TRAIL signalling regulation by ezrin

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TRAIL signalling regulation by ezrin

Régulation de la signalisation TRAIL par l'ezrine

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Résumé

Objectifs: La cytokine TRAIL (TNF Related Apoptosis Inducing Ligand) suscite un intérêt majeur en thérapie anti-cancéreuse grâce à sa capacité à induire l'apoptose des cellules cancéreuses tout en épargnant les cellules saines. L'association du récepteur Fas et de l'actine *via* l'ezrine, une protéine de la famille ERM (Ezrin, Moesin, Radixin), régule les premières étapes de l'induction de l'apoptose par FasL. Au cours de mon projet de thèse, nous avons voulu déterminer le rôle que pouvait jouer l'ezrine au cours de l'apoptose induite par TRAIL, dans des lymphomes B ou des cellules cancéreuses adhérentes (HeLa, HCT116 et SW480).

Matériel et Méthodes: Des approches biochimiques et moléculaires nous ont permis d'étudier et de déterminer l'implication de l'ezrine et sa phosphorylation dans la régulation de la mort induite par TRAIL.

Résultats: Ce travail démontre que l'ezrine peut réguler de manière négative l'apoptose induite par TRAIL et FasL. Cette activité inhibitrice est régulée par la phosphorylation/déphosphorylation sur la serine 66 ainsi que sur la tyrosine 353. Néanmoins cette régulation n'affecte ni la formation, ni l'activation du DISC (Death Inducing Signalling Complex). Des mutations de ces résidus par une alanine (S66A) ou un acide aspartique (Y353D) augmente sélectivement la capacité de TRAIL à induire l'apoptose. Au contraire, des mutations ponctuelles de ces résidus permettant de mimer la phosphorylation de l'ezrine sur la serine 66 (S66D) ou l'expression d'un variant non phosphorylable sur la tyrosine 353 (Y353F) protègent les cellules cancéreuses de l'apoptose induite par TRAIL. De manière concordante, l'utilisation du H89, un inhibiteur de PKA, kinase responsable de la phosphorylation de la serine 66 augmente la sensibilité des cellules cancéreuses à TRAIL, alors qu'au contraire, un activateur de PKA (8bromocyclic AMP) rend ces

mêmes cellules plus résistantes à TRAIL. Enfin, l'association de TRAIL et du cisplatine permet de dépasser l'inhibition de l'apoptose par l'ezrine.

Conclusion: L'ensemble de nos résultats démontre que la phosphorylation de l'ezrine sur la serine 66 ou la tyrosine 353 module la sensibilité des cellules cancéreuses à l'action cytotoxique de TRAIL. Cette résistance induite par l'ezrine peut cependant être dépassée en associant TRAIL au cisplatine.

Discipline: Sciences de la vie

Mots clés: Cancer, TRAIL, TRAIL-R, ezrine, actine, cytosquelette, phosphorylation, chimiothérapie

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Summary

Background and Aim: TRAIL has sparked a growing interest in oncology due to its ability to selectively trigger cancer cell death while sparing normal cells. The Fas/actin association through ezrin, a member of the ERM protein family, has been reported to regulate early steps of Fas-mediated apoptosis. In this project, we addressed the role of ezrin regarding TRAIL-induced cell death in B lymphoma cell lines, or adherent cancer cell lines (HeLa WT, HCT116, SW480).

Methods: Molecular and biochemical approaches were employed to study the relevance of ezrin and its phosphorylation status in TRAIL signaling.

Results: We found that ezrin displays a negative function towards TRAIL- and Fas-mediated apoptosis and that the ezrin-mediated TRAIL-induced cell death inhibition led to ezrin activation through phosphorylation/dephosphorylation events at serine 66 and tyrosine 353, but is mainly independent of TRAIL DISC (Death Inducing Signalling Complex) formation or activation. Mutations of these residues to alanine (S66A) or aspartic acid (Y353D) selectively enhanced TRAIL-induced cell death, whereas point mutations mimicking ezrin phosphorylation on S66 (S66D) or a nonphosphorylable variant on Y353 (Y353F) strongly protected cancer cells from apoptosis induced by TRAIL. Moreover, inhibition of the ezrin serine 66 PKA target site, using H89, increased cancer cell sensitivity to TRAIL, while treatment with 8bromocyclic AMP, a PKA activator, decreased TRAIL-induced cell death. In addition, combined TRAIL/cisplatin treatments abrogated ezrin-mediated inhibition of TRAIL-induced apoptosis.

Conclusions: Altogether our findings show that ezrin phosphorylation at serine 66 or tyrosine 353 differentially modulates cancer cell sensitivity to TRAIL-induced cell death. We also provide evidence that ezrin-mediated resistance to TRAIL can be overcome by a combined treatment cisplatin and TRAIL.

Discipline: Life Sciences

Keywords: Cancer, TRAIL, TRAIL-R, ezrin, actin cytoskeleton, posphorylation, chemotherapy

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List of abbreviations

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β2A: beta 2 adrenergic receptor

AIDS: acquired immune deficiency syndrome

AIF: apoptosis inducing factor

AKT: activated protein kinase

AMP: adenosine monophosphate

APAF: apoptotic protease activating factor

APC: adenomatous polyposis coli

APS: ammonium persulfate

ATP: adenosine-5'-triphosphate

B

8B: 8-bromoadenosine 3′,5′-cyclic monophosphate

BAD: Bcl-2 antagonist of cell death

BAK: Bcl-2 antagonist killer 1

BAX: Bcl2-associated X protein

Bcl-2: B cell lymphoma 2

Bcl-X_L: Bcl-2 related gene X, long isoform

BH: Bcl-2 homology

BID: BH3-interacting domain death agonist

BIK: Bcl-2 interacting killer

BIM: Bcl-2 interacting mediator of cell death

BIR: baculovirus IAP repeat

BOK: Bcl-2 related ovarian killer

BSA: bovine serum albumin

C

CD: cluster of differentiation

Cdc: cell division control

CDDP: cisplatin, cisplatinum or cis-diamminedichloroplatinum

Cdk5: cyclin-dependent kinase 5

cDNA: complementary DNA

C-ERMAD: carboxy-terminal Ezrin Radixin Moesin associated domain

c-FLIP: cellular FLICE like inhibitory protein

CFTR: cystic fibrosis transmembrane conductance regulator

CREB: cAMP response element binding

D

DD: death domain

DED: death effector domain

DIABLO: direct inhibitor of apoptosis-binding protein with low pI

DISC: death inducing signalling complex

DMEM: dulbecco's modified eagle's medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DR: death receptor

DTT: dithiothreitol

E

EBP50: Ezrin Radixin Moesin binding protein 50

ECL: enhanced chemiluminescence

EDTA: Ethylene diamine tetraacetic acid

EGF: epidermal growth factor

E3KARP: sodium hydrogen exchanger type 3 kinase A regulatory protein

Endo G: endonuclease G

ERK: extracellular signal-regulated kinase

ERM: Ezrin Radixin Moesin

F

FACS: fluorescence-activated cell sorter

FADD: Fas-associated protein with death domain

FasL: Fas ligand

FBS: fetal bovine serum

FCS: fetal calf serum

FERM: four point one Ezrin Radixin Moesin

G

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GPI: glycosyl phosphatidyl inositol

GRK2: G protein-coupled receptor kinase 2

H

HDAC: histone-deacetylase

HGF: hepatocyte growth factor

His: histidine

HIV: human immunodeficiency virus

HRP: horseradish peroxidise

HUVEC: human umbilical vein endothelial cells

I

IAP: inhibitor of apoptosis protein

ICAM: inter-cellular adhesion molecule

IFN: interferon

IgG: immunoglobulin G

IkB: inhibitor of kB

IKK: inhibitor of kB kinase

IL: interleukine

IP_3: inositol 1,4,5-trisphosphate

IS: immunological synapse

IZ: isoleucine zipper

J

JNK: c-Jun N-terminal kinase

K

KBTBD2: Kelch-repeat and BTB domain containing 2

kDa: kilodalton

L

LB: luria bertani

LOK: lymphocyte-oriented kinase

LPS: lipopolysaccharide

LZ: leucine zipper

M

MAPK: mitogen activated protein kinase

Mcl-1: myeloid cell leukemia 1

MDM2: mouse double minute 2

MDR: multidrug resistance

MHC: major histocompatibility complex

MOMP: mitochondrial outer membrane permeabilization

MP: milk powder

mRNA: messenger RNA

N

NEMO : NFkB essential modifier

N-ERMAD: amino-terminal Ezrin Radixin Moesin associated domain

NF2: neurofibromatosis type 2

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

NHE: sodium hydrogen exchanger

NHL: non Hodgkin's lymphoma

NK: natural killer

NP40: noninet P-40

NSCLC: non-small cell lung cancer

O

OMM: outer mitochondrial membrane

OPG: osteoprotegerin

ORT: orthovanadate

P

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PE: phycoerythrin

PFA: paraformaldehyde

Pgp: P-glycoprotein

PI3K: phosphoinositide triphosphate kinase

PIP_2: phosphatidylinositol 4,5-bisphosphate

PKA: protein kinase A

PKC: protein kinase C

PLAD: pre-ligand assembly domain

PMA: phorbol 12-myristate 13-acetate

PTB: polypyrimidine tract binding

PUMA: p53-upregulated modulator of apoptosis

R

Rb: retinoblastoma

RhoGAP: Rho GTPase-activating protein

RhoGDI: Rho GDP-dissociation inhibitor

RhoGEF: Rho guanine nucleotide exchange factor

rhTRAIL: recombinant human TRAIL

RIP: receptor interaction protein

RNA: ribonucleic acid

ROCK: Rho-associated protein kinase

RPMI: roswell park memorial institute

S

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SH2: Src homology 2

shRNA: small hairpin RNA

siRNA: small interfering RNA

Smac: second mitochondria-derived activator of caspases

Syk: spleen tyrosine kinase

T

TBE: tris borate EDTA

TBS: tris buffered saline

TCR: T cell receptor

TEMED: N, N, N', N'-tetramethylethylendiamine

THR: Threonine

TNF: tumor necrosis factor

TNFR: TNF receptor

TRADD: TNF receptor associated death domain

TRAF: TNF receptor associated factor

TRAIL: TNF-related apoptosis inducing ligand

TRAIL-R: TNF-related apoptosis inducing ligand receptor

TWEAK: TNF-like weak inducer of apoptosis

U

UV: ultraviolet

V

VDAC: voltage-dependent anion channel

VSV: vesicular stomatitis virus

W

WT: wild-type

W/V: weight/volume

WWOX: WW-domain containing oxidoreductase

X

XIAP: X-linked inhibitor of apoptosis protein

Z

ZAP70: zeta-chain associated protein kinase of 70 kDa

1. Introduction

1.1. Cancer

Cancer is a general name for a group of over 100 diseases, characterized by abnormal, uncontrolled cell growth, and which is the result of pleiotropic and multi-factorial events (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Normally, cells of a healthy body grow, multiply, and die in a tightly controlled manner. However, when normal processes break down, cancer can develop. When cancer begins, cells grow and divide without any control, develop strategies to inhibit cell death signalling pathways, and ultimately form an abnormal mass of cells called a tumour. Sometimes cells originating from this tumour acquire the capacity either to invade nearby tissues, or to penetrate the blood vessels through which they can move to other parts of the body, where they develop a new mass of tumour cells. This process is termed metastasis and is a characteristic of malignant tumours (Klein, 2008; Chiang and Massagué, 2008). Most are solid tumours except leukemia, which starts in blood-forming tissue and causes the formation of abnormal blood cells.

