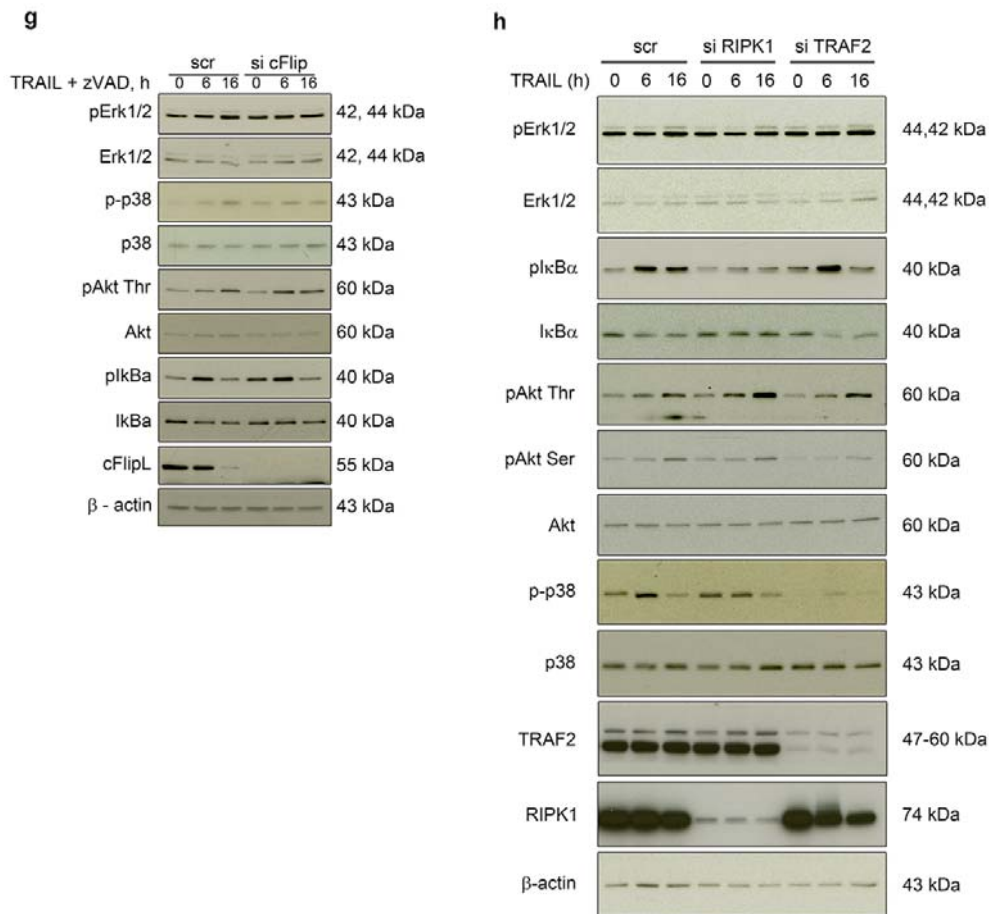


Figure 16 continued. Role of FADD, caspase 8, cFlip, RIPK1 and TRAF2 in the triggering of non-apoptotic pathways.

d- Impact of caspase 8 knock down on the phosphorylation level of Erk1/2. BJELR cells were transfected either with siRNAs targeting *caspase 8* mRNA (“si Casp8”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1µg/ml) during 1, 2, 3, 4, 6h or 16h. Apoptotic cells were discarded by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent replicates. **e-** Impact of caspases activity on the recruitment of RIPK1 to the DISC. BJELR cells were either pretreated with 20µM pan-caspase inhibitor (“zVAD”) of vehicle (“DMSO”) for 1h and then either left untreated (“0”) or treated with TRAIL (1µg/ml) for 30 min. Immunoprecipitation of caspase 8 (caspase 8 IP) was performed and co-immunoprecipitation of the DISC components and RIPK1 was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. Image corresponds to one representative experiment out of two independent biological replicates. **f-** TRAIL treatment induces RIPK1 cleavage. BJELR cells were either left untreated (“0”) or treated with TRAIL (1µg/ml) for 6 or 16 h. Cells were collected for Western blot. Total and cleaved levels of RIPK1 were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of at least two independent biological replicates. **g -** Impact of cFlip knock down on the activation of Erk1/2, Akt, p38 MAPK and regulators of NF- κ B signaling (I κ B α). BJELR cells were transfected either with siRNAs targeting *cFlip* (“si cFlip”) mRNA or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were pretreated with 20µM pan-caspase inhibitor (zVAD) for 1h and then either left untreated (“0”) or treated with TRAIL (1µg/ml) during 6h or 16h. Cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one

representative experiment out of two independent replicates. **h-** Impact of RIPK1 and TRAF2 knock down on the activation of Erk1/2, Akt, p38 MAPK and regulators of NF- κ B signaling (I κ B α). BJELR cells were transfected either with siRNAs targeting *RIPK1* ("si RIPK1") or TRAF2 ("si TRAF2") mRNA or non-targeting scramble siRNAs ("scr"). Forty-eight hours post-transfection BJELR cells were pretreated with 20 μ M pan-caspase inhibitor (zVAD) for 1h and then either left untreated ("0") or treated with TRAIL (1 μ g/ml) during 6h or 16h. Cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α -Tubulin, loading control. Image corresponds to one representative experiment out of 3 independent replicates.

6. Role of TRAIL receptors in the activation of pro- and anti-apoptotic signaling in response to TRAIL

Despite our limited knowledge regarding the molecular mechanisms of tumor-selective action of TRAIL, the molecular basis of TRAIL-induced pro-survival pathways activation and generation of resistance, a number of clinical trials have been launched in order to evaluate the potential of TRAIL in cancer therapy. For that, several approaches were developed, among them: pro-apoptotic receptor antagonists (PARAs) including recombinant human ligand (rhTRAIL) targeting both TRAIL receptors²⁴⁸; agonistic monoclonal antibodies targeting DR4 or DR5^{18,249-254}, TRAIL receptor specific peptides^{89,166} and TRAIL-receptor selective mutants^{74,255,256}. Initially it was considered that TRAIL death receptors trigger apoptotic cell death, while decoy receptors compete for TRAIL binding with death receptors, thus diminishing apoptotic response. However, it was demonstrated that the overexpression of DcR2 leads to the activation of NF- κ B and Akt in a ligand-independent manner^{84,290}. Therefore, even though DcR2 lacks a functional death domain, it encodes the cytoplasmic region that appears to be an important signaling region for the transduction of non-apoptotic signaling²⁹⁰. This observation suggested that the role of TRAIL receptors is more complex than was originally thought. Indeed, it was shown that DR5 can trigger pro-survival pathways in cancer cell lines resistant to TRAIL-induced apoptosis, while leading to apoptosis in cancer cells sensitive to TRAIL-mediated death¹²². Furthermore, a recent report shows that endogenously expressed TRAIL induces DR5-mediated activation of Akt, increasing migration and invasion of cancer cells¹¹⁵. These results seriously challenge the safe use of DR5 agonistic antibodies in anti-cancer therapy. On the other hand, there are many cancer cell lines that express both death receptors: DR4 and DR5; however, only one is the main apoptotic receptor in these experimental systems, while the role of the second one in these cellular context remains unclear. Briefly, TRAIL signals for cell death through DR4 in pancreatic cancer cell lines (Colo357, Panc89, PT45)²⁹⁷, chronic lymphocytic leukemia and primary lymphoid malignancies^{73,74}, whereas apoptotic signaling in human glioblastoma cell lines (D247MG, LN18, LN71, LN443, T98G U343MG, U87MG, U138MG and U343MG)²⁹⁸, colon and breast cancer cell lines (SK-MES, Colo205 and MDA-MB-231)²⁵⁵ is transduced through DR5. Altogether, given the potential of TRAIL to trigger pro-survival pathways, it is very important to understand the role of individual TRAIL receptors in order to evaluate possible apoptotic and pro-survival action of agonistic TRAIL receptor antibodies and rhTRAIL in different cellular contexts.

As previously mentioned, we observed that heterocomplexes composed by DcR2, DR4 and DR5 are formed in response to TRAIL treatment in BJELR cells. In this cellular scenario, which receptor(s) participate in the triggering of non-apoptotic cascades remain elusive. To shed light into these issues, we characterized the role of DcR2, DR4 and DR5 in the activation of non-apoptotic signaling cascades in response to TRAIL. Following our initial observation that DcR2 knock down leads to an increased rate of cell death upon TRAIL treatment, we hypothesized that this decoy receptor may prevent apoptosis by two different mechanisms. Firstly, DcR2 recruitment to the DISC can impede the assembly of the proficient apoptotic DISC (Fig 17a). This can be achieved by two different mechanisms: (i) lack of DcR2 in the DISC can lead to the recruitment of additional death receptors followed by increased FADD and caspase 8 recruitment and cleavage (Fig 17a (b)) or (ii) DcR2 depletion may not affect the number of

death receptors in the DISC in which case the recruitment of FADD will not differ from wild type cells; however the absence of Dcr2 will increase the proximity between neighboring FADD molecules and that of caspase 8 enhancing caspase 8 cleavage (Fig 17a (c)). Secondly, given that Dcr2 is a part of TRAIL-induced non-apoptotic platform we hypothesize that this receptor supports the formation of non-apoptotic signaling complex/es leading to an efficient activation of non-apoptotic pathways upon TRAIL challenge (Fig 17a (d-f)).

Figure 17

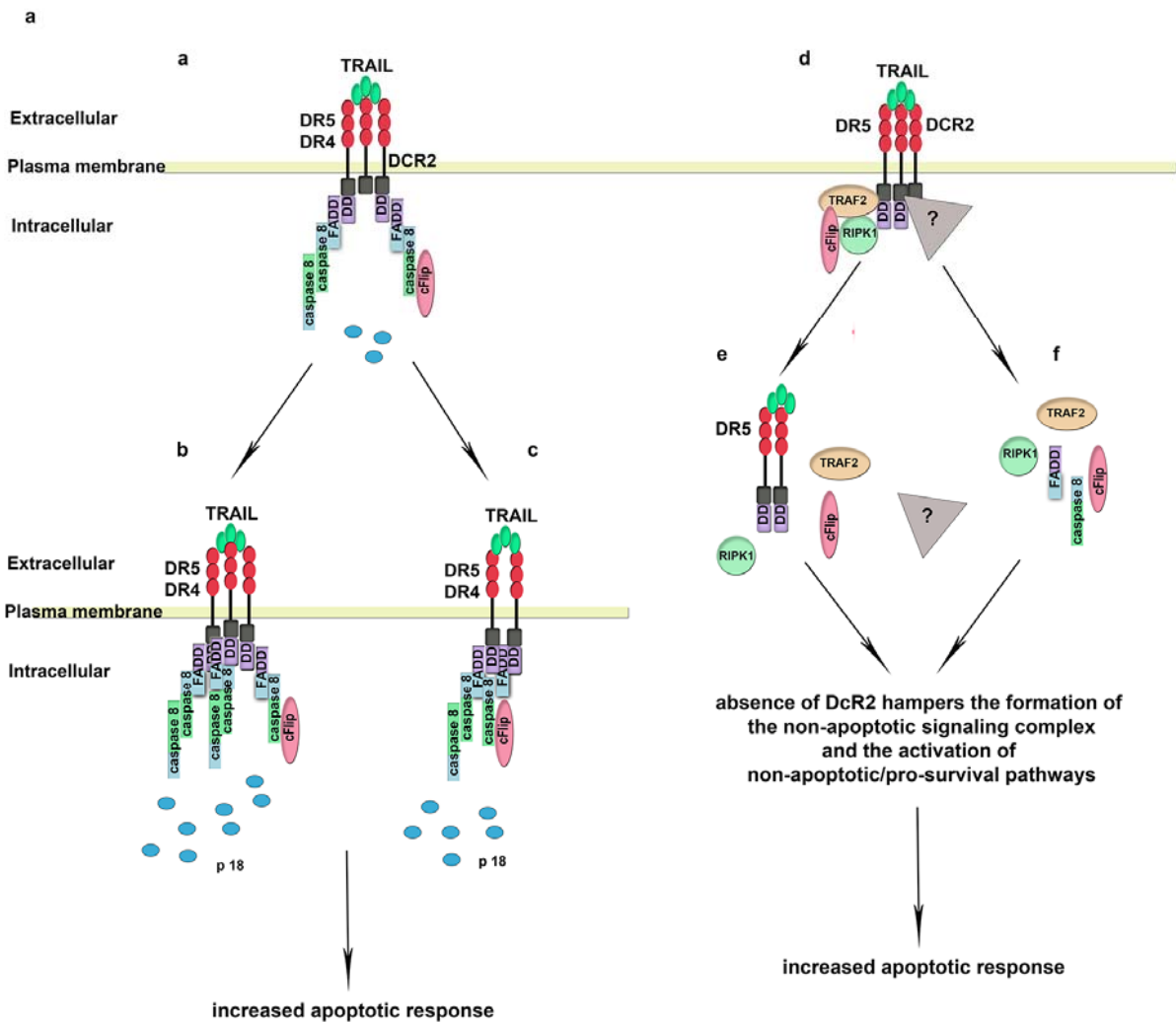
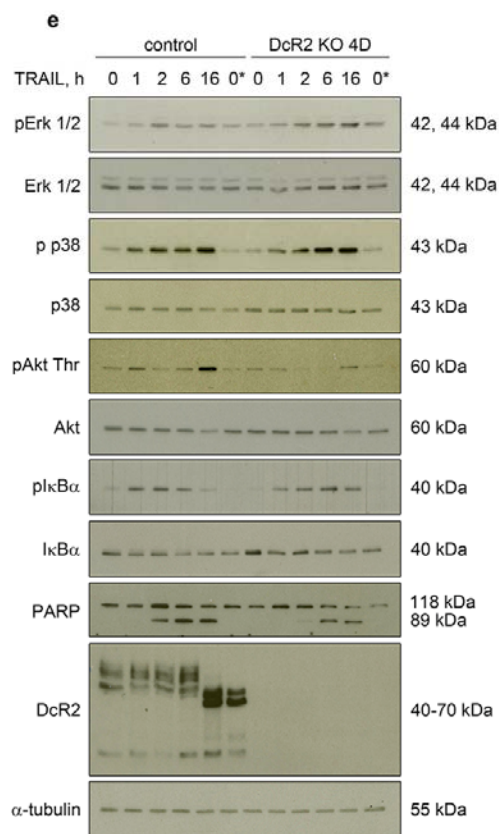
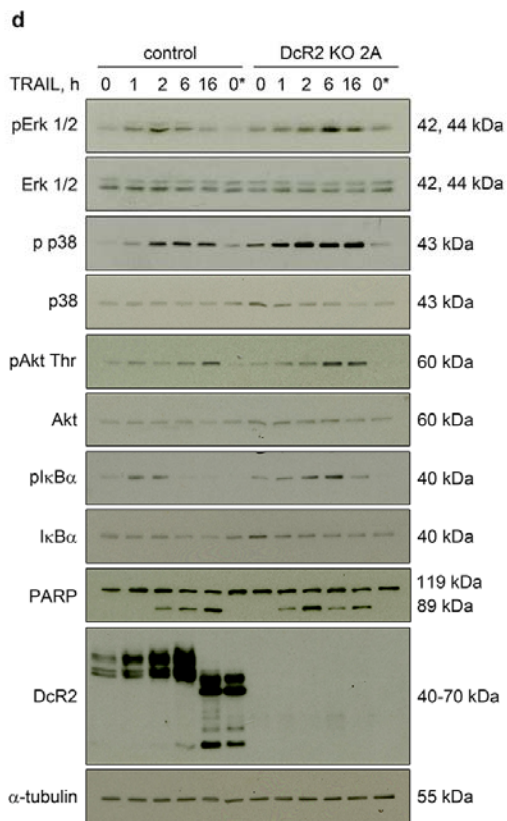
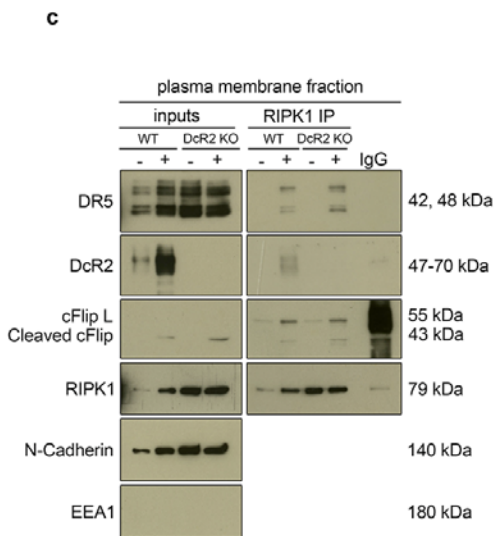
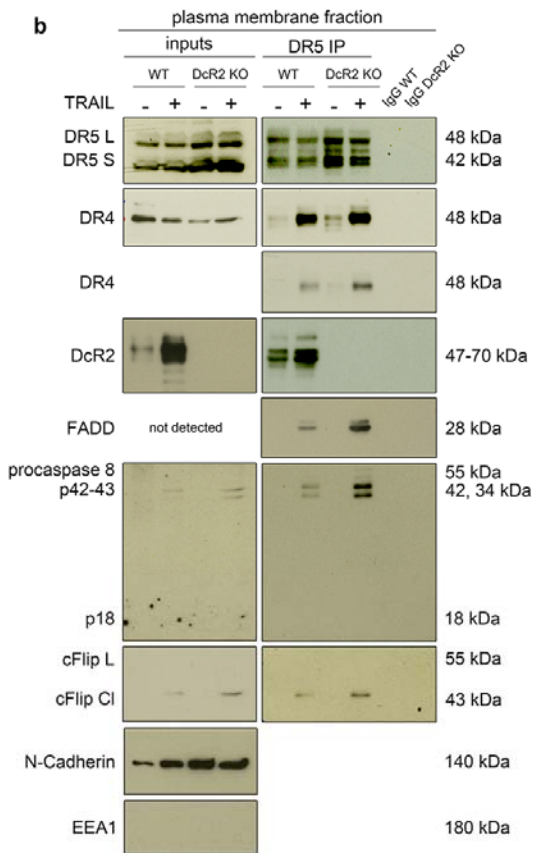


Figure 17. Role of Dcr2 in the regulation of TRAIL signaling. **a-** A model depicting Dcr2 regulation of TRAIL signaling. **(a)** DISC composition in the presence of Dcr2; **(b)** Lack of Dcr2 in the DISC can lead to the recruitment of additional death receptors followed by increased FADD and caspase 8 recruitment and cleavage; **(c)** Dcr2 depletion may not affect the number of death receptors in the DISC in which case the recruitment of FADD will not differ from the wild type cells, however the absence of Dcr2 will increase the proximity between neighboring FADD and consequently caspase 8 molecules that will enhance the efficiency of caspase 8 cleavage. **(d)** Composition of the non-apoptotic signaling complex; **(e and f)** Absence of Dcr2 in the TRAIL-induced non-apoptotic signaling platform may affect its formation, thus hampering the activation of non-apoptotic and pro-survival pathways.

In that regard, it was shown that DcR2 can lead to NF- κ B and Akt activation in a ligand-independent manner^{84,290}. Interestingly, a recent report¹¹⁵ and our data suggest that TRAIL-induced activation of Akt occurs independently of FADD and TRAIL receptor death domain. Therefore, potentially DcR2 that lacks death domain can recruit components that lead to the activation of Akt resulting in the negative regulation of TRAIL-induced apoptosis. To test this hypothesis, DcR2 knock out cells were generated using CRISPR/Cas 9 technology. To study whether clustering of DcR2 to DR5 affect DISC composition we performed DR5 immunoprecipitation from the plasma membrane fraction of wild type and DcR2 knock out BJELR cells followed by the identification of DR4, FADD, caspase 8 and cFlip by Western blot. Our preliminary data indicated an increased level of DR4, FADD, cleaved caspase 8 and cleaved cFlip co-immunoprecipitated with DR5 upon TRAIL treatment in DcR2 knock out cells as compared to wild type cells (Fig 17b). This result suggests that DcR2 may exert negative regulation of TRAIL-induced apoptosis by hampering recruitment of other death receptors as well as FADD, that in turn decreases caspase 8 and cFlip recruitment and/or cleavage. In contrast, when DR5 immunoprecipitation was performed from the whole cell lysate, we did not observe any difference in FADD recruitment between wild type and DcR2 KO cells, while the difference in DR4 recruitment as well as caspase 8 and cFlip cleavage was still detected. Therefore, given these contradictory results further experiments are required in order to evaluate the actual role of DcR2 in the modulation of DISC composition (see “Ongoing work and perspectives”). Further, following our second hypothesis we analyzed whether DcR2 affects formation of the non-apoptotic signaling complex. For that RIPK1 immunoprecipitation was performed from the plasma membrane fraction isolated from wild type and DcR2 knock out BJELR cells followed by the comparative analysis of the recruitment pattern of DR5, DcR2, FADD, caspase 8 and cFlip. Notably, our initial results suggest that there are no differences in TRAIL-induced association of DR5 and cFlip with RIPK1 in the presence or absence of DcR2 (Fig 17c). Furthermore, we did not observe co-IP of RIPK1 neither with FADD nor with caspase 8 in DcR2 knockout cells coinciding with the result observed in wild type (Fig 17c).

Figure 17 continued. Role of DcR2 in the regulation of TRAIL signaling. b- BJELR wild type (“WT”) or DcR2 knock out (“DcR2 KO”) cells were either left untreated (“-”) or challenged with TRAIL (1 μ g/ml) for 30 min (“+”). Further plasma membrane fraction was extracted from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of DR5 (DR5-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of DISC and secondary complex components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. **c-** Role of DcR2 in the regulation of the composition of the TRAIL-induced non-apoptotic signaling complex. BJELR wild type (“WT”) or DcR2 knock out (“DcR2 KO”) cells were either left untreated (“-”) or challenged with TRAIL (1 μ g/ml) for 30 min (“+”). Further plasma membrane fraction was extracted from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of RIPK1 (RIPK1-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of DISC and secondary complex components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. **d and e** – Role of DcR2 in the triggering of non-apoptotic signaling. BJELR wild type (“control”), BJELR DcR2 knock out clone 2A (DcR2 KO 2A) (**d**) and BJELR DcR2 knock out clone 4D (DcR2 KO 4D) (**e**) cells were either left untreated (“0” – collected just before the treatment and “0*” – collected 16h after the beginning of exposure to TRAIL in TRAIL treated samples) or treated with TRAIL (1 μ g/ml) for 1, 2, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α -Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.



Overall, our initial results suggest that DcR2 has no major role in the formation of this signaling platform (see “Ongoing work and perspectives”). Further we studied whether DcR2 is involved in the triggering of non-apoptotic pathways in response to TRAIL. For that we analyzed phosphorylation levels of Erk1/2, p38MAPK, Akt and I κ B α upon TRAIL treatment in wild type and DcR2 knock out BJELR cells. Activation of Erk1/2, p38MAPK, NF- κ B and late wave of Akt signaling induced in response to TRAIL were not affected by DcR2 knock out suggesting that the triggering of these pathways occurs independently of DcR2 (Fig 17d,e). Interestingly, we found that the early wave of Akt activation is DcR2-mediated (Fig 17d, e). Importantly, blocking Akt signaling in our experimental model increases cell death upon TRAIL treatment indicating that this pathway plays a pro-survival role in our cellular system. Therefore, DcR2 may exert its non-apoptotic function not only by affecting the formation of an efficient apoptotic DISC but also via activation of Akt signaling at early time points of response to TRAIL.

Importantly, Erk1/2, Akt and I κ B α were also activated in DR4 depleted cells following TRAIL challenge (Fig 18a). Given that DR4 is neither required for apoptotic nor for the activation of pro-survival pathways we aimed to unravel at which level DR4-mediated signaling is blocked. To shed light on this issue we first questioned whether DR4 can form a DISC independently of DR5 by recruiting FADD and caspase 8. Interestingly, DR4 immunoprecipitation from the plasma membrane fraction of wild type and DR5 knock out BJELR cells revealed that FADD and caspase 8 cannot be recruited to DR4 in the absence of DR5 suggesting that DR4 by itself has no potential to form a DISC (Fig 18b). The absence of the DISC formation upon TRAIL treatment in DR5 KO BJELR cell was also confirmed in caspase 8 immunoprecipitation experiments. Notably, DR5 knock out does not prevent the association of DR4 with DcR2 supporting that DR4 is presented at the cell surface, binds TRAIL, assembles with DcR2, however does not recruit FADD and caspase 8 (Fig 18b). Therefore, we hypothesized that the death domain of DR4 bears mutation that prevents its association with FADD. Indeed, several mutations in the death domain of DR4 that prevent apoptotic cell death in cancer cells were reported: Arg441Lys, Ala420Val and Pro376Leu^{299,300}. However, sequencing DR4 cDNA from BJELR cells did not reveal any mutation in the death domain, suggesting that the inability of DR4 to recruit FADD is not due the mutated death domain (Fig 18c). Instead, we detected a point mutation within the extracellular cysteine rich domain of DR4 – A683C (Glu228Ala) (Fig 18c). This mutation was reported for the human breast cancer cell line BT474 and shown not to affect cell surface expression of DR4³⁰¹. Importantly, nucleotide change in the position 683 leads to the substitution of the large negatively charged amino acid glutamate by the small uncharged amino acid alanine. It was suggested that this replacement might lead to changes in the extracellular cysteine rich domain of DR4 and result in the insufficient interaction with TRAIL³⁰². The fact that DR4 associates with DcR2 upon TRAIL treatment in the plasma membrane (Fig 18b) suggests that DR4 binds TRAIL, however DR4/TRAIL interaction might be negatively affected by the mutation and may not be sufficient for the effective receptor clustering required for the formation of the functional DISC. Indeed, quantitative studies of the TRAIL-DISC composition revealed that recruitment of one FADD molecule requires several death receptors¹⁰². Therefore, if Glu228Ala mutation affects efficient receptor clustering³⁰², this may lead to the lack of FADD recruitment and DISC formation. These results rise obvious conundrum: if DR4 forms heterocomplexes with DR5 but cannot recruit FADD and caspase 8 then why it does not exert a function of a “decoy” receptor. We

speculate that DR4 expression at the plasma membrane might be low, therefore its presence at the DISC may not have any significant effect on TRAIL signaling. Overall, our results suggest that DR4 plays neither apoptotic (due to its inability to recruit FADD) nor non-apoptotic function in BJELR cells. Further studies are required to unravel the molecular mechanisms that prevent DR4 functioning, particularly the impact of Glu228Ala mutation on structural characteristics of the DISC.

Figure 18

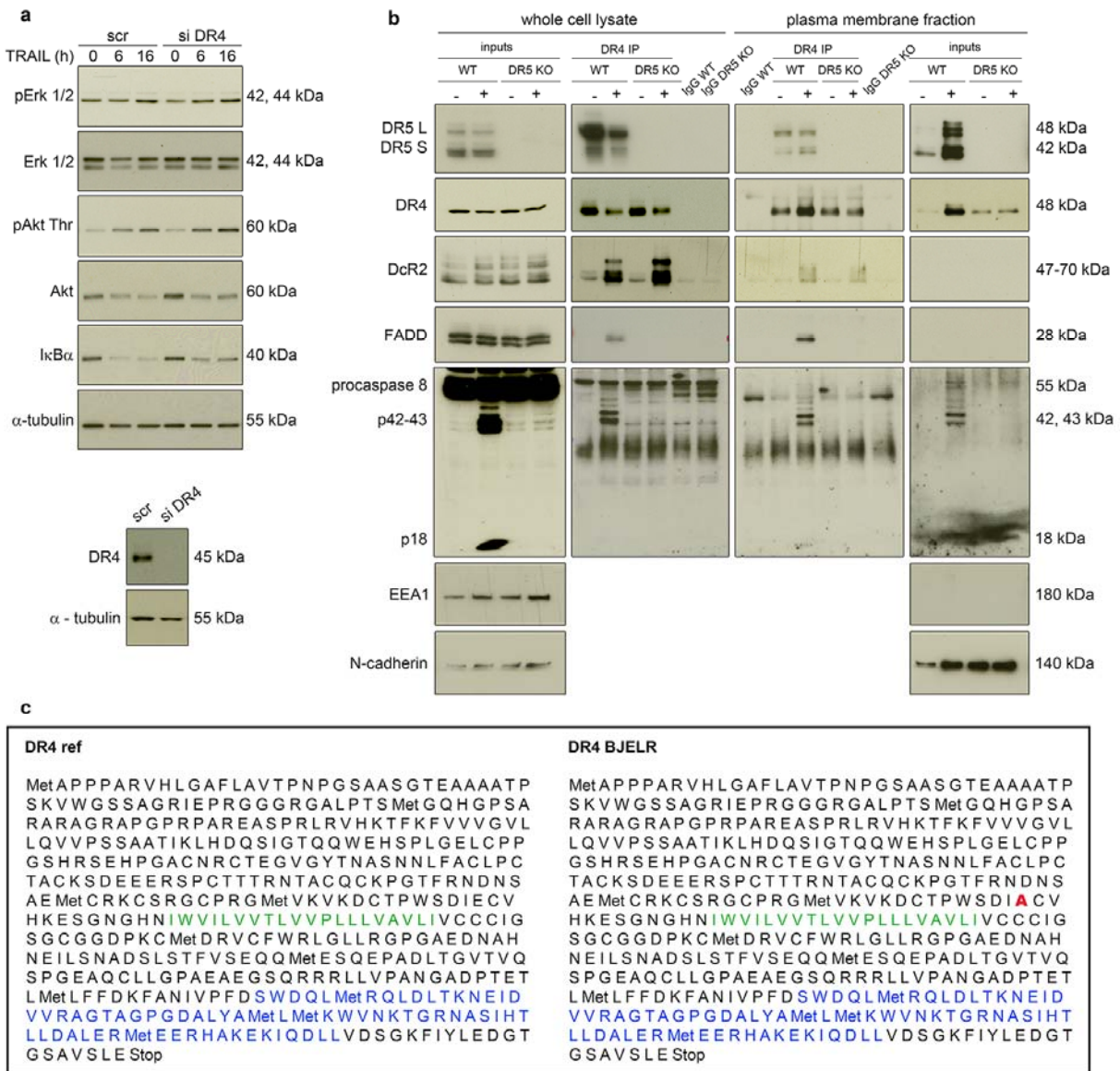


Figure 18. Role of DR4 in the modulation of TRAIL-mediated signaling. **a-** Requirement of DR4 for the activation of non-apoptotic pathways. Total and phosphorylated levels of Erk1/2, Akt and regulator of NF-κB signaling (IκBα in transfected cells surviving TRAIL DR4 were analyzed. BJELR cells were transfected either with siRNAs targeting *DR4* (“si DR4”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1μg/ml) during 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204),

Given that DR4 is not required for the triggering of non-apoptotic pathways, while DcR2 is required only for the activation of Akt signaling at early time points of response to TRAIL, we hypothesized that DR5, besides being the main apoptotic receptor in the system, should also be the one triggering non-apoptotic pathways. Following this hypothesis we first performed DR4/DcR2 double knock down to study the efficiency of TRAIL-induced activation of non-apoptotic pathways in the presence of DR5 alone (Fig 19a). Interestingly, siRNA-mediated depletion of DR4 and DcR2 did not prevent TRAIL-induced activation of Erk1/2, Akt and degradation of I κ B α , suggesting that indeed activation of these pathways is DR5-mediated. Moreover, double knock down of DR5 and DcR2 blocked TRAIL-induced Erk1/2, Akt and I κ B α phosphorylation suggesting that these pathways cannot be activated in the absence of DR5 (Fig 19a). Furthermore, exposing BJELR cells to TRAIL mimetic peptides targeting DR5 also resulted in the activation of Erk1/2, Akt and I κ B α (Fig 19b). In order to finally validate the role of DR5 in the triggering of non-apoptotic pathways we generated DR5 knock out cell line using CRISPR/Cas9 technology. Importantly, TRAIL-induced activation of Erk1/2, p38MAPK and I κ B α was abrogated in DR5 knock out cells as compared to control wild type cells (Fig 19c). Importantly, DR5 knock out resulted in the blockage of late (16 hours) but not early (2-6 hours) wave of Akt induction, suggesting that DcR2-mediated activation of Akt signaling at early time points of response to TRAIL is DR5-independent. Overall, our results show that DR5 is capable of triggering both functionally different signaling outcomes (apoptotic and non-apoptotic pathways) in clonal populations of cancer cells independently of other TRAIL receptors.

Figure 18 continued. Role of DR4 in the modulation of TRAIL-mediated signaling. Akt (Thr308) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Efficiency of DR4 knock down at 48 h post-transfection was analyzed by Western blot. α - Tubulin, loading control. **b** – DISC composition in wild type and DR5 knock out BJELR cells. Wild type (WT) and DR5 knock out (DR5 KO) BJELR cells were either left untreated (“-”) or challenged with TRAIL (1 μ g/ml) for 30 min (“+”). Further plasma membrane fraction was extracted from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of Death Receptor 4 (DR4-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of DISC components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. **c**- Comparison between amino acid sequence of DR4 expressed in BJELR cells (DR4 BJELR) and reference sequence (DR4 ref). The transmembrane domain is marked in green. The death domain is marked in blue. The mutated amino acid is marked in red.

Figure 19. Role of DR5 in the triggering of non-apoptotic pathways upon exposure to TRAIL. **a**- Requirement of TRAIL receptors for the activation of non-apoptotic pathways. Total and phosphorylated levels of Erk1/2, Akt and regulator of NF- κ B signaling (I κ B α) in transformed cells surviving TRAIL or DR5-specific ligand-M1D-treatment upon DR4/DcR2 and DR5/DcR2 double knock down were analyzed. BJELR cells were transfected either with siRNAs targeting *DR4* and *DcR2* (“DR4/DcR2 KD”) or *DR5* and *DcR2* mRNA (“DR5/DcR2 KD”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1 μ g/ml) or M1D (10 μ M) during 6h (“6”). Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308) and I κ B α (Ser32/36) were analyzed. α -Tubulin, loading control. Efficiency of DR4/DcR2, DR5/DcR2 double knock down at 48 h post-transfection was analyzed by Western blot. α - Tubulin, loading control. **b** – Impact of M1D (DR5 specific ligand) treatment on the activation of non-apoptotic pathways. BJELR cells were either left untreated (“0”) or treated with M1D (10 μ M) for 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control.

Figure 19

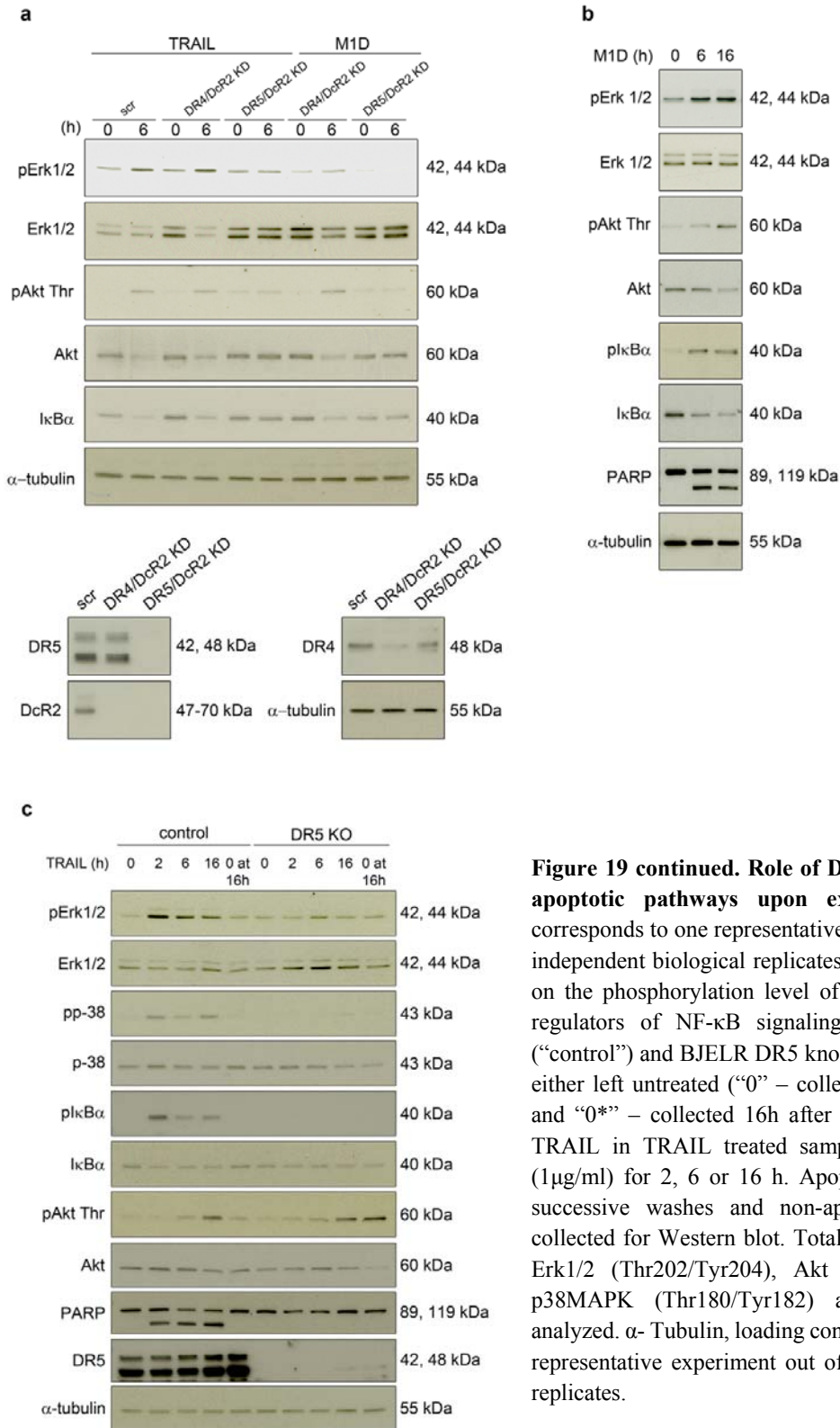
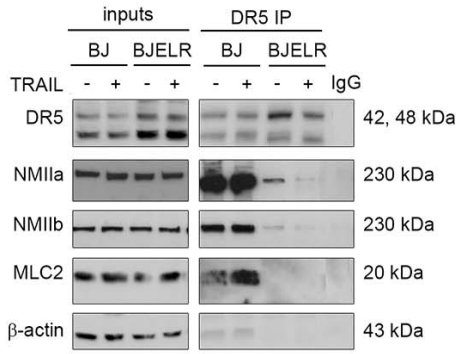


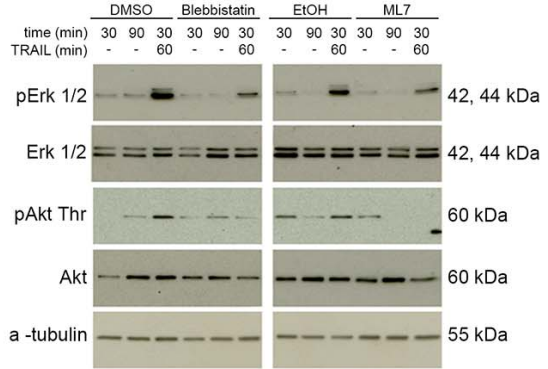
Figure 19 continued. Role of DR5 in the triggering of non-apoptotic pathways upon exposure to TRAIL Image corresponds to one representative experiment out of at least five independent biological replicates. **c-** Impact of DR5 knock out on the phosphorylation level of Erk1/2, Akt, p38MAPK and regulators of NF-κB signaling (IκBα). BJELR wild type (“control”) and BJELR DR5 knock out (“DR5 KO”) cells were either left untreated (“0” – collected just before the treatment and “0*” – collected 16h after the beginning of exposure to TRAIL in TRAIL treated samples) or treated with TRAIL (1μg/ml) for 2, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.

Supplementary figure 1

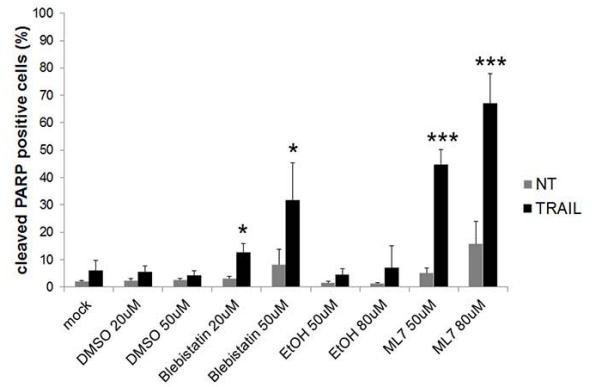
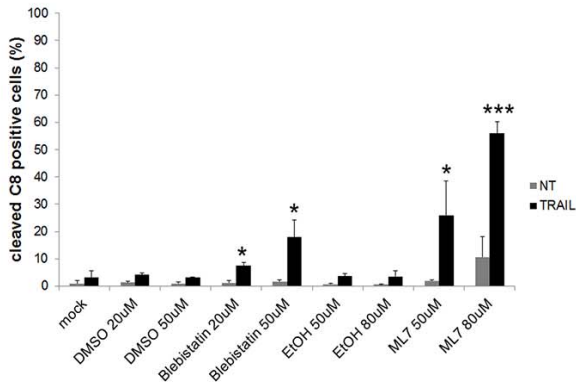
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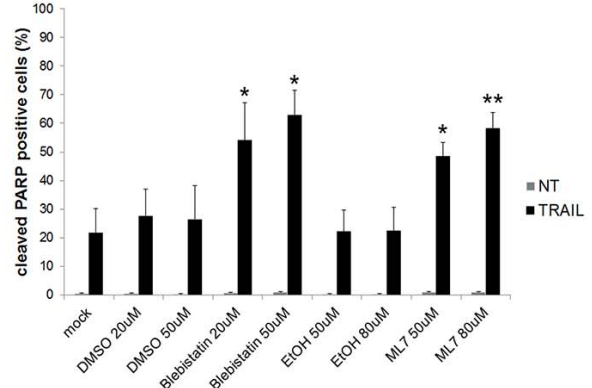
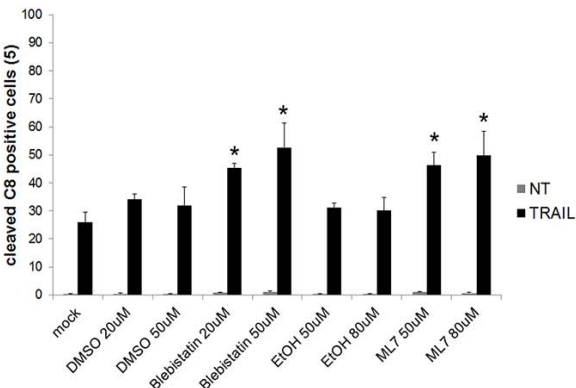
d



b



c



Supplementary figure 1. NMIs – novel DISC interactors in normal cells. **a-** Co-immunoprecipitation of NMII, actin and MLC2 with the DISC in BJ and BJELR cells. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Immunoprecipitation of Death Receptor 5 (DR5-IP) was performed and co-immunoprecipitation of DISC components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. This figure was taken from the PhD thesis of Dr. C. Schulz. **b-** Impact of Blebbistatin and ML7 treatment on sensitivity to TRAIL-induced cell death in BJ cells. BJ cells were either pretreated with Blebbistatin (20 and 50µM), DMSO (vehicle for blebbistatin) or ML7 (50 and 80µM), ethanol (vehicle for ML7) for 1 hour or left untreated (mock). Further, cells populations were either left untreated (“NT”) or treated with TRAIL (1µg/ml) for 4 hours. The percentage of cells with positive labeling for cleaved caspase 8 and cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. **c-** Impact of Blebbistatin and ML7 treatment on sensitivity to TRAIL-induced cell death in BJELR cells. BJELR cells were either pretreated with Blebbistatin (20 and 50µM), DMSO (vehicle for blebbistatin) or ML7 (50 and 80µM), ethanol (vehicle for ML7) for 1hour or left untreated (mock). Further, cells populations were either left untreated (“NT”) or treated with TRAIL (1µg/ml) for 4 hours. The percentage of cells with positive labeling for cleaved caspase 8 and cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. **d-** Impact of Blebbistatin and ML7 treatment on TRAIL-induced activation of non-apoptotic pathways in BJ cells. BJ cells were either pretreated with Blebbistatin (50µM), DMSO (vehicle for blebbistatin) or ML7 (80µM), ethanol (vehicle for ML7) for 30min. Further, cells populations were either left untreated (“-”) or treated with TRAIL (1µg/ml) for 60 min. Cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204) and Akt (Thr308) were analyzed. α - Tubulin, loading control.

DISCUSSION

Cancer cells response to TRAIL. Since the discovery of TRAIL in 1995, this cytokine has been under intense focus due to its unique tumor selectivity, an ability to induce apoptosis in cancer cells, while sparing normal cells^{158,159}. Early investigations of TRAIL also identified another unique characteristic of this cytokine^{158,257}. TRAIL is expressed in a wide range of tissues, including cells of immune system suggesting that this cytokine has a variety of potential physiological functions^{158,303} (for details see “Introduction: Functional role of endogenous TRAIL”). Indeed, several reports indicated that TRAIL is a natural component of the tumor-surveillance system in mammals that was shown to participate in the control of tumor progression and T cells-mediated immunity in graft-versus-tumor reaction after allogenic hematopoietic cell transplantation^{85,153-157}. These attractive therapeutic features of TRAIL led to the development of numerous TRAIL-based approaches in order to evaluate the potential of TRAIL in anti-cancer therapy. Unfortunately, contrary to all expectations, clinical trials revealed only few cases of stable disease and partial response, while in the majority of cases tumors appeared to be resistant to treatment. The resistance to TRAIL-induced cell death can be developed through two main mechanisms: either via the downregulation or silencing of components crucial for TRAIL-induced cell death or through the activation of pro-survival pathways. These mechanisms allow cancer cells to escape apoptosis and promote tumor progression. In that respect, proliferative effects of TRAIL were reported for cancer cell populations *in vitro* and *in vivo*, suggesting that the response to TRAIL ranges from apoptotic cell death to the induction of cell proliferation and survival. TRAIL-mediated proliferative effects were also reported for the non-tumorigenic scenario, such as rheumatoid arthritis. In that respect, it was reported that TRAIL induces a dual response in synoviocytes from human rheumatoid arthritis patients: initially TRAIL activates cell death while, it supports proliferation in the resistant surviving population¹³⁹. Evidences of ligand-induced biphasic response suggest that cells can switch their response to TRAIL from pro-apoptotic to pro-survival upon exposure to this cytokine.

Along those lines, we found that clonal populations of transformed cells (BJELR cells) undergo fractional killing upon TRAIL treatment, meaning that only a part of the cell population dies, whereas the other part of the population survives TRAIL treatment¹⁸³. Moreover, we showed that TRAIL survivors acquire and maintain resistance to TRAIL-induced death upon repetitive and constant exposure to this ligand. Importantly, when ligand treatment is discontinued, cells revert to the initial response within several days. This phenotype of reversible resistance suggests that TRAIL-induced resistance is maintained in a TRAIL-dependent manner and is not due to the selection of inherently resistant clones within the initial population¹⁸³. In line with our findings, fractional killing response and establishment of TRAIL-induced reversible resistance was also reported for cancer cells lines (MCF10A), suggesting that this phenotype is not restricted to stepwise transformation systems¹⁷⁶. Furthermore, treatment of cancer cells populations with other anticancer drugs can also lead to the survival of a small subpopulation of drug resistant cells. Interestingly, when drug treatment is discontinued the population reverts to the initial response, suggesting that acquired resistance to anticancer drugs has a reversible nature and does not require a stable genetic mutation¹⁷⁶. This phenomenon was called “re-treatment response”¹⁷⁸. In that respect, it was shown that some patients with non-small lung carcinoma that initially respond well to the treatment with EGFR tyrosine kinase

inhibitors later show therapy failure. Interestingly, an efficient response to EGFR tyrosine kinase inhibitors is observed again after “drug holiday”^{180,181}. This and other results¹⁸² indicate an establishment of a reversible “drug tolerant state” upon exposure to anticancer drugs. Overall, these observations suggest that cancer cells can develop non-genetic mechanisms to survive, proliferate and escape cell death by the acquisition of resistance upon drug treatment.

Mechanisms of non-genetic heterogeneity in populations of cancer cells. Mechanisms leading to the non-genetic intra-tumor heterogeneity can be generally divided into “cell autonomous” and “non-cell autonomous”³⁰⁴. For example, any solid tumor always contains regions that are differentially exposed to the microenvironment; some of them are located in hypoxic regions, while others in regions of normal oxygenation. This differential localization can affect a cancer cell phenotype in the “non-cell autonomous” way³⁰⁵. Interestingly, decades ago it was shown that Rous sarcoma virus (RSV), which contains one of the most potent oncogenes – pp60^{src}, could cause aggressive tumors when injected in the wing of a chicken, whereas injection of RSV into a chicken embryo did not result in the tumor development³⁰⁶. These results suggest that an oncogene is not sufficient for tumor development and that the microenvironment affects cell fate decisions³⁰⁵.

On the other hand, “cell autonomous” mechanisms of heterogeneity in isogenic cell populations can rely on the cell-to-cell variability due to natural stochastic fluctuations in the expression level of the components of biochemical reactions^{175,307,308} and altered epigenetic states¹⁷⁸. Similar mechanisms of response to stress, starvation and changing environmental conditions due to the non-genetic heterogeneity within the population is also observed in bacteria (toxin – antitoxin system³⁰⁹, cannibalism by sporulating bacteria³¹⁰⁻³¹²). Interestingly, it was observed that bacteria cells that persist antibiotic treatment are “dormant”. Notably, most of bactericidal antibiotics act by inhibiting the transcription or translation machinery. Given that cells in the dormant state have little transcription and translation, the drug is unable to affect the function of its targets³¹³. These mechanisms allow bacteria to survive and withstand unfavorable environmental conditions and repopulate. These reports let us to hypothesize that something similar to these approaches undertaken in bacteria populations to ensure survival can be also observed in populations of cancer cells. Interestingly, it was suggested that cancer cells can also use “dormancy” as an adaptive strategy during periods of stress. In this respect, it was shown that melanomas³¹⁴ as well as colorectal tumors³⁰⁸ contain relatively dormant, slow-cycling sub-population of cells that play a crucial role in tumor propagation potential after chemotherapy.

Interestingly, it was suggested that natural differences in the expression level and activity of pro- and anti-apoptotic proteins is the main cause of cell-to-cell variability that eventually determines cell fate in response to TRAIL¹⁷⁵. In that regard, it was reported that two newborn sister cells are very similar in the protein level, as they inherited it from the parental cell. Nonetheless, after several cell division due to the performance of protein biosynthesis machinery each sister cell accumulates differences in protein levels, therefore becoming as similar with its sister cell as with any other cell randomly chosen from the population¹⁷⁵. It was reported that two sister cells behave the same in response to TRAIL (‘time-to-death’) up to 50h after «birth» (approximately two cell divisions); however, later they behave as two randomly chosen cells from the population. The variability in time-to-death arises from pre-MOMP

processes that largely vary within cells of the population. Therefore, the internal state of each individual cell or “dynamic internal protein profile” will lead to a particular cell fate decision towards death or survival. Importantly, it has been reported that in the presence of caspase inhibitor entire population enter the state of reversible resistance¹⁷⁶. This result leads to several crucial conclusions that help to understand the nature of TRAIL-induced fractional killing and reversible resistance: (i) TRAIL-induced resistance is independent of cell death and/or factors released from dying cells, (ii) reversible resistance is established not only due to the stochastic cell-to-cell variability, otherwise repetitive treatment should result in fractional killing and not in the resistance of entire population; (iii) reversible resistance is TRAIL-induced. Notably, numerous reports showed that, besides its role in apoptosis, TRAIL can also trigger activation of pro-inflammatory (NF- κ B) and pro-survival signaling (Erk1/2, p38MAPK, JNK, Akt/PI3K)¹¹⁶. Along those lines, we found that Akt, NF- κ B, p38MAPK and Erk1/2 signaling is activated at early time points after TRAIL treatment suggesting that the triggering of non-apoptotic pathways as well as activation of apoptotic response occur within initial population. Moreover, we reported that Akt, p38MAPK and Erk1/2 play a pro-survival role in our experimental model. These signaling pathways are involved in the regulation of a huge variety of cellular functions such as cell cycle, survival and death, glucose metabolism, protein synthesis, transcription, etc^{315,316}. To control these functions, Akt, p38MAPK and Erk1/2 require an interaction with cytosolic, as well as nuclear targets. We hypothesize that, perhaps, Akt, p38MAPK and Erk1/2-mediated targeting of their cytoplasmic substrates might be required for the initial cell survival upon TRAIL treatment, while prolonged TRAIL stimulation may be needed for Akt, p38MAPK and Erk1/2-mediated signaling through their nuclear targets involving modifications of transcriptional and epigenetic landscape, leading to the establishment of resistance to TRAIL-induced cell death. Interestingly, it was suggested that the magnitude and duration of Erk1/2 signaling appears to be crucial for the final physiological outcome³¹⁷. It was shown that differentiation of rat pheochromocytoma 12 (PC-12) cells is induced by the ability of nerve growth factor to provide sustained Erk1/2 activation. Furthermore, sustained and prolonged Erk1/2 activation appears to be necessary for the maintenance of the differentiated state and might be crucial for Erk1/2 translocation to the nuclei³¹⁷. Moreover, it was reported that some NF- κ B – responsive genes require short 15 min stimulation, while other genes require at least 2 hours of persistent stimulation with TNF α ³¹⁸. These results suggest that duration of signaling pathways stimulation can indeed determine rapid short-term and stable long-term responses³¹⁸ that in turn enables an initial and durable reaction to the external stimuli. Based on this, we hypothesize that initially activation of both pro- and anti-apoptotic pathways can occur within the same cell but ultimately the internal state of the cell (time of pre-MOMP processes, for example) will determine the final outcome: death or survival (Figure 20a). Furthermore, repetitive TRAIL treatment will lead to the maintenance of only those cells that can trigger pro-survival pathways. In turn, constant TRAIL-induced activation of non-apoptotic pathways may inhibit apoptosis, therefore leading to the establishment of TRAIL-resistant population of cancer cells (Figure 20b). Given the transient nature of resistance, it is possible to hypothesize that TRAIL can mediate transcriptional changes that may rely on transient epigenetic modifications induced by ligand mediated pro-survival pathways. For instance, it was reported that constitutively active Erk1 increases percentage of cells (from 10% to 70%) that express HDAC4 in the nucleus in C2C12 myoblast cells³¹⁹. Notably, histone deacetylase 4 (HDAC4) is an enzyme that catalyzes the removal of acetyl groups from core histones,

which leads to the establishment of a compact chromatin structure that is generally associated with repressed gene transcription. Along those lines, it was reported that TRAIL treatment can lead to the downregulation of pro-apoptotic proteins (caspase 8 and Bax), upregulation of anti-apoptotic (cFlip, Bcl-2, XIAP) and pro-survival proteins¹²³. Thus, we hypothesize that TRAIL can induce changes in the epigenetic status of the cells that support survival upon permanent exposure to the cytokine. However, when TRAIL treatment is discontinued and thus, the factor that ensures selective pressure is removed, a few cell cycles result in the re-establishment of a natural stochastic cell-to-cell variability (Figure 20c). Therefore, a new exposure to TRAIL leads to fractional killing (Figure 20a). Overall, these data indicate that two processes might control the fractional killing response and the establishment of TRAIL-induced resistance: (i) stochastic fluctuations in the level of specific proteins and (ii) TRAIL-induced activation of pro-survival pathways. Using stochastic processes for cell fate decisions seems to be contradictory with the evolutionary paradigm of developing to perfection. However, what is “perfection” in an ever-changing environment? Therefore, making stochastic processes in the basis of life and death decision: is that a nonsensical “flipping coin” approach or a very useful strategy, in which “dynamic internal protein profile” gives a survival advantage in a variety of possible environmental changing conditions?

Role of TRAIL receptors in the regulation of fractional killing response. Despite our limited understanding of the molecular mechanisms of TRAIL-induced activation of non-apoptotic signaling a number of pro-apoptotic receptor antagonists (PARAs) including rhTRAIL, agonistic monoclonal antibodies targeting DR4 (mapatumumab) or DR5 (lexatumumab, apomab, AMG-655, LBY135, CS-1008) and death receptors specific peptides have been developed and clinical trials were launched¹⁶⁶. The idea of using TRAIL death receptors agonistic antibodies is based on the initial discovery that death receptors DR4 and DR5 trigger apoptosis upon exposure to TRAIL, while decoy receptors compete for TRAIL binding, thus, lowering apoptotic response to TRAIL. However, the level of death and decoy receptors expression does not always correlate with the level of cell death observed upon TRAIL challenge. There are cancer cell lines that express death receptors but are resistant to TRAIL-induced cell death¹²². On the other hand, cancer cell lines expressing decoys can be sensitive to TRAIL-mediated cell death³²⁰. Interestingly, it was suggested that the function of DcR2 is not restricted to the competition for ligand binding, but that the overexpression of DcR2 leads to Akt and NF- κ B activation in a ligand-independent manner^{84,290}. Moreover, it was shown that DR5 mediates TRAIL-induced activation of non-apoptotic pathways in TRAIL-resistant NSCLC cell lines, promotes skeletal metastasis in a breast cancer xenograft mouse model, mediates proliferation of pancreatic cancer cell lines and promotes migration and invasion of K-RAS-driven lung and pancreatic cancer^{113-115,122}. Therefore, the role of TRAIL receptors and/or mechanisms that regulate their function appears to be much more complex than was originally thought. Based on this we hypothesized that TRAIL receptors on their own can serve as a bifurcation point towards activation of apoptotic and pro-survival pathways and fractional killing regulation.

Therefore, in my PhD thesis we focused on studying the molecular mechanisms of fractional killing, in particular the role of individual TRAIL receptors in the activation of death *vs* survival pathways and their participation in the formation of TRAIL-mediated pro- and anti-apoptotic signaling platforms. In that regard, we found that TRAIL receptors – DR4, DR5 and DcR2 form heterocomplexes upon

exposure to TRAIL, which in turn recruit FADD, caspase 8 and cFlip. Further characterization of TRAIL-Rs function revealed that even though DR4 is recruited to the DISC, it is neither required for the triggering of apoptotic nor for the activation of non-apoptotic pathways upon TRAIL-treatment. Interestingly, DcR2 sensitizes cancer cell to TRAIL-induced apoptosis suggesting its pro-survival function in our cellular system. Given that DcR2 lacks the functional death domain required for FADD recruitment and apoptotic signaling activation we hypothesized that this receptor plays its non-apoptotic function by hampering efficient DISC formation. In that regard, it was previously reported that DcR2 overexpression significantly changes DISC composition through decreasing the level of TRAIL-induced DR5 and DR4 co-immunoprecipitation and lowering FADD recruitment and caspase 8 cleavage⁸³. Notably, our preliminary data indicates that DcR2 knock out in our experimental model also leads to enhanced TRAIL-induced DR5 and DR4 association, caspase 8 and cFlip processing at the plasma membrane. These results suggest that indeed DcR2 exerts a non-apoptotic function by changing DISC composition. On the other hand, it was reported that the overexpression of DcR2 leads to the triggering of non-apoptotic pathways such as Akt and NF- κ B in a ligand-independent manner^{84,290}. Notably, we found that DcR2 is responsible for Akt activation at early time points of response to TRAIL treatment, whereas it does not affect TRAIL-mediated activation Erk1/2, p38MAPK, NF- κ B and the late wave of Akt induction. In that respect, it was recently reported, that TRAIL-mediated activation of Akt occurs in Rac1-mediated manner independently of TRAIL receptors death domain¹¹⁵. Furthermore, it was shown that Rac1 is recruited to DR5 via membrane-proximal domain. Notably, Rac1 is the GTP-ase of the Rho family that control cytoskeletal dynamics via its interaction with a number of effector proteins³²¹. Given that DcR2 lacks its functional death domain, the early wave of Akt activation may take place through this mechanism. Altogether, our results strongly suggest that, contrary to the originally proposed idea that DcR2 regulates TRAIL-induced apoptosis through competition for ligand binding, this receptor forms heterocomplexes with death receptors upon exposure to the cytokine and appears to be rather “regulatory” than “decoy”. In this respect, we found that DcR2 plays an important role in the modulation of fractional killing response by the activation of Akt signaling and by exerting negative regulation of proficient DISC formation.

Importantly, we revealed that DR5 is the main apoptotic receptor in our experimental model. Moreover, to our surprise we found that TRAIL-induced non-apoptotic and pro-survival pathways, such as Erk1/2, p38MAPK, NF- κ B and the late wave of Akt are DR5-mediated and independent of DcR2 and DR4. Notably, activation of Akt at early time points of response to TRAIL is DR5-independent, suggesting that its activation requires only DcR2. These results rise an obvious question: What is the possible mechanism by which DcR2 mediates an early wave of Akt induction, while the activation of Akt at 16 hours of TRAIL treatment occurs in DcR2-independent and DR5-mediated manner? Recent report demonstrated that Rac1, shown to mediate TRAIL-induced activation of Akt, requires the membrane proximal domain of TRAIL receptors for its recruitment. In those lines, we hypothesized that Rac1 could potentially associate with both DcR2 and DR5. However, at early time points of stimulation with TRAIL DR5 may predominantly associate with FADD, whereas DcR2 that does not recruit FADD due to the absence of the functional death domain may potentially interact with Rac1. Taking into account that FADD and Rac1 bind to different domains of TRAIL receptors, they should not directly compete for the

binding to DR5. Thus, we hypothesize that the association of FADD with DR5 may either induce conformational changes that prevent Rac1 binding to the membrane proximal domain of DR5 or simply spatially restrict Rac1 recruitment. Given that continuous TRAIL treatment leads to the generation of resistance and that this resistance is observed already after 16 hours of TRAIL treatment, we hypothesized that the composition or formation of the apoptotic DISC at this stage is hampered by not yet identified mechanisms. Therefore, we speculate that at late time points of TRAIL treatment, DR5 lacks its ability to recruit FADD and becomes available for Rac1 binding leading to DR5-mediated late wave of Akt activation.

Given that DR5 triggers both functionally different signaling outcomes we analyzed whether it participates in the formation of different signaling platforms. In that respect, TNF α stimulation leads to the assembly of pro-survival and pro-apoptotic complexes that are temporary and spatially separated. Furthermore, formation and functioning of the apoptotic complex II is regulated by the performance of the non-apoptotic complex I (see “Introduction”). Therefore, to further elucidate the molecular basis of fractional killing we analyzed formation, composition and localization of TRAIL-induced signaling platforms. Our preliminary data suggest that TRAIL challenge results in the formation of distinct DR5-comprising complexes: (i) the non-apoptotic signaling platform containing DR5, DcR2, RIPK1, TRAF2 and cFlip and the apoptotic DISC comprising of DR5, DcR2, DR4, FADD, caspase 8 and cFlip. Contrary to the differential localization of TNF α -induced pro- and anti-apoptotic complexes, both TRAIL-stimulated complexes may be formed at the plasma membrane. Given that DR5 triggers dual signaling, we hypothesize that the formation of these signaling platforms lies in the basis of TRAIL-induced fractional killing response. Notably, according to our preliminary data both TRAIL-induced signaling platforms have common components, such as TRAIL receptors, while there are also components that are present exclusively in one or the other complex, such as RIPK1 and FADD. Thus, we questioned whether the recruitment of differential components to DR5 is a stochastic process based on the competition for binding to TRAIL-Rs between death domain containing proteins that are differentially presented in two complexes (FADD and RIPK1). Alternatively there may be another regulatory mechanism that controls formation of plasma membrane-located functionally different signaling complexes in isogenic populations of cancer cells. Importantly, the plasma membrane is not a homogenous cellular compartment, as it contains micro-domains enriched in sphingolipids and cholesterol called lipid rafts. Lipid rafts present specific signaling platforms for the activation of various pathways by bringing different signaling molecules in close proximity, favoring specific protein-protein interactions and regulating availability and accessibility to different substrates^{189,220}.

Therefore, we hypothesized that the assembly of apoptotic and non-apoptotic signaling complexes can take place either in different cells or within the same cell but in different plasma membrane compartments. Interestingly, it was reported that co-localization of TRAIL-induced DISC with lipid raft and non-raft domains of the plasma membrane plays a crucial role in the regulation of TRAIL-induced activation of apoptotic and non-apoptotic pathways. In that respect, it was suggested that RIPK1 and cFlip mediate non-raft localization of the DISC of NSCLC cells resistant to ligand-mediated death and that depletion of either RIPK1 or cFlip leads to the redistribution of the DISC from non-raft to lipid raft

domains and to the switch of TRAIL signaling from survival to death. Importantly, the recruitment of differential components to signaling complexes located in non-raft or lipid raft domains can be determined by their distinct availability within these different plasma membrane compartments. In that regard, it was shown that CD45 (a receptor-linked protein tyrosine phosphatase)-induced Lck (Src family kinase known to participate in T-cell neoplasms) phosphorylation and activation is inhibited by cytoskeleton-mediated localization of Lck within lipid rafts, suggesting that indeed localization of signaling molecules within non-raft and raft fractions of the plasma membrane plays a crucial role in the regulation of receptor-mediated signaling. Moreover, it was reported that active caspase 8 is localized in different cellular compartments depending on the nature of the stimuli. Briefly, CD3 stimulation (proliferation stimuli) leads to the redistribution of a small portion of active caspase 8 to the lipid raft domains, while FAS treatment (apoptotic stimuli) results in the profound activation of caspase 8 exclusively in the cytosolic fraction²³⁰. Consequently, differential availability of caspase 8 substrates depending on whether activated caspase 8 is located in the cytosol or lipid rafts might determine a final cell fate outcome. Based on mentioned above reports it is possible to hypothesize that TRAIL-induced apoptotic and non-apoptotic signaling platforms may be formed within the same cell but in different plasma membrane and/or cellular compartments leading to the activation of both pro- and anti-apoptotic signaling. However, due to the cell-to-cell variability one signaling will eventually overcome the other one. In that regard, it was shown that the time individual cells of the clonal population spend on pre-MOMP processes upon TRAIL stimulation largely varies from one cell to the other¹⁷⁵. Apparently, similar cell-to-cell differences may also exist in the dynamics of non-apoptotic pathway activation. Indeed, analysis of individual cells exposed to HGF stimulation revealed a significantly heterogeneous single cell behavior in terms of Akt recruitment to the plasma membrane (an event crucial for Akt binding to PIP3 leading to Akt phosphorylation) within isogenic populations³²². Moreover, the time of NF- κ B translocation to the nuclei upon TNF α treatment also largely varies between different cells of the clonal population^{323,324}. Therefore, we hypothesize that the time that each individual cell takes to initiate one or the other signaling before it reaches a “no-return point” (commitment) and magnitude of the signaling may be a key in cell death or cell survival outcome. On the other hand, similarly to TNFR1/TNF α signaling²⁸, DR5-mediated activation of cell death and cell survival pathways upon TRAIL treatment can also be sequential and highly regulated.

Overall, we found that DR5 triggers both functionally different signaling outcomes that lead either to cell death or cell survival. What is the biological significance of the fact that single cytokine can use one receptor to trigger dual signaling through the formation of two separated complexes? Perhaps, it has an important adaptive value. The explanation could be that if the first signaling prevails the second one is not required. Let's assume that TRAIL-induced apoptosis is a primary response. If ligand stimulated death does not reach its end point it can be a signal for the secondary response to be activated. This secondary response can include two outcomes either triggering of pro-inflammatory response (NF- κ B) via expression of interleukins^{165,176} or activation of anti-apoptotic, pro-survival and pro-migratory pathways (NF- κ B, Erk1/2, p38MAPK, Akt)^{116,325,326}. In the first case if cell do not die due to the ability of many tumors to inhibit apoptotic signaling at different levels of the cascade, this should alarm the immune system and activate immune response. What is the biological significance of such an

adaptation? Given that TRAIL is a natural component of a tumor-surveillance system it could have developed a mechanism of the secondary response, attraction of immune system and elimination of cancer cells. On the other hand, cancer cells could have also developed their adaptations in order to survive in response to TRAIL. Therefore, if cancer cells do not undergo cell death upon stimulation it can serve as a signal that these cells will have a survival advantage. Therefore, TRAIL-induced activation of pro-survival pathways in these cells may be a justified mechanism of cancer expansion. On the other hand, let's assume that the primary response to TRAIL is the triggering of pro-survival pathways. In this case if the proper activation of pro-survival genes cannot take place upon cytokine stimulation the secondary apoptotic response will be initiated in order to eliminate these cells, and maintain only those cells that can successfully activate pro-survival pathways and, thus might have advantage in the future survival of tumor cell populations.

Altogether, we found that clonal cancer cell populations that respond in fractional killing upon TRAIL treatment trigger apoptotic and non-apoptotic/pro-survival pathways. Furthermore, our results support that DcR2 may exert its non-apoptotic function not only by affecting DISC composition, but also due to its ability to trigger the early wave of Akt activation upon exposure to TRAIL. Moreover, we found that the induction of apoptosis as well as Erk1/2, p38MAPK, NF- κ B and the late wave of Akt signaling is DR5-mediated. Overall our results suggest that clinical application of recombinant human TRAIL as well as death receptor agonists can be seriously challenged due to the fact that stimulation of DcR2 and DR5 can lead to the activation of pro-survival pathways upon exposure to these compounds. Pro-survival actions of rhTRAIL and death receptor agonists can result in cancer cells proliferation and migration leading to tumor expansion and progression. Moreover, it was reported that TRAIL induces mutations via caspase 8-dependent mechanism in cells surviving TRAIL treatment. It was suggested that a certain level of active caspase 8 can be insufficient to kill the cell, however it is sufficient to activate CAD (Caspase-activated DNase), which induces DNA damage³²⁷. Therefore, fractional killing may also give rise to a genetically heterogeneous population of cancer cells that might differentially respond to further antitumor treatment. Altogether, understanding of how a single receptor can regulate fractional killing by triggering functionally different signaling outcomes can help to develop combined therapies targeting components promoting pro-survival signaling activation and allow the use of rhTRAIL/TRAIL-Rs agonists against tumors that respond in fractional killing, without a risk to generate tumor progression.

Figure 20

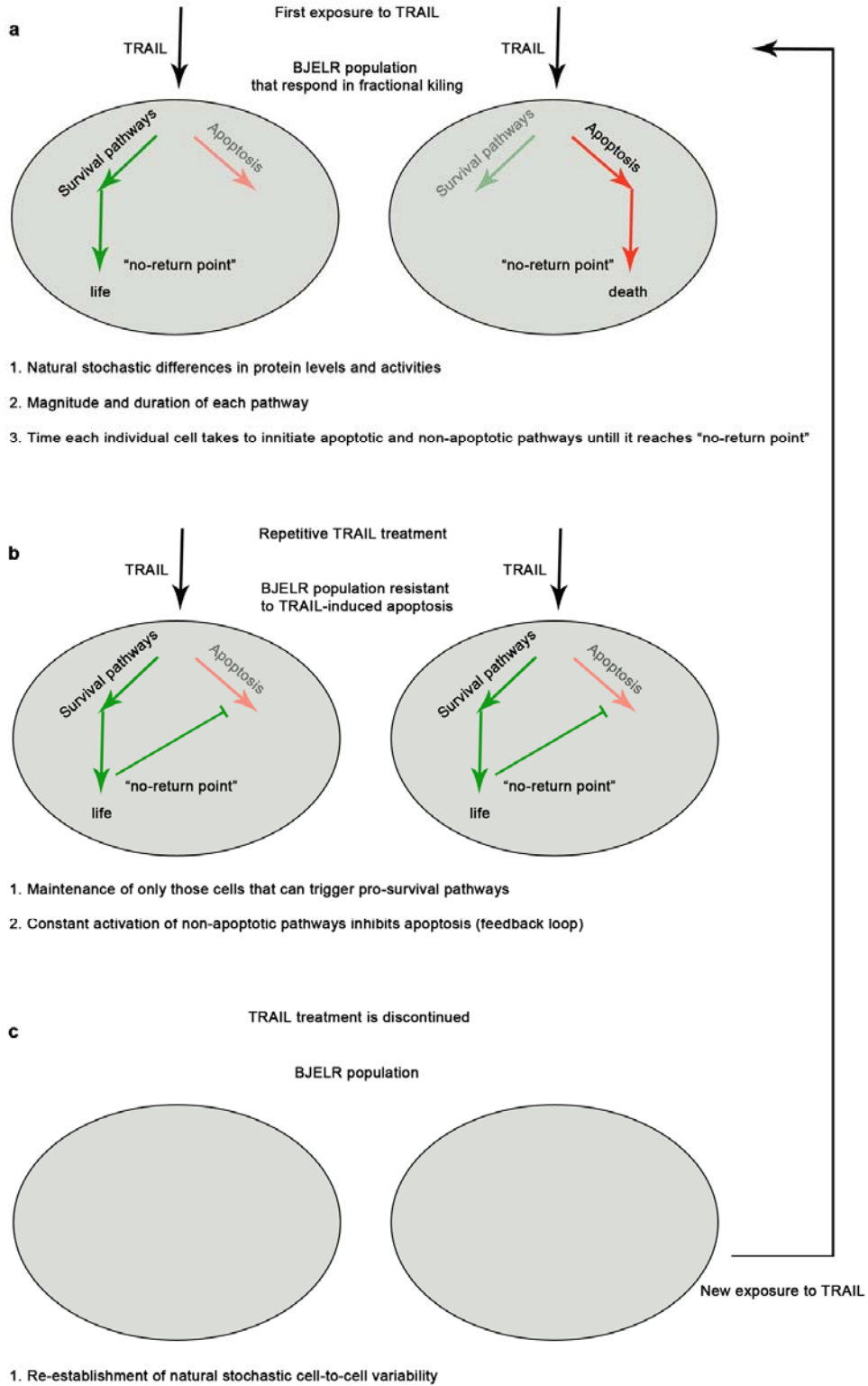


Figure 20. Model explaining a possible mechanism of fractional killing response and establishment of reversible resistance upon TRAIL treatment. See text for details.

ONGOING WORK AND PERSPECTIVES

Role of DcR2 in the regulation of fractional killing upon TRAIL treatment

Our results indicate that TRAIL receptors play a crucial role in the regulation of fractional killing in response to TRAIL. We showed that the activation of Akt signaling is blocked in DcR2 KO cells at early time points of TRAIL response. Our preliminary data indicate that DcR2 inhibits the formation of a proficient apoptotic DISC by hampering the recruitment of death receptors and FADD and abolishing caspase 8 and cFlip cleavage at the plasma membrane. Contrary, when DcR2 immunoprecipitation was performed from the whole cell lysate we did not observe any differences in the recruitment of FADD in wild type cells as compared to DcR2 KO cells, while an increased level of DR4 association as well as enhanced level of caspase 8 and cFlip cleavage was still detected. Taking into account these contradictory results, further experiments will be performed to unravel the role of DcR2 in FADD recruitment to the DISC.

Mentioned above experiments indicated an increased level of TRAIL-induced caspase 8 and cFlip cleavage at the DISC of DcR2 KO cells. Notably, our experimental set up does not allow to analyze whether DcR2 also affects caspase 8 and cFlip recruitment to the DISC. To shed light on this aspect we propose to block caspase 8 cleavage using pan caspase inhibitor followed by DR5 immunoprecipitation from wild type and DcR2 knock out cells. Further Western blot analysis will allow to unravel whether DcR2 alters caspase 8 and cFlip recruitment to the DISC. Overall, our ongoing work is focused on the final characterization of the impact of DcR2 on the composition of the DISC.

On the other hand, our preliminary data indicate that blocking caspase 8 cleavage may support formation of the non-apoptotic signaling complex by enhancing caspase 8 and RIPK1 association. Given that DcR2 modulates caspase 8 cleavage we hypothesized that DcR2 may also affect the formation of the non-apoptotic signaling complex. Nonetheless, our preliminary results indicated that DcR2 does not affect DR5/RIPK1/cFlip co-immunoprecipitation at the plasma membrane, results that need further validation by an additional independent biological replicates. Notably, our data suggest that caspase 8/RIPK1 association can be detected when caspase 8 is immunoprecipitated from the whole cell lysate, suggesting that internalization of the non-apoptotic complex may be required for caspase 8/RIPK1 assembly. Therefore, to unravel the impact of DcR2 depletion on the association of caspase 8 with RIPK1 it is necessary to perform caspase 8 immunoprecipitation from the whole cell lysate of wild type and DcR2 KO BJELR cells and analyze the efficiency of TRAIL-stimulated caspase 8 and RIPK1 co-immunoprecipitation in these cellular contexts. These results will allow to better understand the molecular mechanisms that underlie DcR2-mediated regulation of fractional killing.

Molecular mechanisms of DR5-mediated regulation of fractional killing

Our results show that a single death receptor (DR5) triggers apoptotic as well as non-apoptotic pathways (Erk1/2, Akt late wave, p38MAPK, I κ B α) in populations of BJELR cells. Our preliminary data suggest

that DR5 may regulate fractional killing response by the formation of two different signaling platforms that are located at the plasma membrane. Briefly, DR5 immunoprecipitation from the plasma membrane fraction revealed the association of DR5 with RIPK1 as well as other DISC components (FADD, caspase 8, DR4, DcR2, cFlip) upon TRAIL treatment. On the other hand, RIPK1 immunoprecipitation indicated TRAIL-induced binding of RIPK1 to DR5, DcR2, TRAF2 and cFlip at the plasma membrane. Our results provide initial evidences that both FADD and caspase 8 are not associated with RIPK1 and TRAF2 at the plasma membrane. However, caspase 8 immunoprecipitation from the whole cell lysate leads to the detection of RIPK1 and TRAF2 being associated with this caspase, suggesting that the formation of RIPK1/TRAF2/caspase8/FADD complex may occur in cellular compartments distinct from the plasma membrane. Therefore, in order to validate that RIPK1 and TRAF2 association with caspase 8 and FADD, indeed does not take place at the plasma membrane we will perform FADD and caspase 8 immunoprecipitation from the plasma membrane fraction as well as from the whole cell lysate and analyze the co-immunoprecipitation patterns of RIPK1 and TRAF2 in these conditions. If the result is confirmed, this suggests that TRAIL induces formation of two different plasma membrane-associated complexes and that RIPK1, TRAF2 and cFlip are recruited to TRAIL receptors independently of FADD and caspase 8. Furthermore, it will indicate that internalization of the non-apoptotic signaling platform may be required for its association with caspase 8 and FADD.

To shed light on these aspects we propose to block internalization by using different approaches such as TRAIL treatment at 4°C, clathrin heavy chain depletion, expression of dynamin mutants (depending on the type of internalization taking place in BJELR cells) followed by caspase 8 and FADD immunoprecipitation from the whole cell lysate. Notably, our preliminary data suggest that blocking internalization by incubating cells at 4°C prevents TRAIL-induced I κ B α phosphorylation. However, given that enzymatic reactions can be slowed down at 4°C the observed effect is not necessarily directly related to the block of internalization. Therefore, we propose to further analyze the role of internalization in the activation of non-apoptotic pathways such as Erk1/2, Akt, p38MAPK, I κ B α by using alternative methods of preventing receptors internalization (e.g. clathrin heavy chain depletion, expression of dynamin mutants, etc). These results will help elucidating whether there is a correlation between the impact of internalization on the co-immunoprecipitation of the non-apoptotic signaling complex with caspase 8 and FADD and the activation of non-apoptotic pathways. Altogether, this data will help to better understand the molecular mechanisms that regulate the formation and function of TRAIL-induced signaling platforms.

Role of lipid rafts and the cytoskeleton in the modulation of TRAIL-induced signaling

If confirmed that TRAIL induces formation of two different complexes at the plasma membrane, we propose to analyze whether they are formed within distinct plasma membrane compartments, such as raft and non-raft domains. To study this it is required to separate the raft fraction from the non-raft fraction and perform DR5, RIPK1, caspase 8 and FADD immunoprecipitation from these fractions. Further Western blot analysis will allow to identify whether DR5 recruits distinct components depending

on whether it is immunoprecipitated from raft or non-raft domains. Overall, these results will unravel the molecular mechanisms that allow spatial regulation of TRAIL signaling.

It was shown that the cytoskeleton plays an important role in the formation and maintenance of lipid raft domains^{235-237,239-242}. Moreover, the role of the cytoskeleton in the regulation of TRAIL and FasL signaling was suggested (for details see “Introduction: Role of cytoskeleton in cell fate decisions”). Indeed, previous data of our lab suggest that the association of NMIIIs with the DISC of normal cells may be required for the maintenance of normal cell resistance to TRAIL-induced cell death. Moreover, our preliminary data support that NMIIIs play a crucial role in the triggering of TRAIL-stimulated Erk1/2 and Akt signaling in BJ cells. Given that TRAIL can induce formation of the non-apoptotic signaling platform in BJELR cells, we propose to study whether the same anti-apoptotic complex is formed in BJ cells. If the formation of the anti-apoptotic signaling complex takes place in this cellular context we will further proceed with the analysis of whether NMIIIs affect the composition of this complex. This data will allow to evaluate the role of the cytoskeleton, in particular NMIIIs, in the activation of TRAIL-induced non-apoptotic pathways.