Several stimuli can induce the transformation of normal cells into tumour cells. For instance, DNA damage or alterations in the genetic material of cells by environmental or internal factors can cause cancer (Mena et al, 2009). Likewise carcinogens can promote formation of cancer by directly inducing DNA damage, and can include ultraviolet, gamma, and X-rays, and tobacco (Mena et al, 2009). These mutations can occur in genes responsible for the cell division process, apoptosis, DNA repair, or tumour suppression. Once mutations occur in some of these cryptic genes, cells become unable to control their proliferation, to engage suicide programs and are often resistant to conventional chemotherapies. For instance, p53 can

regulate different cellular processes, such as cell cycle arrest, senescence, apoptosis (Lane, 1992; Levine and Oren, 2009), angiogenesis (Teodoro et al, 2007), and autophagy (Maiuri et al, 2010), to name a few. P53 is the most commonly mutated tumour suppressor gene in human cancers (Vazquez et al, 2008). Mutations in the gene coding for p53 leads to the loss of its tumour suppressive function, and then results in its inactivation or malfunction, causing cancer development (Brosh and Rotter, 2009; Goh et al, 2011). The fact that most cancer cells are defective in p53, together with the multiple roles of p53 in cell physiology, have led to the consideration that p53 is one of the most important players in the development of cancer (Bálint and Vousden, 2001; Meek, 2009). Mutations can also induce an increase in the activity of growth factors, growth factor receptors, or transduction pathways, leading to increase in the activation of pro-growth signal pathways, which results in an uncontrolled cell growth (Mendelsohn and Baselga, 2003; Takeuchi and Ito, 2010). Some viruses can also divert the cellular machinery and play a decisive role in cancer development, such as human papillomavirus, hepatitis B and C, Epstein-Barr virus, and human immunodeficiency virus (HIV) (Mena et al, 2009; Moore and Chang, 2010). Last but not least, genetic inheritance can predispose to cancer, such as in the case of Bloom Syndrome (Ding et al, 2009), Fanconi anemia (Garcia et al, 2009), or mutations in BRCA1 (Antoniou et al, 2003; Thompson and Easton, 2004), all of which increase a person's chance of developing a cancer during their lifetime.

The transformation of normal cells into tumour cells is a complex multistep process, which requires dynamic alteration of the genome and breaking down of intracellular checkpoints (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). This process can be viewed as a "somatic evolution", because during carcinogenesis normal cells evolve to become malignant, accumulating genetic and epigenetic changes and acquiring new phenotypic properties that confer upon them

survival advantages, ultimately leading to emergence of a malignant population (Gillies and Gatenby, 2007; Fang et al, 2008). Several essential alterations in cell physiology, termed the "hallmarks of cancer", which are found in almost every type of human cancer, have been described by Hanahan and Weinberg, and are involved in the malignant process, including resistance to apoptosis, genomic instability, uncontrolled proliferation, aberrant cell cycle, cellular invasion and metastasis, angiogenesis, inflammation, and abnormal metabolism (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). In addition, during the cell progression towards malignancy, most cancer cells can acquire two more phenotypic properties that confer new survival advantages: the capability to evade immune surveillance, and to suppress immune reactivity (Cavallo et al, 2011). Recently, the role of the tumour microenvironment in determining tumour malignancy has received renewed interest, with the suggestion that environmental conditions may drive the selection of a cancerous phenotype (Gillies and Gatenby, 2007). The tumour microenvironment is characterized by hypoxia, low blood supply, and acidity. Under anaerobic conditions, tumour cells produce energy using glycolysis instead of using the aerobic metabolism, and the glycolysis is constitutively upregulated, a phenomenon known as the "Warburg Effect" (Warburg, 1956; Gillies and Gatenby, 2007). Elevated level of glycolysis in turn induces increased acid production that, together with hypoxia, contributes to create an extracellular environment that is not permissive for the growth of normal cells. In order to survive in the unfavourable environment created by themselves, tumour cells upregulate several proton extrusion mechanisms (Sennoune et al, 2004), such as the V-ATPase (Ma et al, 2011; Fais et al, 2007), the Na+/H+ exchanger (NHE) (Slepkov et al, 2007) and the carbonic anhydrase (Robertson et al, 2004). Therefore, the peculiar features of hypoxia and acidosis characterizing the tumour microenvironment produce a selective pressure that favours the survival of tumour cells, which are resistant to

acidosis and have upregulated glycolysis (Gillies and Gatenby, 2007). These observations led Gatenby and Gillies to propose an additional new hallmark of cancer: increased glucose consumption through increased glycolysis, which contribute to confer a metabolic advantage to tumour cells (Gillies and Gatenby, 2007; Fang et al, 2008).

Solid tumours have been treated by surgery for the past 4000 years (Pavet et al, 2011). X-rays were discovered at the end of the nineteenth century (Eisenberg, 1992), and it was observed that cancer cells were susceptible to death by radiations, leading to radiotherapy becoming another major therapeutic approach (Connell and Hellman, 2009). The third main approach that is currently used to treat cancer is chemotherapy, using cytotoxic chemical compounds. At present, surgery, radiation and/or chemotherapy represent the standard treatment for the majority of cancers. Patients often receive a combination of these therapies. However, chemotherapy drugs currently used for treating cancers can be very aggressive and highly toxic for patients. Moreover, a significant proportion of patients are not cured from the disease because of chemoresistance and/or relapse. Thus, novel chemotherapeutic compounds are always being pursued. Alternative approaches are also under investigation, including non conventional chemotherapy (Jacquemin et al, 2010; Mérino et al, 2007; Luciani et al, 2004a; De Milito et al, 2006; De Milito et al, 2010; Marino et al, 2010), immunotherapy (Sato et al, 2009), or gene therapy (Zhang et al, 2011). Despite the great advances that have been made, and the great efforts of the scientific community in finding effective treatments for cancer, the disease still causes death for millions of people per year worldwide (Thun et al, 2010; American Cancer Society, 2007; IARC, 2008). More than 12 million new cases and 7.6 million cancer deaths have been estimated to have occurred in 2007 (American Cancer Society, 2007; Thun et al, 2010). By 2030, it is projected that there will be 26 million new cancer cases and 17 million cancer

deaths per year (IARC, 2008; Thun et al, 2010). New investigations are therefore necessary for the design of novel and innovative therapeutic strategies to cure cancer, and which are less invasive for patients in order to enhance their quality of life.

In light of these considerations, research in the field of apoptosis is of great interest in order to find new ways to kill malignant cells.

1.2. Apoptosis

Cells have several means to trigger cell death, and the literature has multiple examples of number of different modes of cell death, including apoptosis, autophagy, necrosis, anoikis, cannibalism, and pyroptosis. Apoptosis is the most renowned of these, being a highly conserved and tightly controlled type of programmed cell death process, first described in 1972 by Currie and colleagues (Kerr et al, 1972), which eliminates superfluous or irreparably damaged cells from multicellular organisms (Danial and Korsmeyer, 2004; Strasser et al, 2011). This mechanism regulates not only the correct development of organs in mammals, but also the homeostasis and integrity of tissues in adult organisms throughout their lifetime (Meier et al, 2000; Chowdhury et al, 2006; Strasser et al, 2011). Aberrant regulation of the cell death process, such as deficiency or excess in apoptosis, can lead to the development of several diseases, such as autoimmune diseases (Oliveira and Gupta, 2008; Hotchkiss et al, 2009), neurodegenerative disorders (reviewed in Cavallucci and D'Amelio, 2011), or cancer (Cory and Adams, 2002; Vazquez et al, 2008; Hotchkiss et al, 2009; Hanahan and Weinberg, 2011).

Apoptosis is characterized by reduction in cellular and nuclear volume, condensation and fragmentation of DNA, preservation of organelle structure and plasma membrane integrity, and blebbing of the plasma membrane which generates apoptotic bodies that are engulfed by phagocytes, thereby avoiding an inflammatory response in surrounding tissues (Strasser et al, 2011) (**Figure 1.1**). Apoptosis is morphologically distinct from the other types of cell death. For example, during necrosis the organelles swell, the cell volume increases, mitochondria produces reactive oxygen species, the ATP is depleted, and the lysosomes and the plasma membrane are disrupted, resulting in the release of cellular contents into the microenvironment, which induces the activation of an inflammatory response (Golstein and Kroemer, 2007). Alternatively, when cell death occurs with the

features of autopaghy, the cells are characterized by vesicular accumulation, absence of chromatin condensation and cytoplasmic vacuolization (Amelio et al, 2011).

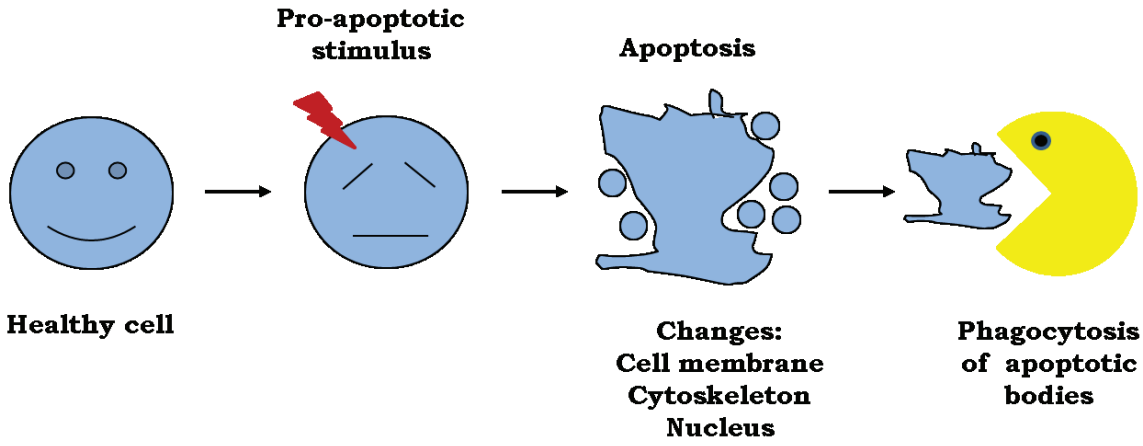

Figure 1.1. Schematic representation of the apoptotic process.

Apoptosis may be initiated following the activation of one of two pathways, the intrinsic and extrinsic apoptotic pathways (**Figure 1.2**). Binding of extracellular ligands to death receptors (DRs) at the cell surface stimulates the extrinsic pathway, whereas cellular damage or stress signals for cell death through the intrinsic pathway (Indran et al, 2011). Both pathways converge on the activation of specific proteins, called caspases, which are highly conserved cysteine-aspartate proteases (Lavrik et al, 2005; Li and Yuan, 2008). The first caspases activated following induction of apoptosis are the initiator caspases, which include caspase-2, -8, -9 and -10 (Chen et al, 2002; Boatright et al, 2003; Bouchier-Hayes et al, 2009; Milhas et al, 2005; Lafont et al, 2010). Once these caspases are activated, they in turn activate the effector caspases-3, -6, -7, which start to dismantle the cell by cleaving and then degradating multiple cellular proteins, leading to the execution of the apoptotic process.

Cells may undergo apoptosis via engagement of the intrinsic pathway. In this case, apoptosis can be initiated by a variety of receptor-independent stimuli, such as UV- or gamma-irradiation, free radicals, viral infections, cellular stress, serum or growth factor withdrawal, heat shock, chemotherapy, DNA damage, etc (**Figure 1.2**). Activation of the intrinsic pathway induces the permeabilization of the mitochondrial outer membrane, leading to the formation of large pores through which soluble apoptogenic factors, such as cytochrome c, Smac-DIABLO and Omi/HtrA2, usually present in the inter-membrane space gain access to the cytosol (Vaux, 2011). Once in the cytosol, they activate the effector caspases in cooperation with cytosolic factors, leading to cell death. Thus, cytoplasmic cytochrome c binds to and activate Apaf-1 (apoptotic protease activating factor 1), and they form a platform termed the apoptosome, which allows recruitment and activation of initiator caspase-9 molecules (Zou et al, 1999; Acehan et al, 2002). Activation of caspase-9 causes the activation of effector caspases, thereby enabling full execution of cell death. Molecules of Smac (second mitochondrial activator of caspases)/Diablo (direct IAP binding protein with low pI) (Du et al, 2000; Verhagen et al, 2000) associate with IAPs (inhibitor of apoptosis proteins) proteins (see paragraph 1.3.6), neutralizing their inhibitory function on caspase-9, -3, and -7 molecules, which are then free to execute apoptosis (Wu et al, 2000; Ekert et al, 2001). In addition to Smac/Diablo, another mitochondrial serine protease protein, HtrA2/Omi, has been identified by virtue of its ability to bind to IAPs (Verhagen et al, 2007; Martins et al, 2002; Hegde et al, 2002). Moreover, other two apoptogenic proteins, AIF (apoptosis inducing factor) and endonuclease G (Endo G), were reported to be released from mitochondria during apoptosis. Once released, AIF and Endo G translocate from the mitochondria to the nucleus to cause DNA fragmentation and cell death in a caspase-independent manner (Joza et al, 2001; Ye et al, 2002; McIlroy et al, 2000; Li et al, 2001). Controversial results have been

reported about HtrA2/Omi, AIF and Endo G, and the physiological significance of their release during apoptosis is still under investigation, and has yet to be established.

The intrinsic pathway of apoptosis can also be promoted through the activity of the endoplasmic reticulum. Indeed, unresolved conditions of stress to the endoplasmic reticulum, resulting in misfolded proteins, stimulates this organelle to activate apoptosis. Multiple pathways may be involved in endoplasmic reticulum-mediated apoptosis. On one hand, apoptosis induced by the endoplasmic reticulum seems to involve the direct activation of initiator caspases at the endoplasmic reticulum level, which can then translocate from the endoplasmic reticulum to the cytosol where they in turn activate caspase-9, leading to the apoptotic caspase cascade (Hitomi et al, 2004; Yukuoka et al, 2008). By contrast, other studies report that the endoplasmic reticulum-mediated apoptosis occurs through cross talk with the mitochondria. In fact, cytochrome c release, loss of mitochondrial membrane potential, and caspase-9 activation has been observed in response to endoplasmic reticulum stressors (Jimbo et al, 2003; Kitamura et al, 2003; Masud et al, 2007). Release of calcium from the endoplasmic reticulum into the cytosol seems to be also required. For example, it has recently been reported that calcium can be directly transmitted into mitochondria following the attachment of mitochondria to the endoplasmic reticulum cisternea, leading to cell death (Rizzuto et al, 2009; Csordas et al, 2010).

The extrinsic pathway is initiated when a specific extracellular death inducing ligand binds to an agonistic transmembrane death receptor at the membrane level (**Figure 1.2**). Currently, six death receptors have been identified: TNFR1, Fas (CD95), TRAIL-R1, TRAIL-R2, DR3, DR6, members of the TNF receptor (TNFR) superfamily. They are characterized by an extracellular domain that is able to engage the ligands, and a cytoplasmic domain, called the death domain, essential

for the transmission of the death signal. Engagement of death receptors drives the recruitment of adaptor proteins and initiator caspase-8 and -10 molecules, which results in the formation of the death-inducing signaling complex (DISC), and caspase activation. Activated caspases-8 and -10 activate the effector caspases in turn, beginning the apoptotic cascade. Activated caspases-8 can also induce mitochondrial damage and reinforce the death signal by activating the intrinsic apoptotic pathway (Elmore et al, 2007). Alternatively, when caspases are inhibited, the death receptor-mediated apoptotic process is blocked, and the death signal induces the activation of another different form of programmed cell death, termed "necroptosis", which resembles necrosis. Indeed, it has recently been proposed that death receptors participate not only in apoptosis but also in necroptosis, which occurs when the apoptotic pathway is blocked, and requires the involvement of RIP1 and RIP3 (receptor interaction protein kinase 1 and 3) (Holler et al, 2000; Degterev et al, 2008; Galluzzi et al, 2009). Upon necroptosis induction, RIP3 has been shown to be recruited to a cytosolic complex formed by RIP1, FADD and caspase-8 (He et al, 2009).

Alternatively, novel intracellular apoptosis-inducing platforms, namely ripoptosomes, and composed by RIP1, FADD and caspase-8, have been recently described, which allow the recruitment and activation of initiator caspases (Ikner and Ashkenazi, 2011; Feoktistova et al, 2011; Tenev et al, 2011). It has been reported that TWEAK (TNF-like weak inducer of apoptosis), toll-like receptor 3 or genotoxic stress could induce the spontaneous formation within the cytosol of the ripoptosome that can stimulate either apoptosis or necroptosis (Ikner and Ashkenazi, 2011; Feoktistova et al, 2011; Tenev et al, 2011).

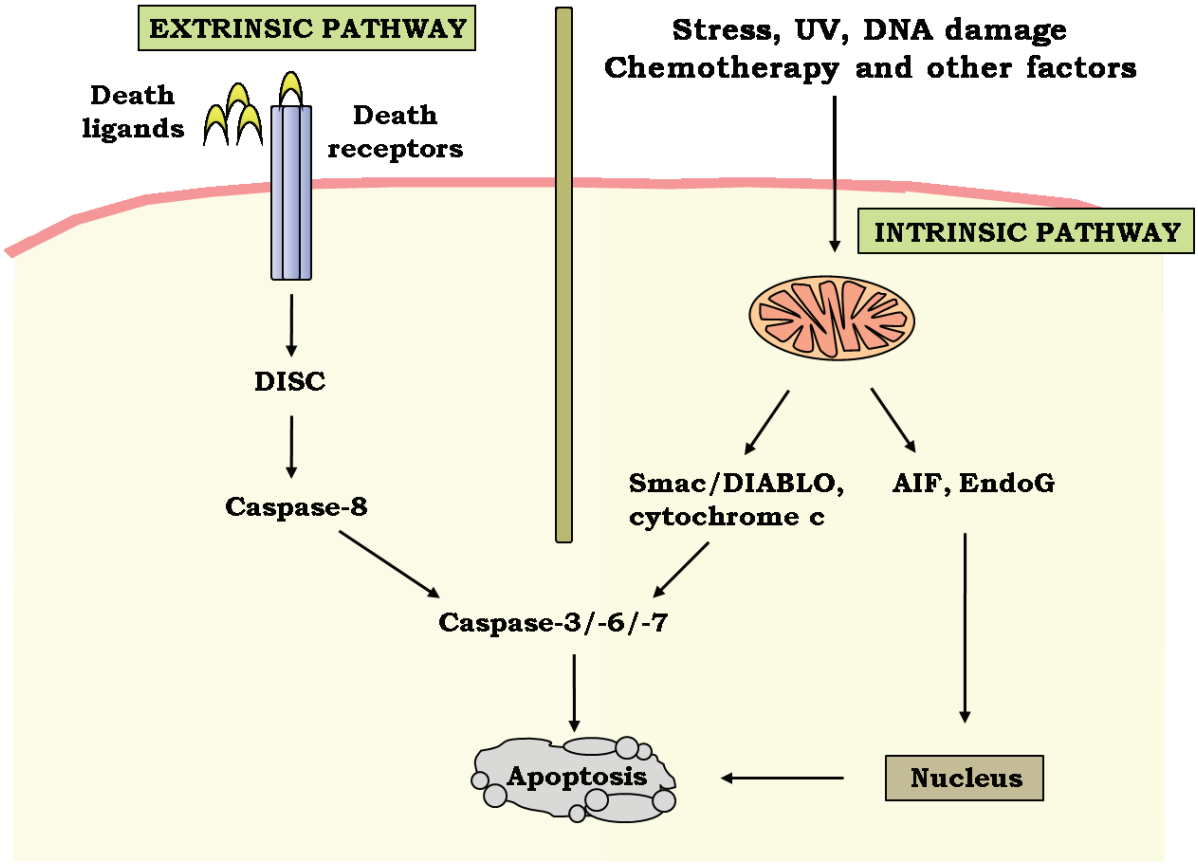

Figure 1.2. Scheme of the intrinsic and extrinsic pathways of apoptosis. The extrinsic pathway (left) starts with the ligation of death ligands to their cognate receptors. This event leads to DISC formation, caspase activation and then cell death. The intrinsic pathway (right) is activated by different cellular and environmental factors, which induce the permeabilization of the mitochondrial outer membrane and the consequent release of cytochrome c, Smac/DIABLO, AIF and Endo G, terminating with execution of apoptosis.

Among the proapoptotic ligands of the TNF superfamily, TRAIL (TNF-Related Apoptosis-Inducing Ligand) is a topic of great interest in recent years for oncologists, because it can trigger cell death in a wide variety of cancer cells while sparing normal cells (Ashkenazi et al, 1999; Lawrence et al, 2001; Ashkenazi, 2002; Mérino et al 2007; Jacquemin et al 2010). For this reason it has been considered as a promising therapeutic agent against malignant diseases (Gura, 1997; Takeda et al, 2004). Moreover, several phase I and II clinical trials with recombinant human TRAIL or agonistic antibodies, which recognize and activate its proapoptotic receptors, are currently underway in patients suffering from different types of malignant diseases (Herbst et al, 2006: Ling et al, 2006; Yee et al, 2007; Tolcher et al, 2007; Plummer et al, 2007; Greco et al, 2008; Leong et al, 2009). Preliminary results indicated that the recombinant human variant of TRAIL and the antibodies are well tolerated in patients, and the side effects on normal cells are mild (see paragraph 1.3.8), increasing interest in using TRAIL in clinical setting.

Extensive research over the last few years has given us a good understanding of the events that occur during the TRAIL signalling pathway, and of the factors involved in its regulation. However, some aspects which could be involved in the transmission of the TRAIL-induced apoptotic signal have not yet been taken into account. In light of these considerations, during this project we investigated the initial steps of the signalling pathway induced by the TRAIL death receptors.

1.3. The TRAIL pathway

1.3.1. TRAIL

Human TNF-Related Apoptosis-Inducing Ligand (TRAIL or Apo2L) has high homology with TNF type α and Fas ligand (FasL), two other members of the tumor necrosis factor (TNF) superfamily. TRAIL was identified by two different groups as a new molecule able to trigger apoptosis in a wide variety of cells (Wiley et al, 1995; Pitti et al, 1996). It is a type II transmembrane protein, composed of 281 amino acid residues, and is coded on the long arm of chromosome 3 (3q26). TRAIL can also be found in a soluble form in which the carboxy-terminal domain can be released into the extracellular milieu following proteolytic cleavage (Mariani and Krammer, 1998). At the protein level, TRAIL possesses an amino-terminal cytoplasmic domain, followed by a transmembrane helix, and terminates in a carboxy-terminal extracellular TNF-like domain. Crystal structure analysis shows that the extracellular domain is characterized by two antiparallel β-sheets (Cha et al, 1999; Hymowitz et al, 2006). TRAIL is usually present in a trimeric form, maintained through a direct association between a zinc atom localized at the interface of the trimer, and the cysteine residue 230 of each monomer (Hymowitz et al, 2006; Bodmer et al, 2000). TRAIL is expressed at the surface of T cells, dendritic cells, natural killer cells, macrophages, monocytes and neutrophils. Its expression at the mRNA level can be induced by type I interferons (Almasan and Ashkenazi, 2003).

1.3.2. TRAIL receptors

The TRAIL signaling system is very complex. TRAIL has five receptors: two agonistic receptors, TRAIL-R1 (DR4), and TRAIL-R2 (DR5, Killer, TRICK2), two decoy receptors, TRAIL-R3 (DcR1, LIT) and TRAIL-R4 (DcR2, TRUDD), and a soluble receptor called osteoprotegerin (OPG) (**Figure 1.3**). These receptors, with the exception of OPG, are type I transmembrane proteins, characterized by the presence of cysteine-rich domains in their extracellular domain (**Figure 1.3**), and coded on chromosome 8, position 8p21-22.