Overall, these experiments will help to better understand the molecular mechanisms by which TRAIL receptors (death and decoys) regulate fractional killing upon TRAIL treatment. Furthermore, these studies will allow to further decorticate the molecular mechanisms underlying the triggering of death and survival pathways through a single death receptor in isogenic populations of cancer cells helping to better understand the complexity of TRAIL-induced signaling. It will provide insights for evaluating whether combined therapies targeting the components promoting pro-survival signaling activation would allow the use of rhTRAIL and DR5 agonistic antibodies to promote TRAIL-mediated killing while avoiding TRAIL-induced tumor progression.

MATERIALS AND METHODS

1. Antibodies

Antibodies used for Western blot that recognize indicated proteins were purchased from Cell Signaling (p38MAPK 9212, p-p38MAPK (Thr180/Tyr182) 9211, DR5 3696, DcR2 8049, DcR1 4756, p44/42MAPK 9102, p-p44/42MAPK (Thr202/Tyr204) 9101, Akt 9272, pAkt (Ser473) 9271, pAkt (Thr308) 4056, pI κ B α (Ser32/36) 9246, I κ B α 4814, PARP 9542, NEMO 2685, NEMO 2695, caspase-8 9746, RIPK1 3493, TRAF2 4724, Anti-mouse IgG – HRP-linked antibody 7076, Anti-rabbit IgG – HRP-linked antibody 7074), Santa Cruz Biotechnology (TNFR1 sc-8436, β -actin sc-1615, TRAF2 sc-7187, α -tubulin sc-32293), Millipore (DR4 AB16955, mouse anti-rabbit light chain specific HRP conjugated monoclonal antibody MAB201P), BD Pharmingen (FADD 556402, N-Cadherin 610920) AdipoGen (cFlip AG-20B-0056-C100), Abcam (anti - EEA1 antibodies AB2900). Antibodies for DR5 (AF631), DR4 (AF347), DcR2 (AF633), TRAF2 (AF3277) immunoprecipitation and normal goat IgG (AB-108-C) were purchased from R and D systems, for caspase 8 immunoprecipitation (sc-6136) - from Santa Cruz Biotechnology, for RIPK1 (51-6559GR) immunoprecipitation - from BD Pharmingen, normal mouse IgG – from Jackson ImmunoResearch (015-000-003). For flow cytometry assays, Cleaved caspase 8 (12602), Cleaved PARP (6987) and Cleaved PARP (8978) were purchased from Cell signaling, Mouse IgG1 (IC002G), Mouse IgG2B (IC0041G), DcR1 (FAB6302P), DcR2 (FAB633G), DR5 (FAB6311G) from R and D systems, DR4 (804-297TD-T100) from Enzo Life Sciences.

2. Inhibitors

P38MAPK inhibitor PD169316 was purchased from Calbiochem (513030), MEK inhibitor U0126 – from Promega (V112A), PI3K inhibitor LY294002 – from Cell Signaling (9901), inhibitor of I κ B α phosphorylation Bay-11-7082 – from Sigma (B5556). Initially we established the most effective inhibitors concentrations for our experimental model (BJELR cells). Moreover, we analyzed the time frame of effective inhibitor-induced downregulation of the phosphorylation of Erk1/2, Akt, p38MAPK and I κ B α in order to determine the optimal time for the beginning of TRAIL treatment and its maximal possible duration. The final working concentrations of inhibitors required for an efficient inhibition of kinases phosphorylation in BJELR cells were the following: PD169316 - 25 μ M, U0126 - 20 μ M, LY294002 - 50 μ M, Bay-11-7082 - 40 μ M. All of them effectively inhibited phosphorylation of corresponding kinases after 1 hour of treatment and sustained this effect for 6 hours.

Pan caspase inhibitor Z-VAD-FMK purchased from R and D systems (FMK001) was used to block apoptosis. The final working concentrations of Z-VAD-FMK used for BJELR cells was 100 μ M. These conditions ensured an effective block of apoptotic cell death up to 20 hours upon TRAIL treatment.

Blebbistatin (B0560) and ML7 (I2764) were purchased from Sigma and used to inhibit NMII's function. The final working concentration of Blebbistatin used for BJ and BJELR cells was 50 μ M, of ML7 - 80 μ M.

Given that PD169316, U0126, LY294002, Bay-11-7082, Z-VAD-FMK and Blebbistatin were reconstituted in DMSO; and ML7 in 50% ethanol, solvent control was used in each experiment to

monitor any DMSO- or ethanol-related effects. In order to monitor the efficiency of inhibitors treatment during the duration of TRAIL treatment we control an effect of each inhibitor in the beginning of TRAIL treatment as well as when exposure to TRAIL was terminated.

Given that all mentioned above inhibitors either effectively inhibited kinases phosphorylation (PD 169316, U0126, LY294002, Bay-11-7082, ML7) or blocked apoptosis (Z-VAD-FMK) after 1 h treatment, cells were exposed to 1 hour of treatment with indicated inhibitors prior to TRAIL treatment.

3. Cell lines

BJ and BJELR cells from the stepwise tumorigenesis system (BJ-derived model) were obtained from Dr. Hahn and cultured in DMEM (1 g/l glucose) + Med 199 (4:1)+10% FCS heat-inactivated. BJ cells were split every 48 hours at 0.5×10^6 /petri dish. BJELR cells were split every 48 hours at 1×10^6 /petri dish. The clonal population of BJELR cells was generated by single cell cloning (Dr. Valeria Pavet). Several clones of BJELR cells were used to test their sensitivity to TRAIL-induced apoptosis. Importantly, no significant differences were observed in apoptotic response between different clones. Clonal population of BJELR cells used for experiments was maintained in culture for no more than 15 passages. When 15th passage was reached new BJELR cell were de-frozen. Importantly, BJELR cells should never exceed 80% confluency, as this impacts their sensitivity to TRAIL-induced apoptosis. For selection, medium of BJELR cells was freshly supplemented with $100 \mu\text{g mL}^{-1}$ hygromicine, $400 \mu\text{g mL}^{-1}$ G418 and $0.5 \mu\text{g mL}^{-1}$ puromycin. All cells were grown at 37°C, 5% carbon dioxide (CO₂) and constant humidity. Cells stocks were prepared by freezing BJ and BJELR cells in 90%FCS +10%DMSO at -80°C. The following day frozen cells were transferred into the liquid nitrogen for a long term storage.

4. siRNA-mediated knock down

siRNA smartpools or single siRNA used for the knock down experiments were purchased from Dharmacon. For DR4 knock down a pool of two individual siRNA – J-008090-09-0005 and J-008090-08-0005 was used. DR5 knock down was achieved utilizing pool of three single siRNA – J-004448-06-0005, J-004448-07-0005 and J-004448-08-0005. DcR2 knock down was performed using individual siRNA J-008092-14. For DcR1 depletion we used Smartpool siRNA – L-008091-00. Smartpool siRNA were used for RIPK1 – L-0044445-00-0005, TRAF2 – L-005198-00-0005 and FADD – L-003800-00-0005 knock down. For caspase 8 knock down individual siRNA – J-003466-16 was utilized. Transfection with the non-targeting scrambled siRNA - D-001810-01-20 was used in each experiment in order to monitor any possible effects of the transfection itself. Initially all individual siRNA targeting all mentioned above mRNA were analyzed on whether they exert any off-target effects towards other DISC components. Based on this, for further experiments we selected only those siRNA that did not express any off-target effects at least towards DISC members. Further, for each siRNA we analyzed an optimal concentration of siRNA and duration of transfection required for an efficient knock down. The working concentration of siRNA used for the knock down of aforementioned proteins was 30nM. The optimal knock down using 30nM siRNA was reached at 48h post-transfection. siRNA were transfected into BJ and BJELR cells using RNAi Max Lipofectamine (13778150, Invitrogene). Below we provide

the transfection protocol based on the calculations done for 1 well (surface area 903mm²) of a 6 wells plates:

Mix1: 4µl siRNA (20µM stock) + 250µl Opti-MEM (Opti-MEM Reduced serum medium, GlutaMAX Supplemented, ThermoFisher Scientific, 51985034). Mix1 was incubated for 15 min at room temperature.

Mix2: 11.25µl RNAi Max Lipofectamine (13778150, Invitrogene) + 250µl Opti-MEM. Mix2 was incubated for 15 min at room temperature.

Further, Mix1 and Mix2 were combined, incubated at room temperature for 20 min and placed into the well. For transfection we used 0.25*10⁶/ of BJELR cells per well (surface area 903mm²) and 0.15*10⁶/ of BJ cells per well (surface area 903mm²). The suspension of trypsinized BJ or BJELR cells was added to the transfection mix (reverse transfection). The final volume was 2.5ml/well. 24 hours post-transfection medium was carefully replaced by 2ml of fresh pre-warmed medium and cells were incubated for additional 24h. 48h post-transfection cells were used for experiments. TRAIL treatment was performed in 1ml of medium. Notably, 1ml of medium was carefully removed one hour before TRAIL treatment to avoid any negative effects of this manipulation on further experiments.

5. CRISPR-Cas9-mediated knock out of DR5 and DcR2

Knock out cell lines were generated using double nickase plasmids. Notably, each double nickase plasmid contains a pair of plasmids each of which leads to the single strand nick, while both of them generate double strand break. Double strand breaks can be repaired by either homology directed repair or non-homologous end joining leading to frame shift mutations.

BJELR cells were transfected with 200ng/ml of DR5 double nickase plasmid (Santa Cruz, sc-4010002-NIC), DcR2 double nickase plasmid (Santa Cruz, sc-403389-NIC) or control double nickase plasmid (Santa Cruz, sc-437281-NIC) using Lipofectamine LTX (15338100, Invitrogene). Below we provide the transfection protocol based on the calculations done for 1 well (surface area 903mm²) of a 6 wells plates: Mix1: 180µl Opti-MEM + 3µl Plus reagent (15338100, Invitrogene). Mix1 was incubated for 15 min at room temperature. Further double nickase plasmid was added to the Mix1, mixed well and incubated for 20min at room temperature.

Mix2: 7.2µl Lipofectamine LTX (15338100, Invitrogene) + 180µl Opti-MEM. Mix2 was incubated for 20 min at room temperature.

Further, Mix1 and Mix2 were combined, mixed well, incubated at room temperature for 20 min and placed into the well containing BJELR cells that were growing at a regular conditions (direct transfection). Notably BJELR cells were prepared 24 hours prior to transfection at a confluency of 0.15*10⁶/well (surface area 903mm²). The final volume in the well after adding transfection mix was 2.5ml.

Twenty-four hours post-transfection cells were trypsinized using 0.05% Trypsin-EDTA (ThermoFisher Scientific, 25300062) and subjected to single cell sorting. Single cells were seeded in 96 wells plates (four plates per plasmid) using fluorescent-activated cell sorting technique (FACS) based on the GFP expression in transfected cells. Before sorting each well of 96 wells plates was filled with 50µl of filtered conditioned medium. Conditioned medium was obtained from BJELR cells growing at a regular growing

conditions (see section “Cell lines”) for 24 hours. Importantly, after single cell sorting 96 wells plates were centrifuged at 1000rpm for 4min. This procedure facilitated attachment of the cells. Twenty-four hours after sorting each well was checked for the presence of the cells. Only wells containing 1 cell were marked as positive and were used for further procedures and analysis. The same day single cell positive wells were supplemented with additional 50µl of filtered conditioned medium. Cell culture medium was then replaced by filtered conditioned medium (100µl) every 48 hours. When single cell clones reached semi-confluency in 96well plates they were transferred into 24 well plates. Importantly, for this manipulation we used conditioned medium. Our observations suggest that conditioned medium facilitates cells recovery. When clones reached confluency in 24 wells plates they were split into two wells of 6 wells plates, one of which was used to maintain the clone in culture and the second one for Western blot analysis for the presence or absence of DR5 or DcR2 protein. Further, CRISPR/Cas9 target sites in DR5 and DR2 knock out positive clones were analyzed by sequencing in order to characterize mutations induced by CRISPR/Cas9.

6. Apoptosis measurement

Apoptotic cell death was determined by flow cytometry approach by measuring the percentage of cells displaying positive staining for cleaved PARP or cleaved caspase 8. For that BJELR cells were plated in a density $1,5 \cdot 10^5$ /well of 6 wells plates, BJ cells – $0,75 \cdot 10^5$ /well of 6 wells plates. Forty-eight hours later cells were either left untreated or treated with TRAIL for different time points depending on the experiment. Notably, TRAIL treatment was performed in 1ml of medium. 1ml of medium was carefully removed one hour before TRAIL treatment to avoid any negative effects of this manipulation on further experiments. Further, cells were collected by trypsinization and centrifuged. A pellet of cells was re-suspended in 50µl of 1xPBS and fixed with 1ml of methanol (kept at -20°C prior to use) for 5min at room temperature. Fixed cells were either stored at -20°C (can be stored for several days) or subjected to the immunolabeling. For that methanol was discarded by centrifugation and cells were washed once with 1ml of 1xPBS followed by blocking with 0.5% BSA in 1xPBS (80µl final volume) for 1h at 21°C and 650rpm (done in thermomixer) and staining with anti-cleaved PARP or anti-cleaved caspase 8 antibodies for 1h at 21°C and 650rpm in dark (done in thermomixer). After staining cells were washed once in 1ml of 0.5 % BSA in 1xPBS, re-suspended in 300µl of 0.5 % BSA in 1xPBS and immediately analyzed by FACS. Results presented in histograms derived from at least three independent biological replicates.

7. Surface levels of TRAIL receptors

Expression of TRAIL receptors at the surface of BJ and BJELR cells was measured by flow cytometry approach. Cells were trypsinized, centrifuged at 1100rpm for 5min, re-suspended in the blocking solution (5% FCS in 1xPBS) and counted. $1 \cdot 10^5$ cells were transferred into FACS tubes and incubated with the blocking solution (5% FCS in 1xPBS, 80µl) for 1h on ice. Further cells were subjected to the staining with antibodies recognizing DR4, DR5, DcR1 and DcR2. Appropriate IgGs were used to control background binding. Staining was performed for 1h on ice in dark. Each sample was additionally labeled

with DAPI which allows discrimination of viable cells and, thus ensures an analysis of surface staining. Consequently, expression of TRAIL receptors were measured only in viable DAPI negative cells. Resulting mean (or median) fluorescence intensities (MFI) of the viable cells were taken for analysis. Values were normalized according to the respective auto fluorescence and the signal from IgGs.

8. Immunoprecipitation

BJ cells were plated at a confluency 0.5×10^6 / petri dish. BJELR cells were plated at a confluency 1×10^6 / petri dish. Twenty-four hours later the medium was replaced by fresh medium. Forty-eight hours later cells were either left untreated or treated with TRAIL at regular growing conditions (37°C , 5% CO_2) or at 4°C for different time points depending on the experiment. Cells were washed in plate with 1xPBS, collected in 1xPBS, centrifuged at 1100rpm for 5min at 4°C and lysed in 1ml (for 20×10^6 cells – number of cells used per sample) of freshly prepared lysis buffer (30mM Tris-HCl pH7.4, 150mM NaCl, 5mM KCl, 10% glycerol, 2mM EDTA pH8,0) supplemented with EDTA-free protease inhibitor cocktail (Roche, 11873580001), phosphatase inhibitor (PhosStop, Roche, 04906845001) and 0.9% Triton-X100 (BioRad). Lysates were incubated on ice for 30 min followed by 30min centrifugation at 14000 rpm at 4°C . Further supernatants were subjected for precleaning with equilibrated in lysis buffer Pure proteome Protein G magnetic beads (Millipore, LSKMAGG02) for 2h at 4°C (15 μl beads for 1ml of lysate). Binding of antibodies to equilibrated beads were performed in the proportion: 1 μg of antibodies for 2 μl beads, for 3-4 hours at 21°C and 1000rpm (in the thermomixer). Further, after preclearing lysates were quantified using Protein assay (BioRad, 500-0006), adjusted to the same concentrations using lysis buffer and subjected to DR4, DR5, DcR2, TRAF2, RIPK1, caspase 8 or IgG immunoprecipitation using anti-DR4, anti-DR5, anti-DcR2, anti-TRAF2, anti-RIPK1, anti-caspase 8, normal goat IgG or normal mouse IgG bound to the Pure proteome Protein G magnetic beads. Immunoprecipitation with normal goat IgG or normal mouse IgG was used to control background binding. Immunoprecipitation was performed overnight at 4°C on the rotating shaker to ensure proper mixing of the sample content. For caspase 8, DR4, DR5, DcR2, TRAF2 and RIPK1 IP immunoprecipitation we used 5 μg of antibodies + 10 μl beads for 20×10^6 cells. After immunoprecipitation samples were washed 5 times with the ice-cold lysis buffer and eluted. Elution was performed using 60 μl of 1 fold concentrated Laemmli buffer (2-fold concentrated buffer- 125mM Tris-HCl pH 6.8; 4% SDS; 10% 2-mercaptoethanol; 10% glycerol – was diluted to 1 fold using lysis buffer) supplemented with 20mM DTT (10 μl of 1M DTT/500 μl of 1 fold elution buffer). Elution was done at 75°C , 1000rpm for 15 min (in the thermomixer). Samples were subjected for the Western blot analysis. Each sample was divided for 2 or 3 gels.

Antibodies cross-reactivity. Importantly, DR5 immunoprecipitation experiments showed a pre-ligand association of DR5 with DcR2 that is further enhanced upon TRAIL treatment. It was reported (by R and D Systems, data sheet for DR5 antibodies) that DR5 antibodies that we used for immunoprecipitation experiments show approximately 2% cross-reactivity with rhDcR2. Based on this we analyzed whether DR5 antibodies cross-reacted with DcR2 in our experimental set up. For that we performed DR5 immunoprecipitation from wild type and DR5 knock out BJELR cells. Western blot analysis revealed DcR2 signal in wild type as well as in DR5 knock out sample, suggesting that the detection of DcR2 in

DR5 immunoprecipitation prior to TRAIL treatment may be due to the cross-reactivity of DR5 antibodies with DcR2 and not due to the actual pre-ligand assembly of these two receptors.

Immunoprecipitation of DR4 also indicated a pre-ligand assembly of DR4 with DR5. It was reported (by R and D Systems, data sheet for DR4 antibodies) that DR4 antibodies that we used for immunoprecipitation experiments show approximately 10% cross-reactivity with rhDR5. To unravel whether DR4 cross-reacted with DR5 in our experiments we generated DR4 knock out BJELR cells. Further experiments are required in order to evaluate whether DR4 signal in DR5 IP prior to TRAIL treatment is due to the cross-reactivity of DR4 antibodies with DR5.

9. Extraction of the plasma membrane fraction form BJELR cells

Extraction of the plasma membrane fraction was performed using Plasma membrane protein extraction kit (BioVision K268-50). BJELR cells were plated at a confluency 1×10^6 / petri dish. Twenty-four hours later the medium was replaced by fresh medium. Forty-eight hours later cells were either left untreated or treated with TRAIL at regular growing conditions (37°C, 5% CO₂) for different time points depending on the experiment. Cells were washed in plate with 1xPBS, collected in 1xPBS, centrifuged (at 1100rpm for 5min at 4°C) and re-suspended in 2ml (for 40×10^6 cells – number of cells used per sample) of the Homogenization buffer supplemented with 8µl of the Protease Inhibitors Cocktail (reconstituted in 500µl of DMSO). Cells re-suspended in the Homogenization buffer were homogenized in Dounce homogenizer (Weaton 1ml, Loose pestle 0.089-0.14mm) on ice. Each sample was passed through the homogenizer 100 times resulting in a complete lysis (90% of cells, verified by checking through the microscope). Further procedures were performed as indicated in the protocol available with the Kit. Briefly, obtained homogenate was transferred to a 1.5ml micro-centrifuge tube (2ml of homogenate were divided into two tubes) and centrifuged at 700 x g for 10min at 4°C. Supernatant was transferred to a new micro-centrifuge tube and centrifuged at 10000 x g for 30min at 4°C. Supernatant represented the cytosol fraction, while the pellet contained proteins from the plasma membrane and cellular organelles membranes. Therefore, the pellet was used for further manipulations. The pellet was re-suspended in 200µl of the Upper Phase Solution followed by the addition of 200µl of the Lower Phase Solution. The sample was well mixed and incubated on ice for 5min. Further, the sample was centrifuged at 1000 x g for 5min at 4°C. The upper phase was carefully transferred into a new tube and kept on ice. To maximize the yield we performed the second extraction from the remaining lower phase by adding 100µl of the Upper Phase Solution to the sample. As before, sample was well mixed and centrifuged at 1000 x g for 5min at 4°C. The upper phase was carefully collected and combined with the upper phase obtained after the first extraction. Further, we extracted the combined upper phase by adding 100µl of the Lower Phase Solution. Sample was well mixed and centrifuged at 1000 x g for 5min at 4°C. We carefully collected the upper phase and diluted it in 5 volumes of water. The sample was incubated for 5min on ice and centrifuged at 15000 x g for 10min at 4°C. The pellet represented the plasma membrane fraction. The pellet of the plasma membrane fraction was re-suspended in 500µl of lysis buffer/tube (30mM Tris-HCl pH7.4; 150mM NaCl; 5mM KCl; 10% glycerol; 2mM EDTA pH8.0) supplemented with EDTA-free protease inhibitor cocktail (Roche, 11873580001), phosphatase inhibitor (PhosStop, Roche, 04906845001) and 1% Triton-X100 (BioRad)). To ensure the proper reconstitution of the pellet samples

were well mixed and incubated on ice for 20-30min. Given that after homogenization each sample was divided into two tubes, the final volume of each sample after reconstitution was 1ml. Further, immunoprecipitation assays using plasma membrane fraction were performed as described in the “Immunoprecipitation” section. For RIPK1 immunoprecipitation from the plasma membrane fraction we used 2.5µg of antibodies + 5µl beads for 40*10⁶ cells; for DR4 and DR5 - 5µg of antibodies + 10µl beads for 40*10⁶ cells. Samples were subjected for the Western blot analysis. Each sample was divided for 2 (RIPK1 IP, DR4 IP) or 3 gels (DR5 IP). Anti - N-cadherin antibodies were used as a plasma membrane marker, while anti - EEA1 antibodies, marker of early endosomes, were used in order to verify the purity of the plasma membrane fraction.

10. Western blot assay

Samples for WB were washed in plate 3 times in 1xPBS and collected in plate by adding RIPA buffer (10mM Na₂HPO₄, 150mM NaCl, 2mM EDTA, 1% IGEPAL, 1% Deoxicolate Na, 0.1 % SDS, 10mM NaF) supplemented with EDTA-free protease inhibitor cocktail (Roche, 11873580001) and phosphatase inhibitor (PhosStop, Roche, 04906845001). RIPA samples were lysed on ice for 30min, centrifuged at 14000rpm for 30min at 4°C and the DNA pellets were discarded. Further samples were either stored at -80°C or used for Western blot assay. For that, first of all samples were quantified using Protein assay (BioRad, 500-0006) and adjusted to the same concentration using RIPA buffer supplemented with EDTA-free protease inhibitor cocktail (Roche, 11873580001) and phosphatase inhibitor (PhosStop, Roche, 04906845001). Further samples were supplemented with the loading buffer and incubated for 10min at 100°C (protein denaturation step). Proteins were separated by SDS-PAGE in the Min—PROTEAN Tetra Cell Run at 120V in 1xTris Glycine buffer (10x Tris Glycine buffer (11): 30.2g Tris Base, 188g Glycine, 50ml SDS 20%) and transferred into the nitrocellulose membrane in Criterion Blotter at 100V for 45min in 1xTris Glycine buffer that does not contain SDS. Nitrocellulose membranes were stained with Ponceau S to control equal protein loading and transfer. Further membranes were washed with 1xPBS+0.1% Tween20 to remove Ponceau S staining. After this membranes were blocked using 5% milk in 1xPBS+0.1% Tween20 for 1h at room temperature. Further membranes were incubated with required primary antibodies either in 5% milk in 1xPBS+0.1% Tween20 or in 5% BSA in 1xPBS+0.1% Tween20 overnight at 4°C at head-to-head shaker. After this membranes were washed three times (7 min each wash) in 1xPBS+0.1% Tween20 and incubated with corresponding secondary peroxidase-conjugated antibodies. After incubation with secondary antibodies membranes were washed three times (7 min each wash) in 1xPBS+0.1% Tween20. Immunoreactive bands were visualized by chemiluminescence using ECL Western blotting substrate (Pierce, 32209), SuperSignal West Pico Chemiluminescent substrate (Pierce, 34080) or Luminata Forte Western HRP Substrate (Millipote, WBLUF0500) and exposed to Carestream Kodak BioMax MR film (Z350389-50EA), Amersham Hyperfilm ECL (28906835) or Amersham Hyperfilm MP (28906842). Further Western blot films were scanned and processed in Adobe Photoshop CS4. Images presented in the manuscript correspond to one representative experiment out of at least two biological replicates.

11. Preparation of competent bacteria

The single colony of XL1 blue was used to inoculate 20 ml of LB medium (liquid bacteria culture). The liquid bacteria culture was grown overnight at 37°C (“overnight pre-culture”). 10 ml of pre-culture were used to inoculate 100 ml of pre-warmed (37°C) LB medium. Bacteria culture was grown at 37°C 190rpm until OD reached 0.48 and chilled on ice. Further bacteria culture was centrifuged for 10min at 3500rpm at 4°C. The pellet was re-suspended in 30ml of ice-cold TFBI buffer (For 100ml: 1.2092g RbCl₂, 0.8094g MnCl₂, 0.2944g CH₃CO₂K, 1ml 1M CaCl₂, 15ml 100% glycerol, adjust pH 5.8 using 0.2 M acetic acid) and incubated on ice for 1 – 2 hours. Further bacteria culture was centrifuged again for 10min at 3500rpm at 4°C. Bacteria pellet was re-suspended in 4ml ice-cold TFBII buffer (For 100ml: 7.5ml 1M CaCl₂, 0.1209g RbCl₂, 0.2930g MOPS, 15ml 100% glycerol, adjust pH to 7.0). Eventually, aliquots of 100ul were dispensed in precooled 1.5ml tubes. Bacteria were frozen in liquid nitrogen and stored at -80°C for at least one year.

12. Bacteria transformation

An aliquot of competent bacteria were thrown on ice. Further, 1-100 ng of DNA were added to the competent bacteria and quickly mixed followed by 30 min incubation on ice. In order to optimize DNA uptake bacteria were subjected to the heat shock by the incubation at 42°C for 90 seconds followed by 5 min incubation on ice. Consequently, 500 microliters of LB buffer together with the cells were transferred into 10ml falcon and incubated at 37°C for 30-45 min (with vigorous shaking). After this bacteria were plated on the agar plates containing selection antibiotics and grown at 37°C for 12-16 hours.

13. Reverse transcription reaction

RNA was isolated using Trizol reagent (Invitrogen 15596-026). Briefly, BJELR cells growing in a Petri dish were washed once in 1xPBS. Further, 1ml of Trizol reagent was added into the Petri dish and cells were collected into a micro-centrifuge tube. Further samples were mixed by pipetting up and down until the homogenous sample was obtained (3-4 times). Samples were either kept at -80°C for a long term storage or used for RNA extraction. For this, 200µl of chloroform was added per 1ml of the initial volume of Trizol reagent. Tubes were vigorously shaken by hand for 15sec and incubated at room temperature for 2-3min. After this, samples were centrifuged at 10000rpm for 15min at 4°C. Consequently, the aqueous phase (upper phase) that contains RNA was collected (500µl to avoid contamination) and transferred into a fresh tube. For RNA precipitation 500µl of isopropanol was added to the samples and incubated at room temperature for 10min followed by the centrifugation at 10000rpm for 30min at 4°C. Supernatant was discarded and the pellet containing RNA was washed once with 1ml of 75% ethanol. Samples were mixed by vortexing and centrifuged at 6000rpm for 5min at 4°C (RNA washing step). The RNA pellet was dried for 5-10min and reconstituted by adding 50µl of RNA-ase free water. Samples were either stored at -80°C (for a long term storage) or used for reverse transcription reaction.

The protocol for reverse transcription reaction is provided below:

Mix 1: 1µl dNTP (10mM each, ThermoScientific R0141, R0151, R0161, R0171) + 1µl of 20mM oligo dT + 3µg of RNA + H₂O to the final volume of 12µl. The reaction mix 1 was heated at 65°C for 5 min (denaturation step) and chilled on ice.

Mix 2: 4µl Superscript buffer (SuperScript II Reverse Transcriptase Invitrogen 18064-022) + 2µl 0.1M DTT + 1µl Ribonuclease inhibitor + 0.5µl Superscript Reverse Transcriptase (SuperScript II Reverse Transcriptase Invitrogen 18064-022). The reaction mix 1 was well mixed with the reaction mix 2 and incubated at 44°C for 1h (reverse transcription). Further reaction was inactivated by heating at 70°C for 15min. All reactions were performed in the PCR machine.

14. PCR and cloning

cDNA obtained in RT reaction was diluted 10 times prior to PCR reaction. Primers used for DR4 cDNA amplification were the following: pair 1 – forward atggcgccaccaccagctagag and reverse gtcactccagggcgtacaat; pair 2 – forward ggaactttccggaatgacaa and reverse tcaactccaaggacacggcagagc. PCR Mix 1 and Mix 2 were prepared as follows.

Mix 1: 1µl dNTP (10mM each) + 1µl 20µM Forward primer + 1µl 20µM Reverse primer + 5µl cDNA (1/10 dilution of RT product) + H₂O to the final volume of 25µl.

Mix 2: 5µl high fidelity polymerase buffer (Roche 14676700) + 19.25µl H₂O + 0.75µl high fidelity polymerase (Roche 14528824). Further Mix 1 was combined with Mix 2 and subjected to the PCR reaction. Efficiency of the PCR reaction was verified by subjecting PCR products to the gel electrophoresis (2% agarose gel in 1xTBE buffer supplemented with 5µl of Ethidium Bromide/100ml) at 100V in 1xTBE buffer. The required band was purified from the gel using MiniElute gel extraction Kit (Qiagen, 28604). Purified PCR fragments were cloned into the pGEM-T vector (pGEM-T easy vector system Promega A1360). The ligation was performed using the following reaction conditions: 0.5µl of pGEM-T vector + 5µl buffer + 1µl T4 ligase + 3.5µl PCR product. The reaction was performed overnight at 4°C. Following day competent XL1 blue bacteria were transformed with the constructs as described in the “Bacteria transformation” section. Importantly, agar plates were supplemented with 100µl LB + 10µl XGal (100mg/ml) + 10µl IPTG (1M). After overnight growth at 37°C white colonies (PCR product efficiently integrated into the pGEM-T vector) were selected for the preparation of liquid culture (1 colony for 3.5ml of LB supplemented with ampicillin). Plasmid purification was performed using NucleoSpin Plasmid Kit (Macherey-Nagel, 740588.50). Sequencing of DR4 cDNA was done in the AGTC Biotech utilizing T7 and SP6 primers.

15. Statistical analysis

Results represented in the histograms show the mean value +/- Standard Deviation (SD) of at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student's t-test, ****P* value < 0.0005, ***P* value < 0.005, **P* value < 0.05.

EXTENDED SUMMARY

Introduction. The discovery of TRAIL (Tumor necrosis factor-Related Apoptosis-Inducing Ligand; TNFSF10) in the mid 90's opened an opportunity for cancer-selective therapy, as this cytokine selectively induces apoptosis in a broad range of human cancer cell lines while sparing normal tissue²⁴⁹. TRAIL is a TNF-superfamily member that binds as a homotrimer to TRAIL receptors (TRAIL-Rs), inducing their oligomerization. Four TRAIL-specific receptors have been described, two death receptors (DR4 and DR5) that are able to trigger apoptosis, and two decoy receptors that bind TRAIL but do not trigger apoptotic signaling. Once TRAIL-TRAIL-Rs complexes are formed, the recruitment of the adaptor molecule FADD takes place via its death domain (DD). In turn, FADD allows the recruitment of procaspase 8 and/or 10 forming the death inducing signaling complex (DISC). Importantly, besides the fact that TRAIL can rapidly and selectively induce apoptosis in cancer cells, it was reported that cytokine binding to TRAIL-Rs also activates pro-survival pathways in cancer cells resistant to TRAIL-induced cell death (Erk1/2, p38MAPK, Akt, NF- κ B)²⁸⁶. Contrary to the well-described molecular mechanisms modulating TRAIL-induced cell death, those underlying TRAIL-induced survival remain largely elusive. Notably, the role of a secondary complex comprised of FADD, TRADD, RIPK1, TRAF2, NEMO and the function of c-Flip(p43) in TRAIL-induced survival signaling has been suggested¹¹⁹. In that regard, a number of studies were performed to unravel the role of the secondary complex in TRAIL-induced survival activation in cell lines fully resistant to TRAIL-induced apoptosis¹²². However, the exact composition of the secondary complex, the mechanisms underlying its formation and activation, and whether other not yet identified complexes promote cancer cell survival in response to TRAIL remains unclear.

Despite our limited knowledge regarding the molecular mechanisms of tumor selective action of TRAIL, the basis of TRAIL-induced pro-survival pathways and how generation of resistance takes place, a number of clinical trials have been launched in order to evaluate the potential of pro-apoptotic receptor antagonists (PARAs) in cancer therapy¹⁶⁶. In that regard, different approaches have been tested including recombinant human ligand (rhTRAIL) that targets all TRAIL receptors, agonistic monoclonal antibodies targeting DR4 or DR5 as well as TRAIL receptor specific peptides. Importantly, initially it was considered that death receptors would trigger apoptotic cell death while decoy receptors compete for TRAIL binding with death receptor, thus diminishing the apoptotic response. However, it has been later reported that DcR2 overexpression leads to Akt activation in a ligand-independent manner⁸⁴. Moreover it was also reported that, besides its well characterized apoptotic-inducing function, DR5 can trigger the activation of pro-survival pathways in cancer cell lines resistant to TRAIL-induced apoptosis¹²². Furthermore, recent study shows that endogenously expressed TRAIL leads to DR5-mediated activation of Akt that increases migration and invasion of cancer cells¹¹⁵. Collectively, all these observations suggest that the role of TRAIL receptors in TRAIL-induced signaling is more complex than originally thought. In that regard, it has been shown that even though numerous cancer cell lines express both DR4 and DR5 at the plasma membrane, only one receptor mediates TRAIL-induced apoptosis while the role of the other death receptor in this cellular context remains unclear^{73,328,329}.

Finally, an important feature to highlight is that according to their response to TRAIL, cancer cells can be grouped into three classes; (i) the entire population commits to apoptosis or (ii) is inherently resistant to cell death induced by TRAIL and (iii) only a fraction of the cell population dies while another activates survival pathways (referred to as “fractional killing”¹⁷⁴) and establish a resistant status that reverts to the initial response upon ligand release (referred to as “reversible resistance”). Notably, the role of each individual TRAIL receptor in mediating apoptotic and non-apoptotic responses in populations of cancer cells displaying fractional killing and reversible resistance upon TRAIL challenge remains largely elusive.

Goal. In order to shed light into this matter, in my PhD thesis we aimed to (i) identify the role of individual TRAIL receptors and (ii) characterize the molecular mechanisms orchestrating the activation of TRAIL-induced apoptosis *vs* non-apoptotic signaling in populations of cancer cells undergoing fractional killing and reversible resistance upon TRAIL challenge.

Results. In our project we used stepwise tumorigenesis models in which normal cells are transformed into cancer cells by the introduction of hTERT, SV40 early region, HRasV12²⁷⁷; which allow the comparison between cancer cells and their normal progenitors. We and others²⁷⁹ observed that normal cells from epithelial (HEK) and mesenchymal (BJ) origins are resistant to TRAIL-induced cell death whereas sensitivity to ligand-induced apoptosis is acquired upon transformation. Recently we reported that, following TRAIL challenge, only a fraction of the stepwise cancer cell population (BJELR) dies while another survives, activates pro-survival cascades and proliferates¹⁷³; referred to as fractional killing¹⁷⁶. Based on this observation we hypothesized that TRAIL receptors regulate fractional killing by exerting differential function in the activation of apoptotic or pro-survival cascades upon TRAIL treatment. We have shown that DR4, DR5 and DcR2 are expressed on the cell surface of BJELR cells (Fig 1a) and form heterocomplexes upon TRAIL treatment (Fig 1b). Thus, taking this into account we aimed to identify the role of each of these receptors in the triggering of pro- and anti-apoptotic pathways. By depleting DR5 from BJELR cells we have observed that this death receptor is the main apoptotic mediator in our cellular model (Fig 1c). Contrary, even though DR4 is recruited to the DISC, we revealed that it is neither required for apoptotic signaling nor for the activation of non-apoptotic cascades in BJELR cells (Fig 1c, 2a). Notably, using DR5 knock out BJELR cells we revealed that DR4 binds DcR2 upon TRAIL challenge independently of DR5; however, DR4 lacks the ability to recruit FADD and caspase 8 upon ligand binding (Fig 2b). In that regard, several mutations that block the pro-apoptotic function of DR4 have been described in the DR4 death domain of different cancer cell lines^{299,300}. Importantly, we confirmed that DR4 expressed in BJELR cells does not bear any mutations in the death domain that may explain its inability to form a proficient DISC upon TRAIL challenge (Fig 2c). Instead, we identified a point mutation in DR4 extracellular domain - Glu228Ala (Fig 2b). Although, this mutation has been observed in breast cancer cell lines its biological impact on TRAIL-induced signaling remains unclear²¹⁸. The fact that in our cellular model DR4 is expressed at the plasma membrane and it clusters with DcR2 upon TRAIL challenge suggests that Glu228Ala mutation neither affects shuttling of DR4 to the plasma membrane nor ligand binding or interaction with other TRAIL-receptors located at the plasma membrane. Future experiments aiming to re-express wild type DR4 in BJELR DR5-DR4 double knock out cell lines will help elucidating whether Glu228Ala mutation is indeed responsible for the inability to recruit an efficient DISC upon TRAIL challenge. Overall, our results suggest that DR4 plays neither apoptotic (due to its inability to recruit FADD) nor non-apoptotic function in BJELR cells. Further

studies are required to unravel the molecular mechanisms that prevent DR4 functioning, particularly the impact of Glu228Ala mutation on the structural characteristics of the DISC.

Following our initial observation that DcR2 knock down leads to an increased cell death, we have shown that DcR2 depletion enhances caspase 8 cleavage upon TRAIL treatment. Notably, our preliminary data suggest that DcR2 exerts negative regulation of TRAIL-induced apoptosis by hampering recruitment of other death receptors as well as FADD that in turn decreases caspase 8 and cFlip cleavage (Fig 3a). Furthermore, we found that DcR2 knock out results in the block of Akt activation at early time points of response to TRAIL, while activation of other non-apoptotic pathways is DcR2-independent (Fig 3b-c). Altogether, our results suggest that DcR2 may support fractional survival of cancer cells by regulating TRAIL signaling at two different levels: (i) DR5 and DcR2 association leads to the formation of the less efficient apoptotic DISC; (ii) DcR2 stimulation results in the activation of the early wave of Akt that may support cancer cell survival.

Given that our data indicates that neither DR4 nor DcR2 are essential for the activation of non-apoptotic signaling in our model we hypothesized that, besides its apoptotic role, DR5 also mediates the triggering of pro-survival cascades in an autonomous fashion upon exposure to TRAIL. To our surprise, this hypothesis was validated by different experimental approaches (knocking down DR4/DcR2, knocking out DR5 and using DR5 specific ligand-M1D) demonstrating that a given death receptor – DR5 – can trigger functionally different signaling outcomes such as apoptosis, pro-survival and pro-inflammatory pathways, thus supporting fractional killing and the establishment of reversible resistance in clonal populations of cancer cells (Fig 4a-c). Based on our results, we hypothesized that TRAIL stimulation leads to the formation of distinct DR5-signaling complexes supporting either apoptosis or survival activation. In that regard, our preliminary data indicate that TRAIL may induce formation of two different plasma membrane located DR5-associated complexes: (i) the DISC, which includes TRAIL-Rs, FADD, caspase 8 and Flip (Fig 5a); and (ii) the non-apoptotic signaling platform that consists of DR5, DcR2, RIPK1, TRAF2 and cFlip, but does not contain FADD and caspase 8 (Fig 5b). Interestingly, immunoprecipitation experiments targeting Caspase 8 from the whole cell lysate revealed that RIPK1, TRAF2, FADD as well as TRAIL receptors co-immunoprecipitate with this caspase following TRAIL challenge (Fig 5c). Based on this data we hypothesized that the non-apoptotic signaling complex may undergo internalization which in turn leads to the recruitment of caspase 8 possibly through its interaction with cFlip via DED (death effector domain) of both proteins. On the other hand, during internalization RIPK1/TRAF2/cFlip can potentially dissociate from TRAIL receptors, which in turn may lead to the association of RIPK1/TRAF2/cFlip with FADD. This event can further result in the recruitment of caspase 8 via DED of FADD molecule. Thus, caspase 8 immunoprecipitation leads to the pull down of caspase 8 from two different complexes, leading to the identification of the components that form a DISC on one hand and the non-apoptotic signaling platform/s on the other hand.