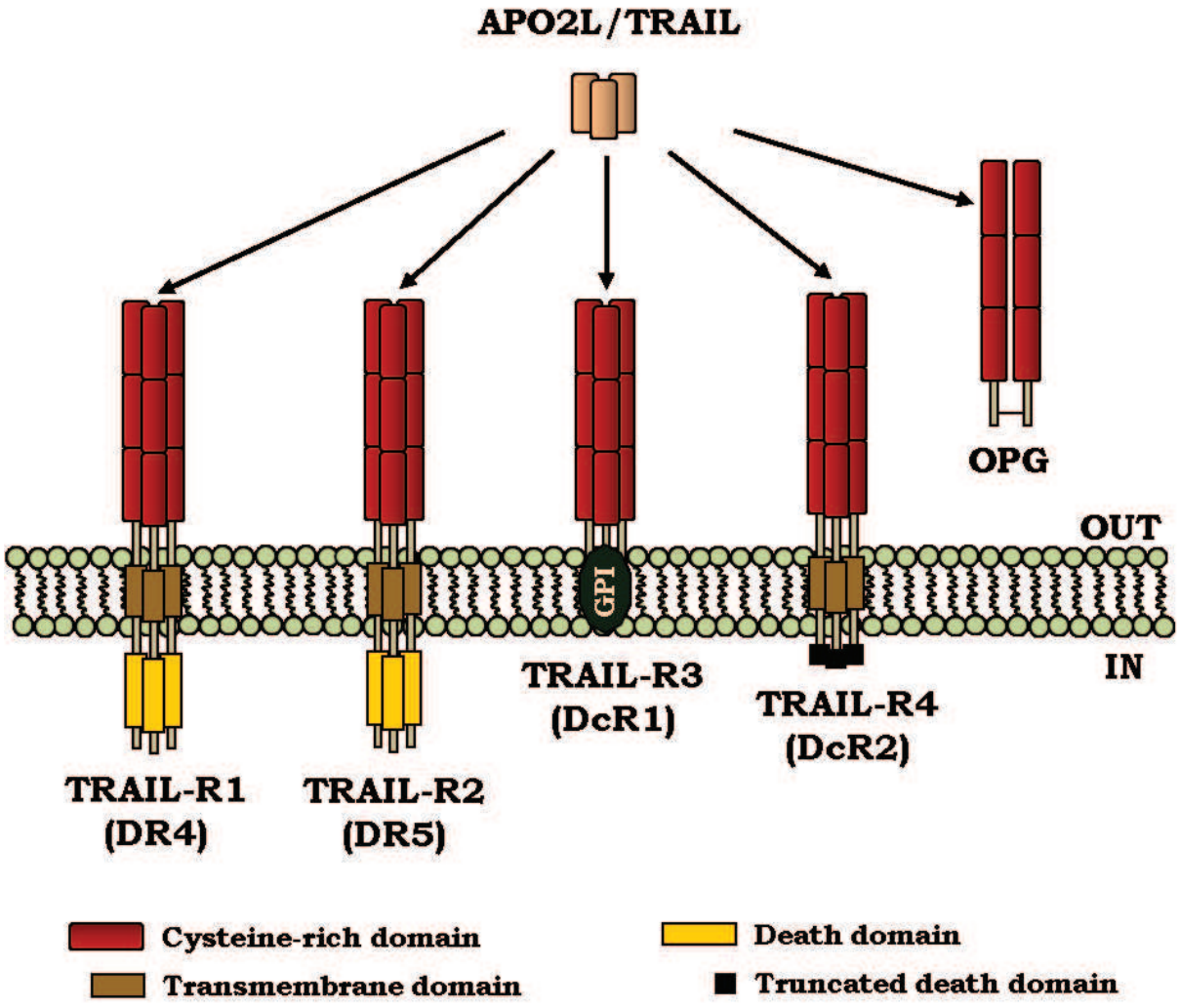

Figure 1.3. TRAIL receptors. Five TRAIL receptors have been identified. All possess three cystein rich regions in the extracellular part. TRAIL induces apoptosis upon binding to TRAIL-R1 and TRAIL-R2, which have a death domain of 80 amino acids in the citopasmic region, crucial for transmit apoptotic signal. TRAIL has also two decoy receptors, TRAIL-R3 with a GPI anchor and TRAIL-R4 with a truncated death domain, that do not signal for apoptosis. One soluble receptor, the OPG, is also present.

TRAIL induces apoptosis following its binding to TRAIL-R1 (Pan et al, 1997a) and TRAIL-R2 (Walczak et al, 1997; Chaudary et al, 1997; Wu et al, 1997), which have 58% sequence homology with each other. Both receptors consist of an amino-terminal extracellular domain containing three cysteine-rich domains, a transmembrane domain and a carboxy-terminal cytoplasmic domain (**Figure 1.3**). The intracellular domain is characterized by a Death Domain (DD) of 80 amino acid residues that is conserved in each of the death receptors of this family, and is essential for triggering apoptosis (**Figure 1.3**). Two TRAIL-R2 isoforms have been described, one long and one short, both of which are able to transduce apoptotic signaling. The short isoform differs from the long because it lacks a sequence of 23 amino acids located between the transmembrane domain and the first cysteine-rich domain (Screaton et al, 1997). In mice, only one agonistic receptor has been identified (Wu et al, 1999). This receptor, known as mTRAIL-R2 or mDR5, is more similar to the human TRAIL-R2 than to human TRAIL-R1 (Wu et al, 1999). It signals for apoptosis in mouse and human cell lines, in response to both mouse and human TRAIL, through its cytoplasmic DD (Wu et al, 1999).

When TRAIL binds to TRAIL-R3 (Pan et al, 1997b; Degli-Esposti et al, 1997a) or TRAIL-R4 (Degli-Esposti et al, 1997b; Marsters et al, 1997), it does not induce apoptosis in target cells. Indeed, although these receptors possess an extracellular domain that is very similar to that of the agonistic death receptors, they differ in the intracellular part. TRAIL-R3 lacks the cytoplasmic and transmembrane domains, and it is anchored to the plasma membrane through the Glycosyl-Phosphatidyl-Inositol (GPI), whereas TRAIL-R4 contains a truncated, nonfunctional DD (**Figure 1.3**). Thus, these receptors are unable to transmit an apoptotic signal, and consequently they protect cells from TRAIL-induced apoptosis. The mechanisms through which TRAIL-R3 and TRAIL-R4 carry out their inhibitory function are still under investigation. TRAIL-R3 can inhibit TRAIL-induced cell death by competing

with the death receptors for TRAIL binding, which leads to sequestering of TRAIL in lipid rafts (Mérino et al, 2006) (**Figure 1.4**). Whereas in cells over-expressing TRAIL-R4, addition of TRAIL induces association of TRAIL-R4 with TRAIL-R2 impeding caspase-8 recruitment and activation (Mérino et al, 2006) (**Figure 1.4**). The TRAIL-R4-mediated TRAIL inhibition is also described to involve the formation of ligand-independent complexes between TRAIL-R2 and TRAIL-R4 through special extracellular domains called PLAD (pre-ligand assembly domains) (Clancy et al, 2005). Although a weak interaction between TRAIL-R4 and TRAIL-R2 has been observed, it has been clearly demonstrated that the heterotypic interaction mostly occurs in a ligand-dependent manner (Mérino et al, 2006). TRAIL-R4 has also been suggested to be able to transmit an antiapoptotic signal through the activation of the transcription factor NF-kB (Degli-Esposti et al, 1997b) or Akt in HeLa cells (Lalaoui et al, 2011) (**Figure 1.4**). In mice, two decoy receptors are present, called mDcTRAIL-R1/mDcR1 and mDcTRAIL-R2/mDcR2, and these are highly homologous to the human decoy receptors (Schneider et al, 2003).

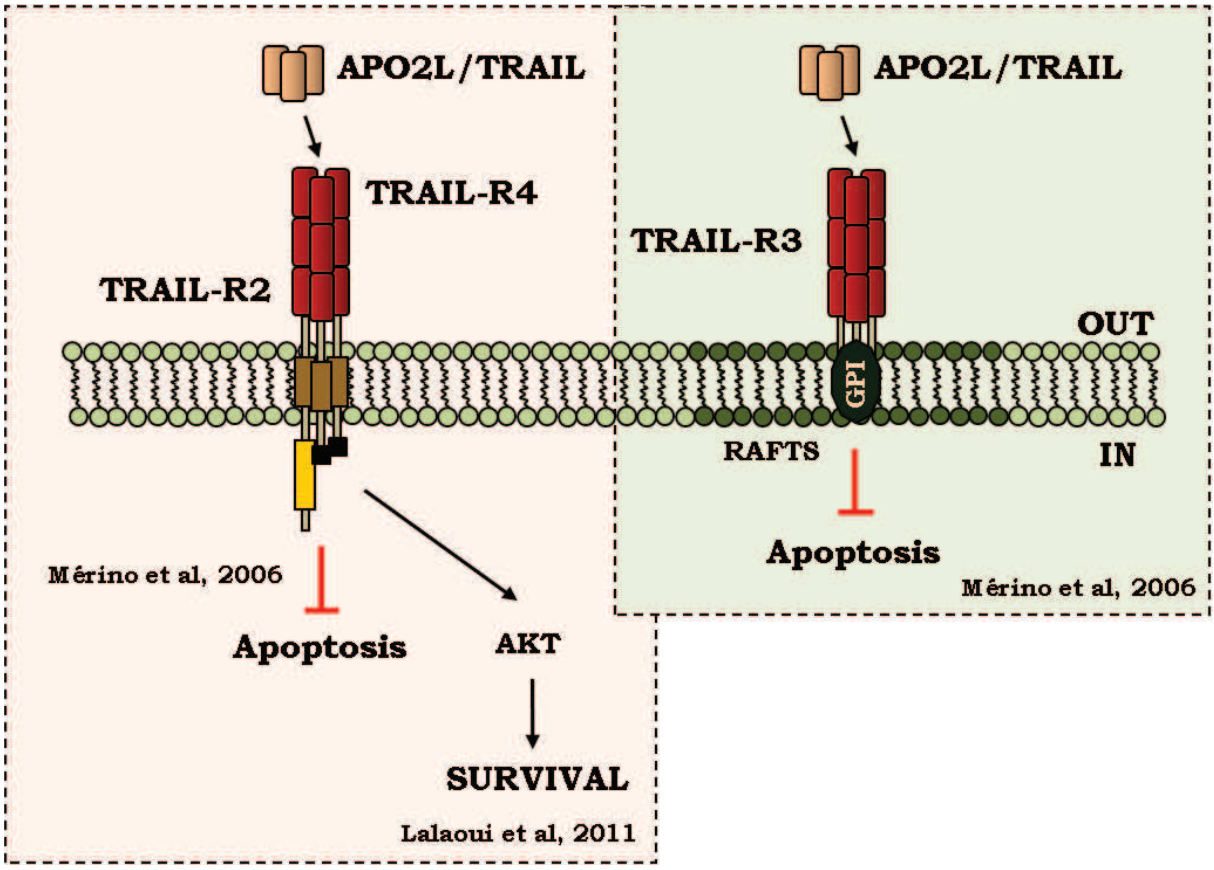

Figure 1.4. Inhibition of TRAIL-induced apoptosis by TRAIL-R4 and TRAIL-R3. Stimulation with TRAIL leads to the association of TRAIL-R4 with TRAIL-R2, which induces cell resistance to apoptosis and transmits an antiapoptotic signal (left panel). Alternatively, addition of TRAIL favors its sequestering in lipid rafts by TRAIL-R3 (right panel).

TRAIL can also bind to osteoprotegerin (OPG) (Emery et al, 1998) (**Figure 1.3**), the only soluble receptor, and which has the lowest affinity for TRAIL. TRAIL binding to OPG has been proposed to regulate osteoclastic differentiation and survival (Zauli et al, 2004, 2008; Vitovski et al, 2007). However, the physiological role of this receptor in the TRAIL pathway remains to be better elucidated.

Initially it was thought that TRAIL receptors were expressed on the cell surface in a monomeric form, and that engagement of TRAIL-R1 and TRAIL-R2 by trimeric TRAIL promoted trimerization of these receptors. Further studies revealed that in the absence of ligand, TRAIL-R1 and TRAIL-R2, as well as Fas, TNFR1 and TNFR2, in fact reside on the plasma membrane in pre-assembled complexes, maintained by

the reciprocal association between the PLAD domains located in the first cysteine-rich domain in the extracellular part of each receptor, and which interact with each other, mediating the association between monomers (Papoff et al, 1999; Chan et al, 2000; Siegel et al, 2000, Clancy et al, 2005).

TRAIL-R1 and TRAIL-R2 have been reported to be post-translationally modified through O-glycosylation and palmitoylation (Wagner et al, 2007; Pan et al, 1997b; Rossin et al, 2009), and these two events are responsible for the modulation of the receptor clustering. Indeed, the level of expression of O-glycosylation enzymes in cancer cells has been correlated with cellular sensitivity to TRAIL-induced apoptosis (Wagner et al, 2007). Furthermore, TRAIL-R2 O-glycosylation at specific sites, which are highly conserved in TRAIL-R1, is reported to be required for TRAIL-R1 and TRAIL-R2 clustering, and subsequently to trigger DISC formation and caspase-8 activation. The N-glycosylation of TRAIL-R1 (Pan et al, 1997b) involves a specific site, which is not conserved in TRAIL-R2 (Sheridan et al, 1997), and its pharmacological inhibition has been described to alter the clustering of TRAIL-R1, but not TRAIL-R2 (Yoshida et al, 2007). Although these post-translational modifications have been correlated with sensitivity to TRAIL-induced apoptosis, the molecular mechanisms through which they modulate the TRAIL signalling pathway have not yet been fully elucidated.

The literature also demonstrates that TRAIL-R1, similar to Fas (Chakrabandhu et al, 2007) but contrary to TRAIL-R2, is constitutively palmitoylated on a triplet of cysteine residues located between the receptor's transmembrane and DD region (Rossin et al, 2009). This post-translational modification was described to be required for the trimerization of TRAIL-R1, which is necessary for the further transduction of the apoptotic signal (Rossin et al, 2009).

1.3.3. The apoptotic extrinsic pathway and the DISC formation

Binding of trimeric TRAIL to TRAIL-R1 or TRAIL-R2 induces further clustering of the receptors into aggregates of high molecular weight, leading to the concomitant formation of the DISC (Death-Inducing Signalling Complex), composed of the adaptor protein FADD (Fas-Associated protein with Death Domain) and the initiator caspases-8 and -10 (Walczak and Sprick, 2001). Activation of TRAIL-R1 or TRAIL-R2 by TRAIL drives the recruitment of FADD that translocates to the DISC where its DD interacts directly with the DD of TRAIL-R1 or TRAIL-R2 (Kischkel et al, 2000; Sprick et al, 2000). FADD then recruits caspase-8 and -10 molecules to the complex through the interaction of its amino-terminal DED (Death Effector Domain) with the DED domain of the initiator caspases (Kischkel et al, 2000; Sprick et al, 2000). Within this complex, caspases-8 and -10 are activated.

Caspases are a family of cysteine proteases that play an essential role during apoptosis due to their ability to cleave several different substrates (reviewed in Li and Yuan, 2008). They are synthesized in cells as inactive proforms, and therefore need to be activated during apoptosis. Two groups of apoptotic caspases exist: initiator and effector caspases. In response to apoptotic stimuli, initiator caspases (caspase-8, -10, and -9) are activated. Once activated, they activate the effector caspases (caspase-3, -6, and -7). Activation of effector caspases generally occurs through proteolytic cleavage, which takes place on specific aspartate residue (Cohen et al, 1997), localized in a short segment that connects the large and small subunits of the caspases's catalytic domain. However, the mechanism of initiator caspase activation seems to be more complex and it is not yet completely understood. Several hypothesis have been proposed. The first model, the induced-proximity model, postulates that FADD promotes the clustering of initiator caspases within the DISC, which increases their local concentration and allows their reciprocal trans-activation through proteolytic cleavage (Salvesen and Dixit, 1999). Thereby,

initiator caspases have a weak proteolytic activity and can therefore auto-process themselves only when they are brought within close proximity of each other in the DISC. Further studies on caspase-8 and -9 have led to the assumption that initiator caspases are activated after dimerization at the DISC or apoptosome level, and their subsequent cleavage serves only to stabilize the active dimers (Boatright et al, 2003; Donepudi et al, 2003; Renatus et al, 2001; Micheau et al, 2002). Based on this model, called the proximity-induced dimerization model (Boatright et al, 2003), the recruitment of initiator caspases within the DISC or the apoptosome increases their local concentration, which promotes their dimerization (Boatright et al, 2003; Renatus et al, 2001). Nowadays, this model is considered the principal mechanism of activation for initiator caspases. However, the induced proximity model was refined once again in 2005 by Chao and coauthors. They proposed a third model: the induced conformation model, which was based on studies with caspase-9, where they engineered a constitutive dimeric caspase-9 (Chao et al, 2005). Their hypothesis highlights the importance of the conformation of the active site during the process of activation. Consistent with their theory, binding of monomers of inactive caspase-9 to the apoptosome induces a conformational change, most likely at the level of the caspase-9 active site, which is the prerequisite that favors caspase-9 activation (Chao et al, 2005). This model can be also extended to the other initiator caspases, caspase-8 and -10. Recently, a new mechanism has been proposed that involves the ubiquitylation of caspase-8 (Jin et al, 2009). This study showed that stimulation with TRAIL induces caspase-8 polyubiquitylation at its carboxy-terminal region, promoted by the E3 ubiquitin ligase CUL3 within the DISC, leading to caspase-8 activation. The polyubiquitylation of caspase-8 can be reverted by the deubiquitinase enzyme A20, which is also present in the TRAIL DISC. Once the caspase-8 is ubiquitylated, the ubiquitin-binding protein p62/sequestosome-1 associates with caspase-8 and promotes the translocation of

caspase-8 from the DISC to intracellular ubiquitin-rich protein regions, where the caspase-8 is fully activated (Jin et al, 2009).

Both caspase-8 and -10 can be recruited to the TRAIL DISC where they are activated with similar kinetics, and can work independently of each other (Kischkel et al, 2001; Wang et al, 2001; Sprick et al, 2002). It is still controversial whether these caspases have redundant or exclusive roles during apoptosis. Evidence in the literature shows that caspase-10 over-expression can compensate for deficiency in caspase-8 in TRAIL-treated Jurkat cells (Kischkel et al, 2001; Wang et al, 2001), whereas another study reported that caspase-10 cannot functionally substitute caspase-8 (Sprick et al, 2002).

Once the initiator caspases are activated, cleaved forms of caspase-8 and caspase-10 are released in the cytosol where they activate the effector caspases-3, -6 and -7, ultimately leading to cell death (**Figure 1.5**).

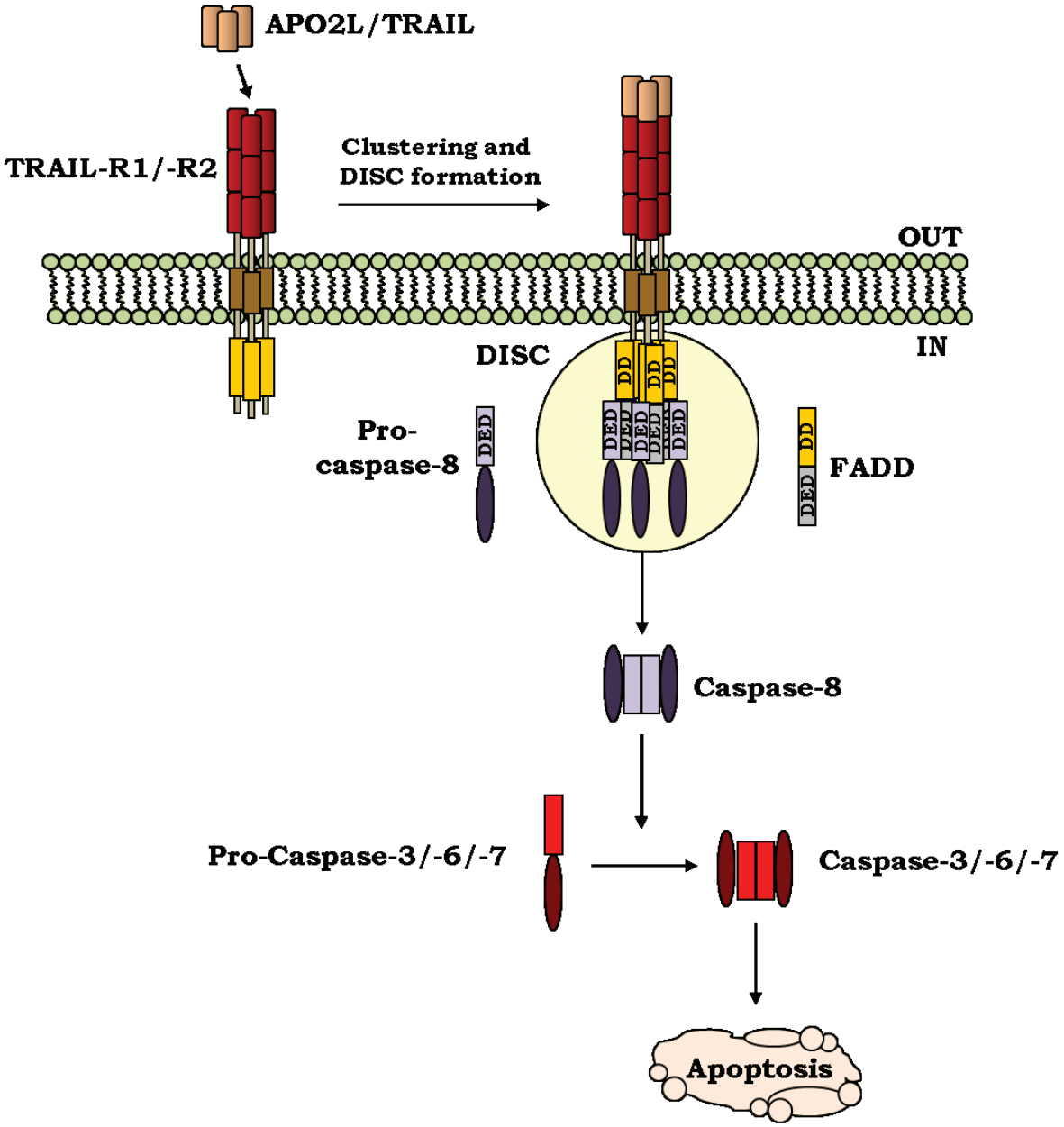

Figure 1.5. The TRAIL apoptotic extrinsic pathway. Binding of a TRAIL trimer to TRAIL-R1 or TRAIL-R2 leads to the clustering of receptors and recruitment of the adaptor FADD, which in turn recruits and activates caspase-8, to form the DISC. Active molecules of caspase-8 then activate the effector caspases-3, -6 and -7 and directly induce apoptosis.