Further, we set to evaluate the role of the canonical DISC components (FADD, caspase 8, cFlip) and members on the anti-apoptotic signaling complex (RIPK1 and TRAF2) in the activation of non-apoptotic cascades following exposure to TRAIL. Briefly, we observed that FADD, caspase 8 and RIPK1 are essential for the triggering of I κ B α and p38MAPK phosphorylation. Moreover, Erk1/2 induction requires FADD, cFlip as well as caspase 8. Additionally, cFlip is required for p38MAPK phosphorylation. Together, these results indicate that the activation of these three non-apoptotic cascades upon TRAIL treatment relies on the canonical DISC members as well as RIPK1. Contrary, our data supports that activation of Akt signaling is

independent of both the canonical DISC and secondary complex components suggesting that additional yet unidentified TRAIL-induced signaling platforms are assembled following ligand binding. In those lines, a recent report shows that endogenously expressed TRAIL lead to the DR5-mediated Akt activation independently of DR5 death domain and canonical DISC members ¹¹⁵.

Conclusions. Overall, our results show that (i) DcR2 negatively regulate TRAIL-induced apoptosis due to the formation of the less efficient DISC and by modulating the activation of the early wave of Akt signaling; (ii) DR4 cannot autonomously lead to the apoptotic signaling activation nor participate in the triggering of non-apoptotic pathways; (iii) DR5 can trigger both pro-apoptotic and non-apoptotic pathways activation upon TRAIL treatment in a clonal population of cancer cells; (iv) DR5 stimulation may lead to the formation of different signaling complexes that result in the activation of apoptosis, Erk1/2, Akt, p38MAPK and NF- κ B. Overall, our results suggest that clinical application of recombinant human TRAIL as well as death receptor agonists can be seriously challenged due to the fact that the stimulation of DcR2 and DR5 can lead to the activation of pro-survival pathways upon exposure to this compounds. Pro-survival actions of rhTRAIL and death receptor agonists can result in cancer cells proliferation and migration leading to tumor expansion and progression. Future research is required to further decorticate the molecular mechanisms underlying the triggering of death vs survival through a single death receptor helping to better understand the complexity of TRAIL-induced signaling. These results will provide insights for the evaluation of whether combined therapies targeting the components promoting pro-survival signaling activation could allow the use of rhTRAIL/DR5 agonistic antibodies to promote TRAIL-mediated killing while avoiding TRAIL-induced tumor progression.

Perspectives. Previous reports demonstrated that the formation and functionality of different signaling complexes may be strongly dependent on the spatial localization of its components ²³⁰. For instance, it was reported that subcellular localization of caspase8 determines its role in the triggering of cell differentiation or cell death in T-cells. Aggregation of a small portion of active caspase8 and cFlip within lipid rafts upon TCR stimulation lead to differentiation, whereas a profound Fas-induced caspase 8 activation in cytosolic fraction triggers cell death ²³⁰. Importantly, it was shown that the translocation of death receptors into lipid rafts upon TRAIL treatment leads to the initiation of apoptosis in TRAIL-susceptible NSCLC cells. Contrary, TRAIL-DISC assembly in non-raft phase of the plasma membrane, mediated by RIPK1 and cFlip, leads to the triggering of NF- κ B and Erk1/2 in TRAIL-resistant NSCLC cells ¹²². Our preliminary results show that TRAIL treatment may lead to the formation of two different plasma membrane located DR5-associated complexes. Based on our data and previous reports ¹²² we hypothesize that two scenarios are possible for the localization of TRAIL-induced signaling complexes: either (i) “apoptotic” and “non-apoptotic” complexes are formed in different cells within the population based on cell-to-cell variability that favors pro-death or pro-survival complex formation upon TRAIL treatment or (ii) both complexes are formed within the same cell but in different subcellular compartments. In order to study this several approaches can be used, among them: (i) subcellular protein fractionation followed by the immunoprecipitation of TRAIL-induced signaling complexes from different plasma membrane compartments (raft and non-raft fractions), (ii) laser scanning confocal microscopy in order to unravel the population and subcellular distribution of DR5 co-localization with the known components of the apoptotic DISC, as well as with RIPK1 and TRAF2. These experiments

will allow to shed the light onto the molecular mechanisms that control pro- and anti-apoptotic function of a single death receptor in a clonal population of cancer cells.

Figure 1

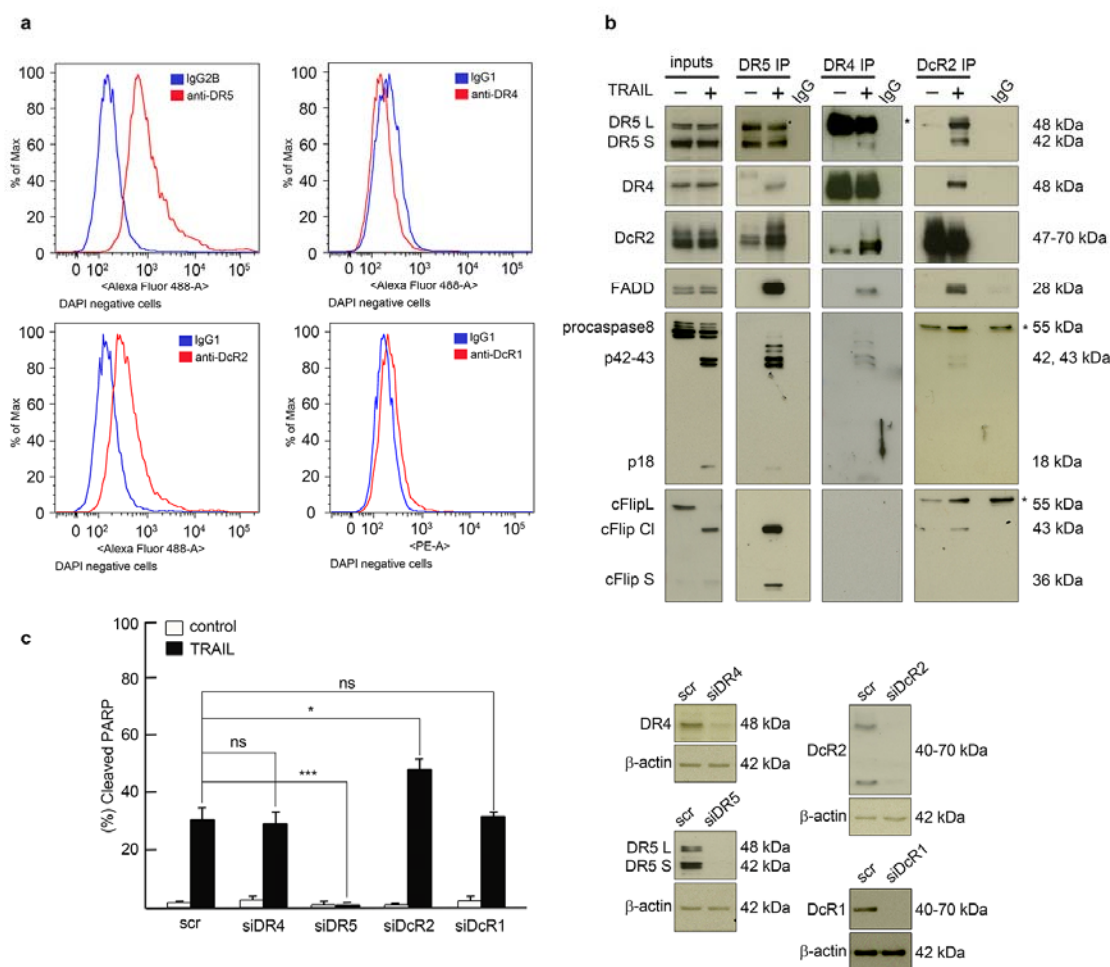


Figure 1. a - Surface levels of TRAIL-Rs in BJELR cells. Surface levels of Death Receptor 5 (“anti-DR5”), Death Receptor 4 (“anti-DR4”), Decoy Receptor 1 (“anti-DcR1”) and Decoy Receptor 2 (“anti-DcR2”) were analyzed by flow cytometry. Isotypic IgG1 or IgG2B labeling was used as a control for background fluorescence. **b** – DISC composition in BJELR cells. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Immunoprecipitation of Death Receptor 5 (DR5-IP), Death Receptor 4 (DR4-IP), and Decoy Receptor 2 (DcR2-IP) was performed and co-immunoprecipitation of DISC components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as a background control. **c** – Impact of TRAIL-Rs knock down on BJELR sensitivity to TRAIL. BJELR cells were transfected either with siRNAs targeting *DR4* (“DR4 KD”), *DR5* (“DR4 KD”), *DcR1* (“DcR1 KD”) or *DcR2* (“DcR2 KD”) mRNA or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection cell populations were either left untreated (control; white bar) or challenged with 1µg/ml TRAIL (TRAIL; black bar) during 1h and the percentage of cells with the positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P* value < 0.0005, ***P* value < 0.005, **P* value < 0.05. Efficiency of DR4, DR5, DcR1 and DcR2 knock down at 48 h post-transfection was analyzed by Western blot. α- Tubulin, loading control.

Figure 2

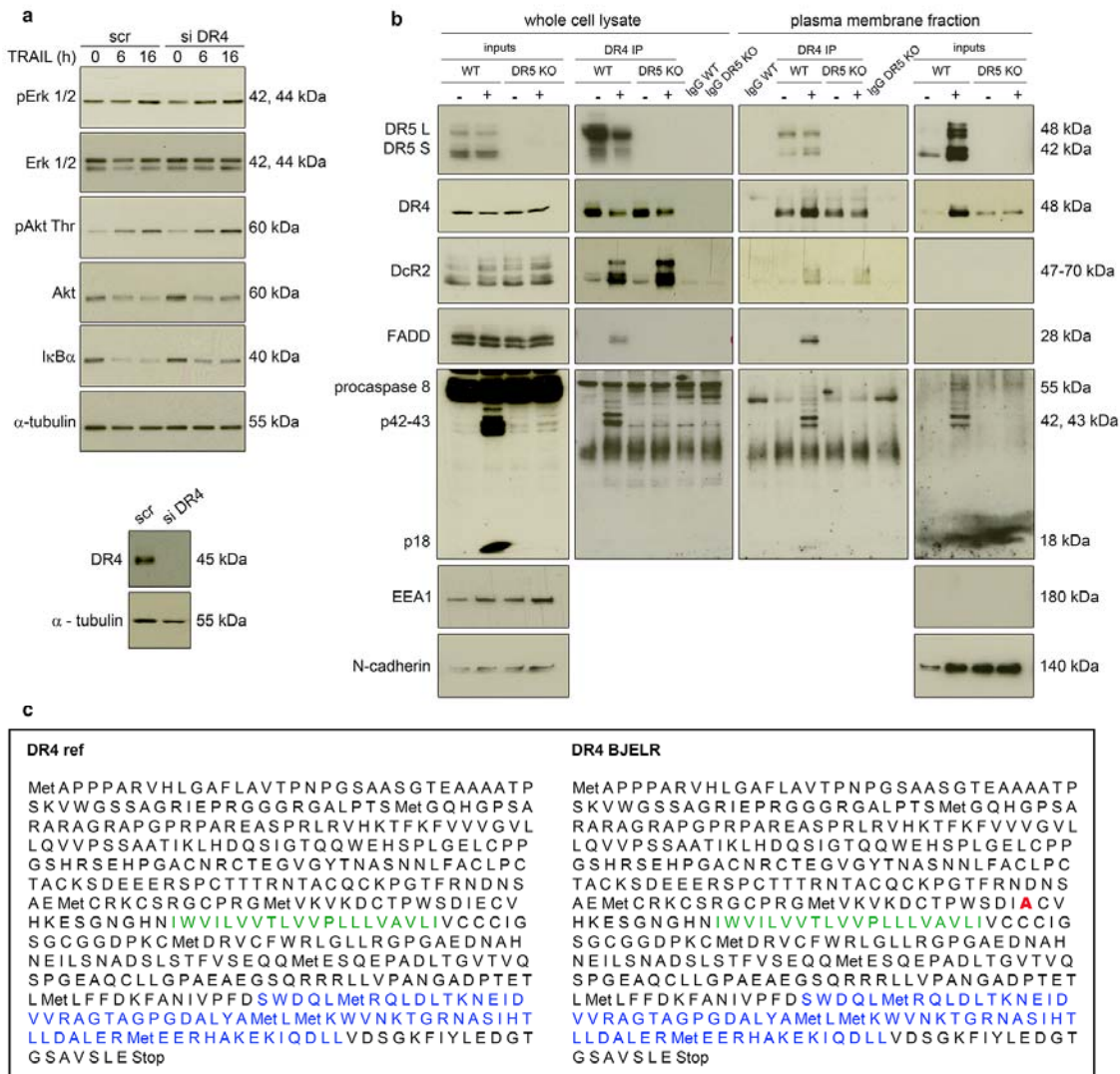


Figure 2. Role of DR4 in the modulation of TRAIL-mediated signaling. **a** – Requirement of DR4 for the activation of non-apoptotic pathways. Total and phosphorylated levels of Erk1/2, Akt and regulator of NF-κB signaling (IκBα in transformed cells surviving TRAIL DR4 were analyzed. BJELR cells were transfected either with siRNAs targeting *DR4* (“si DR4”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1μg/ml) during 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Efficiency of DR4 knock down at 48 h post-transfection was analyzed by Western blot. α-Tubulin, loading control. **b** – DISC composition in wild type and DR5 knock out BJELR cells. Wild type (WT) and DR5 knock out (DR5 KO) BJELR cells were either left untreated (“-”) or challenged with TRAIL (1μg/ml) for 30 min (“+”). Further plasma membrane fraction was extracted from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of Death Receptor 4 (DR4-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of DISC components was analyzed using Western blot approach. Immunoprecipitation using isotopic IgG1 (“IgG1”) was used as background control. **c** – Comparison between amino acid sequence of DR4 expressed in BJELR cells (DR4 BJELR) and reference sequence (DR4 ref). The transmembrane domain is marked in green. The death domain is marked in blue. The mutated amino acid is marked in red.

Figure 3

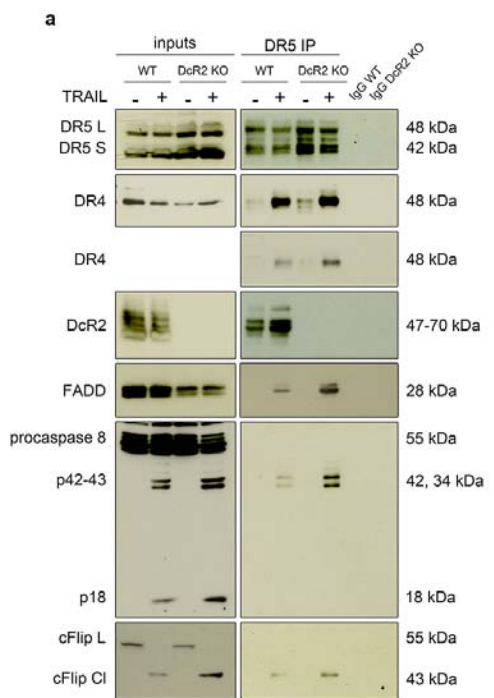


Figure 3. Role of DcR2 in the regulation of TRAIL signaling. **a**- Role of DcR2 in the regulation of DISC composition. BJELR WT (“WT”) or DcR2 knock out (“DcR2 KO”) cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Immunoprecipitation of Death Receptor 5 (DR5-IP) was performed and co-immunoprecipitation of DISC components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1 WT” and “IgG1 DcR2 KO”) was used as background control. **b and c** – Role of DcR2 in the triggering of non-apoptotic signaling. BJELR wild type (“control”), BJELR DcR2 knock out clone 2A (DcR2 KO 2A) (**b**) and BJELR DcR2 knock out clone 4D (DcR2 KO 4D) (**c**) cells were either left untreated (“0” – collected just before the treatment and “0*” – collected 16h after the beginning of exposure to TRAIL in TRAIL treated samples) or treated with TRAIL (1µg/ml) for 1, 2, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.

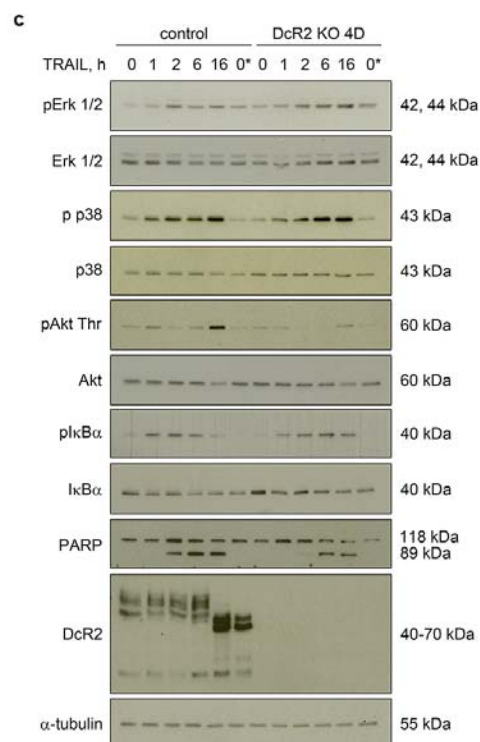
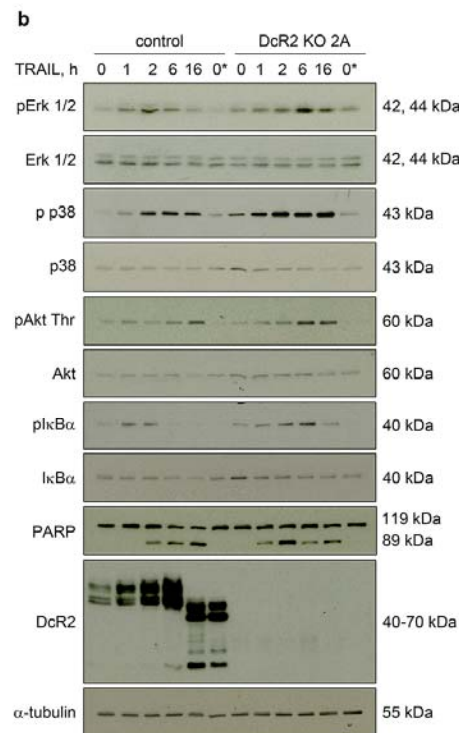


Figure 4

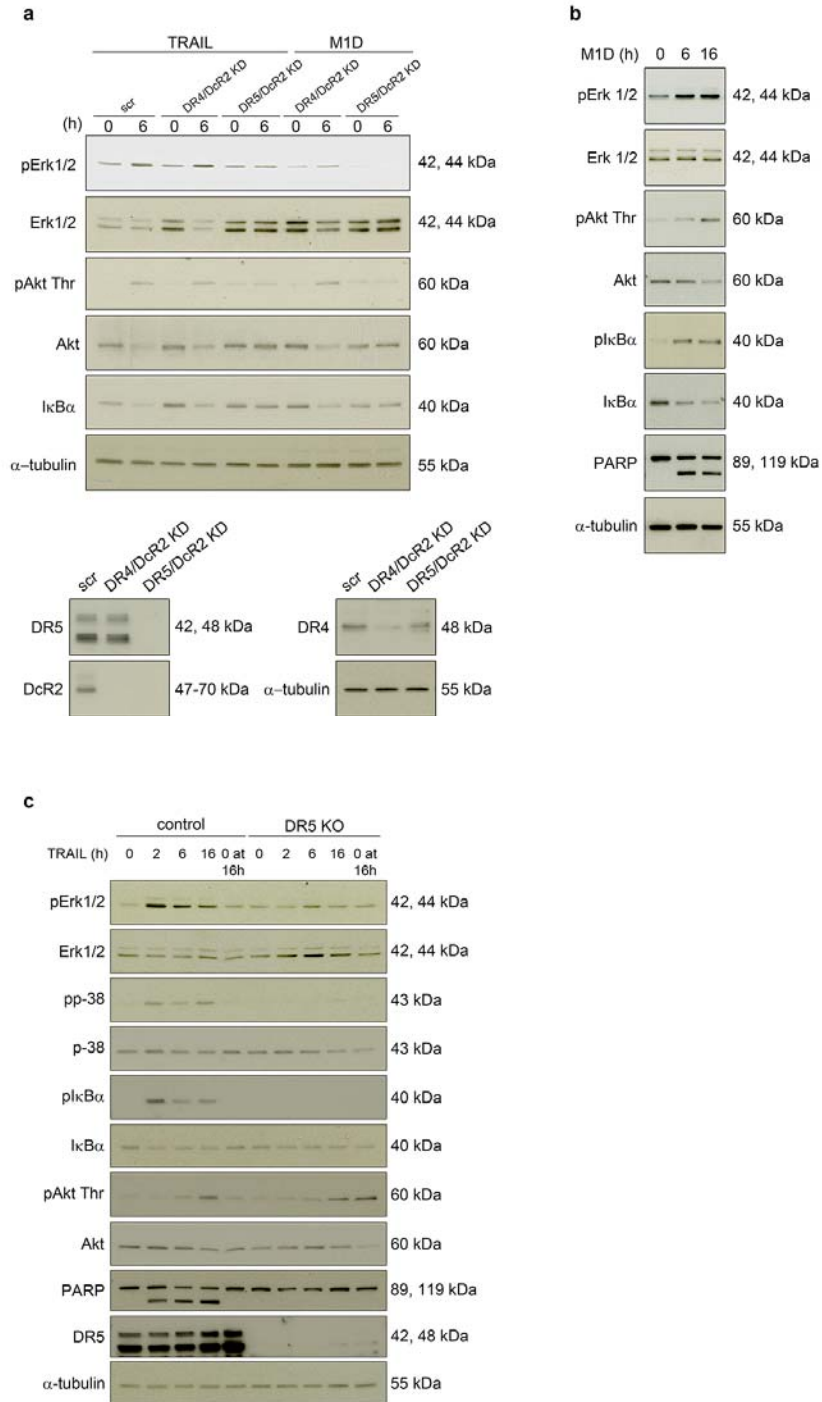


Figure 4. Role of DR5 in the triggering of non-apoptotic pathways upon exposure to TRAIL. **a-** Requirement of TRAIL receptors for the activation of non-apoptotic pathways. Total and phosphorylated levels of Erk1/2, Akt and regulator of NF- κ B signaling (I κ B α) in transformed cells surviving TRAIL or DR5-specific ligand-M1D-treatment upon DR4/DcR2 and DR5/DcR2 double knock down were analyzed. BJELR cells were transfected either with siRNAs targeting *DR4* and *DcR2* (“DR4/DcR2 KD”) or *DR5* and *DcR2* mRNA (“DR5/DcR2 KD”) or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection BJELR cells were either left untreated (“0”) or treated with TRAIL (1 μ g/ml) or M1D (10 μ M) during 6h (“6”). Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308) and I κ B α (Ser32/36) were analyzed. α -Tubulin, loading control. Efficiency of DR4/DcR2, DR5/DcR2 double knock down at 48 h post-transfection was analyzed by Western blot. α -Tubulin, loading control. **b** – Impact of M1D (DR5 specific ligand) treatment on the activation of non-apoptotic pathways. BJELR cells were either left untreated (“0”) or treated with M1D (10 μ M) for 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α -Tubulin, loading control. Image corresponds to one representative experiment out of at least five independent biological replicates. **c-** Impact of DR5 knock out on the phosphorylation level of Erk1/2, Akt, p38MAPK and regulators of NF- κ B signaling (I κ B α). BJELR wild type (“control”) and BJELR DR5 knock out (“DR5 KO”) cells were either left untreated (“0” – collected just before the treatment and “0*” – collected 16h after the beginning of exposure to TRAIL in TRAIL treated samples) or treated with TRAIL (1 μ g/ml) for 2, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α -Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.

Figure 5

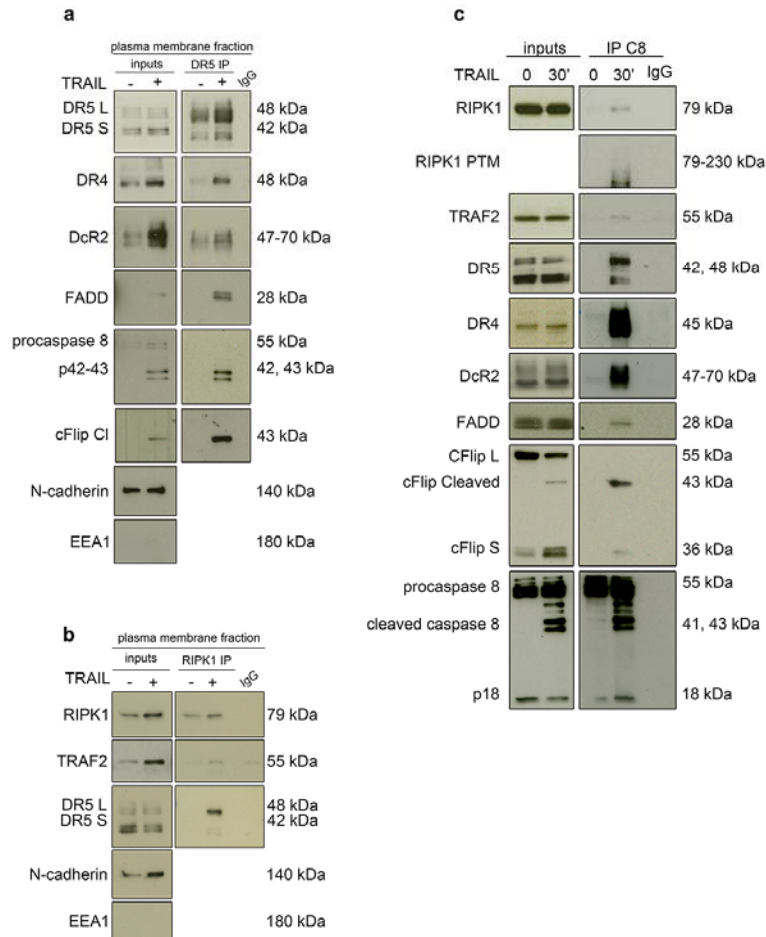


Figure 5. TRAIL induces assembly of the DISC and the non-apoptotic signaling complex at the plasma membrane. a – DISC assembly in BJELR cells takes place at the plasma membrane. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Further plasma membrane fraction was extracted from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of Death Receptor 5 (DR5-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of the DISC components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. **b** – TRAIL-induced non-apoptotic signaling complex is formed at the plasma membrane. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Further plasma membrane fraction was separated from the whole cell lysate using Plasma membrane protein extraction kit (BioVision). Immunoprecipitation of RIPK1 (RIPK1-IP) was performed from the plasma membrane fraction and co-immunoprecipitation of the DISC and secondary complex components was analyzed using Western blot approach. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as a background control. **c**- TRAIL-induced co-immunoprecipitation of caspase 8 with RIPK1 and TRAF2. BJELR cells were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 (“30”) min. Immunoprecipitation of caspase 8 (“c8 IP”) was performed and co-immunoprecipitation of the DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as a background control.

RÉSUMÉ

Introduction. La découverte dans les années 1990 de la cytokine TRAIL (Tumor necrosis factor-Related Apoptosis-Inducing Ligand; TNFSF10) a ouvert de nouvelles possibilités de développement de thérapies anti-cancer ²⁴⁹. Le potentiel de cette protéine réside dans le fait qu'elle peut induire de façon sélective la mort cellulaire programmée, ou apoptose, des cellules humaines cancéreuses, tout en ayant peu d'effet sur les cellules normales. A ce jour quatre récepteurs pouvant se lier spécifiquement à TRAIL ont été identifiés sous la forme d'homotrimères (TRAIL-R). Parmi ces récepteurs, deux sont décrits comme des récepteurs de mort pouvant induire l'apoptose (DR4 et DR5), et deux comme des récepteurs de leurre (DcR1 et DcR2) pouvant se lier à TRAIL sans induire l'apoptose. La trimérisation des récepteurs TRAIL-Rs, en réponse à la liaison de leur ligand, permet la fixation de la protéine FADD (FAS-Associated Death Domain protein) via le domaine de mort (DD pour Death Domain), et le recrutement de la procaspase 8 et / ou 10 pour former un complexe appelé DISC (Death Inducing Signaling Complex). Toutefois il a été reporté que l'activité du complexe TRAIL-TRAIL-R peut aussi stimuler des voies de signalisation de survie cellulaire (Erk1/2, p38MAPK, Akt, NF-κB) ²⁸⁶, conférant une résistance aux cellules cancéreuses. Le mécanisme moléculaire en jeu dans cette voie de signalisation est encore peu connu, mais il semble impliquer un complexe dit secondaire, formé par les protéines FADD, RIPK1, TRAF2, NEMO et cFlip (p43) ¹¹⁹. Malgré de nombreuses études visant à comprendre le rôle de ce complexe anti-apoptotique ¹²², sa composition exacte, les mécanismes précis de sa formation / initiation, ou encore l'identification d'autres complexes impliqués dans la survie des cellules cancéreuses en réponse à TRAIL sont encore peu connus.

Des essais cliniques visant à évaluer le potentiel pro-apoptotique de molécules antagonistes à TRAIL (PARAs) sont aujourd'hui en cours de réalisation, et cela malgré un manque de connaissances approfondies sur les mécanismes moléculaires de la sélectivité tumorale, et de l'induction de la survie cellulaire ¹⁶⁶. Des études initiales ont suggéré que l'activation des récepteurs de mort par TRAIL stimule la mort cellulaire par apoptose, alors que les récepteurs de leurre réduisent l'ampleur de la réponse apoptotique en séquestrant le ligand TRAIL. Des recherches plus récentes nuancent toutefois cette hypothèse en montrant que la surexpression du récepteur de leurre DcR2 conduit à l'activation de la survie cellulaire via Akt, et indépendamment de la présence du ligand TRAIL ⁸⁴. De même le récepteur de mort DR5 peut induire la survie cellulaire de manière TRAIL-dépendante ^{122,115}. Ainsi la transduction du signal TRAIL via les récepteurs TRAIL-Rs se révèle beaucoup plus complexe qu'une compétition ligand-récepteur. Un autre fait marquant est que, dans de nombreuses lignées cellulaires cancéreuses, bien que les deux récepteurs de mort DR4 et DR5 soient exprimés au niveau de la membrane plasmique, seul DR5 induit l'apoptose en présence de TRAIL ^{73,328,329}.

La ou les voies de signalisation activées en réponse à TRAIL permettent d'établir trois catégories de cellules cancéreuses : (i) dans la première catégorie la totalité de la population cellulaire s'engage dans la voie apoptotique, (ii) dans la deuxième catégorie la totalité de la population cellulaire est résistante à la mort cellulaire par apoptose, (iii) et dans la troisième catégorie, seule une fraction de la population cellulaire meurt en réponse à TRAIL, alors que l'autre partie active la survie cellulaire ¹⁸³, on parle de

mort cellulaire fractionnée¹⁷⁴. La résistance des cellules cancéreuses à TRAIL est réversible. En effet lorsque la fraction cellulaire résistante est réexposée à TRAIL, le phénomène de mort fractionnée est à nouveau observé.

Objectifs de la thèse. (i) Déterminer le rôle de chacun des récepteurs TRAIL dans l'activation des voies de signalisation apoptotiques et non-apoptotiques, et (ii) caractériser les mécanismes moléculaires qui en découlent en utilisant des lignées de cellules cancéreuses qui induisent une mort cellulaire fractionnée en réponse à TRAIL.

Résultats. Pour ce projet nous avons utilisé comme modèle de tumorigenèse des cellules normales transformées en cellules cancéreuses en plusieurs étapes définies par l'introduction des éléments génétiques suivants : hTERT, SV40 ER (Early Region) et H-RAS-V12²⁷⁷. Ce modèle permet de comparer les cellules cancéreuses avec les cellules normales parentales. Nous, et d'autres²⁷⁹, avons observé que des cellules normales d'origine épithéliale (HEK) et mésenchymateuse (BJ), au départ toutes résistantes à la mort cellulaire induite par TRAIL, deviennent en partie sensibles à ce ligand lors de la transformation cancéreuse¹⁷³. On parle alors d'un phénotype de mort fractionnée des cellules BJELR, induit par le traitement TRAIL. Basé sur la littérature et ces observations, nous avons émis l'hypothèse que les récepteurs TRAIL régule la mort cellulaire fractionnée en exerçant deux fonctions distinctes : l'activation de l'apoptose d'une part, l'induction de la survie cellulaire d'autre part, en réponse à TRAIL. Tout d'abord nous avons confirmé que les récepteurs DR4, DR5 et DcR2 sont bien exprimés à la surface des cellules BJELR, formant des hétérocomplexes en présence de TRAIL (Fig 1a). Le récepteur DcR1 n'étant pas exprimé à la surface des cellules BJELR, nous n'avons pas poursuivi sa caractérisation. Afin d'identifier le rôle des récepteurs DR4, DR5, DcR2 et de leurs hétérocomplexes (Fig 1b), nous avons utilisé une stratégie de knock out (KO) / knock down (KD). Ainsi nous avons montré que le récepteur DR5 est le principal médiateur de la réponse apoptotique dans notre modèle cellulaire (Fig 1c). Le récepteur DR4, bien que normalement recruté au niveau du complexe DISC avec DR5, n'est pas nécessaire à l'induction de l'apoptose, ni à l'activation des voies de signalisation non-apoptotiques dans les cellules BJELR (Fig 1c, 2a). Dans les cellules KO pour DR5, DR4 forme un complexe avec DcR2 en présence de TRAIL (Fig 2b). Cependant, dans ce contexte, DR4 perd sa capacité à recruter la protéine FADD et la caspase 8, bloquant la formation d'un complexe DISC fonctionnel. Dans différentes lignées cellulaires cancéreuses, des mutations inhibant la fonction pro-apoptotique de DR4 ont déjà été mises en évidence au niveau du domaine DD du récepteur^{299,300}. Nous avons donc analysé la séquence du récepteur DR4 dans les cellules BJELR et n'avons détecté aucune mutation dans le domaine DD (Fig 2c). Nous avons en revanche identifié une mutation ponctuelle dans le domaine extracellulaire de DR4 (Glu228Ala) (Fig 2c). Bien que cette mutation ait été observée dans des lignées cellulaires issues de cancers du sein, son potentiel impact biologique sur la signalisation TRAIL-dépendante reste à clarifier²¹⁸. D'après nos précédents résultats, nous savons que la mutation Glu228Ala n'interfère pas avec la localisation de DR4 à la membrane plasmique, n'inhibe pas la formation de la liaison ligand-récepteur ou les interactions avec les autres récepteurs de TRAIL. Afin de déterminer si cette mutation impacte la formation d'un complexe DISC fonctionnel après un traitement avec TRAIL, nous proposons d'exprimer une copie sauvage de DR4 dans les cellules BJELR double KO pour DR4 et

DR5. De plus nos résultats suggèrent que DR4, dans les cellules BJELR, ne joue ni un rôle pro-apoptotique (dû à son incapacité à recruter FADD), ni un rôle anti-apoptotique. L'élucidation du mécanisme moléculaire demande une étude approfondie des conséquences de la mutation Glu228Ala sur les caractéristiques structurales du DISC.

Dans la poursuite de notre projet à comprendre le rôle des récepteurs TRAIL lors de la mort cellulaire fractionnée, nous avons généré des cellules KD pour DcR2, et avons observé une augmentation de la mort cellulaire. De plus la déplétion du récepteur DcR2 augmente le clivage de la caspase 8 en présence de TRAIL. Nos données préliminaires suggèrent en effet que DcR2 régule négativement l'apoptose induite par TRAIL en affectant le recrutement d'autres récepteurs de mort, et de FADD (Fig 3a). Ainsi il en découle une réduction du clivage de la caspase 8 et de cFlip. Enfin nous avons montré que le KO DcR2 inhibe la première vague d'activation de la voie Akt, alors que l'activation des autres voies non-apoptotiques est indépendante de DcR2 (Fig 3b,c). Ainsi nos résultats suggèrent que DcR2 est impliqué dans le phénotype de mort fractionnée des cellules cancéreuses en régulant la voie de signalisation TRAIL à deux niveaux : (i) l'association entre DR5 et DcR2 induit la formation d'un complexe DISC peu actif. (ii) L'activation de DcR2 via TRAIL active Akt et induit la survie des cellules cancéreuses.

Nos résultats suggèrent que ni DR4, ni DcR2, ne sont essentiels pour l'activation de la signalisation non-apoptotique dans notre modèle cellulaire. De plus nous avons démontré par plusieurs approches expérimentales (double KD DR4/DcR2, KO DR5, utilisation du ligand M1D spécifique de DR5) que le récepteur DR5, en plus de son rôle apoptotique, pouvait induire la survie cellulaire en réponse à TRAIL (Fig 4a-c). Ainsi un récepteur de mort tel que DR5 peut activer des voies de signalisation fonctionnellement distinctes telles l'apoptose, la survie cellulaire et la voie pro-inflammatoire. Cet aspect multifonctionnel de DR5 est en cohérence avec le phénotype de mort cellulaire fractionnée, et l'établissement d'une résistance réversible dans des populations clonales de cellules cancéreuses. On pourrait donc imaginer que la cytokine TRAIL stimule la formation de complexes de signalisation incluant DR5, mais de composition et fonctions distinctes. Dans cette direction, nous avons obtenu des données préliminaires montrant que TRAIL peut induire la formation de deux complexes associant DR5 et localisés au niveau de la membrane plasmique : (i) le DISC qui inclut TRAIL-Rs, FADD, caspase 8 et cFlip (Fig 5a) ; et (ii) un complexe de signalisation non-apoptotique composé de DR5, DcR2, RIPK1, TRAF2 et cFlip, mais qui ne contient ni FADD, ni la caspase 8 (Fig 5b). De plus nous avons montré par co-immunoprécipitation, à partir de lysats de cellules, que la caspase 8 interagit avec RIPK1, TRAF2, FADD et TRAIL-Rs en présence de TRAIL (Fig 5c). Ainsi nous proposons que le complexe de signalisation non-apoptotique est internalisé en présence de TRAIL, conduisant au recrutement de la caspase 8 via son interaction avec cFlip, probablement par l'intermédiaire de leurs domaines DED (death effector domain) respectifs. Toutefois il est possible que lors de l'internalisation du complexe RIPK1/TRAF2/cFlip, celui-ci se dissocie des récepteurs de TRAIL, engendrant la formation d'un complexe RIPK1/TRAF2/cFlip/FADD. La caspase 8 pourrait ensuite être recrutée directement par la protéine FADD. Pour résumer, les expériences ont mis en évidence l'existence de deux complexes distincts : le DISC, et un complexe de signalisation non-apoptotique.

Nous avons ensuite étudié le rôle des composants canoniques du DISC (FADD, caspase 8, cFlip), et celui des membres du complexe de signalisation non-apoptotique (RIPK1 et TRAF2) lors de l'activation des cascades non-apoptotiques par TRAIL. En bref nous avons observé que FADD, la caspase 8, et cFlip, sont essentiels pour la phosphorylation de I κ B α and p38MAPK, deux protéines impliquées dans la signalisation de voies non-apoptotiques. De plus l'activation des kinases Erk1/2 nécessitent l'activité des protéines FADD et caspase 8. Pour conclure l'activation de ces trois cascades de signalisation non-apoptotiques requiert la formation d'un complexe DISC canonique, ainsi qu'un complexe DISC recrutant RIPK1. D'autres complexes, indépendants du DISC et des protéines du complexe secondaire, pouvant activer des voies non-apoptotiques en réponse à TRAIL, restent encore à identifier. En effet dans notre modèle l'activation de la voie Akt en réponse à TRAIL est indépendante du DISC et des protéines du complexe secondaire. En complément, l'expression endogène de TRAIL dans l'étude de von Karstedt montre que l'activation de Akt ne nécessite pas un récepteur DR5 avec un domaine DD fonctionnel, ni l'activité des composants canoniques du DISC ¹¹⁵.