1.3.4. The apoptotic intrinsic pathway

Cells sometimes require further signal amplification through the mitochondrial (intrinsic) pathway in order to undergo TRAIL-induced apoptosis. This phenomenon, described initially for the Fas signalling pathway (Scaffidi et al, 1998), has led to the classification of cells into two types, namely type I and type II cells. In type I cells, caspase-8 is activated and processed in the DISC in quantities that are sufficient to directly activate the effector caspases, and thereby to promote apoptosis. In contrast, in type II cells, the quantities of active caspase-8 generated at the DISC are limited, and therefore cells need an amplification loop through the mitochondria for further transduction of the apoptosis signal and execution of apoptosis (Scaffidi et al, 1998; Barnhart et al, 2003). Further research, however, has revealed that the differences in DISC formation and caspase-8 activation are not the only molecular factors that can discriminate between type I and type II apoptotic signalling. Indeed, increasing caspase-8 activity, for example by down-regulating c-FLIP, an inhibitor of caspase-8 (see paragraph 1.3.6), does not automatically convert type II into type I cells (Wilson et al, 2009). This role could be attributed to the protein XIAP, which binds to and inhibits caspase-3, -7 and -9 (Deveraux et al, 1997; Riedl et al, 2001) (see paragraph 1.3.6). Accordingly, combined inhibition of c-FLIP and XIAP enhanced apoptosis in type II colorectal cancer cells (Wilson et al, 2009). Moreover, XIAP was identified as a critical discriminator between type I and type II apoptosis signalling during death receptor-induced apoptosis (Jost et al, 2009). It was reported that loss of XIAP sensitized hepatocytes (type II cells) but not thymocytes (type I cells) to Fas-mediated apoptosis (Jost et al, 2009). In the absence of XIAP, induction of apoptosis by Fas was shown to occur through a caspase-dependent process and did not involve the mitochondria (Jost et al, 2009). Thus, genetic deletion of XIAP in hepatocytes

changed the Fas apoptosis phenotype of these cells from type II to type I (Jost et al, 2009).

Ligation of TRAIL to its cognate death receptors, TRAIL-R1 and TRAIL-R2, triggers for apoptosis through DISC formation, where proforms of initiator caspases (caspase-8 and /or -10) are recruited and activated. Active molecules of caspase-8/-10 are then released into the cytosol where they directly activate the effector caspases-3, -6, and -7 (type I pathway). In type II cells, however, activated caspase-8 engages the mitochondrial apoptotic pathway for full effector caspases activation and a complete execution of apoptosis. The transition from the death receptor-mediated extrinsic pathway to the mitochondrial apoptotic pathway is achieved through the caspase-8-mediated processing of the protein Bid (BH3-interacting domain death agonist), a BH3-only member of the Bcl-2 family (Li et al, 1998; Luo et al, 1998) (**Figure 1.6**).

Following TRAIL stimulation, Bid is cleaved by caspase-8 at the aspartate residue 60 within the cytosol (Li et al, 1998; Gross et al, 1999), resulting in the generation of its truncated form of 15 kDa, tBid, containing the carboxyl-terminal part of the protein (**Figure 1.6**). Recent evidence has shown that caspase-10 is also able to cleave and thereby activate Bid during Fas ligand-induced apoptosis (Fischer et al, 2006; Milhas et al, 2005). Caspase-3 (Slee et al, 2000) and other proteases, such as granzyme B (Sutton et al, 2000), cathepsins (Stoka et al, 2001; Reiners et al, 2002; Cirman et al, 2004) and calpains (Chen et al, 2001; Mandic et al, 2002) are also reported to be involved in the processing of Bid during death ligand-independent apoptotic pathways. Truncated Bid has a potent pro-apoptotic activity (Li et al, 1998; Gross et al, 1999), but the literature also describes a pro-apoptotic role for full-length Bid (Sarig et al, 2003), for example in a model of cell death termed anoikis (Valentijn and Gilmore, 2004). Moreover, full length Bid can

accumulate and be processed directly at the mitochondrial membrane. It has recently been demonstrated that caspase-8 can also be activated at the mitochondria following apoptosis induction by Fas ligand in type II cells (Gonzalvez et al, 2008; Scorrano, 2008). Indeed, engagement of Fas by its cognate ligand induces the enrichment and insertion of proforms of caspase-8 into the mitochondrial membrane (Gonzalvez et al, 2008). Caspase-8 has been found integrated in special microdomains of the mitochondrial outer membrane rich in cardiolipin, a membrane lipid uniquely present in mitochondria, where caspase-8 dimerises and then becomes activated (Gonzalvez et al, 2008). This new mechanism of caspase-8 activation describes the mitochondria as a platform for the activation of caspase-8 following Fas ligand stimulation, where caspase-8 directly gains access to cleave its substrate Bid. This mechanism was also reported to be crucial for apoptosis induction in type II cells. Indeed, abrogation of caspase-8 translocation to mitochondria, through depletion of mature cardiolipin, inhibited caspase-8 activation, prevented Bid cleavage, and blocked apoptosis (Gonzalvez et al, 2008). A recent paper identified a macromolecular complex formed by caspase-8 and Bid on the mitochondrial membrane within which caspase-8 cleaves Bid following Fas ligand or TRAIL stimulation (Schug et al, 2011). As previously described for caspase-8, the caspase-8/Bid complex was localized to mitochondrial membrane microdomains enriched in cardiolipin, which was found to be necessary for the function and stabilization of the complex (Schug et al, 2011). The authors described the formation of caspase-8/Bid mitochondrial complexes as a way through which type II cells concentrate caspase-8 and its substrate (Bid) at the place where it is needed most, on the surface of mitochondria, during death receptor-induced apoptosis.

Hence, in type II cells, once the caspase-8 is activated by TRAIL, it in turn activates the effector caspases and the BH3-only protein Bid. Thus, activation of caspase-8 induces the cleavage of Bid, leading to the release of its truncated form, tBid (**Figure 1.6**). The truncated Bid then accumulates at the mitochondria, facilitated by its interaction with MTCH2/MIMP (Zaltsman et al, 2010), and inserts into the mitochondrial outer membrane (OMM) where it induces the activation of Bax and Bak. Active forms of Bax and Bak in turn promote the destruction of the mitochondrial outer membrane (Jourdain and Martinou, 2009), and the concomitant formation of large pores, resulting in the release of pro-apoptotic proteins into the cytosol, including cytochrome c, Smac/DIABLO and Omi/HtrA2. Cytochrome c interacts with Apaf-1 (apoptotic protease activating factor 1) to form the apoptosome where caspase-9 molecules are activated, leading to activation of effector caspases, and then cell death (**Figure 1.6**). Smac/DIABLO and Omi/HtrA2 neutralize the action of IAPs (inhibitor of apoptosis proteins) proteins, which function as inhibitors of caspases (see paragraph 1.3.6), thereby enabling full activation of caspase-9, -3, and -7 and consequently induction of apoptosis (**Figure 1.6**).

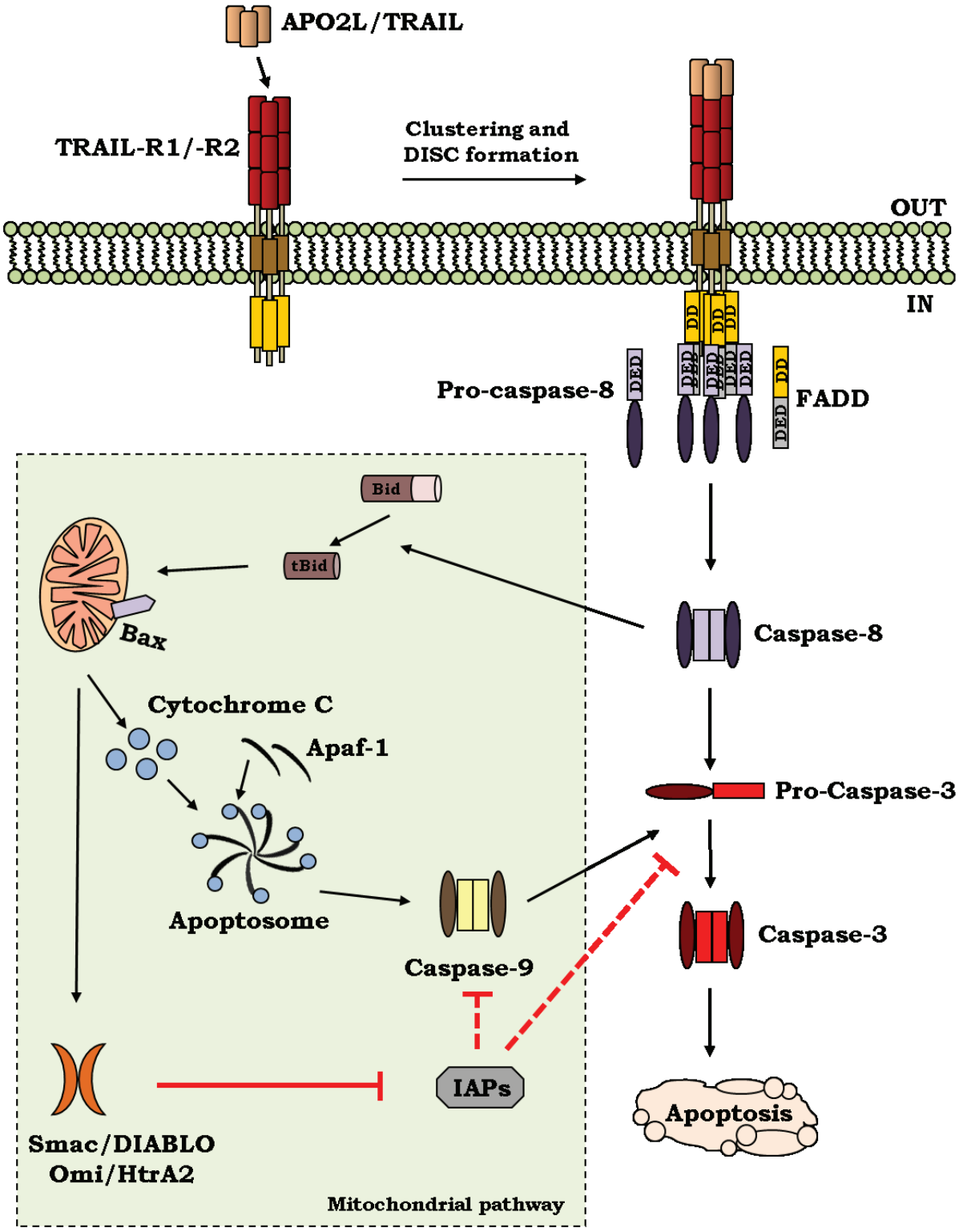

Figure 1.6. The TRAIL apoptotic intrinsic pathway. Binding of a TRAIL trimer to TRAIL-R1 or TRAIL-R2 leads to the clustering of receptors and recruitment of the adaptor FADD, which in turn recruits and activates caspase-8, to form the DISC. In type I cells, active molecules of caspase-8 then activate the effector caspases-3, -6 and -7, and directly induce apoptosis. In type II cells, the mitochondrial pathway is also engaged through the cleavage of Bid by caspase-8, that translocates to the mitochondria, where it induces the Bax-mediated release of cytocrome c and Apaf-1. These events leads to the activation of caspase-9, which in turn activates the effector caspases.

The intrinsic pathway is controlled by members of the Bcl-2 (B cell lymphoma-2) family. Bcl-2 proteins can act as pro- or anti-apoptotic regulators, and they share one or more of the four characteristic BH (Bcl-2 homology) domains that present a high similarity at the amino acid level, and are called BH1, BH2, BH3, BH4 (Youle and Strasser, 2008; Martinou and Youle, 2011). Members of this family have been grouped into three classes (**Figure 1.7**).