Conclusions. Nos résultats ont permis de mettre en évidence que (i) le récepteur DcR2 régule négativement la voie apoptotique activée par TRAIL, d'une part par la formation d'un DISC peu actif, et d'autre part en modulant potentiellement la première vague d'activation de la voie de signalisation Akt ; (ii) le récepteur DR4 seul, en présence de TRAIL, ne peut pas activer les voies de signalisation apoptotiques et non-apoptotiques ; (iii) le récepteur DR5 est impliqué dans l'activation de voies de signalisation apoptotiques et non-apoptotiques ; (iv) le récepteur DR5 pourrait être impliqué dans la formation de différents complexes de signalisation qui activent les voies de l'apoptose, Erk1/2, Akt, p38MAPK, et NF- κ B.

L'activation de la voie Akt par la formation d'hétérocomplexes entre DcR2 et les récepteurs de mort DR4 et DR5, même en absence de ligand, ainsi que le rôle de DR5 dans l'activation de voies pro et anti-apoptotiques, suggèrent que la stratégie visant à activer les récepteurs de mort par des anticorps agonistes peut en fait induire l'activation de la survie cellulaire, et donc promouvoir la progression tumorale. Ainsi il est important de continuer à décortiquer les mécanismes moléculaires pour comprendre comment l'activation d'un même récepteur peut induire soit la mort cellulaire, soit la survie dans une lignée cellulaire clonale. Ces recherches permettront d'évaluer plus efficacement des thérapies combinées visant spécifiquement à inhiber les voies de survie cellulaire, et à activer l'apoptose de cellules cancéreuses (anticorps agonistes rhTRAIL/DR5).

Perspectives. De nombreuses études ont montré que la formation et la fonction de différents complexes de signalisation dépendent de la localisation de ses composants dans la cellule ²³⁰. Par exemple la concentration de caspase 8 et c-Flip au sein de rafts lipidiques de la membrane plasmique, activée par le complexe TCR (T-cell receptor), induit la différenciation des lymphocytes T, alors que l'activation cytosolique de la caspase 8 par FAS induit la mort cellulaire ²³⁰. La translocation des récepteurs de mort au sein de rafts lipidiques en réponse à TRAIL active l'apoptose dans les cellules NSCLC sensibilisées à TRAIL, alors que l'assemblage du TRAIL-DISC en dehors des rafts lipidiques par RIPK1 conduit à l'activation des voies NF- κ B et Erk1/2 dans des cellules NSCLC résistantes à TRAIL ¹²². Nos données

préliminaires montrent que TRAIL peut conduire à la formation de deux complexes associant DR5, localisés à la membrane plasmique. A la lumière de toutes ces données, nous proposons deux scénarios possibles pour expliquer l'activation de deux voies de signalisation "opposées" par le DISC en réponse à TRAIL dans une lignée de cellules clonales : (i) la variabilité de cellule à cellule va favoriser dans une cellule donnée, soit la formation de DISC pro-apoptique, soit la formation de DISC pro-survie après traitement par TRAIL, ou (ii) les deux complexes sont formés dans une même cellule mais dans des compartiments subcellulaires distincts. Afin d'étudier ces possibilités nous avons initié plusieurs approches : (i) le fractionnement subcellulaire (incluant les fractions contenant ou non les rafts lipidiques de la membrane plasmique) suivi par une immunoprécipitation des complexes activés par TRAIL, (ii) l'utilisation de la microscopie confocale à balayage pour analyser la distribution du récepteur DR5 au sein de la membrane plasmique et sa co-localisation avec des protéines du DISC apoptotique, ainsi que les protéines RIPK1 et TRAF2. Grâce à ces approches nous aurons une meilleure compréhension des mécanismes moléculaires contrôlant les fonctions pro et anti-apoptotiques assurées par un seul récepteur de mort dans une population clonale de cellules cancéreuses.

Figure 1

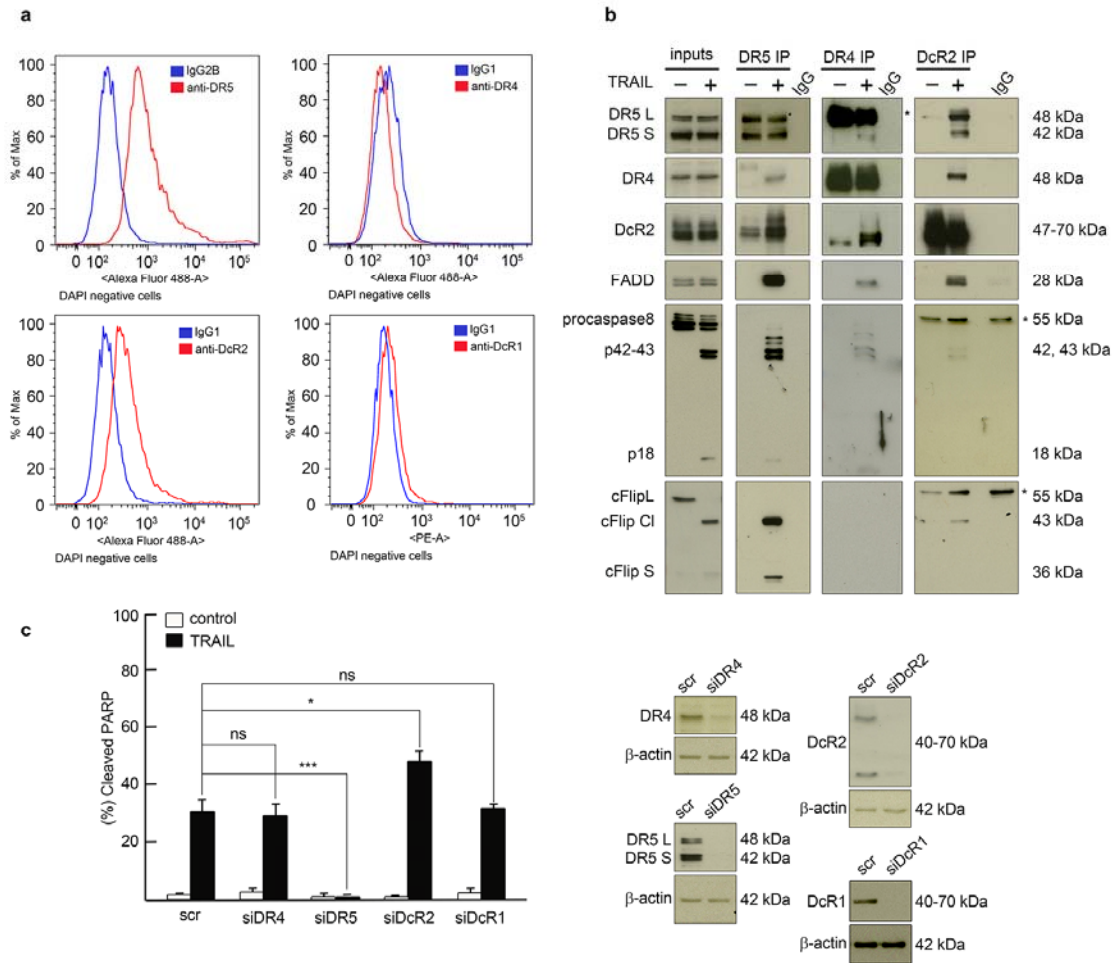


Figure 1. a – Quantification des récepteurs de TRAIL (TRAIL-Rs) à la surface des cellules BJELR. La quantification du récepteur de mort 5 (“anti-DR5”), du récepteur de mort 4 (“anti-DR4”), et des récepteurs de leurre 1 (“anti-DcR1”) et 2 (“anti-DcR2”) à la surface des cellules a été analysée par cytométrie en flux. Les marquages isotypiques par IgG1 ou IgG2B ont été utilisés comme contrôles pour différencier le signal fluorescent positif du bruit de fond. **b** – Composition du DISC dans les cellules BJELR. Les cellules BJELR ont été non-traitées (“-”) ou traitées avec TRAIL (1µg/ml) pendant 30 min (“+”). La co-immunoprécipitation des composés du DISC a été réalisée suite aux immunoprécipitations de DR5 (“DR5-IP”), DR4 (“DR4-IP”), et DcR2 (“DcR2-IP”), et analysée par Western blot. L’immunoprécipitation de IgG1 (“IgG1”) a été utilisée pour contrôler le bruit de fond expérimental. **c** – Impact de la réduction des TRAIL-Rs par knock down (KD) sur la sensibilité des cellules BJELR à TRAIL. Les cellules BJ et BJELR ont été transfectées soit avec des siRNAs ciblant les ARNm *DR4* (“DR4 KD”), *DR5* (“DR5 KD”), *DcR1* (“DcR1 KD”) et *DcR2* (“DcR2 KD”), soit avec un mélange de siRNAs sans cibles (“control”). 48h après la transfection les populations de cellules ont été soit traitées avec TRAIL (1µg/ml) (TRAIL ; barre noire) pendant 1h, soit non traitées durant le même laps de temps (contrôle ; barre blanche). Les cellules positives pour le clivage de PARP ont été analysées par cytométrie en flux. Les histogrammes représentent la moyenne + /- la déviation standard (SD) d’au moins trois réplicas biologiques indépendants. La signification statistique a été calculée en utilisant le test t de Student non-apparié et bilatéral, ****P value* < 0.0005, ** *P value* < 0.005, * *P value* < 0.05. La réduction des niveaux protéiques résultant des KD pour DR4, DR5, DcR1 et DcR2 a été contrôlée par Western blot 48h après la transfection. α-Tubuline, contrôle de chargement.

Figure 2

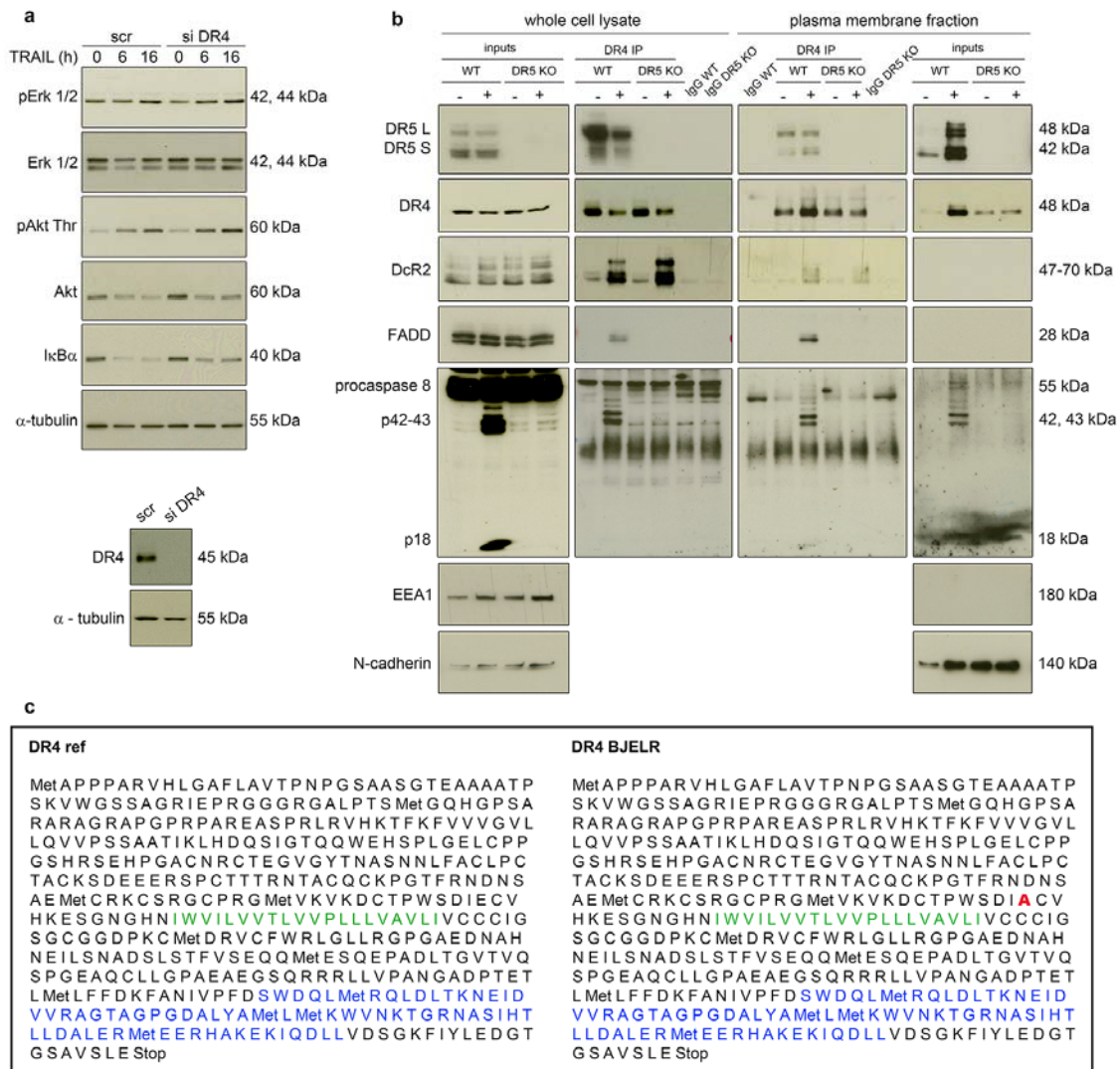


Figure 2. a – Rôle de DR4 dans l’activation des voies non-apoptotiques. Les niveaux totaux et phosphorylés des protéines Erk1/2, Akt et IκBα (régulateur de la voie NF-κB) ont été analysés dans des cellules KD pour DR4, transformées, et résistantes à TRAIL. Les cellules BJELR ont été transfectées soit par des siRNAs adressés contre DR4 (“si DR4”), soit par des siRNAs contrôles sans cibles (“scr”). 48h après la transfection les cellules BJELR ont été soit traitées avec TRAIL (1µg/ml) pendant 6h (“6”), soit non traitées (“0”). Les cellules apoptotiques ont été éliminées par des lavages successifs, et les cellules non-apoptotiques attachées au support ont été collectées pour des analyses par Western blot. Les niveaux totaux et phosphorylés des protéines Erk1/2 (Thr202/Tyr204), Akt (Thr308), et IκBα (Ser32/36) ont été ainsi analysés. α-Tubuline, contrôle de chargement. **b** – Composition du DISC dans les cellules BJELR sauvage et knock out (KO) pour DR5. Les cellules BJELR sauvages et KO pour DR5 ont été non-traitées (“-”) ou traitées avec TRAIL (1µg/ml) pendant 30 min (“+”). La fraction contenant la membrane plasmique a été isolée du lysat cellulaire total en utilisant le kit d’extraction Plasma membrane protein extraction kit (Bio Vision). L’immunoprécipitation de DR4 (“DR4-IP”) a été réalisée à partir de la fraction contenant les protéines de la membrane plasmique. La co-immunoprécipitation des composés du DISC a été analysée par Western blot. L’immunoprécipitation de IgG1 (“IgG1”) a été utilisée pour contrôler le bruit de fond expérimental. **c** – Comparaison de la séquence protéique de DR4 exprimée dans les cellules BJELR (“DR4 BJELR”) à sa séquence de référence (“DR4 ref”). Le domaine transmembranaire est indiqué en vert. Le domaine de mort est signalé en bleu. L’acide aminé muté est marqué en rouge.

Figure 3

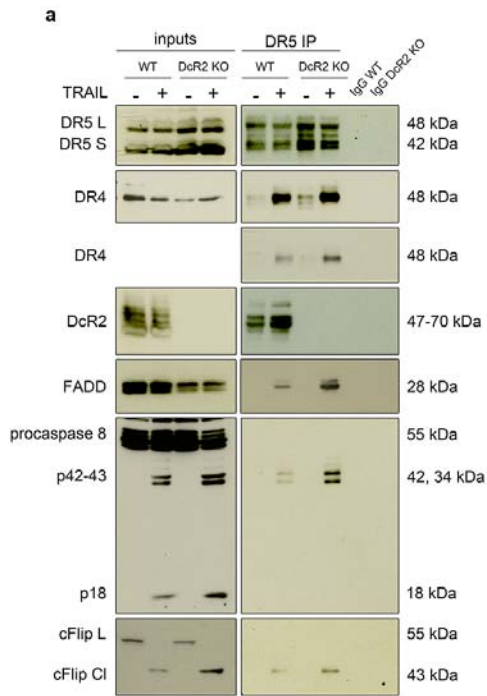


Figure 3. a – Rôle du récepteur DcR2 dans la régulation de la composition du DISC. Les cellules BJELR sauvages (“WT”) ou KO pour DcR2 (“DcR2 KO”) ont été non-traitées (“-”) ou traitées avec TRAIL (1 µg/ml) pendant 30 min (“+”). La co-immunoprécipitation des composés du DISC par l’intermédiaire de DR5 (“DR5-IP”) a été analysée par Western blot. L’immunoprécipitation de IgG1 (“IgG1”) a été utilisée pour contrôler le bruit de fond expérimental. **b and c** – Rôle du récepteur DcR2 dans l’induction de la signalisation non-apoptotique. Les cellules BKELR sauvages, les cellules KO pour DcR2 issues du clone 2A (**b**) et issues du clone 4D (**c**) ont été soit non-traitées (“0” – cellules collectées à temps T=0 ; “0*” – cellules collectées à temps T=16h), soit traitées avec TRAIL (1 µg/ml) pendant 1h (“1”), 2h (“2”), 6h (“6”) et 16h (“16”). Les cellules apoptotiques ont été éliminées par des lavages successifs, et les cellules non-apoptotiques attachées au support ont été collectées pour des analyses par Western blot. Les niveaux totaux et phosphorylés des protéines Erk1/2 (Thr202/Tyr204), Akt (Ser473), Akt (Thr308), p38MAPK (Thr180/Tyr182), et IκBα (Ser32/36) ont été ainsi analysés. a- Tubuline, contrôle de chargement. Ici la figure correspond à une expérience représentative de trois répliques biologiques indépendants.

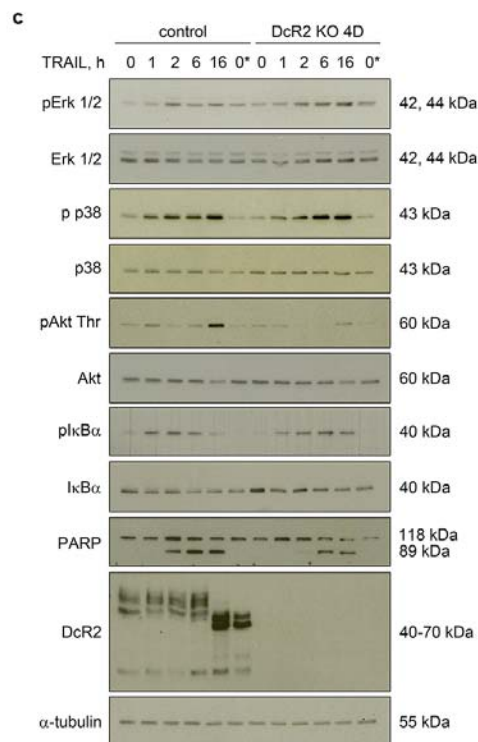
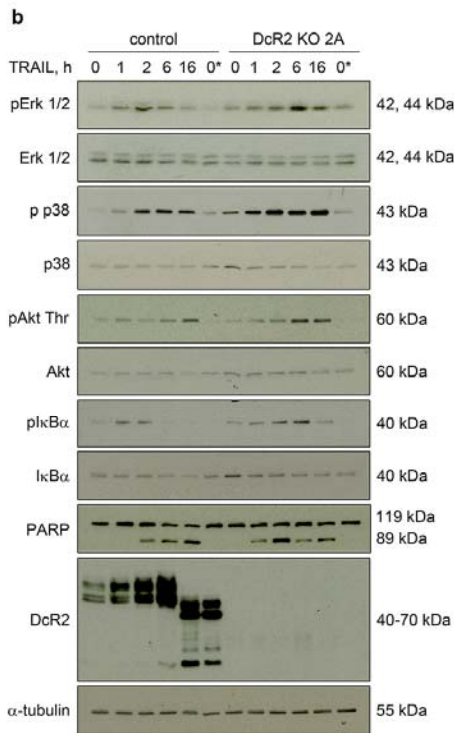


Figure 4

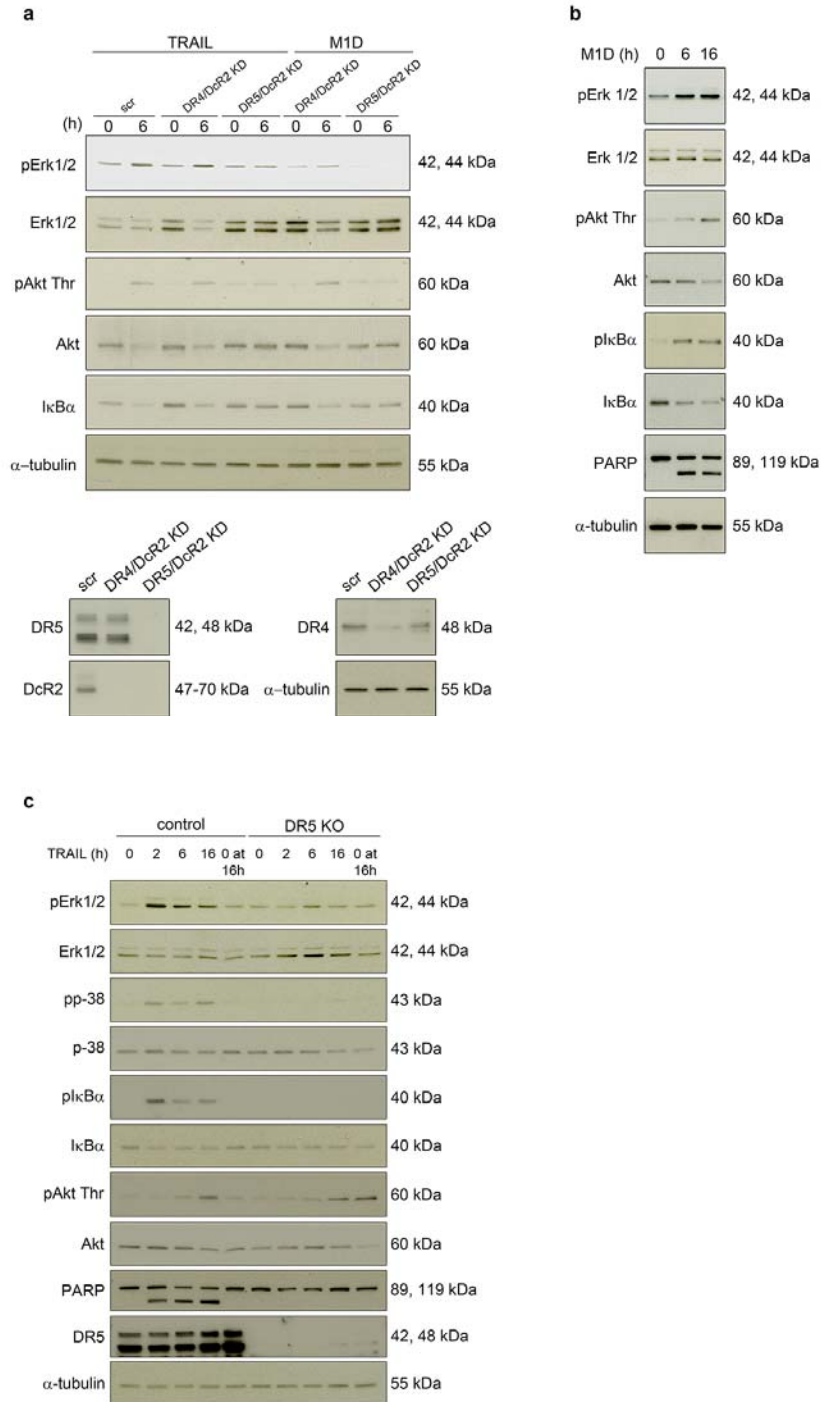


Figure 4. a – Rôle des récepteurs de TRAIL pour l'activation des voies de signalisation non-apoptotiques. Les niveaux totaux et phosphorylés des protéines Erk1/2, Akt et I κ B α (régulateur de la voie NF- κ B), ont été analysés dans des cellules transformées double KD DR4/DcR2, DR5/DcR2, et résistantes au traitement par TRAIL ou par le ligand M1D, qui se lie spécifiquement à DR5. Les cellules BJELR ont été transfectées soit avec des siRNAs dirigés contre les ARNm *DR4* et *DcR2* ("DR4/DcR2 KD"), soit contre les ARNm *DR5* et *DcR2* ("DR5/DcR2 KD"), soit avec un mélange de siRNAs sans cible ("control"). 48h après la transfection, les cellules BJELR ont été soit traitées avec TRAIL (1 μ g/ml) ou M1D (10nM) pendant 6h ("6"), soit non traitées ("0"). Les cellules apoptotiques ont été éliminées par des lavages successifs, et les cellules non-apoptotiques attachées au support ont été collectées pour des analyses par Western blot. Les niveaux totaux et phosphorylés des protéines Erk1/2 (Thr202/Tyr204), Akt (Thr308), et I κ B α (Ser32/36) ont été ainsi analysés. α - Tubuline, contrôle de chargement. La réduction des niveaux protéiques résultant des doubles KD DR4/DcR2 et DR5/DcR2 a été contrôlée par Western blot 48h après la transfection. a- Tubuline, contrôle de chargement. **b** – Impact du traitement par M1D (ligand spécifique de DR5) sur l'activation des voies non-apoptotiques. Les cellules BJELR ont été traitées avec M1D (10 μ M) pendant 6h ("6") ou 16h ("16"), ou non traitées ("0"). Les cellules apoptotiques ont été éliminées par des lavages successifs, et les cellules non-apoptotiques attachées au support ont été collectées pour des analyses par Western blot. Les niveaux totaux et phosphorylés des protéines Erk1/2 (Thr202/Tyr204), Akt (Ser473), Akt (Thr308), p38MAPK (Thr180/Tyr182), et I κ B α (Ser32/36) ont été ainsi analysés. a- Tubuline, contrôle de chargement. Ici la figure correspond à une expérience représentative d'au moins cinq répliques biologiques indépendants. **c** – Impact de la perte de fonction de DR5 sur les niveaux de phosphorylation de Erk1/2, Akt, p38MAPK et I κ B α (régulateur de la voie NF- κ B). Des cellules BJELR sauvages ou KO pour DR5 ont été soit traitées avec TRAIL (1 μ g/ml) pendant 2h ("2"), 6h ("6"), ou 16h ("16"), soit non traitées ("0" – cellules collectées à temps T=0 ; "0*" – cellules collectées à temps T=16h). Les cellules apoptotiques ont été éliminées par des lavages successifs, et les cellules non-apoptotiques attachées au support ont été collectées pour des analyses par Western blot. Les niveaux totaux et phosphorylés des protéines Erk1/2 (Thr202/Tyr204), Akt (Ser473), Akt (Thr308), p38MAPK (Thr180/Tyr182), et I κ B α (Ser32/36) ont été ainsi analysés. α - Tubuline, contrôle de chargement. Ici la figure correspond à une expérience représentative de trois répliques biologiques indépendants.

Figure 5

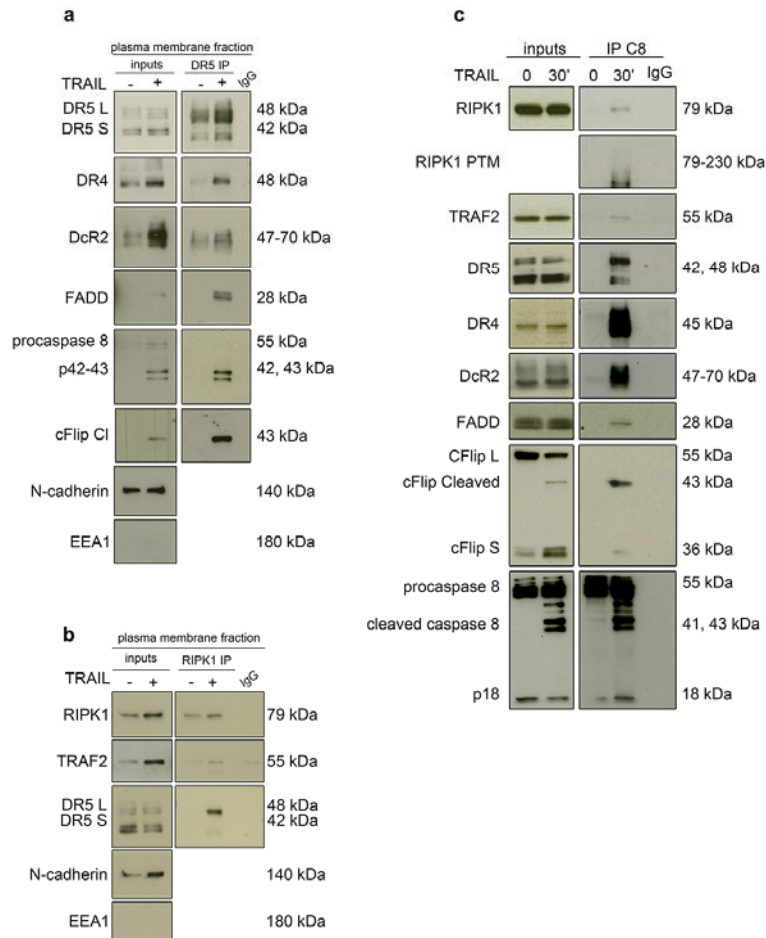


Figure 5. a – La formation du DISC est localisée à la membrane plasmique dans les cellules BJELR. Les cellules BJELR ont été non-traitées (“-“) ou traitées avec TRAIL (1µg/ml) pendant 30 min (“+“). La fraction contenant la membrane plasmique a été isolée du lysat cellulaire total en utilisant le kit d’extraction Plasma membrane protein extraction kit (Bio Vision). L’immunoprécipitation de DR5 (“DR5-IP“) a été réalisée à partir de la fraction contenant les protéines de la membrane plasmique. La co-immunoprécipitation des composés du DISC a été analysée par Western blot. L’immunoprécipitation de IgG1 (“IgG1“) a été utilisée pour contrôler le bruit de fond expérimental. **b** – La formation du complexe non-apoptotique induit par TRAIL est localisée à la membrane plasmique. Les cellules BJELR ont été non-traitées (“-“) ou traitées avec TRAIL (1µg/ml) pendant 30 min (“+“). La fraction contenant la membrane plasmique a été isolée du lysat cellulaire total en utilisant le kit d’extraction Bio Vision : Plasma membrane protein extraction kit. L’immunoprécipitation de RIPK1 (“RIPK1-IP“) a été réalisée à partir de la fraction contenant les protéines de la membrane plasmique. La co-immunoprécipitation des composés du DISC et du complexe secondaire a été analysée par Western blot. L’immunoprécipitation de IgG1 (“IgG1“) a été utilisée pour contrôler le bruit de fond expérimental. **c** – Analyse des interactions entre la caspase 8, RIPK1 et TRAF2. Les cellules BJELR ont été non-traitées (“-“) ou traitées avec TRAIL (1µg/ml) pendant 30 min (“+“). La co-immunoprécipitation des composés du DISC, et du complexe secondaire, par l’intermédiaire de la caspase 8 (“c8-IP“), a été analysée par Western blot. L’immunoprécipitation de IgG1 (“IgG1“) a été utilisée pour contrôler le bruit de fond expérimental.

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Publication 1

Plasminogen activator urokinase expression reveals TRAIL responsiveness and supports fractional survival of cancer cells

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/TNFSF10/Apo2L) holds promise for cancer therapy as it induces apoptosis in a large variety of cancer cells while exerting negligible toxicity in normal ones. However, TRAIL can also induce proliferative and migratory signaling in cancer cells resistant to apoptosis induced by this cytokine. In that regard, the molecular mechanisms underlying the tumor selectivity of TRAIL and those balancing apoptosis *versus* survival remain largely elusive. We show here that high mRNA levels of *PLAU*, which encodes urokinase plasminogen activator (uPA), are characteristic of cancer cells with functional TRAIL signaling. Notably, decreasing uPA levels sensitized cancer cells to TRAIL, leading to markedly increased apoptosis. Mechanistic analyses revealed three molecular events taking place in uPA-depleted cells: reduced basal ERK1/2 prosurvival signaling, decreased preligand decoy receptor 2 (DcR2)-death receptor 5 (DR5) interaction and attenuated recruitment of DcR2 to the death-inducing signaling complex upon TRAIL challenge. These phenomena were accompanied by increased FADD and procaspase-8 recruitment and processing, thus guiding cells toward a caspase-dependent cell death that is largely independent of the intrinsic apoptosis pathway. Collectively, our results unveil *PLAU* mRNA levels as marker for the identification of TRAIL-responsive tumor cells and highlight a key role of uPA signaling in 'apoptosis *versus* survival' decision-making processes upon TRAIL challenge.

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/TNFSF10/Apo2L) is a member of the TNF family that induces apoptosis in a large variety of cancer cells while sparing normal ones.¹ This cytokine binds as homotrimer to the extracellular domains of its plasma membrane-bound receptors inducing their oligomerization. Four TRAIL-specific receptors have been described, comprising two death receptors (DRs) that are able to trigger apoptosis (TNFRSF10A/TRAILR1/DR4, TNFRSF10B/TRAILR2/DR5) and two decoy receptors (DcRs) that bind the ligand but do not trigger apoptotic signaling (TNFRSF10C/TRAILR3/DcR1, TNFRSF10D/TRAILR4/DcR2).^{2–5} TRAIL binding to DR4 and DR5 induces the formation of the death-inducing signaling complex (DISC) owing to the recruitment of the specific adaptor protein FADD, which in turn engages initiator procaspase-8 and/or procaspase-10. DISC formation enables the autocatalytic cleavage and further activation of procaspases, thus triggering the death-executing cascade.^{6,7} Once an apoptosis-proficient DISC is formed, procaspases are

activated to generate two possible scenarios: either the caspase cascade triggered by initiator caspases is sufficient to commit the cell to apoptosis (referred to as type I cells) or the additional activation of the intrinsic apoptosis pathway is required to induce cell death (type II cells).⁸ Notably, the final cell fate upon TRAIL challenge depends on multiple cellular factors including sequestration of the ligand by DcR1 and/or DcR2 at the cell surface, which can trigger survival signaling,^{9–11} the recruitment of the non-functional procaspase homolog cFlip^{12–14} and the relative levels of proteins that regulate the activation of the intrinsic mitochondrial pathway and/or modulate the activity of executor caspases.^{15,16}

Importantly, it was also demonstrated that TRAIL induces proliferative, survival, migratory and inflammatory signals via activation of NF- κ B, PI3K/Akt, MAPK and JNK.¹⁷ The existence of non-apoptotic TRAIL signaling was demonstrated for apoptosis-resistant cancer cells, primary childhood leukemia and non-tumorigenic scenarios such as rheumatoid arthritis.^{17–20} Therefore, depending on their response to

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Abbreviations: *PLAU*, plasminogen activator urokinase; uPA, urokinase plasminogen Activator; uPAR, urokinase plasminogen activator receptor; DISC, death-inducing signaling complex; TRAIL-R, TRAIL receptor

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TRAIL, cancer cells can be grouped into three classes: (i) the entire population commits to apoptosis, (ii) is inherently resistant to cell death induced by TRAIL or (iii) only a part of the cell population dies, while a significant fraction survives the treatment and may even proliferate (referred to as 'fractional killing').²¹ From the mechanistic point of view, it was shown that activation of survival pathways by TRAIL involves assembly of a secondary signaling complex that retains the DISC components FADD and caspase-8, and recruits RIP1, TRAF2, TRADD and NEMO/IKK.²² However, contrary to the well-known cellular mechanisms mediating TRAIL-induced cell death, the understanding of how TRAIL and TRAIL receptors induce the formation of this secondary complex and the triggering of non-apoptotic cascades is still in its infancy.

Taken together, the numerous cellular factors modulating the response to TRAIL and the dual nature of the signaling triggered by this cytokine may lead to the generation of resistant populations. In that regard, even though combining TRAIL-based approaches with multiple therapeutic agents improves the apoptotic response and diminishes the chances to generate resistance,²³ only proteasome inhibitors were shown to revert acquired TRAIL resistance.²⁴ Therefore, the identification of tumor features predicting the response of cancer cells to TRAIL, the understanding of the mechanisms orchestrating the outcome and the characterization of molecular targets that will improve the initial apoptotic response are important goals to achieve.

By comparative transcriptome profiling of stepwise tumorigenesis systems,^{25,26} we observed that *PLAU* mRNA levels increase in tumorigenesis models and primary human tumors relative to normal tissue and correlate with sensitivity to TRAIL-induced cell death. We demonstrate that diminishing urokinase plasminogen activator (uPA) levels leads to decreased ERK1/2 survival signaling, thus lowering the cellular threshold to trigger TRAIL-induced apoptosis. Importantly, we show that uPA depletion alters the DISC composition by reducing the preligand and TRAIL-induced DcR2–DR5 interaction. Collectively, these molecular events lead to the formation of an apoptosis-proficient DISC, resulting in pronounced caspase-dependent apoptosis and preventing the generation of resistant populations. All in all, our work unveils an intricate cross-talk between uPA and TRAIL signaling, which highlights the use of *PLAU* mRNA as a marker of response and as a potential target for improving the apoptogenic action of TRAIL.

Results

Stepwise human tumorigenesis systems as cellular model to study TRAIL-induced signaling. Although the natural process of carcinogenesis is highly complex and specific to each cell type and/or carcinogenic insult, *in vitro* models recapitulate the basic events necessary for cellular transformation.^{25,26} In these models, normal primary human cells are transformed in a stepwise manner by the introduction of the catalytic subunit of telomerase (*hTERT*), the early region of the SV40 virus (SV40 ER) and the activated allele of *H-ras* (*H-rasV12*) (Figure 1a). These cellular systems display several hallmarks of tumorigenesis²⁷ and allow comparing cancer cells with their normal progenitors, which

is difficult to achieve with cancer cell lines derived from primary human tumors. We observed that normal cells from epithelial and mesenchymal origins (human embryonic kidney (HEK), foreskin fibroblasts (BJ), respectively) are resistant to TRAIL-induced cell death, whereas sensitivity to apoptosis is acquired along the transformation process, with transformed cells displaying the major apoptotic rate (Figure 1b). Therefore, in line with previous reports,^{28–31} our data indicate that stepwise tumorigenesis systems recapitulate the tumor selectivity reported for TRAIL. Interestingly, we observed that transformed cells from stepwise models display fractional killing even when exposed to high doses of TRAIL (>60-fold above the IC₅₀; Figures 1b and c (population 1) and Supplementary Figures 1a and b). Furthermore, cells surviving the first challenge remained resistant to further TRAIL treatments (Figure 1c, populations 2 and 3) and continued to proliferate in the presence of the cytokine (Figure 1d and Supplementary Figure 1c). Notably, when TRAIL treatment was discontinued, the resistant cell population reverted to the initial apoptotic rate (Figure 1c, population 4). The reversible nature of resistance suggests that it is not based on the selection of inherently resistant clones within the initial population, and that its establishment and maintenance is TRAIL-dependent. Taken together, these results indicate that stepwise models are valuable cell systems for the identification of genes whose altered expression is characteristic of tumor cells, and whose products may modulate apoptosis *versus* survival signaling triggered by this cytokine.

***PLAU* mRNA levels correlate with TRAIL-induced apoptosis in cancer cells.** To identify genes over-expressed in cancer cells relative to normal ones and whose encoded proteins may modulate the response to TRAIL, we compared the transcriptomes of normal, immortalized and transformed cells from stepwise models (HEK *versus* HA1ER; BJ *versus* BJELR; HA1E *versus* HA1ER; BJEL *versus* BJELR). Genes with increased expression in transformed cells relative to normal and immortalized ones were selected (fold change >2.5; *P*-value <0.01). From 316 genes that showed higher expression in HA1ER than in HEK cells, 39 also displayed a significant upregulation from the immortalization to transformation step (HA1ER *versus* HA1E; Figure 2a and Supplementary Figure 2a). A similar analysis of the BJ model revealed a set of 20 genes with such characteristics (Figure 2a and Supplementary Figure 2b). A comparison of the two systems identified four genes showing high expression levels in transformed cells from both stepwise models (Figure 2a). Among those, only *PLAU* mRNA was significantly increased in a panel of primary human tumors from different tissue origins as compared with normal tissue (Figure 2b; IST database, see Materials and Methods). In line with this, *in silico* analysis revealed that *PLAU* mRNA was significantly increased in breast cancer as compared with normal breast tissue, particularly in tumors showing a Her2+, PgR– and/or p53 mutant phenotype (Figure 2b and Supplementary Figure 2c). Moreover, we observed that higher levels of *PLAU* mRNA correlate with a reduced relapse-free survival in breast cancer patients (Figure 2c), as previously reported for its encoded protein uPA.^{32,33}

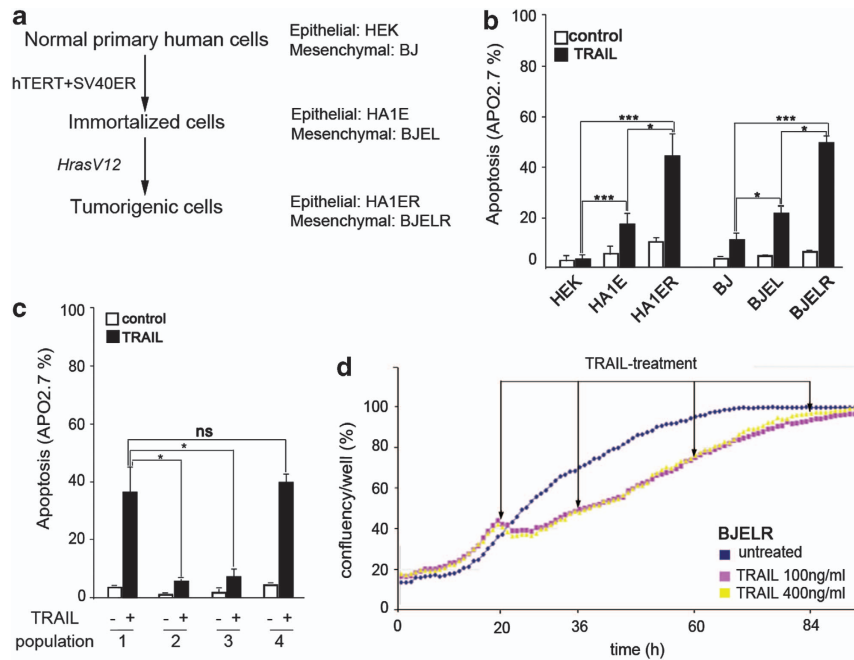


Figure 1 RAS-derived stepwise tumorigenesis models recapitulate TRAIL-tumor-selective apoptosis and display fractional killing and proliferation upon treatment. (a) Scheme representing RAS-derived stepwise tumorigenesis models generated from human foreskin fibroblast (BJ) and human embryonic kidney cells (HEK). Genetic elements introduced for stepwise transformation are indicated. (b) Apoptosis observed in populations of normal, immortalized and RAS-transformed cells derived from HEK and BJ stepwise tumorigenesis models. Basal cell death in untreated populations (control; white bars) and cell death after 16 h of treatment with 1 μ g/ml of TRAIL (TRAIL; black bars) is displayed. Apoptosis was analyzed by flow cytometry as the percentage of APO 2.7-positive cells. Histograms represent the mean \pm S.D. from at least three independent biological replicates. (c) Apoptosis was analyzed as in (b) after 16 h of treatment with 1 μ g/ml TRAIL in populations of BJELR-naive cells (population 1), resistant cells obtained from 24 (population 2) or 48 (population 3) hours of pre-exposure to TRAIL (500 ng/ml) and revertant cells (population 4) obtained from a pretreatment of BJELR cells with TRAIL (500 ng/ml) during 48 h, followed by a release from exposure to the cytokine during 12 days before receiving a new dose of 1 μ g/ml TRAIL during 16 h. Histograms represent the mean \pm S.D. from two independent biological replicates, representative of at least three independent experiments. Statistical significance in (b and c) was calculated by applying two-tailed, unpaired Student's *t*-test, ****P*-value < 0.0005, **P*-value < 0.05. (d) Proliferation of BJELR cells upon sequential treatment with TRAIL. Cells were plated and allowed to proliferate during 20 h before receiving a first dose of TRAIL (100 or 400 ng/ml, as indicated), were maintained under continuous exposure to the cytokine and further received sequential treatments at the indicated time points (arrows). Untreated cells were grown in parallel as proliferation controls. Images were acquired every hour using INCUCYTE and confluency was evaluated as the percentage of the surface covered by cells. The mean value obtained from two independent wells per time point is displayed and is representative of four independent biological replicates

Importantly, previous studies have revealed a correlation between high uPA levels and poor response of human tumors to standard cancer therapies.^{34–36} To elucidate a potential link between *PLAU* expression and response to TRAIL, these two features were first correlated in a study of 11 breast and prostate cancer cell lines. Interestingly, a direct correlation between *PLAU* mRNA levels and TRAIL-induced apoptosis was observed (Figure 2d). A similar analysis was performed with a publically available transcriptome data set of 20 breast cancer cell lines.³⁷ These cell lines were classified into two groups according to their apoptotic response toward TRAIL (death rate higher or lower than 50%) and *PLAU* mRNA levels for each group were assessed from the transcriptome data sets (Figure 2e). As observed in our initial study (Figure 2d), cell lines triggering prominent apoptosis presented higher levels of *PLAU* mRNA (Figure 2e). Taken together, these data demonstrate that high *PLAU* mRNA levels are indicative for cancer cells that exhibit a functional TRAIL apoptotic signaling cascade, independently of their genetic background, tissue origin and the transformation events that led to the malignancy.

PLAU and PLAUR expression support cell survival upon TRAIL challenge. A positive correlation between *PLAU*

expression and TRAIL-induced apoptosis does not *a priori* imply that uPA supports the apoptotic response to TRAIL. Indeed, uPA and its membrane-bound receptor urokinase plasminogen activator receptor (uPAR) have been primarily documented as promoting the proliferation and survival of cancer cells,^{38–40} however, other reports propose that uPA/uPAR may support cell death.^{41–43} Notably, the previously analyzed cancer cell lines and stepwise transformed cells with high expression of *PLAU* mRNA show fractional killing (Figures 1b, d and 2d and Supplementary Figure 1). Therefore, we evaluated whether uPA was functionally involved in the response to TRAIL, either by supporting apoptosis or survival upon exposure to this cytokine. Interestingly, increased apoptosis was observed in BJELR cells upon depletion of uPA or its membrane-bound receptor uPAR (Figures 3a and b and Supplementary Figures 3a and b). Comparable results were obtained when uPA or uPAR were depleted in HA1ER cells (Supplementary Figures 3c–e). These data indicate that uPA and uPAR are factors that counterbalance TRAIL-induced apoptosis in *H-rasV12* tumorigenic cells independently of their genetic background and highlight their functional relevance in the generation of resistant populations upon TRAIL challenge.

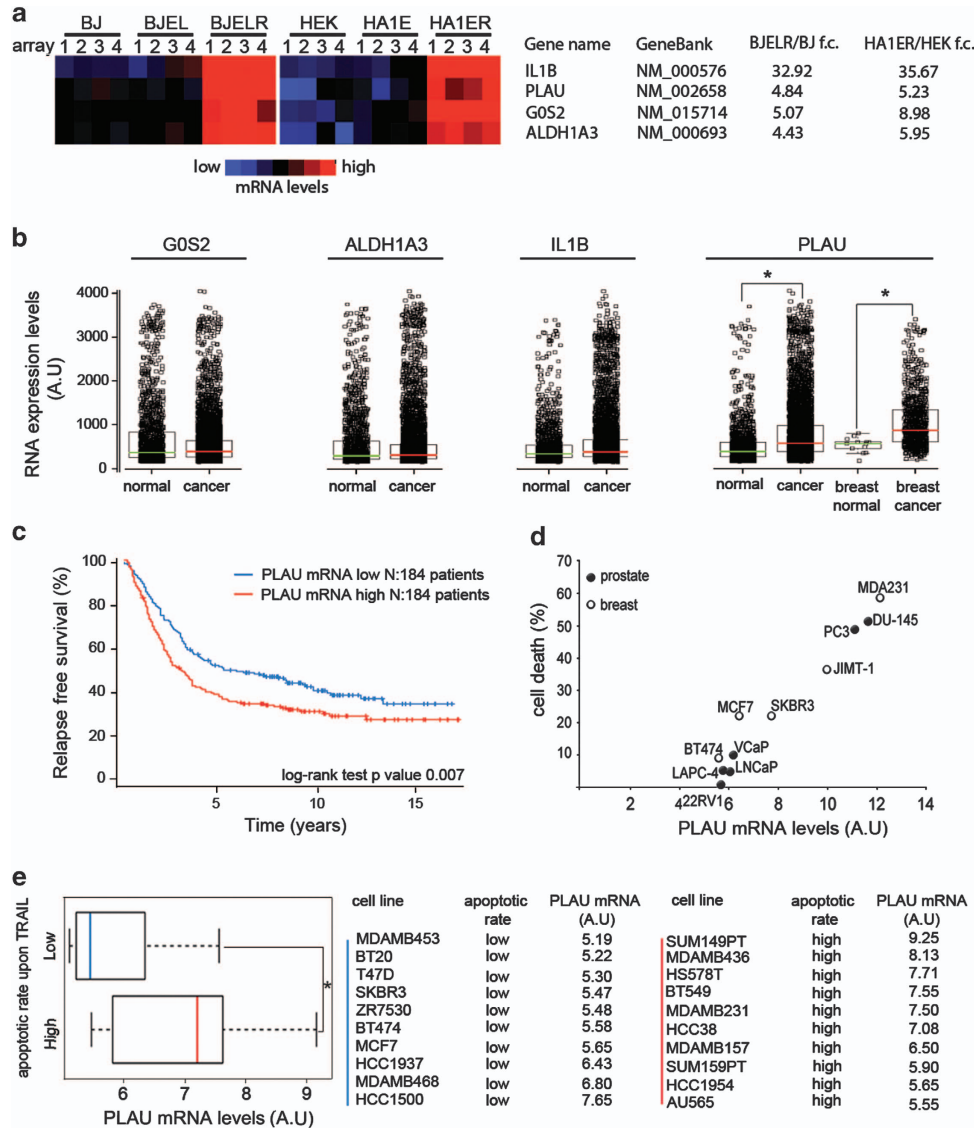


Figure 2 *PLAU* mRNA level increases during neoplastic transformation and is a tumor feature whose expression correlates with the rate of apoptosis triggered in response to TRAIL. (a) Expression heatmaps corresponding to the four upregulated genes common between HEK and BJ RAS-derived stepwise models. *IL1B*: interleukin 1 β ; *PLAU*: plasminogen activator urokinase; *G0S2*: G0/G1 switch 2 and *ALDH1A3*: aldehyde dehydrogenase 1 family, member A3. Results from four independent transcriptome datasets ('array 1–4') are displayed. Mean fold change ('f.c.') differences in their expression between normal and transformed cells from each model are indicated. (b) *G0S2*, *IL1B*, *ALDH1A3*, and *PLAU* mRNA levels were analyzed for 1840 healthy and 6806 malignant human primary samples from different tissue origin using the GeneSapiens database (<http://www.genesapiens.org>). Box plots displaying median and interquartile range (25–75%) of mRNA levels in each data set are shown. For *PLAU* mRNA, expression in normal and malignant breast cancer samples is depicted. Statistical significance of the difference between groups was calculated with two-tailed unpaired Student's *t*-test. **P*-value < 0.05. (c) Relapse-free survival curve of breast cancer patients according to *PLAU* mRNA levels. Three hundred and sixty-eight patients were ranked according to the expression level of *PLAU* mRNA and divided into 184 *PLAU* mRNA high and 184 *PLAU* mRNA low patients. The percentage of patients with relapse-free survival is plotted along 15 years. Statistical significance between the two groups is shown as log-rank test, *P*-value < 0.007. (d) TRAIL-induced cell death in breast and prostate human cancer cell lines was evaluated by CellTiter-Glo luminescence assay and levels of *PLAU* mRNA were extracted from Affymetrix GeneChip U133 Plus 2.0 array-based transcript profiling (see Materials and Methods). Correlation plot between cell death upon TRAIL treatment and *PLAU* mRNA levels is displayed (Pearson's correlation $r = 0.82$, two-tailed *P*-value < 0.003). The average of three technical replicates representative of three independent biological experiments is shown. (e) Correlation between *PLAU* mRNA expression and the apoptotic response to TRAIL of a publically available data set of breast cancer cell lines.³⁷ Twenty breast cancer cell lines were grouped according to whether they were reported to display an apoptotic rate higher ('High') or lower ('Low') than 50% upon TRAIL treatment.³⁷ *PLAU* mRNA levels in each group were calculated from the transcriptome data set and are displayed as box plots of the median and interquartile range (25–75%). **P*-value < 0.05. Cell lines classified as showing 'Low' and 'High' apoptotic rate upon TRAIL and the corresponding *PLAU* mRNA levels in arbitrary units ('AU') are specified on the right

uPA depletion lowers the cellular threshold for triggering caspase-dependent TRAIL-induced apoptosis. It is generally believed that low doses of TRAIL induce a poor apoptotic response and may generate resistance, whereas exposure to high levels of this cytokine results in extensive

cell death, indicating the existence of a cellular threshold for triggering apoptosis (referred hereafter as 'sensitivity'). To evaluate whether uPA knockdown modifies sensitivity to TRAIL-induced cell death, uPA-depleted cells were exposed either to high doses of TRAIL during different time points

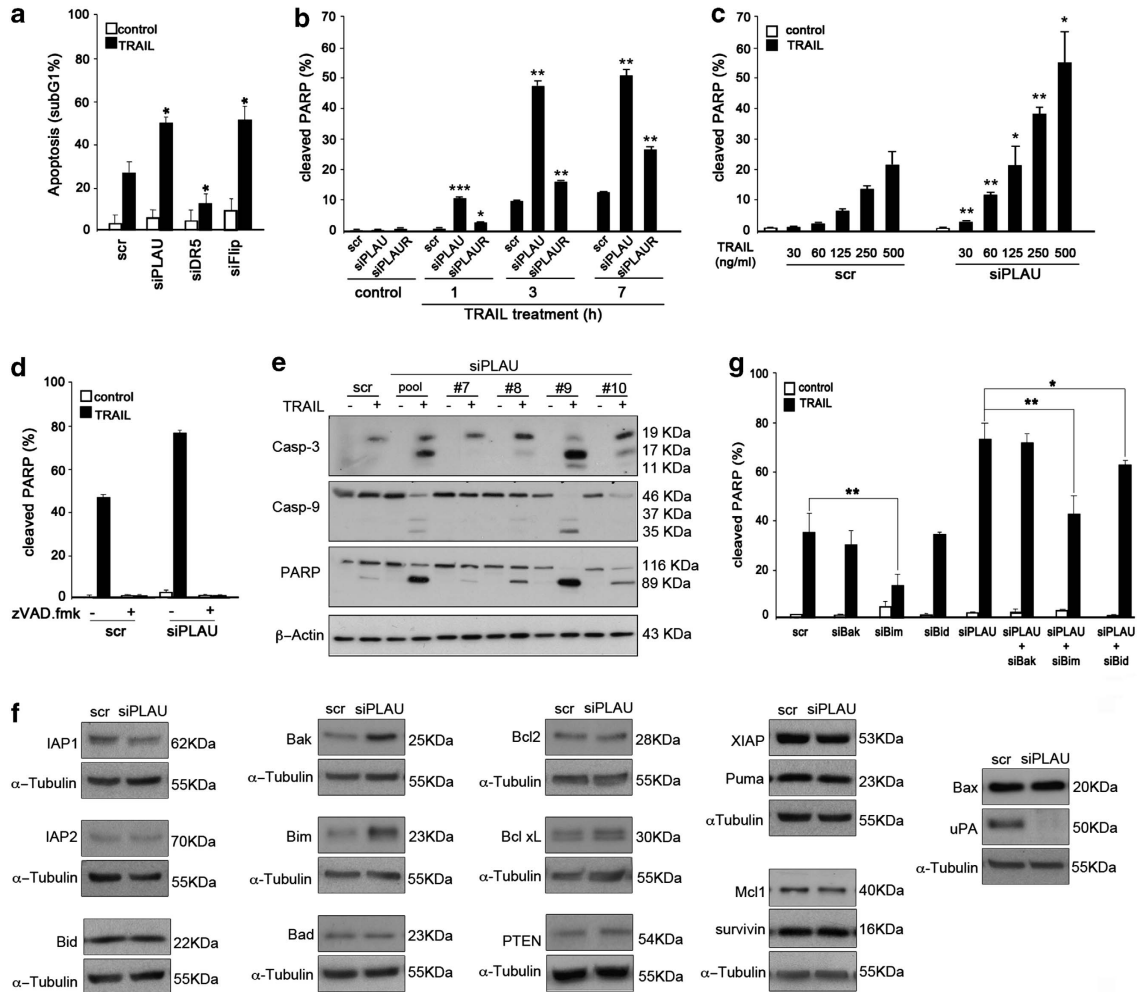


Figure 3 uPA expression is required for cell survival upon TRAIL treatment in RAS-derived stepwise tumorigenic cells. (a) Effect of uPA depletion on the response of BJELR cells to TRAIL. Cells were transfected with pooled siRNAs targeting *PLAU* mRNA ('siPLAU'), *Death Receptor 5* mRNA ('siDR5'), *cFlip* mRNA ('siFlip') or non-targeting scramble siRNAs ('scr'). Forty-eight hours after transfection, cells were either left untreated as control of basal cell death or challenged with 1 μ g/ml TRAIL during 16 h. Flow cytometry analysis was performed to assess the percentage of cells displaying sub-G1 DNA. Results from at least two independent biological replicates are displayed as histograms (mean \pm S.D.). (b) Time course analyzing the effect of uPA or uPAR depletion on the apoptotic response of BJELR cells to TRAIL. Cells were transfected with pooled siRNAs targeting *PLAU* mRNA ('siPLAU'), *PLAUR* mRNA ('siPLAUR') or non-targeting scramble siRNAs ('scr'). Forty-eight hours after transfection, cells were either left untreated or challenged with 1 μ g/ml TRAIL. Apoptosis was determined by flow cytometry as the percentage of cells displaying positive labeling for cleaved PARP at 1, 3 and 7 h after treatment. Results from two independent biological replicates are displayed as histograms (mean \pm S.D.) and are representative of at least three independent experiments. Statistical significance between either knock down relative to scramble-transfected cells was calculated. (c) Effect of uPA depletion on the sensitivity threshold of BJELR cells to trigger TRAIL-induced apoptosis. BJELR cells were transfected with pooled siRNAs targeting *PLAU* mRNA ('siPLAU') or non-targeting scramble siRNAs ('scr'). Forty-eight hours after transfection, cells were either left untreated (white bars) or treated with increasing doses of TRAIL (30, 60, 125, 250 or 500 ng/ml TRAIL, black bars) during 3 h. Apoptosis was analyzed by flow cytometry as the percentage of cells displaying positive labeling for cleaved PARP. Results obtained from two independent biological replicates are displayed as histograms (mean \pm S.D.). Statistical significance between uPA knockdown relative to scramble-transfected cells at the corresponding dose of TRAIL was calculated. (d) Caspase dependency of TRAIL-induced cell death in uPA knockdown cells. BJELR cells were transfected either with pooled siRNAs targeting *PLAU* mRNA ('siPLAU') or non-targeting scramble siRNAs ('scramble'), and 48 h after transfection, cells were either pretreated with 100 μ M zVAD.fmk caspase inhibitor or vehicle (dimethylsulfoxide (DMSO)) for 1 h and were either left unchallenged or treated with 1 μ g/ml of TRAIL for 5 h. Apoptosis was analyzed by flow cytometry as the percentage of cells displaying positive labeling for cleaved PARP. Results obtained from two independent biological replicates are displayed as histograms (mean \pm S.D.). (e) Generation of cleaved products of caspase-3, caspase-9 and PARP at early time points of the response to TRAIL. BJELR cells were transfected either with pooled siRNAs targeting *PLAU* mRNA ('siPLAU pool'), four individual siRNAs targeting different regions of the *PLAU* mRNA ('no. 7, no. 8, no. 9 and no. 10', see Supplementary Figure S4b) or non-targeting scramble siRNAs ('scr'). Forty-eight hours after transfection, cells were left untreated ('-') or challenged with 1 μ g/ml TRAIL ('+') during 45 min and total cell extracts were processed for western blot. Caspase-3 ('Casp-3'), caspase-9 ('Casp-9') and PARP cleavage were analyzed. β -Actin, loading control. (f) Cellular levels of pro- and antiapoptotic proteins reported to modulate TRAIL-induced apoptosis upon uPA knockdown. BJELR cells were transfected either with pooled siRNAs targeting *PLAU* mRNA ('siPLAU') or non-targeting siRNAs ('scr'), and 48 h after transfection, total cell extracts were processed for western blot analyzing the indicated pro- and antiapoptotic proteins. uPA level in control samples and upon knock down is shown. α -Tubulin, loading control. (g) Requirement of the intrinsic mitochondrial pathway for triggering TRAIL-induced apoptosis in uPA-depleted cells (top panel). Cells were transfected with pooled siRNAs targeting *PLAU* mRNA ('siPLAU'), *Bak* mRNA ('siBak'), *Bim* mRNA ('siBim'), *Bid* mRNA ('siBid'), non-targeting scramble siRNAs ('scr') or double-transfected ('siPLAU + siBak', 'siPLAU + siBim' and 'siPLAU + siBid'). Forty-eight hours after transfection, cells were either left untreated or challenged with 1 μ g/ml TRAIL during 7 h. Apoptosis was determined by flow cytometry as the percentage of cells displaying positive labeling for cleaved PARP. Results obtained from two biological replicates are displayed as histograms (mean \pm S.D.) and are representative of at least three independent experiments. Statistical significance in (a, b, c and g) was calculated by applying two-tailed, unpaired Student's *t*-test, ****P*-value < 0.005; ***P*-value < 0.005; **P*-value < 0.05

(Figure 3b) or to increasing doses of the cytokine (Figure 3c), and the percentage of apoptotic cells was analyzed. uPA depletion resulted in a ~4-fold increase of sensitivity for triggering cell death upon TRAIL challenge, supporting the notion that diminishing uPA levels reduces the cellular threshold for triggering TRAIL-induced cell death (Figures 3b and c).

Although it is widely accepted that apoptosis is the main type of cell death triggered through DRs of the TNF family, it was reported that TNF and TRAIL could trigger caspase-independent necroptosis.⁴⁴ To elucidate the molecular mechanisms underlying sensitization to TRAIL upon uPA knockdown, its dependence on caspase activity was analyzed. We observed that TRAIL-induced cell death was abolished in uPA knockdown and control samples in the presence of the pancaspase inhibitor zVAD.fmk (Figure 3d). Moreover, western blot assays revealed that uPA depletion promoted the generation of cleaved products of caspases-3, caspase-9 and PARP at earlier time points of exposure to TRAIL as compared with controls (Figure 3e and Supplementary Figure 4a). These results were obtained by using an siRNA pool as well as four individual siRNAs targeting different regions of *PLAU* mRNA (Figure 3e and Supplementary Figure 4b), supporting that the increase in apoptosis was neither due to a synthetic nor to an individual off-target effect of these siRNA molecules. Taken together, our results demonstrate that the increased cell death observed upon uPA knockdown proceeds through the classical caspase-dependent TRAIL-induced apoptosis cascade.

Enhanced sensitivity to TRAIL-induced apoptosis upon uPA knockdown could originate from an imbalance of pro- and/or antiapoptotic proteins regulating the response to TRAIL. Indeed, alterations in levels of Bcl-2 family members have been previously reported upon uPAR knockdown in cancer cells.^{38,45} In that regard, increased levels of proapoptotic Bim and Bak were observed, indicating that uPA knockdown changes the expression pattern of Bcl-2 family members to a proapoptotic setting (Figure 3f). Therefore, we investigated whether a cross-talk between the extrinsic and intrinsic apoptotic pathways was required for the sensitization effect observed upon uPA knockdown. By concomitantly decreasing the levels of either Bak, Bim, Bid and of PLAU, we showed that the enhanced sensitivity to TRAIL-induced apoptosis upon uPA depletion was significantly diminished by reducing Bim, whereas a decrease of Bak levels did not have any significant effect (Figure 3g and Supplementary Figure 4c). Moreover, Bid-mediated activation of the intrinsic mitochondrial apoptotic cascade had only a mild effect on the sensitization observed upon uPA depletion, suggesting that other molecular mechanisms besides the activation of the intrinsic mitochondrial cascade underlie the sensitization to TRAIL in uPA-depleted cells. Previous reports described the involvement of MAPK, Akt and NF- κ B signaling in cell survival upon TRAIL challenge.¹⁷ In this context, we observed that populations of BJELR cells surviving TRAIL treatment display increased levels of ERK1/2, Akt and I κ B α phosphorylation as compared with those in the initial naive cell population (Figure 4a). Among these cascades, ERK1/2 and Akt were described as modulated by the uPA/uPAR system.^{39,46,47} Therefore, abnormal ERK1/2 and/or Akt phosphorylation could account for the enhanced apoptotic response to TRAIL

in uPA-depleted cells. To substantiate this hypothesis, total and phosphorylated ERK1/2 and Akt were analyzed upon uPA knockdown and, whereas no differences in Akt phosphorylation were found, basal ERK1/2 phosphorylation decreased in the whole cell population (Figures 4b and c). Importantly, blocking ERK1/2 phosphorylation in BJELR cells by using U0126 MEK1/2 inhibitor (Supplementary Figure 4d) enhanced their apoptotic response to TRAIL (Figure 4d). These data suggest that the ERK1/2 cascade supports cell survival upon exposure to TRAIL in this model; thus, decreased basal ERK1/2 signaling as a consequence of uPA depletion accounts – at least partially – for the enhanced sensitivity to TRAIL-induced cell death.

uPA depletion alters DcR2 recruitment to the DISC-promoting caspase-8 processing. uPA knockdown resulted in a higher number of cells displaying caspase-8 cleavage upon TRAIL treatment (Figure 5a), suggesting that levels of DISC components and/or DISC composition itself could be affected upon uPA depletion. In that regard, no changes in total protein levels of DR4, DR5, DcR1, cFlip, FADD or procaspase-8 were observed, whereas a mild decrease on total DcR2 was evidenced (Figure 5b, lane 1 *versus* 3 and Supplementary Figure 5a). However, no differences were detected when surface levels of DR4, DR5, DcR1 and DcR2 were analyzed by flow cytometry (Figure 5c). Therefore, we analyzed whether increased caspase-8 activation originated from changes of the DISC composition. For that, the DISC was immunoprecipitated by using anti-DR5 antibodies and the co-immunoprecipitation of cognate DISC members was revealed by western blot. We observed that DcR2 displayed preligand association to DR5⁴⁸ (Figure 5b, lane 5), which was further enhanced by exposure to the cytokine (Figure 5b, lane 6 *versus* 5), whereas DcR1 was not detected at the DISC (not shown). Moreover, recruitment of FADD, DR4 as well as recruitment and cleavage of procaspase-8 and cFlipL (but not FlipS) were entirely TRAIL-dependent (Figure 5b, lane 6 *versus* 5). Interestingly, uPA knockdown resulted in lower preligand association of DcR2 with DR5 (Figure 5b, lane 7 *versus* 5) and in a reduced recruitment of DcR2 to the DISC after TRAIL exposure (Figure 5b, lane 8 *versus* 6). The antiapoptotic role of DcR2 in stepwise models was confirmed by DcR2 knockdown followed by TRAIL challenge (Figures 5d and e and Supplementary Figures 5b and c). Interestingly, neither uPA nor uPAR co-immunoprecipitated with DR5, suggesting that these molecules are not components of the DISC in our experimental conditions. Concomitant to the decrease of DcR2 recruitment upon uPA knockdown, enhanced FADD and caspase-8 recruitment and cleavage were observed (Figure 5b, lane 8 *versus* 6). Taken together, these results show that increased apoptotic response to TRAIL in uPA-depleted cells arises – in part – from the reduced interaction of the antiapoptotic DcR2 with DISC components.

Discussion

Despite the conceptually attractive therapeutic features of the TRAIL signaling pathway – such as its unique tumor

selectivity – only few cases of stable disease and partial remission were observed in clinical trials, suggesting that human tumors are largely resistant to TRAIL-based monotherapies.⁴⁹ These disappointing results were rather unexpected, given the well-documented tumoricidal effects of TRAIL *in vitro* and the cancer-protective activities of the TRAIL cascade in mouse knockout studies. Indeed, the TRAIL pathway is a natural component of the tumor-surveillance system in mammals that was shown to modulate

tumor onset/progression and to participate in T-cell-mediated immune defense against tumors in allogeneic graft-versus-tumor settings.¹⁶ Facing TRAIL challenge, cancer cells may need to develop strategies to survive and proliferate *in vivo* by acquiring resistance to this cytokine at early steps of the neoplastic transformation. Indeed, activation of survival cascades supporting evasion from apoptosis is one of the hallmarks of cancer.²⁷ In that regard, acquisition of resistance to TRAIL-induced cell death during neoplastic transformation may be based on two general mechanistic principles:²³ (i) key components of the TRAIL pathway may be silenced (e.g., epigenetically) and/or mutated, resulting in a non- or subfunctional signaling cascade and (ii) cancer cells may hijack a functional TRAIL-apoptotic pathway *in vivo* through the activation of survival cascades that counterbalance TRAIL-induced cell death. In such a scenario, cancer cells would ‘hit two birds with one stone’ by simultaneously evading from the apoptotic TRAIL challenge imposed by immune surveillance and gaining survival advantage through shifting the TRAIL response from apoptosis to survival/migration. It is therefore of major importance to identify tumor-related features of those cancer cells that have maintained a functional TRAIL-apoptotic pathway and to assess the relative impact of the associated apoptogenic and survival/proliferative functions of this cascade. By following such an approach, we identified *PLAU* mRNA as a molecule whose high expression is characteristic of cancer cells with a functional TRAIL-apoptotic cascade and whose level has a direct correlation with the sensitivity of cancer cells to trigger TRAIL-induced apoptosis. Surprisingly, we faced an apparent conundrum, as depletion of uPA in these apoptosis-responsive cells enhanced cell death upon cytokine challenge. In that regard, we observed that TRAIL-sensitive cells expressing high levels of *PLAU* mRNA triggered fractional killing upon TRAIL challenge and survivor cells displayed increased prosurvival signaling. Thus, that uPA and uPAR depletion

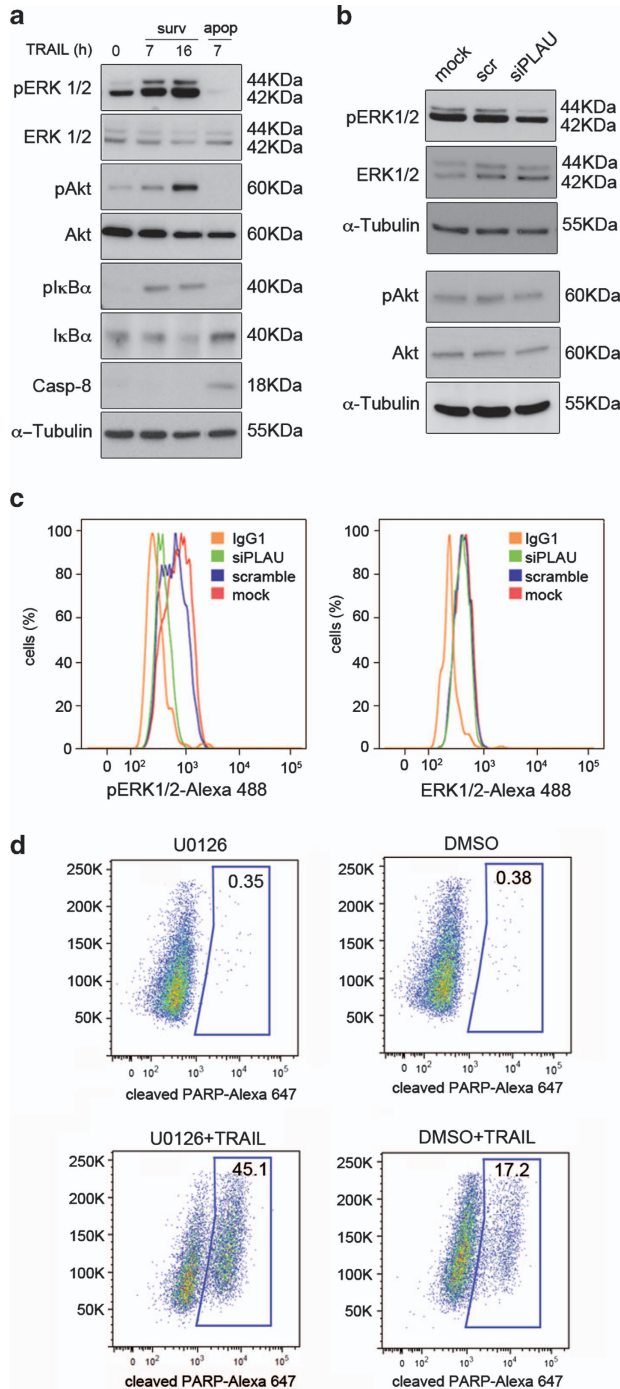
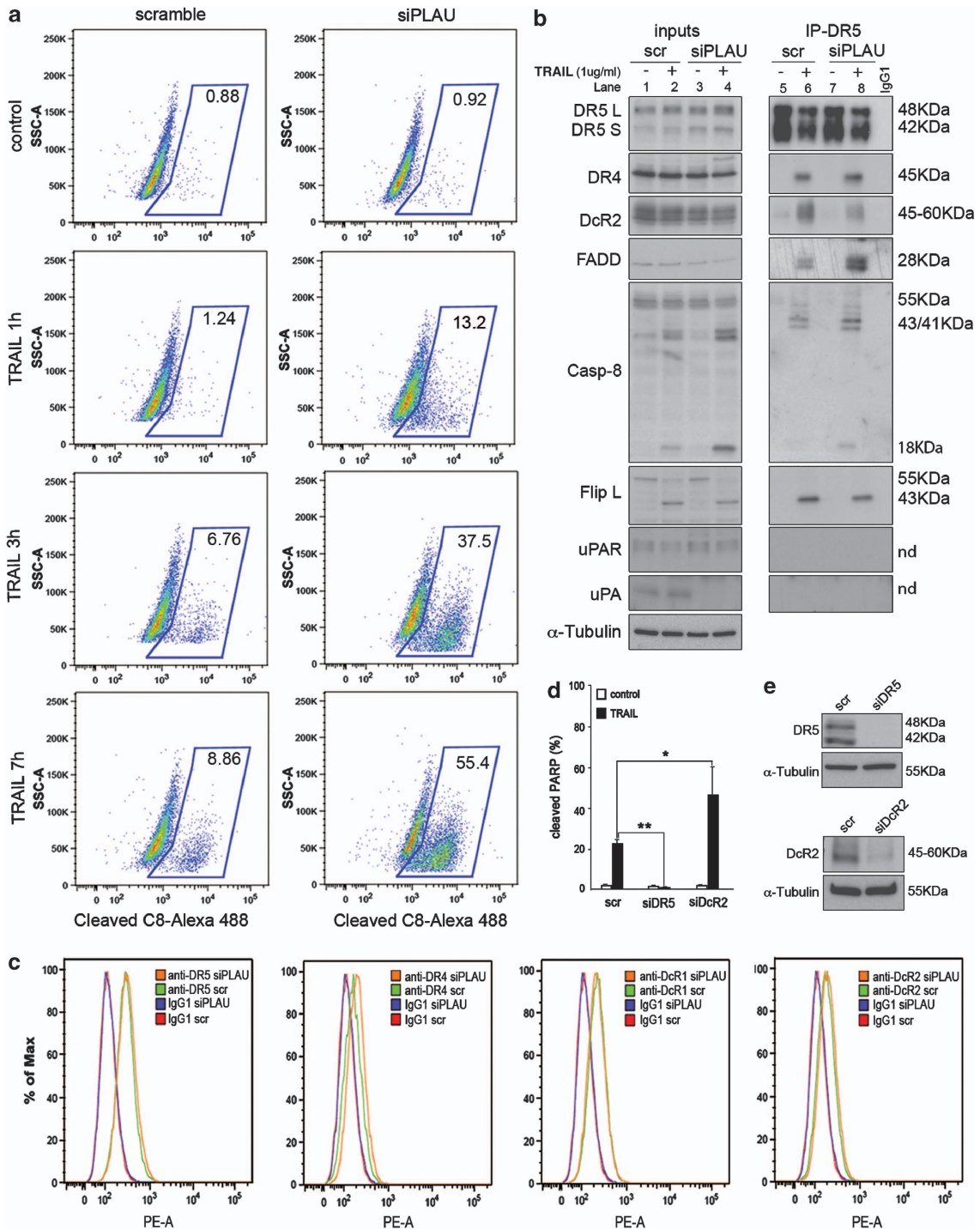


Figure 4 uPA knockdown affects ERK1/2 survival signaling. (a) Phosphorylation levels of ERK1/2, Akt and regulators of NF- κ B signaling (I κ B α) in transformed cells surviving TRAIL treatment. BJELR cells were either left untreated (‘control’) or treated with TRAIL (1 μ g/ml) during 7 or 16 h. Apoptotic cells (‘apop’) were collected by successive washes and non-apoptotic attached cells (‘surv’) were harvested for western blot analysis. Total and phosphorylated levels of ERK1/2 (Thr202/Tyr204), Akt (Ser 473) and I κ B α (Ser32/36) were analyzed. Caspase-8 cleavage (‘Casp-8’) was evaluated in each cell population. α -Tubulin, loading control. (b) Phosphorylation status of ERK1/2 and Akt upon uPA knockdown. BJELR cells were left non-transfected (‘mock’) or transfected either with pooled siRNAs targeting *PLAU* mRNA (‘siPLAU’) or non-targeting scramble siRNAs (‘scr’). Forty-eight hours after transfection, total and phosphorylated protein levels of ERK1/2 (Thr202/Tyr204) and Akt (Ser 473) were analyzed by western blot analysis. α -Tubulin, loading control. (c) Distribution of total (ERK1/2 Alexa488) and phosphorylated ERK1/2 (pERK1/2-Alexa 488; Thr202/Tyr204) in populations of BJELR cells untreated (‘mock’), scramble transfected (‘scr’) and uPA depleted (‘siPLAU’) was analyzed by flow cytometry. Images represent the distribution of fluorescence within each sample for a representative experiment out of three independent replicates. Isotypic IgG1 (‘IgG1’) was used as control for background fluorescence. (d) Requirement of ERK1/2 signaling for cell survival upon TRAIL challenge. BJELR cells were either treated for 1 h with 20 μ M U0126 MEK1/2 inhibitor or vehicle (DMSO). Apoptosis in untreated samples (‘U0126’, ‘DMSO’) and upon 3 h of treatment with 1 μ g/ml of TRAIL (‘U0126 + TRAIL’, ‘DMSO + TRAIL’) was analyzed by flow cytometry as the percentage of cells with positive labeling for cleaved PARP. Images correspond to one representative experiment out of four independent replicates. Percentage of cleaved PARP-positive cells is indicated in the upper right quadrant

resulted in diminished survival upon challenge with TRAIL highlighted a functional involvement of this cascade in evasion from TRAIL-induced apoptosis. Particularly, we demonstrate that uPA knockdown shifts the profile of Bcl-2 family members to proapoptotic, decreases basal prosurvival ERK1/2 signaling and results in abnormal recruitment of DcR2 to the DISC. The regulation of ERK1/2 phosphorylation by the uPA/uPAR system can involve the interaction between uPAR and

integrins, uPAR and epidermal growth factor (EGF) receptor, as well as cell signaling triggered by soluble uPAR.⁵⁰ In these scenarios, uPAR has a central role in modulating the ERK1/2 cascade. In our experiments, sensitization to TRAIL-induced cell death was also observed upon uPAR knockdown, but surprisingly, no major differences in ERK1/2 phosphorylation were seen (data not shown). This suggests the existence of a uPAR-independent/uPA-dependent modulation of ERK1/2



signaling in this experimental model. Supporting this hypothesis, uPAR-independent uPA signaling has been ascribed its binding to other receptors (e.g., α M β 2-, α 5 β 5-integrin) and/or to its uPAR-independent proteolytic activity.^{51,52}

That depletion of uPA alters the DISC composition by reducing the recruitment of antiapoptotic DcR2 suggests a cross-talk between uPA signaling and the formation of TRAIL receptor complexes. Importantly, regulation of signaling from plasma membrane-bound receptors by uPA/uPAR has been reported. Indeed, it has been shown that binding of uPAR to integrins, FPRL1 and EGF receptors subverts the ligand-dependent signaling toward the formation of new signaling complexes.⁵⁰ Interestingly, neither uPAR nor uPA were found to interact with the DISC in our experimental conditions, suggesting that the modulation of DISC composition/processing in response to TRAIL does not rely on a direct interaction between these molecules and TRAIL receptors.