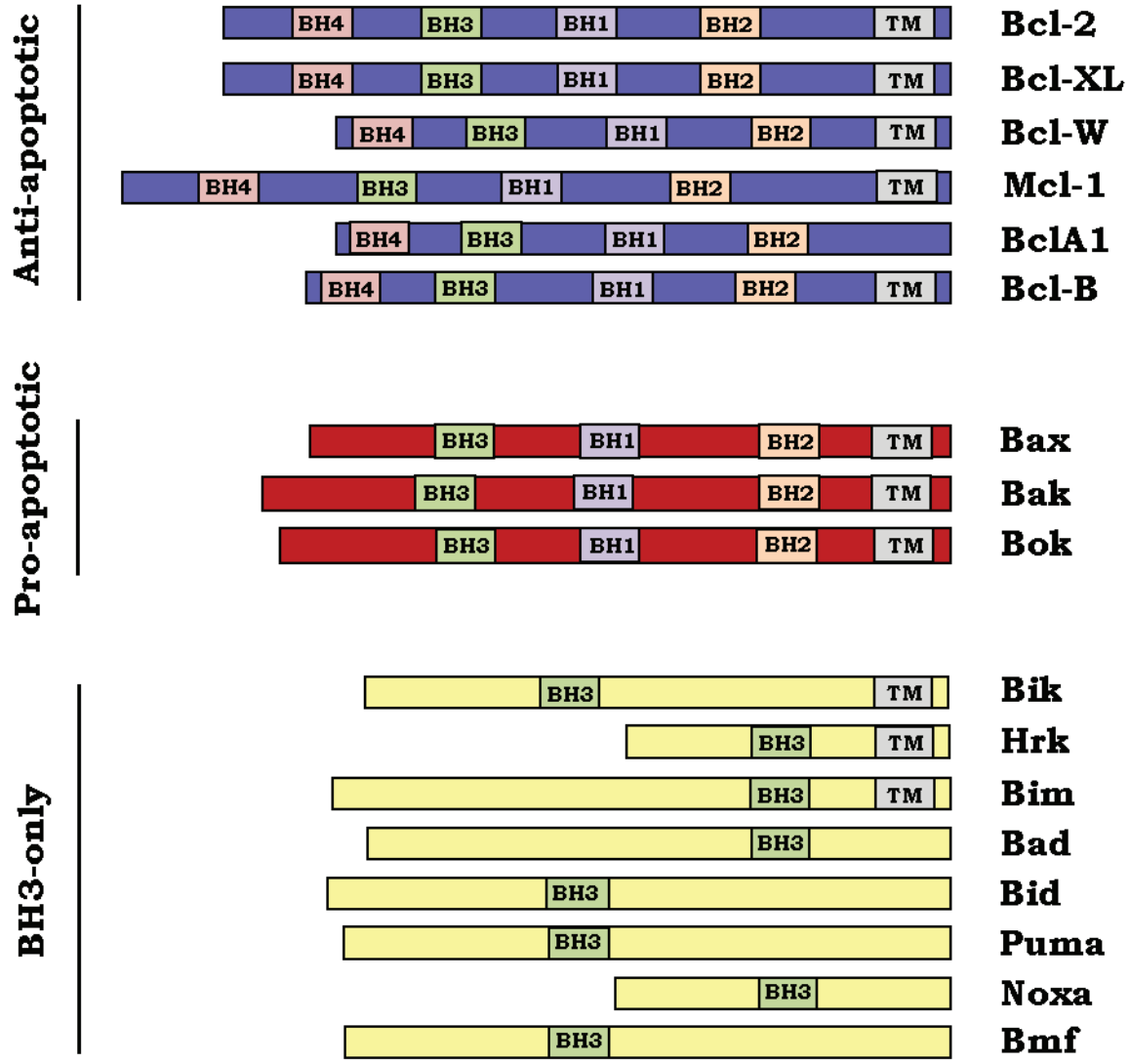

Figure 1.7. Schematic representation of the Bcl-2 family. (Figure adapted from Taylor et al, 2008).

One class inhibits apoptosis, and includes the founding member Bcl-2, as well as Bcl-X_L, Bcl-W, Mcl-1, Bcl-2A1 and Bcl-B, which contain BH domains 1-4 (**Figure 1.7**) (Lindsay et al, 2011). Usually they are associated with the mitochondrial outer membrane through a carboxy-terminal hydrophobic membrane-anchoring tail. They can also be found within the cytosol or integrated in the endoplasmic reticulum membrane. For example, Bcl-2 docks onto the membranes of the mitochondria, the endoplasmic reticulum and nucleus (Lithgow et al, 1994), whereas Bcl-X_L (Hsu et al, 1997), Bcl-W (Wilson-Annan et al, 2003) and Mcl-1 (Nijhawan et al, 2003) have a cytoplasmic localization and translocate to the mitochondria during apoptosis. Their expression can be regulated by different stimuli. For example, growth factors promote cell survival through the induction of Bcl-X_L expression (Grad et al, 2000). Ultraviolet radiation or conditions of cytokine deprivation stimulate the degradation of Mcl-1 by the ubiquitin–proteasome pathway (Zhong et al, 2005; Cuconati et al, 2003). Members of this group function to control and stabilize the integrity of the mitochondrial outer membrane through the direct inhibition of the pro-apoptotic Bcl-2 proteins. Indeed, structural analysis has showed that the BH1, BH2 and BH3 domains form a globular pocket that can interact with the BH3 domain of pro-apoptotic molecules, neutralizing their function (Sattler et al, 1997; Petros et al, 2000; Liu et al, 2003).

Their function is counteracted by the pro-apoptotic Bcl-2 proteins Bax (Bcl-2-associated x protein), Bak (Bcl-2-antagonist killer 1) and Box (Bcl-2-related ovarian killer), belonging to the second class, and which contain three BH domains (BH1, BH2 and BH3) (**Figure 1.7**) (Lindsay et al, 2011). Recent evidence based on structural comparison between Bcl-2 family members also revealed the presence of the BH4 domain (Kvansakul et al, 2008). Members of this group function as effectors of apoptosis because they can directly promote the permeabilization of the mitochondrial outer membrane and the subsequent release of apoptogenic

molecules (Lindsay et al, 2011). They have a redundant role during apoptosis. Indeed, deficiencies in both Bax and Bak are needed in order to induce preservation of mitochondria and cell resistance to apoptotic stimuli (Wei et al, 2001). Bax is predominantly found in the cytosol in a monomeric inactive form in which the carboxy-terminal anchor is masked within the BH3 pocket (Gilmore et al, 2000). Detectable amounts of Bax can also be seen on the mitochondria of nonapoptotic cells. It has recently been proposed by Edlich and co-workers that the cytosolic localization of Bax is regulated by Bcl-X_L, which retranslocates mitochondrial Bax to the cytosol, then preventing its accumulation into the mitochondrial outer membrane that could promote its autoactivation. It has been observed that Bax in the cytosol of nonapoptotic cells continually binds to mitochondria. Once attached to the mitochondrial outer membrane, it interacts with Bcl-X_L which retrotranslocates it back to the cytoplasm (Edlich et al, 2011). In contrast to Bax, Bak is constitutively localized to the mitochondrial outer membrane in nonapoptotic cells where it has been reported to be bound to Mcl-1 and to Bcl-X_L (Willis et al, 2005). Its insertion can be facilitated by the interaction of Bak with VDAC2 (voltage-dependent anion channel isoform 2) (Cheng et al., 2003; Lazarou et al., 2010; Roy et al., 2009). However, the exact role of VDAC2 in the regulation of Bak is still unclear. Indeed, the literature reports that VDAC knockout mice display normal apoptosis, indicating that VDAC can be dispensable during the apoptotic process (Baines et al, 2007). Induction of the mitochondrial pathway stimulates the activation of Bax and Bak. Bax then translocates to the mitochondria and undergoes conformational changes, which favor its insertion into the mitochondrial outer membrane through its carboxy-terminal anchor. Subsequently, Bax and Bak homo- or hetero-oligomerize to form large pores within the mitochondrial outer membrane. These events cause the permeabilization of the mitochondrial outer membrane, and consequently they result in the release of soluble pro-apoptotic

proteins from the mitochondrial intermembrane space to the cytosol, such as cytochrome c and Smac/DIABLO, leading to caspase activation and then to cell death. Bax and Bak also seem to induce fragmentation of the mitochondria (Martinou et al, 2006). The third member of this class, Bok, translocates from the cytosol to mitochondria during apoptosis (Gao et al, 2005) but its function during the apoptotic process is not fully established.

The pro- and anti-apoptotic Bcl-2 proteins are regulated by members of the third group, termed the BH3-only proteins, which contain only the BH3 domain, and include Bid, Bim, Bik, Bad, PUMA and NOXA (**Figure 1.7**) (Lindsay et al, 2011). These proteins function as sensors of apoptotic stimuli. Their expression and activity can be regulated by posttranscriptional and posttranslational modifications. For example, DNA damage can induce the p53-mediated expression of NOXA and PUMA (Oda et al, 2000; Nakano et al, 2001; Yu et al, 2001). Conditions of growth factor deprivation or endoplasmic reticulum stress stimulate the expression of Bim (Dijkers et al, 2000; Puthalakath et al, 2007). Moreover, Bid is activated by caspase-8-mediated proteolysis (Li et al, 1998; Luo et al, 1998). Loss of phosphorylation via the ERK pathway or in response to growth factor deprivation induced the activation of Bim (Akiyama et al, 2003; Ley et al, 2005) or Bad respectively (Zha et al, 1996). Once induced or activated, they interact with the anti-apoptotic partners of the Bcl-2 family, neutralizing their effect and then promoting apoptosis. Some of them, such as Bim, Bid and PUMA, bind to all the anti-apoptotic members, whereas Bad binds Bcl-2, Bcl-X_L and Bcl-W, but not Mcl-1 or A1, and Noxa binds only Mcl-1 and A1 (**Figure 1.8**) (Chen et al, 2005). These proteins can function either as inhibitors of the anti-apoptotic Bcl-2 members, or as activators of the pro-apoptotic proteins Bax and Bak. Based on their specific function, the BH3-only proteins have been divided into two groups: one is composed by Bid, Bim and PUMA (**Figure 1.8**), which are able to directly activate

Bax and Bak, and that have been classified as the BH3 activators; the second, formed by Bad and NOXA (**Figure 1.8**), was referred as the BH3 sensitizers because these proteins promote the activation of Bax and Bak interacting with the anti-apoptotic Bcl-2 proteins and then derepressing the BH3 activator members (Letai et al, 2002; Kuwana et al, 2005; Kim et al, 2006).

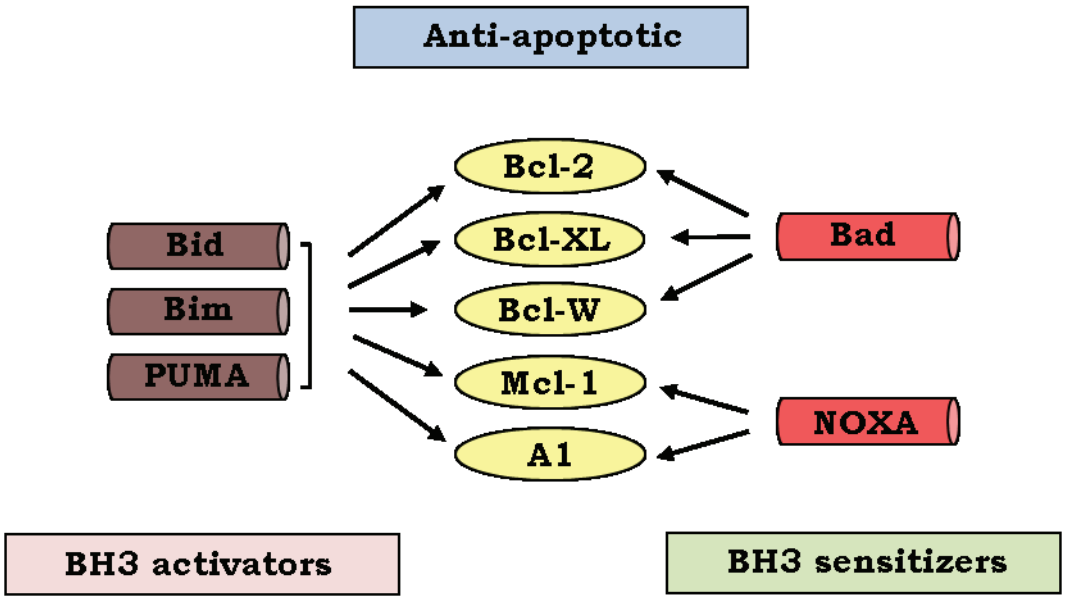

Figure 1.8. Schematic representation of the interaction between the BH3-only proteins and the anti-apoptotic partners. Bid, Bim and PUMA, termed the BH3 activators, are able to bind to all the anti-apoptotic members, whereas Bad and NOXA, referred as the BH3 sensitizers, bind to Bcl-2, Bcl-X_L and Bcl-W or Mcl-1 and A1, respectively.