Finally, several factors have been described to correlate with sensitivity or resistance to TRAIL-induced cell death in tumor cells. For example, the expression of GALNT14 correlates with sensitivity to TRAIL-induced apoptosis in human cancer cell lines and its predictive value is currently tested in phase II clinical trials.⁵³ Expression of the homeoprotein Six1 was shown to correlate with resistance to TRAIL-induced cell death and its depletion sensitized cancer cells to apoptosis by this cytokine.⁵⁴ In this context, we show that high *PLAU* mRNA levels correlate with TRAIL responsiveness and demonstrate that depletion of uPA promotes the apoptogenic action of TRAIL decreasing the risk of generating resistant cells that may turn TRAIL signaling from apoptosis to proliferation/survival. Therefore, uPA/uPAR adds another facet to the TRAIL signaling, which may be considered in therapeutic settings, also in view that TRAIL-apoptotic action is independent of p53.¹ In that regard, we have shown that high levels of *PLAU* mRNA are observed in breast tumors showing Her2+, PgR- and/or p53 mutant phenotypes and that such levels correlate with the apoptotic response of breast cancer cells to TRAIL, suggesting that this type of cancer may express a functional TRAIL cascade. Importantly, first-in-man studies modulating uPA activity⁵⁵ and testing TRAIL-based therapeutics^{1,49} are ongoing. Thus, combinatorial therapies may be envisaged for patients bearing tumors with high *PLAU* expression and p53 mutations, which are expected to have a poor response to standard treatments.^{34–36}

Materials and Methods

Antibodies. Antibodies for western blotting recognizing the indicated proteins were purchased from Cell Signaling (Beverly, MA, USA; caspase-8 1C12 no. 9746, cleaved caspase-3 D175 no. 9661, caspase-9 no. 9502 and PARP no. 9542, DR5 no. 3696, DcR2 no. 8049, DcR1 no. 4756, BID no. 2002, Bcl-2 no. 2872, Bcl-xl no. 2762, PTEN no. 9552, PUMA no. 4976, Bak no. 6947, Bim no. 2933, Bax no. 5023, Bad no. 9239, Mcl1 no. 4572, XIAP no. 2045, survivin no. 2808, IAP1 no. 4952, IAP2 no. 3130, ERK1/2 no. 9102, pERK1/2 no. 9101, Akt no. 9272, pAkt no. 9271, $\text{I}\kappa\text{B}\alpha$ no. 4814, $\text{pI}\kappa\text{B}\alpha$ no. 9246), Santa Cruz Biotechnology (Santa Cruz, CA, USA; β -actin clone sc-1615, α -tubulin sc-32293, uPA sc-14019), Millipore EMD Millipore Corporation (Billerica, MA, USA; DR4 AB16955), Alexis Biochemicals, Enzo Life Sciences (Farmingdale, NY, USA), (cFlip ALX-804-428-c050), BD Transduction Laboratories (San Jose, CA, USA; FADD 610399) and Abcam (Cambridge, UK; uPAR ab3129). For flow cytometry assays, APO2.7-PE was purchased from Beckman Coulter (Fullerton, CA, USA; IM2088U), cleaved PARP (no. 8978; no. 6987) and cleaved caspase-8 (no. 9496) from Cell Signaling. DISC immunoprecipitation assays used anti-DR5 (AF631) purchased from R&D Systems (Minneapolis, MN, USA).

Cell Lines. Cells from stepwise tumorigenesis systems were cultured in DMEM (1 g/l glucose) + Med 199 (4:1) + 10% FCS heat-inactivated (BJ-derived model) and DMEM (1 g/l glucose) + 10% FCS heat-inactivated (HEK-derived model). Culture medium was supplemented with 400 μ g/ml hygromycin (hTERT selection), 400 μ g/ml G418 (SV40 ER selection) and 1 μ g/ml puromycin (*H-rasV12* selection) when required. Expression of SV40 ER and *H-rasV12* was confirmed by western blot. LNCaP, PC-3, DU-145, MDA-231, MCF-7, BT-474, 22Rv1 and SKBR3 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and JIMT-1 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, GmbH, Germany) and grown in recommended media. LAPC-4 cells (a kind gift from Prof. C Sawyer, Los Angeles, CA, USA) were grown in Iscove's medium supplemented with 7.5% FBS supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin and 10 nM R1881. VCaP and PC-3MPro4 cells were received from Adrie van Bokhoven (University Medical Center Nijmegen, Nijmegen, The Netherlands) and Marco Ceccini (University of Bern, Bern, Switzerland), respectively, and cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin.

Microarrays analysis for stepwise tumorigenesis models.

Illumina arrays were normalized by quantiles method in R using lummi package.⁵⁶ Analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team. Briefly, univariate *t*-tests were performed for the different class comparisons (BJ versus BJELR, BJEL versus BJELR, HEK versus HA1ER, HA1E versus HA1ER). The differential expressed genes with a *P*-value < 0.01, false discovery rate < 0.01 and fold change (FC) > 2.5 were selected. Multiple probe i.d.'s were discarded and gene list containing unique names was composed. Hierarchical clustering (complete linkage) and heatmaps were performed using Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and Java TreeView,⁵⁷ respectively.

In silico analysis of *PLAU* mRNA expression. *In silico* data mining for gene expression levels of *PLAU* mRNA in clinical samples was carried out using the *in silico* transcriptomics database (IST) developed at VTT Technical Research Center in Finland. The database covers 113 million data points and is available from the GeneSapiens website (<http://www.genesapiens.org>).⁵⁸

Figure 5 Altered recruitment of inhibitory components to the DISC promotes caspase-8 activation in uPA knockdown cells. (a) Time-course analysis of procaspase-8 cleavage. BJELR cells were transfected either with pooled siRNAs targeting *PLAU* mRNA ('siPLAU') or non-targeting scramble siRNAs ('scramble'). Forty-eight hours after transfection, cell populations were either left untreated or challenged with 1 μ g/ml TRAIL during 1, 3 or 7 h and the percentage of cells displaying cleaved caspase-8 ('C8') was determined by flow cytometry. Images from one representative experiment out of three independent replicates are shown. Percentage of cleaved C8-positive cells is indicated. (b) DISC composition in uPA-depleted cells. BJELR cells were transfected either with pooled siRNAs targeting *PLAU* mRNA ('siPLAU') or non-targeting scramble siRNAs ('scr'), and 48 h after transfection, cell populations were either left untreated or challenged with TRAIL (1 μ g/ml) during 30 min. Immunoprecipitation of Death Receptor 5 (IP-DR5) was performed and co-immunoprecipitation of cognate DISC components, uPA and uPAR, was analyzed by western blot analysis. Immunoprecipitation using isotopic IgG1 ('IgG1') was used as background control. (c) Surface levels of TRAIL-Rs upon uPA knockdown. BJELR cells transfected either with pooled siRNAs targeting *PLAU* mRNA ('siPLAU') or non-targeting scramble siRNAs ('scr'). Forty-eight hours after transfection, surface levels of Death Receptor 5 ('DR5'), Death Receptor 4 ('DR4'), Decoy Receptor 1 ('DcR1') and Decoy Receptor 2 ('DcR2') were analyzed by flow cytometry. Isotypic IgG1 labeling was used as control for background fluorescence in scramble- ('IgG1 scr') and siPLAU- ('IgG1 siPLAU') transfected cells. (d) Antiapoptotic role of DcR2. BJELR cells were transfected either with pooled siRNA targeting *Death Receptor 5* mRNA ('siDR5'), *Decoy Receptor 2* mRNA ('siDcR2') or non-targeting scramble siRNAs ('scr'), and 48 h after transfection, cell populations were either left untreated or challenged with TRAIL (1 μ g/ml) during 3 h. Apoptosis was determined as the percentage of cells with positive labeling for cleaved PARP by flow cytometry. Histograms represent the mean \pm S.D. of three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student's *t*-test, **P*-value < 0.05; ***P*-value < 0.005. (e) Efficiency of DR5 and DcR2 knockdown at 48 h after transfection analyzed by western blot analysis. α -Tubulin, loading control

To determine the expression of *PLAU* mRNA in 14 different cancer cell lines, we utilized the profiling data from the following resources, which have all been generated using Affymetrix GeneChip U133 Plus 2.0 arrays: (1) GlaxoSmithKline from the National Cancer Institute's cancer Bioinformatics Grid (caBIG). This data set included genome profiling data for over 300 cancer cell lines, available through the caArray open source microarray data management system (<https://cabig.nci.nih.gov/tools/caArray>). (2) VTT in-house cell line gene expression profiling database⁵⁸ included data from four breast cancer (JIMT-1, SKBR3, MCF7, BT474) and six prostate cancer (DU-145, PC3, VCAP, LNCaP, LAPC-4 and 22RV1) cell lines. (3) Gene Expression Omnibus (GEO) database. Breast cancer expression set includes breast normal and breast cancer samples from 483 records with GEO series i.d.'s GSE6532, GSE9195, GSE12276, GSE12276 and GSE19615. Only samples with survival information attached were used from the series. From these samples, 79 are annotated as Her2(-) and 36 as Her2(+). Probes were summarized according to improved probe definitions (custom CDF hgu133plus2hsensgcd, version 12)⁵⁹ and data were normalized using the robust multiarray (RMA) average expression measure with R/Bioconductor v.2.9.1. Kaplan-Meier plots were drawn using R standard functions. *P*-values for survival were calculated with χ^2 statistics of the rank sum. For between-group comparisons at the gene level two-tailed Student's *t*-test was used.

Transfection. Smart pool siRNAs targeting *DR5* mRNA (L-004448-00), *DR4* mRNA (L-008090-00), *DcR2* mRNA (L-008092-01), *cFLIP* mRNA (L-003772-00), *PLAU* mRNA (L-006000-00), *PLAUR* mRNA (L-0063388-00), *Bid* mRNA (L-004387-00-0005), *Bak* (J-003305-07) and non-targeting scrambled no. 1 (D-001810-10-05), as well as single siRNAs targeting different regions of *PLAU* mRNA (LU-006000-00) were purchased from Dharmacon, Thermo Scientific (Waltham, MA, USA). siRNA targeting *Bim* (no. 6518) was purchased from Cell Signaling. These siRNAs were transfected into BJELR and/or HA1ER cells by using RNAi Max Lipofectamine following the vendor's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). Twenty-four hours after transfection, the culture medium was replaced and cells were incubated under normal growth conditions (37 °C, 5% CO₂) for additional 24 h.

Apoptosis measurement. Apoptosis was determined either as the percentage of cells displaying sub-G1 DNA content or a positive immunostaining for the APO 2.7 mitochondrial marker, cleaved PARP or cleaved caspase-8 following the manufacturer's instructions. Apoptotic rate in non-treated control cells and upon TRAIL challenge is displayed as the percentage of cells presenting a positive staining for the indicated marker. When displayed as histograms, each data set was derived from at least two independent biological replicates.

Analysis of growth inhibition upon TRAIL. Cell viability following exposure to TRAIL in 11 different cancer cell lines (LNCaP, PC-3, DU-145, MDA-231, MCF-7, BT-474, 22Rv1, SKBR3, VCap and LAPC) was determined by CellTiter-Glo Luminescence Assay following the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, cells were plated in 384-well plates, and 24 h after plating, cells were treated with 400 ng/ml of TRAIL for 48 h and cell viability was measured. The percentage of cell viability was determined as: luminescence value in TRAIL-treated cells/luminescence value in non-treated controls. Percentage of cell death displayed in Figure 2d was calculated as: 100% - % of cell viability.

Growth curve assays. HA1ER and BJELR cells were plated in 48-well plates, and after 20 h, a first dose of TRAIL (100 or 400 ng/ml) was added to the medium. A second, a third and a fourth dose were administered 16, 40 and 64 h after the first treatment. Growth curves were obtained using Incucyte (Essen Instruments, Ann Arbor, MI, USA) under normal culture conditions. Phase-contrast images of cells were taken every hour during 4 days and temporal plots of confluence were established to analyze cell growth. Results are shown as the mean of two technical replicates and are representative of three independent biological repeats.

Western blot assays. Samples were harvested in RIPA buffer, separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk and 0.1% Tween-20 in PBS for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies according to the manufacturer's instructions, followed by incubation with corresponding secondary peroxidase-conjugated antibodies. β -Actin or α -tubulin was used as an internal standard for protein loading. Immunoreactive bands were visualized by enhanced chemiluminescence and subsequent exposure to hyperfilm (Amersham, GE Healthcare, Amersham, UK). Images were scanned and processed by Adobe

Photoshop CS2 software (San Jose, CA, USA). Images from one representative experiment out of at least two biological replicates are displayed.

Surface levels of TRAIL receptors. Antibodies were purchased from Diaclone Immunology Products (Diaclone, Besancon, France; TRAIL R1 854.852.010; TRAIL R2 854.862.010; TRAIL R3 854.892.010 and TRAIL R4 854.972.010) and detection was performed following the manufacturer's instructions.⁶⁰

DISC immunoprecipitation. BJELR cells were plated in a confluency of 10⁶ cells per 10 cm Petri dishes. Twenty-four hours later, cells were exposed to TRAIL 1 μ g/ml during 30 min at regular growing conditions (37 °C; 5% CO₂). Cells were washed with ice-cold PBS and lysed in 1 ml of lysis buffer (30 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM KCl, 10% glycerol, 2 mM EDTA (pH 8.0)) freshly supplemented with complete, EDTA-free protease inhibitor cocktail (Roche, Mannheim Germany; no. 11873580001), phosphatase inhibitors (PhosStop; Roche) and 1% Triton X-100 (Bio-Rad). Lysates were precleared with Pure Proteome Protein G magnetic beads (Millipore; LSKMAGG02) and the DISC was immunoprecipitated by using 10 μ g of anti-DR5 antibody bound to Pure Proteome Protein G magnetic beads (Millipore; LSKMAGG02). Immunoprecipitates were processed for immunoblotting as described in western blot section.

Conflict of Interest

The authors declare no conflict of interest.

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Publication 2

Manuscript in preparation

DR5 induces fractional killing in cancer cells by assembling apoptotic and pro-survival platforms in response to TRAIL

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Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/TNFSF10/Apo2L) is a member of the TNF family that binds to the extracellular domains of its two death (TNFRSF10A/TRAILR1/DR4, TNFRSF10B/TRAILR2/DR5) and two decoy (TNFRSF10C/TRAILR3/DcR1, TNFRSF10D/TRAILR4/DcR2) plasma membrane-bound receptors inducing their clustering^{1,2,3,4}. In turn, receptor clustering promotes the recruitment of the adaptor protein FADD which engages initiator procaspase-8 and/or pro-caspase-10 resulting in the assembly of the death-inducing signaling complex (DISC)^{5,6}. DISC formation enables a proximity-induced autocatalytic cleavage and further activation of procaspases, thus triggering the death-executing cascade^{7,8}. Notably, it was also demonstrated that TRAIL induces the assembly of a secondary signaling complex composed of FADD, Caspase 8, RIP1, TRAF2, TRADD and NEMO/IKK, leading to the activation of proliferative, survival, migratory and inflammatory signaling (NF- κ B, PI3K/Akt, MAPK and JNK cascades)⁹. The activation of these non-apoptotic TRAIL-induced cascades has been reported in different cellular scenarios including apoptosis-resistant cancer cells, primary childhood leukemia and non-tumorigenic scenarios such as rheumatoid arthritis^{10,11,12,13,14}. We and others have shown that the activation of non-apoptotic TRAIL-induced pathways takes place in populations of cancer cells in which only part of the population dies while a fraction survives and proliferate (referred to as 'fractional killing')^{15, 16}. Therefore, a striking divergence in cell fates is observed in populations of cancer cells as not only the time between TRAIL exposure and caspase activation can vary considerably between those cells undergoing apoptosis, but also cell survival and TRAIL-dependent resistance can be established in response to this ligand^{15,16,17,18,19}.

There is considerable evidence supporting that TRAIL induces apoptosis in cancer cells while leaving normal tissues unaffected, thereby providing a valuable tool for the development of cancer-selective therapies²⁰. Indeed, even though our current knowledge regarding the molecular mechanisms underlying TRAIL-induced fractional killing remains fragmentary, multiple clinical trials have been/are being conducted in order to define its therapeutic potential²⁰. Briefly, several TRAIL receptor agonists (TRAs) are being tested ranging from the use of recombinant human ligand (TRAIL) to the application of agonistic antibodies and mimetic peptides that selectively target DR4 or DR5^{21,22, 23}. This latter strategy aims at promoting cell death by selectively triggering the TRAIL cascade via death-inducing receptors while avoiding sequestration of the ligand and/or activation of non-apoptotic signaling by decoy receptors^{24, 25}. However, more recent reports demonstrate that TRAIL triggers the activation of pro-survival pathways by binding to DR5 in cancer cell lines in which the whole population is resistant to TRAIL-induced apoptosis^{10,12}. Furthermore, it was also shown that endogenously expressed TRAIL leads to DR5-mediated activation of Akt promoting tumor cells migration and invasion^{26, 27}. Finally, it has been reported that even though

numerous cancer cell lines express DR4, DR5 and decoy receptors at the plasma membrane, only one death receptor mediates TRAIL-induced apoptosis while the role of the other receptor(s) in the modulation of TRAIL-induced signaling in these cellular scenarios remains unclear²⁸. Collectively, these observations highlight that the role of TRAIL receptors in TRAIL-induced signaling is much more complex than originally anticipated.

In this work we studied the role of individual TRAIL receptors and characterized the molecular platforms participating in the activation of TRAIL-induced fractional killing. For that, we use isogenic stepwise tumorigenesis systems in which normal cells are transformed into tumorigenic ones by the introduction of defined genetic elements^{29, 30}. We and others have reported that normal cells from these models are resistant to TRAIL-induced cell death whereas sensitivity to apoptosis is acquired along the transformation process^{15,31}. Interestingly, we demonstrated that clonal populations of transformed cells from stepwise systems display fractional killing in response to TRAIL and showed that cells surviving the first challenge remained resistant and continued to proliferate in the presence of this cytokine¹⁵. Using this experimental system, we show that DcR2, DR4 and DR5 are expressed at the cellular surface of stepwise tumorigenic cells and form heterocomplexes in response to TRAIL treatment. Our data indicates that, even though DR4 is recruited to the DISC, this receptor does not mediate either apoptosis or to the triggering of non-apoptotic pathways in this cellular context. Moreover, in line with its “decoy” nature, we observed that DcR2 negatively regulates TRAIL-induced apoptosis by modulating the activation of pro-survival Akt signaling upon challenge with TRAIL. Strikingly our results show that, besides being the central pro-apoptotic receptor in our model, DR5 also triggers pro-survival pathways in response to TRAIL. Indeed, binding of TRAIL to DR5 leads to the formation of the canonical pro-apoptotic DISC as well as promotes the assembly of a TRAIL-induced pro-survival complex, thereby activating non-apoptotic Erk, Akt, p38MAPK and NF- κ B signaling cascades. All in all, our work provides evidences for the molecular basis of fractional killing and reveals the dual nature of a single death receptor within populations of cancer cells.

Results

Activation of non-apoptotic pathways support fractional killing in response to TRAIL treatment

In line with our previous report¹⁵, flow cytometry assays assessing the percentage of cells displaying positive labeling for cleaved caspase 8 and cleaved PARP (poly ADP ribose polymerase) showed that exposure of stepwise-derived tumorigenic cells (BJELR) to TRAIL induces fractional killing (Figure 1a-b). Furthermore, phosphorylation levels of Erk1/2, p38 MAPK, Akt and I κ B α increased at early time points after exposure to this cytokine indicating that activation of non-apoptotic cascades takes place within the initial cell population (Figure 1c). Notably, an increased apoptotic rate in response to TRAIL is observed in BJELR cells pre-treated either with MEK1/2 (U0126), PI3 kinase (LY294002) or p38MAP kinase (PD169316) inhibitors blocking Erk1/2, Akt and p38 MAPK phosphorylation, respectively (Figure 1d, Supplementary Figure 1a). In contrast, inhibiting NF- κ B signaling by pre-incubating BJELR cells with Bay 11-7082 (IKK inhibitor) did not modify the apoptotic response to this ligand (Figure 1d, Supplementary Figure 1a). Collectively, these results indicate that activation of NF- κ B signaling does not play a key role in cell survival following TRAIL challenge, whereas activation of Erk1/2, p38MAPK and Akt cascades support fractional killing. Finally, pre-incubation of BJELR cells with pan-caspase inhibitors (zVAD.fmk) resulted in a complete block of cell death in response to TRAIL whereas the activation of non-apoptotic signaling remains unaffected (Figure 1e-f). These results support that the triggering on non-apoptotic cascades is independent of caspase activity in our experimental model.

TRAIL induces the assembly of pro-apoptotic and pro-survival platforms in cancer cells displaying fractional killing

To further elucidate the molecular basis underlying fractional killing in BJELR cells, we set out to identify the molecular platforms assembled in response to TRAIL. Direct immunolabeling of TRAIL receptors followed by flow cytometry showed that DR4, DR5 and DcR2 were expressed at the cell surface, whereas surface expression of DcR1 was not detected (Figure 2a). Furthermore, immunoprecipitation (IP) assays targeting either DR4, DR5 or DcR2 indicated that these receptors form heterocomplexes upon TRAIL treatment, which in turn recruit FADD, cFlip and Caspase 8 (Figure 2b).

The induction of non-apoptotic signaling in response to TRAIL has been previously ascribed to the formation of a “secondary complex” composed by FADD, Caspase 8 as well as RIPK1, TRAF2, TRADD and NEMO⁹. Consequently, we performed IP experiments targeting DR5 and analyzed the co-immunoprecipitation of components of the secondary complex previously described. Our results indicated that both RIPK1 and TRAF2 are recruited to the DISC in BJELR cells following TRAIL challenge, which was confirmed by TRAF2 IP assays (Figure 2c and Supplementary Figure 1b). Moreover, we observed that TRAIL induces the co-IP of RIPK1, TRAF2 and FADD with Caspase 8 in this cellular system (Figure 2d). Altogether, these results support that both pro-apoptotic and pro-survival platforms are assembled in BJELR populations that display fractional killing in response to this tumor-selective ligand.

Role of TRAIL-Rs and TRAIL-Rs binding proteins in TRAIL-induced apoptosis

Our results showed that FADD, Caspase 8, cFlip, RIPK1 and TRAF2 are recruited to the DISC in response to TRAIL treatment. Thus, we set out to shed light into the role of these DISC members in TRAIL-induced apoptosis in our cellular model. For that, each of the aforementioned proteins was knocked down (siRNA-mediated approaches), cells were further challenged with TRAIL, and apoptosis was assessed as the percentage of cells displaying positive labeling for cleaved PARP. Our results show that DR4 knock down does not affect the apoptotic rate in response to this cytokine, whereas DcR2 downregulation enhanced apoptosis and DR5 depletion resulted in a major block of TRAIL induced cell death (Figure 3a). Together, these results support that DR4 does not play a key role in apoptosis induced by this ligand and demonstrate that DcR2 and DR5 are the main “decoy” and “death” receptors, respectively, in BJELR cells. Furthermore, FADD and Caspase 8 knock down blocked TRAIL-induced apoptosis indicating that these are the key adaptor protein and initiator caspase, respectively. Moreover, cFlip depletion increased the apoptotic rate following TRAIL challenge, highlighting the anti-apoptotic function of cFlip in our cellular model (Figure 3b). Similarly, siRNA-mediated downregulation of RIPK1 and TRAF2 increased cell death in response to TRAIL, supporting a pro-survival function of these two proteins in BJELR cells (Figure 3b). In that regard it has been reported that, based on its E3 ligase activity, TRAF2 leads to RIPK1 K63 ubiquitination enabling its anti-apoptotic function whereas de-ubiquitination of RIPK1 switches its function from pro-survival to pro-death³²⁻³⁶. Along those lines, a similar sensitization to TRAIL-induced cell death was observed in TRAF2-RIPK1 double knock down cells as compared to TRAF2 knock down cells, supporting the notion that the increased apoptotic rate upon TRAF2 depletion does not arise from a switch of RIPK1 function from pro-survival to pro-death (Figure 3c). Finally, a complete block of TRAIL-induced apoptosis was observed by pre-incubation of TRAF2, RIPK1 and TRAF2-RIPK1 knock down cells with pan-caspase inhibitors as well as in TRAF2-DR5 and RIPK1-DR5 double knock down cells indicating that, as in wild type conditions, the cell death process is caspase-dependent and DR5 mediated (Figure 3c-d).

Role of TRAIL-Rs and TRAIL-Rs binding proteins in TRAIL-induced non-apoptotic signaling

Next we analyzed the functional role of TRAIL-R binding proteins in the activation of non-apoptotic cascades in BJELR cells. Our data revealed that TRAF2 depletion does not affect the activation of non-apoptotic cascades (Figure 4a) whereas RIPK1, FADD and Caspase 8 are required for the triggering of $\text{I}\kappa\text{B}\alpha$ and p38MAPK phosphorylation following TRAIL challenge (Figure 4a-c). Moreover, TRAIL-induced Erk1/2 phosphorylation was not affected by RIPK1 downregulation whereas the activation of this signaling cascade was delayed upon FADD knock down and abolished under conditions of Caspase 8 depletion (Figure 4a and 4d-e). Finally, TRAIL-induced Akt phosphorylation took place independently of FADD, Caspase 8, RIPK1 and TRAF2 (Figure 4a-c). Taken together, our results support that TRAIL-induced Erk1/2, p38 MAPK, and NF κ B activation relies on components of the canonical DISC and/or TRAIL-induced pro-survival complex formation whereas yet unidentified TRAIL induced signaling platforms are involved in Akt activation in our experimental model.

As previously reported, TRAIL induces the formation of DcR2-DR4-DR5 heterocomplexes in BJELR stepwise tumorigenic cells (Figure 2b). Our results showed that non-apoptotic signaling cascades were activated in DR4 depleted cells following TRAIL challenge (Figure 5a) ruling out a key role of this receptor in TRAIL-induced non-apoptotic signaling. On the other hand, following our initial observation that DcR2 knock down led to an increased rate of TRAIL-induced cell death (Figure 3a), we hypothesized that this decoy receptor may prevent apoptosis by supporting the activation of pro-survival pathways upon TRAIL challenge³⁷. To test this hypothesis, we generated BJELR DcR2 knock out cells and evaluated the activation of non-apoptotic cascades by analyzing the phosphorylation levels of Erk1/2, p38 MAPK, Akt and $\text{I}\kappa\text{B}\alpha$ in response to TRAIL treatment. As observed in wild type cells, TRAIL induced the activation of Erk1/2, p38MAPK and NF- κ B in DcR2 KO cells indicating that this decoy receptor is not essential for the triggering of these non-apoptotic signaling cascades (Figure 5b-c). In contrast, we observed that the early wave of Akt activation was blocked in DcR2 KO cells (Figure 5b-c). In that regard, our results show that blocking Akt signaling increases apoptosis in response to TRAIL treatment, suggesting that this pathway supports cell survival (Figure 1d and Supplementary Figure 1a). Taken together, these results suggest that the anti-apoptotic role of DcR2 relies –at least partially– on the modulation of Akt activation. Interestingly, we observed that DR5 is key for the triggering of non-apoptotic pathways in response to TRAIL challenge in our experimental model. Indeed, this was validated by different experimental approaches. First, diminishing DR4 and DcR2 levels by siRNA mediated knock down followed by exposure to TRAIL resulted in the activation of non-apoptotic cascades (Figure 6a). Moreover, treating wild type cells with TRAIL mimetic peptides targeting DR5³⁸ activated non-apoptotic pathways (Figure 6b). Finally, activation of Erk1/2, p38 MAPK, NF- κ B and late wave of Akt signaling was blocked in DR5 knock out cells exposed to TRAIL treatment (Figure 6c). Collectively these results support the notion that, besides being the key pro-apoptotic receptor in our cellular model, DR5 does also mediate the triggering of non-apoptotic cascades following TRAIL challenge.

Figures legends

Figure 1: TRAIL induces apoptotic and non-apoptotic pathways in BJELR cells. **a-** Apoptotic rate in populations of BJELR cells in response to TRAIL. Cell death in untreated BJELR cells (“control”) and cell death after 6 h of treatment with 1µg/ml of TRAIL (“TRAIL”) is displayed. Apoptosis was analyzed by flow cytometry as the percentage of cells displaying positive labeling for cleaved caspase 8. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. **b-** Apoptotic rate in populations of BJELR cells in response to TRAIL. Cell death in untreated BJELR cells (“control”) and cell death after 6 h of treatment with 1µg/ml of TRAIL (“TRAIL”) is displayed. Apoptosis was analyzed by flow cytometry as the percentage of cells displaying a positive labeling for cleaved PARP. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. **c-** Phosphorylation levels of Erk1/2, Akt, p38MAPK and a regulator of NF-κB signaling (IκBα) in BJELR cells upon TRAIL treatment. BJELR cells were either left untreated (“0”) or treated with TRAIL (1µg/ml) for 1, 2, 3, 4, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and IκBα (Ser32/36) were analyzed by Western blot. α-Tubulin, loading control. Image corresponds to one representative experiment out of at least five independent biological replicates. **d-** Requirement of Erk1/2, Akt and p38MAPK and NF-κB signaling for cell survival upon TRAIL challenge. BJELR cells were either treated for 1 h with 20µM U0126 MEK1/2 inhibitor, 50µM LY294002 PI3 kinase inhibitor, 25µM PD169316 p38MAP kinase inhibitor, 40µM Bay 11-7082 NF-κB inhibitor or vehicle (“DMSO”). Apoptosis in untreated samples (control) and upon 5h of treatment with 1µg/ml of TRAIL (TRAIL) was analyzed by flow cytometry as the percentage of cells with positive labeling for cleaved PARP. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. **e-** Role of caspases cleavage in the activation of non-apoptotic signaling. BJELR cells were either left untreated (“mock”), pretreated with pan-caspase inhibitor (“zVAD”) or vehicle (“DMSO”) for 1h and then either left untreated (“0”) or treated with TRAIL (1µg/ml) for 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308), p38MAPK (Thr180/Tyr182) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates. **f-** Role of caspases cleavage in the induction of apoptosis. BJELR cells were either pretreated with pan-caspase inhibitor (“zVAD”) or vehicle (“DMSO”) for 1h and then either left untreated (“control+DMSO”, “control+zVAD”) or treated with TRAIL (1µg/ml) for 6h (“TRAIL+DMSO”, “TRAIL+zVAD”). Apoptosis was analyzed by flow cytometry as the percentage of cells displaying a positive labeling for cleaved PARP. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates.

Figure 2. Apoptotic and non-apoptotic platforms are assembled in BJELR cells in response to TRAIL. **a-** Surface levels of TRAIL-R in BJELR cells. Surface level of Death Receptor 5 (“anti-DR5”), Death Receptor 4 (“anti-DR4”), Decoy Receptor 1 (“anti-DcR1”) and Decoy Receptor 2 (“anti-DcR2”) were analyzed by flow cytometry. Isotypic IgG1 or IgG2B labeling was used as control for background fluorescence. **b-** DISC composition in BJELR cells. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 min (“+”). Immunoprecipitation of Death Receptor 5 (DR5-IP), Death Receptor 4 (DR4-IP), and Decoy Receptor 2 (DcR2-IP) was performed and co-immunoprecipitation of DISC components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was

used as background control. Image corresponds to one representative experiment out of three independent biological replicates. **c-** Co-immunoprecipitation of RIPK1 and TRAF2 with DR5 upon TRAIL treatment. BJELR cells were either left untreated (“-”) or challenged with TRAIL (1µg/ml) for 30 (“+”) min. Immunoprecipitation of DR5 (“DR5 IP”) was performed and co-immunoprecipitation of DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. Image corresponds to one representative experiment out of three independent biological replicates. **d-** TRAIL-induced co-immunoprecipitation of caspase 8 with RIPK1 and TRAF2. BJELR cells were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 (“30”) min. Immunoprecipitation of caspase 8 (“c8 IP”) was performed and co-immunoprecipitation of DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control.

Figure 3. Role of the DISC and secondary complex components in TRAIL-induced apoptosis. **a-** Impact of TRAIL-R knock down on TRAIL-induced apoptosis in BJELR cells. BJELR cells were transfected either with siRNAs targeting *DR4* (“siDR4”), *DR5* (“siDR5”) or *DcR2* (“siDcR2”) mRNA or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection cell populations were either left untreated (“control”) or challenged with 1µg/ml TRAIL (“TRAIL”) during 1h and the percentage of cells with positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least three independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. Efficiency of DR4, DR5 and DcR2 knock down at 48 h post-transfection was analyzed by Western blot. α -Tubulin, loading control. **b-** Impact of FADD, caspase 8, RIPK1, TRAF2, cFlip knock down on TRAIL-induced apoptosis in BJELR cells. BJELR cells were transfected either with siRNAs targeting *FADD* (“si FADD”), *caspase 8* (“si caspase8 ”), *RIPK1* (“si RIPK1”), *TRAF2* (“si TRAF2”) or *cFlip* (“si cFlip”) mRNA or non-targeting scramble siRNAs (“scr”). Forty-eight hours post-transfection cell populations were either left untreated (“control”) or challenged with 1µg/ml TRAIL (“TRAIL”) during 1h and the percentage of cells with positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least five independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. **c-** Impact of TRAF2 and RIPK1 knock down on TRAIL-induced apoptosis. BJELR cells were transfected either with siRNAs targeting *RIPK1* (“si RIPK1”), *TRAF2* (“si TRAF2”), *RIPK1/TRAF2* (“si RIPK1/TRAF2”) mRNA or non-targeting scramble siRNAs (“scr 30” and “scr 60”). Forty-eight hours post-transfection cell populations were either left untreated or pretreated with zVAD (“zVAD”) for 1h; following, cells were either left untreated (“control” and “control+zVAD”) or challenged with 1µg/ml TRAIL (TRAIL) during 1h (“TRAIL” and “TRAIL+zVAD”) and the percentage of cells with positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least five independent biological replicates. Statistical significance was calculated by applying two-tailed, unpaired Student’s t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. Efficiency of RIPK1, TRAF2 and RIPK1/TRAF2 knock down at 48 h post-transfection was analyzed by Western blot. α - Tubulin, loading control. **d-** Impact of RIPK1 and TRAF2 knock down on TRAIL-induced signaling. BJELR cells were transfected either with siRNAs targeting *RIPK1* (“si RIPK1”), *TRAF2* (“si TRAF2”), *DR5* (“si DR5”), *DR5/TRAF2* (“si DR5/TRAF2”), *DR5/RIPK1* (“si DR5/RIPK1”) mRNA or non-targeting scramble siRNAs (“scr 30” and “scr 60”). Forty-eight hours post-transfection cell populations were either left untreated (“control”) or challenged with 1µg/ml TRAIL during 1h (“TRAIL”) and the percentage of cells with positive labeling for cleaved PARP was analyzed by flow cytometry. Histogram represents the mean +/- Standard Deviation (SD) from at least five independent biological replicates. Statistical significance was calculated

by applying two-tailed, unpaired Student's t-test, ****P value*< 0.0005, ***P value*<0.005, **P value*<0.05. Efficiency of RIPK1, TRAF2, DR5, DR5/TRAF2 and DR5/RIPK1 knock down at 48 h post-transfection was analyzed by Western blot. α - Tubulin, loading control.

Figure 4. Role of the DISC and secondary complex components in TRAIL-induced non-apoptotic pathways.

a- Impact of RIPK1 and TRAF2 knock down on the activation of Erk1/2, Akt, p38 MAPK and the regulator of NF- κ B signaling (I κ B α) in response to TRAIL. BJELR cells were transfected either with siRNAs targeting *RIPK1* ("si RIPK1") or TRAF2 ("si TRAF2") mRNA or non-targeting scramble siRNAs ("scr"). Forty-eight hours post-transfection BJELR cells were pretreated with pan-caspase inhibitor (zVAD) for 1h and then either left untreated ("0") or treated with TRAIL (1 μ g/ml) during 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent replicates. **b-** Impact of FADD knock down on TRAIL-induced phosphorylation of Akt, p38MAPK and regulator of NF- κ B signaling (I κ B α). BJELR cells were transfected either with siRNAs targeting *FADD* mRNA ("si FADD") or non-targeting scramble siRNAs ("scr"). Forty-eight hours post-transfection BJELR cells were either left untreated ("0") or treated with TRAIL (1 μ g/ml) during 6h or 16h. Apoptotic cells were discarded by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates. **c-** Impact of caspase 8 knock down on the induction of Akt, p38MAPK and regulator of NF- κ B signaling (I κ B α). BJELR cells were transfected either with siRNAs targeting *caspase 8* mRNA ("si Casp8") or non-targeting scramble siRNAs ("control"). Forty-eight hours post-transfection BJELR cells were either left untreated ("0") or treated with TRAIL (1 μ g/ml) during 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Akt (Thr308), p38MAPK (Thr180/Tyr182) and I κ B α (Ser32/36) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent replicates. **d-** Impact of FADD knock down on the phosphorylation level of Erk1/2 in response to TRAIL. BJELR cells were transfected either with siRNAs targeting *FADD* mRNA ("si FADD") or non-targeting scramble siRNAs ("scr"). Forty-eight hours post-transfection BJELR cells were either left untreated ("0") or treated with TRAIL (1 μ g/ml) during 1, 2, 3, 4, 6h or 16h. Apoptotic cells were discarded by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated level of Erk1/2 (Thr202/Tyr204) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates. **e-** Impact of caspase 8 knock down on the phosphorylation level of Erk1/2 in response to TRAIL. BJELR cells were transfected either with siRNAs targeting *caspase 8* mRNA ("si Caspase 8") or non-targeting scramble siRNAs ("scr"). Forty-eight hours post-transfection BJELR cells were either left untreated ("0") or treated with TRAIL (1 μ g/ml) during 1, 2, 3, 4, 6h or 16h. Apoptotic cells were discarded by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated level of Erk1/2 (Thr202/Tyr204) were analyzed. α - Tubulin, loading control. Image corresponds to one representative experiment out of three independent replicates.

Figure 5. Role of DcR2 and DR4 in the regulation of TRAIL signaling. **a-** Requirement of DR4 for the activation of non-apoptotic pathways. Total and phosphorylated levels of Erk1/2, Akt and total level of I κ B α in transformed cells surviving TRAIL DR4 were analyzed. BJELR cells were transfected either with siRNAs targeting *DR4* ("si DR4") or non-targeting scramble siRNAs ("scr"). Forty-eight hours post-transfection

BJELR cells were either left untreated ("0") or treated with TRAIL (1 µg/ml) during 6h or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates. Efficiency of DR4 knock down at 48 h post-transfection was analyzed by Western blot. α-Tubulin, loading control. **b and c** – Role of DcR2 in the triggering of non-apoptotic signaling. BJELR wild type and BJELR DcR2 knock out cells clone 2A (**a**) and clone 4D (**b**) were either left untreated ("0" – collected just before the treatment and "0*" – collected 16h after the beginning of exposure to TRAIL in TRAIL treated samples) or treated with TRAIL (1 µg/ml) for 1, 2, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.

Figure 6. Role of DR5 in the activation of non-apoptotic pathways. **a**- Requirement of TRAIL receptors for the activation of non-apoptotic pathways. Total and phosphorylated levels of Erk1/2, Akt and the regulator of NF-κB signaling (IκBα) in transformed cells surviving TRAIL or DR5-specific ligand-M1D-treatment upon DR4/DcR2 and DR5/DcR2 double knock down were analyzed. BJELR cells were transfected either with siRNAs targeting *DR4* and *DcR2* ("DR4/DcR2 KD") or *DR5* and *DcR2* mRNA ("DR5/DcR2 KD") or non-targeting scramble siRNAs ("scr"). Forty-eight hours post-transfection BJELR cells were either left untreated ("0") or treated with TRAIL (1 µg/ml) or M1D (10 µM) during 6h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were harvested for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Thr308) and total level of IκBα were analyzed. α-Tubulin, loading control. Efficiency of DR4/DcR2, DR5/DcR2 double knock down at 48h post-transfection was analyzed by Western blot. α-Tubulin, loading control. **b** – Impact of M1D (DR5 specific ligand) treatment on the activation of non-apoptotic pathways. BJELR cells were either left untreated ("0") or treated with M1D (10 µM) for 6 or 16h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Image corresponds to one representative experiment out of at least three independent biological replicates **c**- Impact of DR5 knock out on the phosphorylation level of Erk1/2, Akt, p38MAPK and the regulator of NF-κB signaling (IκBα) in response to TRAIL. BJELR wild type and BJELR DR5 knock out cells were either left untreated ("0" – collected just before the treatment and "0*" – collected 16h after the beginning of exposure to TRAIL in TRAIL treated samples) or treated with TRAIL (1 µg/ml) for 2, 6 or 16 h. Apoptotic cells were removed by successive washes and non-apoptotic attached cells were collected for Western blot. Total and phosphorylated levels of Erk1/2 (Thr202/Tyr204), Akt (Ser 473), Akt (Thr 308), p38MAPK (Thr180/Tyr182) and IκBα (Ser32/36) were analyzed. α-Tubulin, loading control. Image corresponds to one representative experiment out of three independent biological replicates.

Supplementary figure 1. **a**- Impact of U0126 MEK1/2, LY294002 PI3K, PD169316 p38MAPK and Bay 11-7082 NF-κB inhibitors on the phosphorylation level of Erk1/2, Akt, p38MAPK and IκBα. Western blot assays of total Erk1/2, Akt, p38MAPK, IκBα and phosphorylated Erk1/2 (pErk1/2; Thr202/Tyr204), Akt (Thr 308), Akt (Ser 473), p38MAPK (Thr180/Tyr182), IκBα (Ser32/36) in BJELR cells treated with 20 µM U0126 MEK1/2, 50 µM LY294002 PI3K, 25 µM PD169316 p38MAPK, 25 µM Bay 11-7082 NF-κB inhibitors or vehicle (DMSO) during 1 or 5 h. α-Tubulin, loading control. **b**- Co-immunoprecipitation of DR5 and RIPK1 with TRAF2 in TRAF2 IP upon TRAIL treatment. BJELR cells were either left at 37°C or placed at 4°C for

15 min. Further, BJELR cells kept at 37°C and 4°C were either left untreated (“0”) or challenged with TRAIL (1µg/ml) for 30 min (“30”) or 3h (“3h”). Immunoprecipitation of TRAF2 (“TRAF2 IP”) was performed and co-immunoprecipitation of the DISC and secondary complex components was analyzed by Western blot. Immunoprecipitation using isotypic IgG1 (“IgG1”) was used as background control. Image corresponds to one representative experiment out of three independent biological replicates.

Materials and methods.

Antibodies. Antibodies used for Western blot were purchased from Cell Signaling (p38MAPK 9212, p-p38MAPK (Thr180/Tyr182) 9211, DR5 3696, DcR2 8049, DcR1 4756, p44/42 MAPK 9102, p-p44/42 MAPK (Thr202/Tyr204) 9101, Akt 9272, pAkt (Ser473) 9271, pAkt (Thr308) 4056, plkBα (Ser32/36) 9246, IκBα 4814, PARP 9542, IKKγ 2685, IKKγ 2695, caspase-8 9746, RIPK1 3493, TRAF2 4724, Anti-mouse IgG – HRP-linked antibody 7076, Anti-rabbit IgG – HRP-linked antibody 7074), Santa Cruz Biotechnology (TNFR1 sc-8436, β-actin sc-1615, TRAF2 sc-7187, α-tubulin sc-32293), Millipore (DR4 AB16955, mouse anti-rabbit light chain specific HRP conjugated monoclonal antibody MAB201P), BD Pharmingen (FADD 556402), AdipoGen (cFlip AG-20B-0056-C100). Antibodies for DR5 (AF631), DR4 (AF347), DcR2 (AF633), TRAF2 (AF3277) immunoprecipitation and normal goat IgG (AB-108-C) were purchased from R and D systems, for caspase 8 immunoprecipitation (sc-6136) - from Santa Cruz Biotechnology, for RIPK1 (51-6559GR) immunoprecipitation - from BD Pharmingen, normal mouse IgG – from Jackson ImmunoResearch (015-000-003). For flow cytometry assays, Cleaved caspase 8 (12602), Cleaved PARP (6987), Cleaved PARP (8978) from Cell signaling, Mouse IgG1 (IC002G), Mouse IgG2B (IC0041G), DcR1 (FAB6302P), DcR2 (FAB633G), DR5 (FAB6311G) from R and D systems, DR4 (804-297TD-T100) from Enzo Life Sciences.

Inhibitors. P38 MAPK inhibitor PD 169316 (513030) was purchased from Calbiochem, MEK inhibitor U0126 (V112A) – in Promega, PI3K inhibitor LY294002 (9901) – from Cell Signaling, inhibitor of IκBα phosphorylation Bay-11-7082 (B5556) – from Sigma, General caspase 8 inhibitor Z-VAD-FMK (FMK001) – from R and D systems.

Cell lines. BJELR cells from stepwise tumorigenesis systems (BJ-derived model) were cultured in DMEM (1 g/l glucose) + Med 199 (4 : 1)+10% FCS heat-inactivated.

siRNA-mediated knock down. siRNA smartpools or single siRNA used for the knock down experiments were purchased from Dharmacon. For DR4 knock down pool of two individual siRNA – J-008090-09-0005 and J-008090-08-0005 was used. DR5 knock down was achieved utilizing pool of three single siRNA – J-004448-06-0005, J-004448-07-0005 and J-004448-08-0005. DcR2 knock down was performed using individual siRNA J-008092-14. Smartpool siRNA were used for RIPK1 – L-0044445-00-0005, TRAF2 – L-005198-00-0005 and FADD – L-003800-00-0005 knock down. For caspase 8 knock down individual siRNA – J-003466-16 was utilized. TNFR1 KD was performed using J-005197-08-0005. These siRNA were transfected into BJELR cells using RNAi Max Lipofectamine (13778150, Invitrogene) following vendor’s instruction. The concentration of siRNA used for the knock down of each protein was optimized for each case. 24 hours post-transfection medium was replaced and cells were incubated for additional 24h. 48h post-transfection cells were used for experiments.

CRISPR-Cas9 mediated knock out of DR5 and DcR2. BJELR cells were transfected with 200ng/ml of DR5 double nickase plasmid (Santa Cruz, sc-4010002-NIC), DcR2 double nickase plasmid (Santa Cruz,

sc-403389-NIC) or control double nickase plasmid (Santa Cruz, sc-437281-NIC) using Lipofectamine LTX (15338100, Invitrogen). Each double nickase plasmid contains a pair of plasmids each of which leads to the single strand nick, while both of them generate double strand break. Double strand breaks can be repaired by either homology directed repair or non-homologous end joining leading to the frame shift mutations. Consequently, 24h post-transfection single cells were seeded in 96 well plates using fluorescent-activated cell sorting technique (FACS) based on the GFP expression in transfected cells. After 2-3 weeks single cell clones were lysed and analyzed by Western blot for the presence or absence of DR5 or DcR2. Moreover, the CRISPR-Cas9 target sites in DR5 and DcR2 knock out positive clones were analyzed by sequencing in order to characterize mutations induced by CRISPR-Cas9.

Apoptosis measurement. Apoptotic cell death was determined by flow cytometry approach by measuring the percentage of cells displaying positive staining for cleaved PARP or cleaved caspase 8. For that BJELR cells populations were plated in a confluency 1.5×10^5 / well of 6 well plates. Forty-eight hours later cells were either left untreated or treated with TRAIL for different time points depending on the experiment. Further, cells were fixed followed by blocking with 0.5% BSA in PBS for 1h and staining with anti-cleaved PARP or anti-cleaved caspase 8 antibodies for 1h at RT. Concentration of the antibodies used for staining was optimized for each antibody and according to the number of cells used for each labeling. Results presented in the histograms derived from at least three independent biological replicates.

Surface levels of TRAIL receptors. Expression of TRAIL receptors in the surface of BJELR cells was measured by flow cytometry approach. Cells were trypsinized and counted. 1×10^5 cells were transferred into the FACS tubes and incubated with the blocking solution (5% FCS in PBS) for 1h on ice. Further cells were subjected to the staining with antibodies recognizing DR4, DR5, DcR1 and DcR2. Appropriate IgG isotypes were used as background control. Each sample was additionally labeled with DAPI to detect permeabilized cells. Consequently, expression of TRAIL receptors was measured only in alive DAPI negative cells.

Immunoprecipitation. BJELR cells were plated in a confluency 1×10^6 / petri dish. Forty-eight hours later cells were either left untreated or treated with TRAIL at regular growing conditions (37°C, 5% CO₂) or at 4°C for different time points depending on the experiment. Cells were washed with 1xPBS and lysed in 1ml (for 20×10^6 cells) of freshly prepared lysis buffer (30mM Tris-HCl pH7.4, 150mM NaCl, 5mM KCl, 10% glycerol, 2mM EDTA pH8.0) supplemented with EDTA-free protease inhibitor cocktail (Roche, 11873580001), phosphatase inhibitor (PhosStop, Roche, 04906845001) and 1% Triton-X100 (BioRad)). Lysates were incubated on ice for 30 min followed by 30min centrifugation at 14000 rpm at 4°C. Further lysates were pre-cleaned with equilibrated in lysis buffer Pure proteome Protein G magnetic beads (Millipore, LSKMAGG02) for 2h at 4°C and DR4, DR5, DcR2, TRAF2, caspase8 or IgG were immunoprecipitated using anti-DR4, anti-DR5, anti-DcR2, anti-TRAF2, anti-RIPK1, anti-caspase8, normal goat IgG or normal mouse IgG bound to the equilibrated in lysis buffer Pure proteome Protein G magnetic beads (in proportion - 1µg of antibodies for 2µl beads) overnight at 4°C. The amount of beads and antibodies used for DR4, DR5, DcR2, RIPK1, TRAF2 and caspase 8 immunoprecipitation was optimized for each protein according to the experimental purpose. After immunoprecipitation samples were washed 5 times with the lysis buffer and eluted. Elution was performed using 1 fold concentrated Laemmli buffer [2-fold concentrated buffer- 125mM Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 10% glycerol] and supplemented with 20mM DTT. Elution was done at 75°C for 15 min. Immunoprecipitates were subjected for the Western blot analysis.

Western blot assay. Samples for WB were collected in RIPA buffer, separated by SDS-PAGE and transferred into nitrocellulose membrane. Membranes were blocked using 5% milk in PBS+0.1% Tween 20 for 1h at RT. Further membranes were incubated with required primary antibodies (according to vendor's instructions) overnight at 4°C followed by incubation with corresponding secondary peroxidase-conjugated antibodies. Immunoreactive bands were visualized by chemiluminescence using ECL Western blotting substrate (Pierce, 32209), SuperSignal West Pico Chemiluminescent substrate (Pierce, 34080) or Luminata Forte Western HRP Substrate (Millipore, WBLUF0500) and exposed to Carestream Kodak BioMax MR film (Z350389-50EA), Amersham Hyperfilm ECL (28906835) or Amersham Hyperfilm MP (28906842). Further Western blot films were scanned and processed in Adobe Photoshop CS4. Images presented in the paper correspond to one representative experiment out of at least two biological replicates.

Statistical analysis. Results represented in the histograms show the mean value +/- Standard Deviation (SD) of at least three independent biological replicates.

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Figure 1

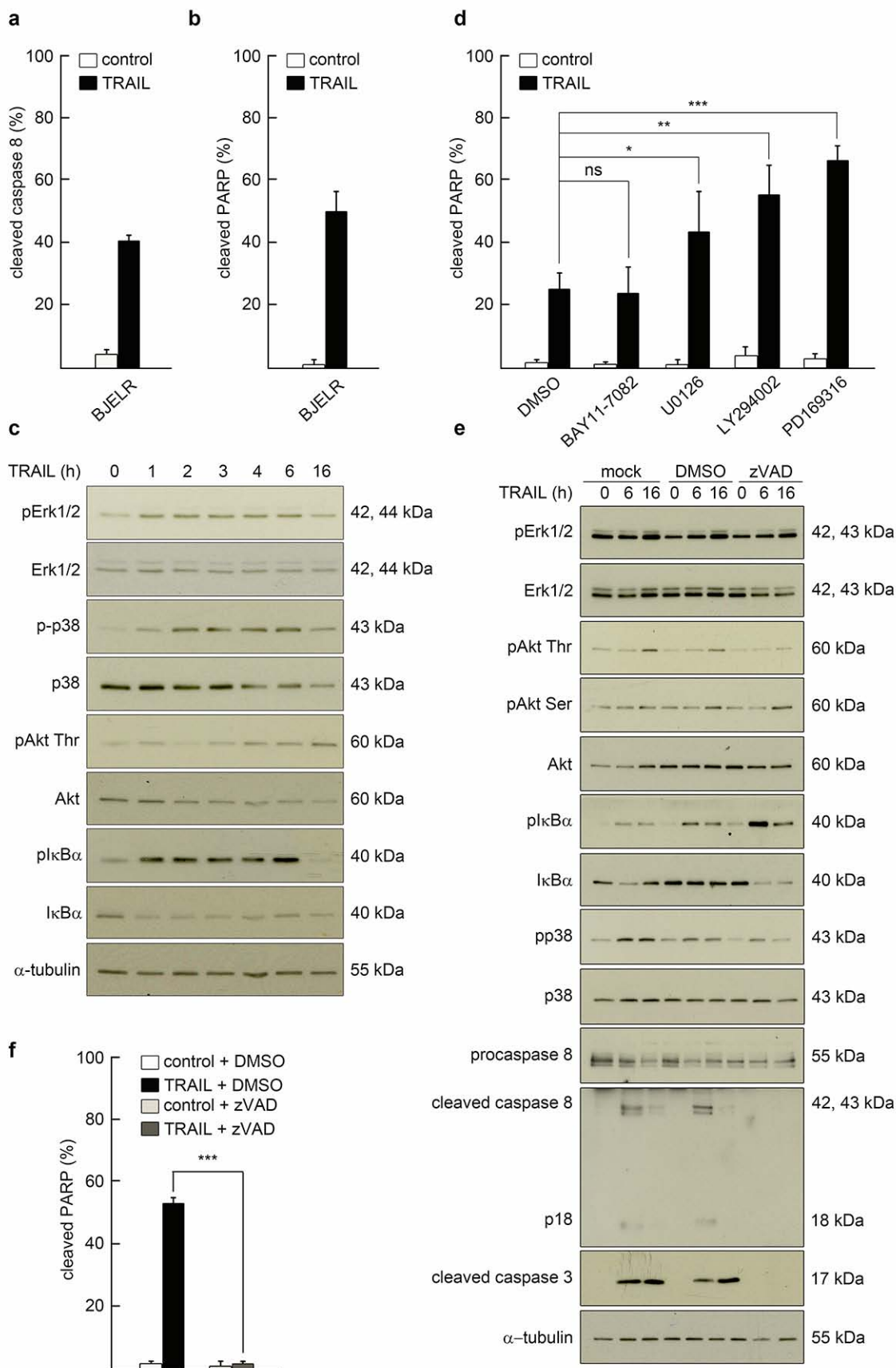
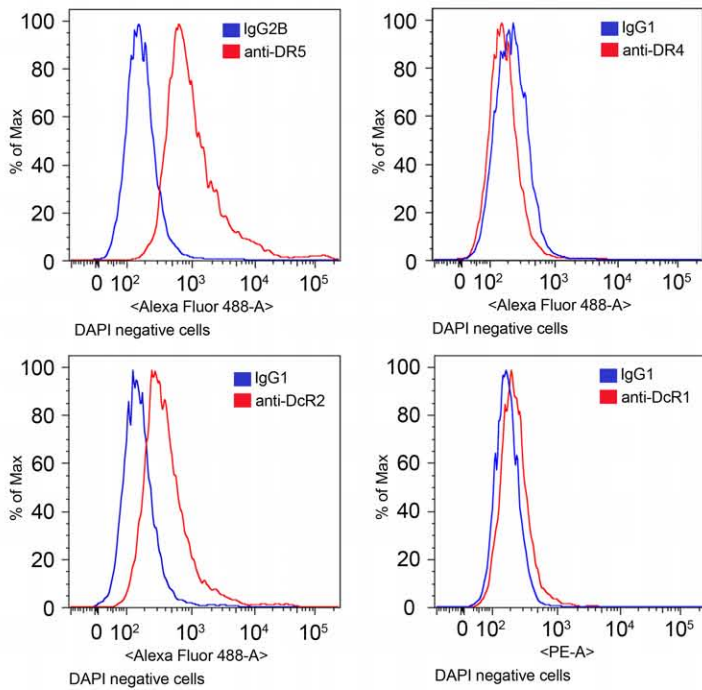
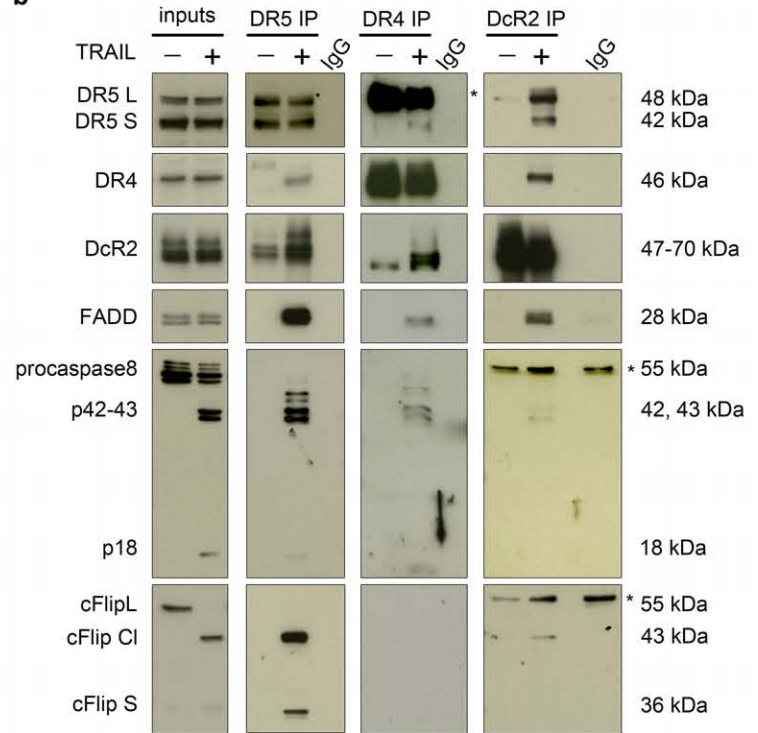


Figure 2

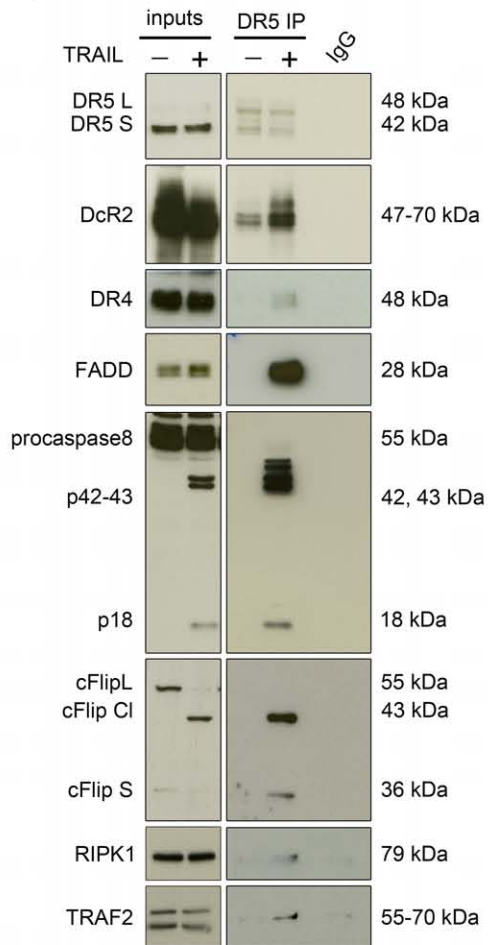
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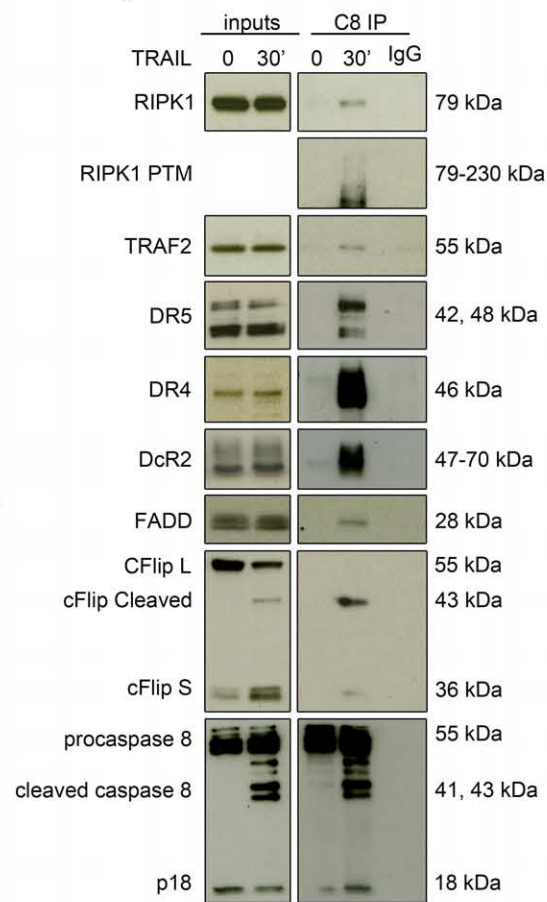


Figure 3

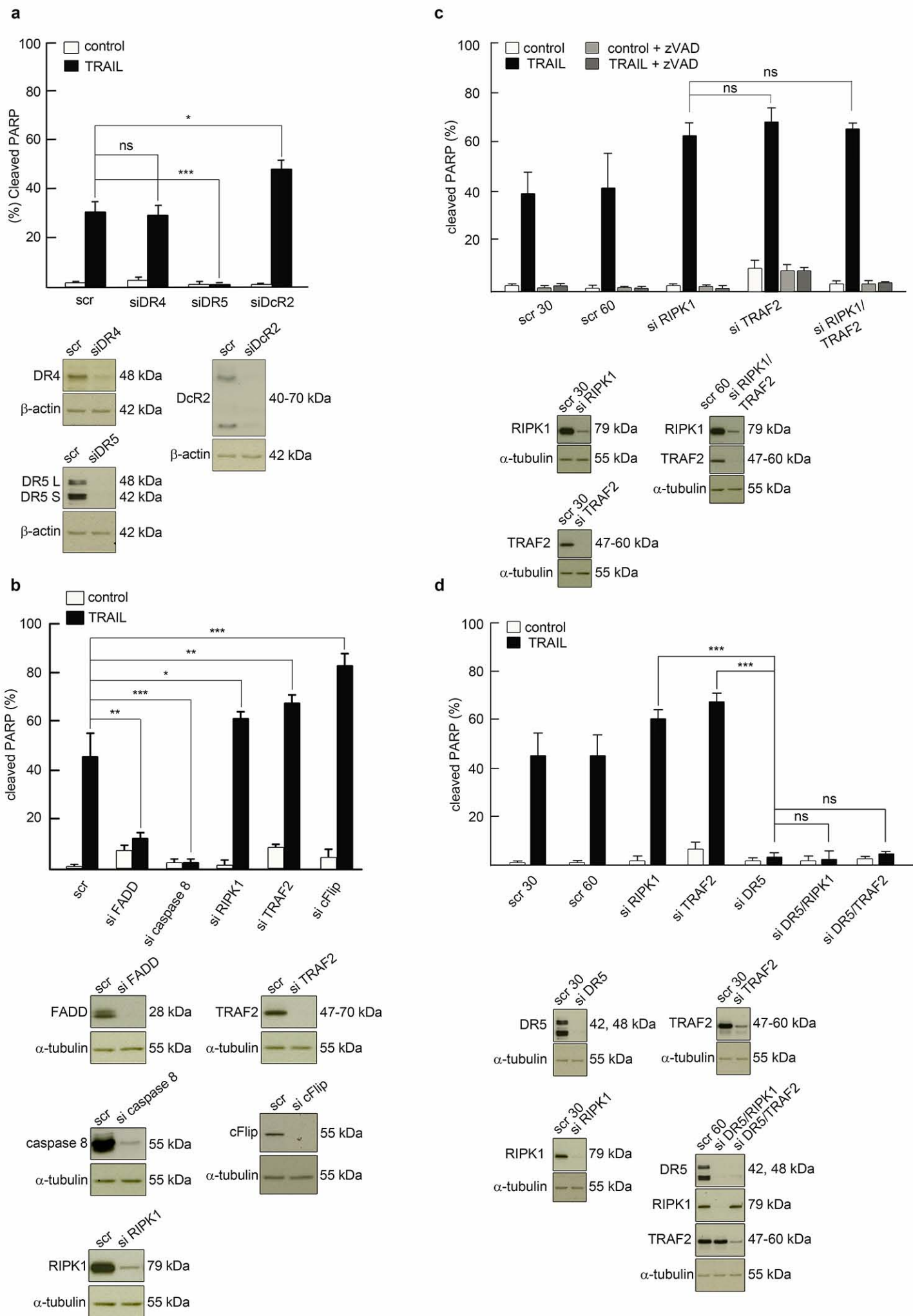


Figure 4

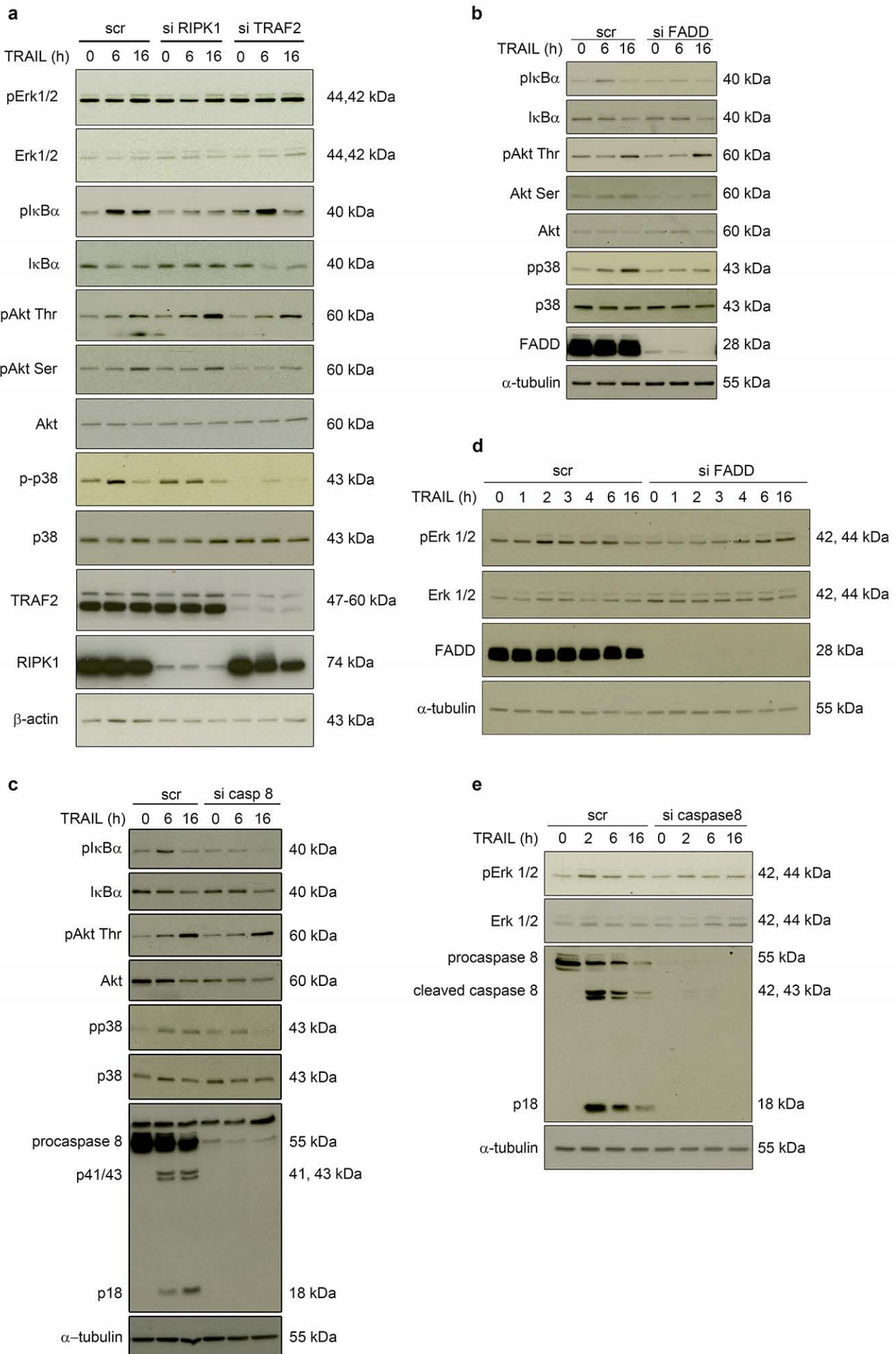
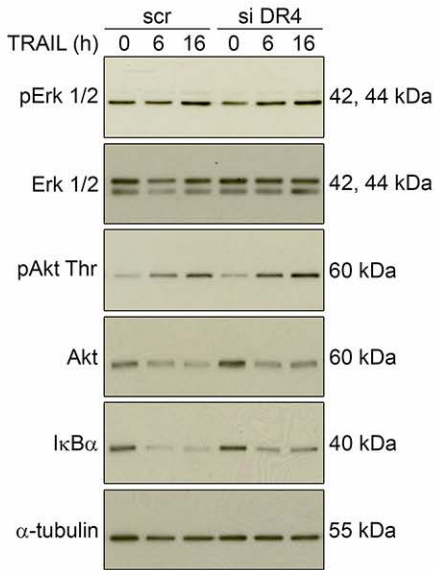
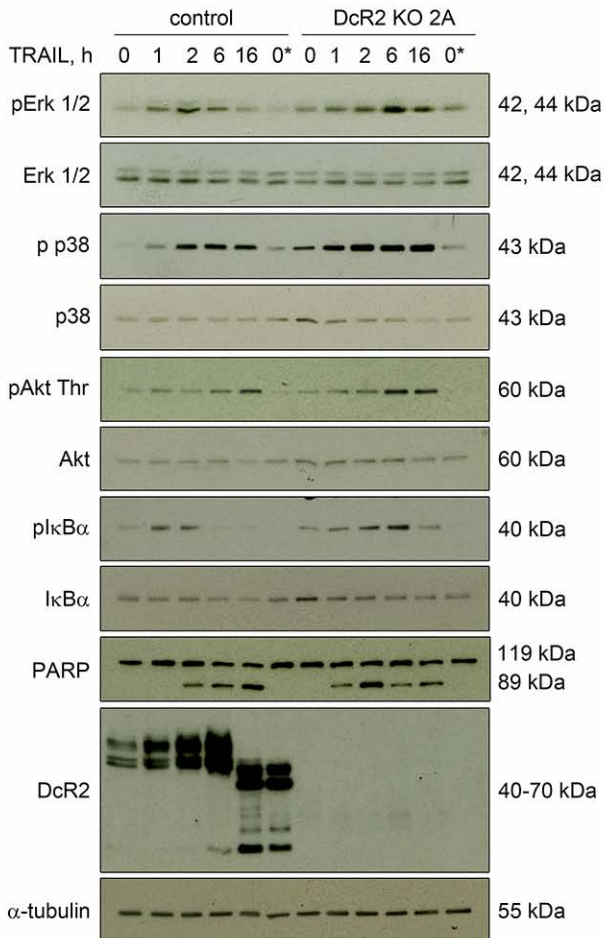


Figure 5

a



b



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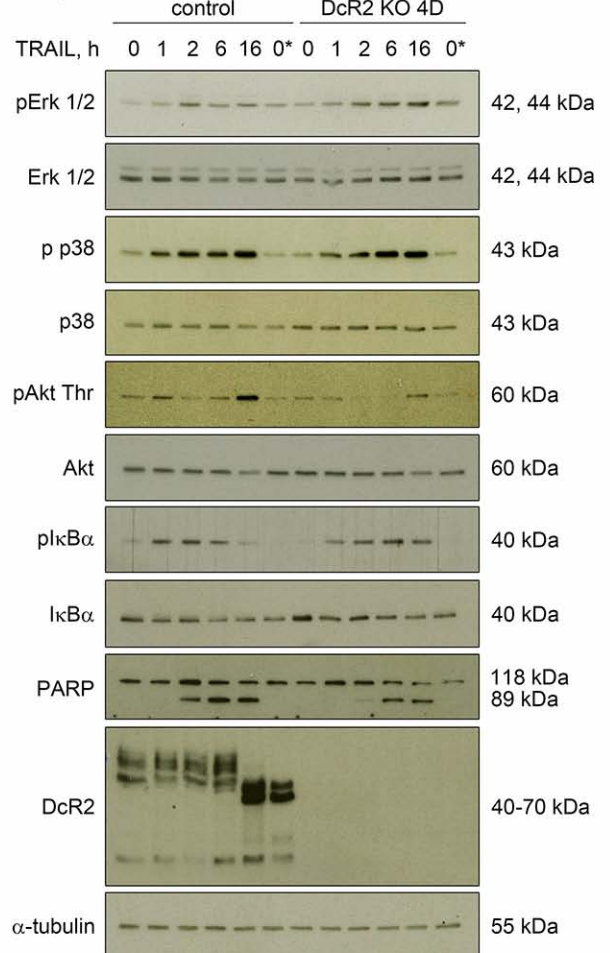
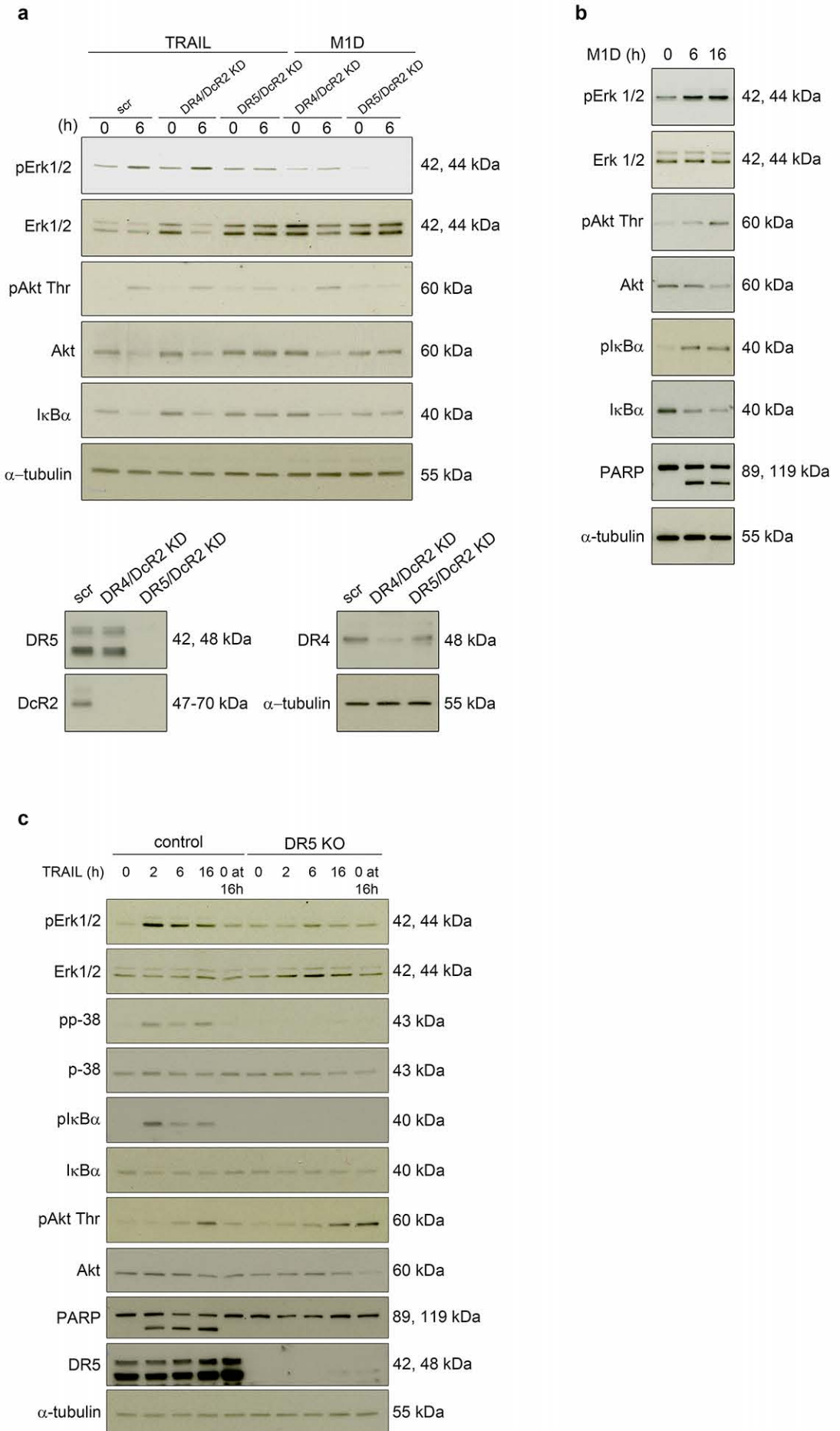


Figure 6



Role of TRAIL-receptors in apoptosis and non-apoptotic signaling in cancer cells populations undergoing fractional killing

Résumé. La spécificité cellulaire de la cytokine TRAIL (Tumor necrosis factor-Related Apoptosis-Inducing Ligand) offre une piste prometteuse pour le développement de thérapies anti-cancer ciblées. En effet TRAIL induit l'apoptose des cellules cancéreuses de manière sélective, tout en épargnant les cellules saines. Dans les cellules cancéreuses la protéine TRAIL, en se liant aux récepteurs de mort, est capable d'induire la formation d'un complexe apoptotique nommé DISC (Death-Inducing Signaling Complex). Toutefois TRAIL peut aussi activer des voies de signalisation de survie cellulaire dans des cellules cancéreuses résistantes à l'induction de la mort programmée après traitement. Les cellules cancéreuses que nous avons étudiées sont caractérisées par un phénotype de mort cellulaire fractionnée en réponse à TRAIL, c'est-à-dire que seule une fraction de la population meurt en présence de TRAIL, alors que l'autre fraction active la survie cellulaire. Dans ce contexte, les fondements moléculaires qui régissent l'activation des voies de l'apoptose ou de la survie cellulaire par TRAIL ne sont pas encore pleinement identifiés. Lors de notre étude nous avons montré que les récepteurs de TRAIL jouent un rôle particulièrement important dans l'engagement de ces cellules vers l'une ou l'autre de ces voies. Nous avons révélé que le récepteur DcR2 régule négativement l'apoptose en présence de TRAIL par la formation d'un complexe apoptotique DISC moins efficace. En parallèle, DcR2 peut aussi déclencher la première vague d'activation de la voie de signalisation Akt. Nous avons aussi démontré que le récepteur DR5 est nécessaire et suffisant pour induire l'apoptose, ainsi que plusieurs voies de signalisation non-apoptotiques (Erk1/2, p38MAPK, NF- κ B, vague d'activation tardive de la voie Akt). Des recherches plus approfondies sont aujourd'hui nécessaires afin de comprendre comment TRAIL, à travers son interaction avec un seul récepteur de mort (DR5), peut induire soit l'apoptose, soit la survie cellulaire, au sein d'une même population clonale de cellules cancéreuses. De telles recherches permettront de mieux appréhender la complexité de la signalisation TRAIL-dépendante, et de proposer des thérapies combinées qui réduiront la progression tumorale induite par TRAIL dans certaines cellules. L'association de composés inhibant l'activation des voies de signalisation pro-survie, avec les anticorps agonistes rhTRAIL/DR5, devrait permettre de promouvoir uniquement la mort cellulaire TRAIL-dépendante.

Abstract. Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) gives a promise for cancer-selective therapy as it exclusively induces apoptosis in tumor cells while sparing normal tissues. TRAIL binds to its death receptors leading to the formation of the Death-Inducing Signaling Complex (DISC) enabling apoptotic signaling activation. Importantly, TRAIL also activates pro-survival pathways in cancer cells resistant to TRAIL-induced cell death. In this study we used clonal populations of cancer cells that respond in fractional killing upon TRAIL treatment, meaning that only a fraction of a clonal cancer cell population dies while another survives, activates non-apoptotic cascades and proliferates (referred to as fractional killing). The molecular basis regulating the activation of apoptotic and survival signaling in this cellular context remains largely unknown. In that regard, we found that TRAIL receptors play an important role in the bifurcation of TRAIL signaling towards activation of death and survival pathways in populations of cancer cells that respond in fractional killing upon TRAIL challenge. We identified that DcR2 negatively regulates TRAIL-mediated cell death by the formation of a less efficient apoptotic DISC and by the activation of Akt signaling at early time points of response to TRAIL. Furthermore, we reported that a single death receptor - DR5 triggers apoptotic and non-apoptotic (Erk1/2, p38MAPK, NF- κ B and late wave of Akt) pathways. Future experiments are required to fully decorticate the molecular mechanisms underlying induction of death vs survival signaling through a single death receptor helping to better understand the complexity of TRAIL-induced signaling. These results will provide insights for evaluating whether combined therapies targeting components promoting the activation of pro-survival signaling would allow the use of rhTRAIL/DR5 agonistic antibodies to activate TRAIL-mediated killing while avoiding TRAIL-induced tumor progression.