Two models for the activation of Bax and Bak have been proposed: the direct and indirect model.

The direct model is based on the assumption that the activation of Bax and Bak is triggered by the BH3 activator proteins Bid, Bim and Puma, which can directly bind to Bax or Bak, inducing their activation. This model proposes that in normal conditions, the BH3 activators are sequestered by the anti-apoptotic Bcl-2

members, then preventing the activation of Bax and Bak (**Figure 1.9.a**) (Letai et al, 2002). An apoptotic stimulus triggers apoptosis through the activation of the BH3 sensitizer members. Thus they bind to the anti-apoptotic proteins, liberating the BH3 activators which can then interact with and activate Bax and Bak, inducing apoptosis (**Figure 1.9.b**) (Letai et al, 2002).

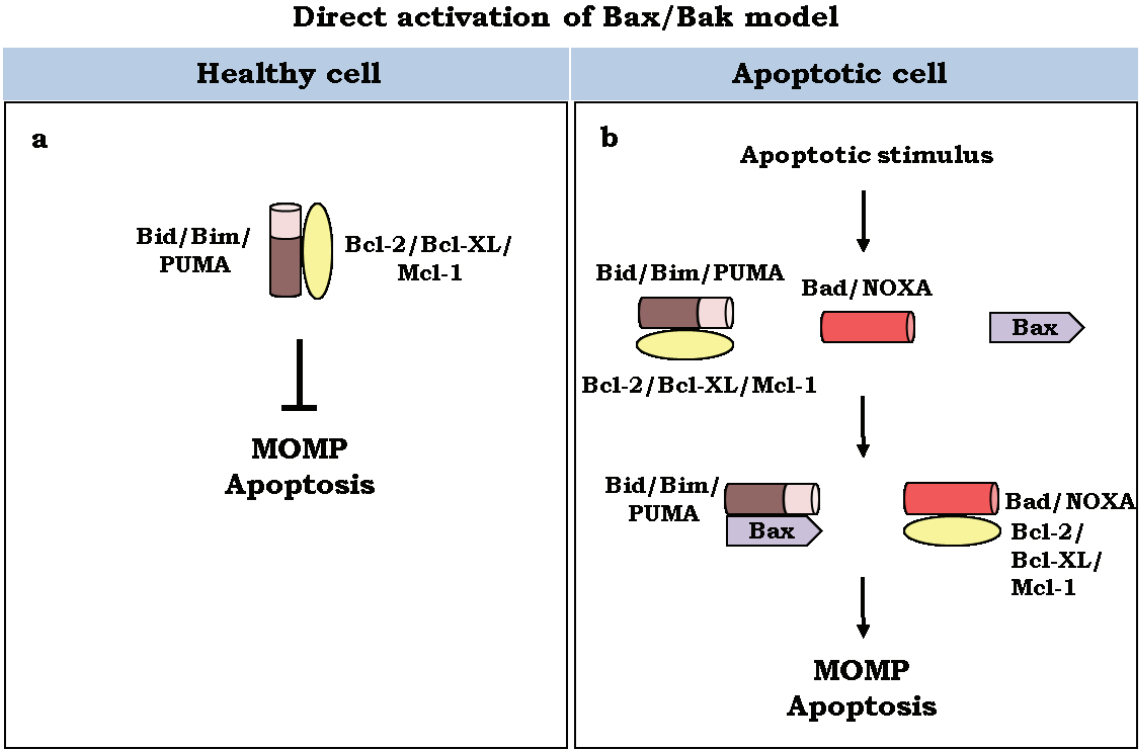

Figure 1.9. Direct model for Bax/Bak activation. (a) In normal conditions, activator BH3 proteins, such as Bid, Bim or PUMA, are sequestered by the anti-apoptotic Bcl-2 proteins. (b) Following an apoptotic stimulus, the BH3 sensitizer proteins are activated and then they associate with the anti-apoptotic members, leading to the release of the activator BH3 proteins. The activators then bind to and activate Bax and Bak, which results in mitochondrial outer membrane permeabilization and apoptosis.

The alternative model, the indirect model of the Bax/Bak activation, assumes that Bax and Bak are sequestered by the anti-apoptotic Bcl-2 proteins in nonapoptotic cells (**Figure 1.10.a**). Following an apoptotic stimulus, the BH3-only proteins are activated and then they bind to and inhibit the anti-apoptotic members, thereby liberating Bax and Bak to drive the permeabilization of the mitochondrial outer membrane and to induce apoptosis (**Figure 1.10.b**) (Willis et al, 2007).

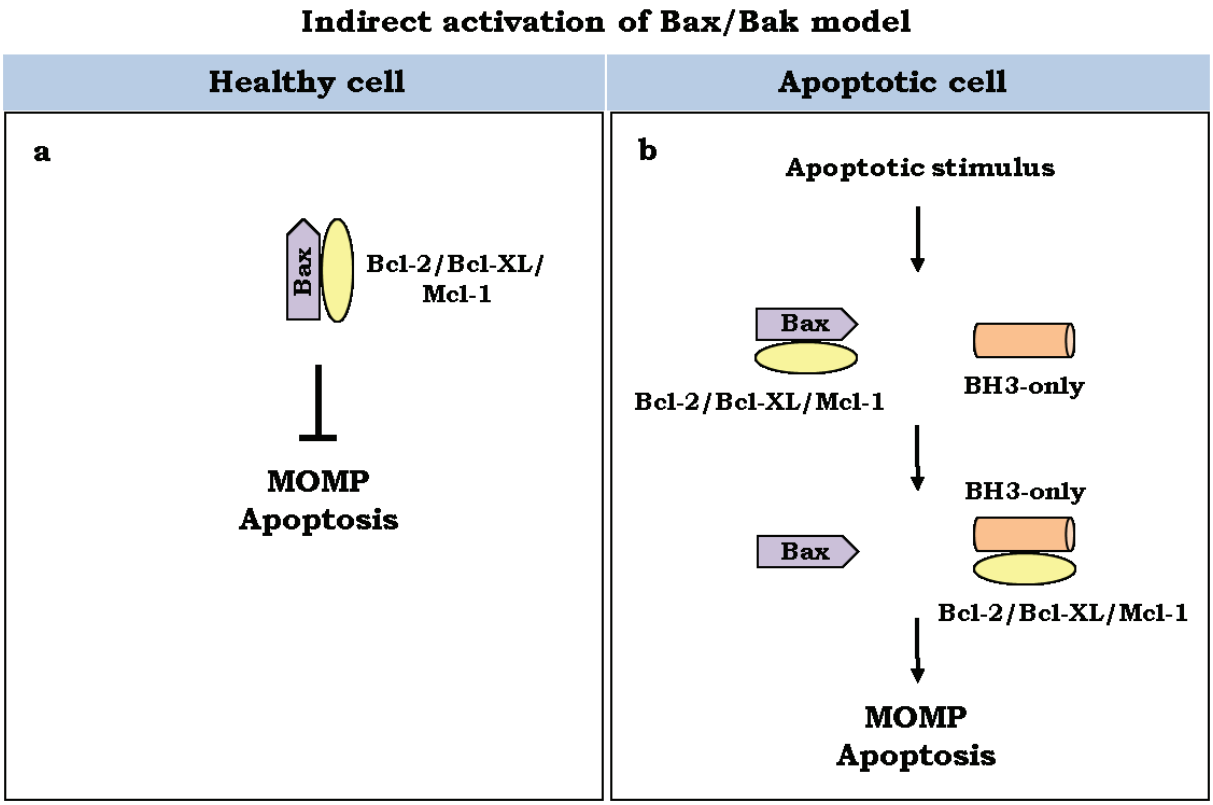

Figure 1.10. Indirect model for Bax/Bak activation. (a) In normal conditions, Bax/Bak is sequestered by the anti-apoptotic Bcl-2 proteins. (b) Following an apoptotic stimulus, the BH3 activator, Bim or Bid, is activated and then it associates with the anti-apoptotic members, releasing Bax/Bak. Bax/Bak then induces the mitochondrial outer membrane permeabilization and then the apoptotic process.

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1.3.5. Nonapoptotic signaling pathways

Binding of trimeric TRAIL to its receptors does not only signals for apoptosis, but can also stimulate nonapoptotic pathways, which includes the activation of NF-kB, Akt, and MAPKs.

For instance, it has been reported that engagement of TRAIL-R1, TRAIL-R2 and TRAIL-R4 by TRAIL can induce the activation of NF-kB (MacFarlane et al, 2003). Activation of NF-kB by TRAIL requires the protein RIP for the activation of the IKK complex (Lin et al, 2000). It has been reported that TRAIL activates NF-kB by promoting the formation of an intracellular secondary complex, called complex II, formed downstream of and physically separated from the DISC, which is composed of FADD, TRADD, caspase-8, caspase-10, RIP, TRAF2 and NEMO (Varfolomeev et al, 2005; Jin and El-Deiry et al, 2006). This event leads to the activation of the IKK complex. Once the IKK complex is activated, it in turn phosphorylates the inhibitor of kB (IkB), and induces its degradation, thereby liberating NF-kB. NF-kB then translocates to the nucleus, where it stimulates the transcription of several anti-apoptotic genes, such as c-FLIP (Micheau et al, 2001; Kreuz et al, 2001), cIAP1 (Wang et al, 1998a), cIAP2 (Chu et al, 1997; Wang et al, 1998a), XIAP (Stehlik et al, 1998) and Bcl-X_L (Chen et al, 2000; Lee et al, 1999a). The biological role of the NF-kB activation induced by TRAIL is still not completely elucidated. Evidence shown that this activation can promote survival, proliferation, invasiveness and metastatic potential of cancer cell lines resistant to TRAIL-induced apoptosis (Ishimura et al, 2006).

Several examples in the literature also highlight the capacity of TRAIL to stimulate the activation of MAPKs. The MAPKs belong to a family of proteins consisting of three main members: the extracellular signal-regulated kinase (ERK), JNK and p38. The role of ERK activation in the TRAIL pathway is still matter of debate. Indeed, the TRAIL-mediated ERK activation was described to be associated

with anti- and pro- apoptotic functions (Tran et al, 2001; Lee et al, 2006a; Frese et al, 2003). The TRAIL-mediated JNK and p38 activation, instead, was mainly associated with pro-apototic signals (Wada et al, 2004).

Lastly, TRAIL can stimulate survival and proliferation of cancer cells through the activation of the PI3K/Akt pathway, leading to inhibition of TRAIL-induced apoptosis. For example, TRAIL has been reported to be able to induce the activation of the phosphatidylinositol-3 kinase in primary human umbilical vein endothelial cells (HUVEC), which in turn leads to the phosphorylation of the Serine/Threonine kinase Akt, and then to its subsequent activation (Secchiero et al, 2003).

Although the molecular mechanisms through which TRAIL triggers apoptosis are well known, the mechanisms leading to the activation and regulation of these nonapoptotic pathways are not yet fully understood. In our hands, for instance, we have so far failed to detect such a soluble complex. Further studies are probably required to better elucidate these mechanisms and determine their biological relevance in order to establish new cancer therapy approaches based on the use of TRAIL.

1.3.6. Regulation of the TRAIL-induced cell death

Sensitivity to TRAIL-induced cell death can be modulated at several levels in the apoptosis signaling pathway. Numerous mechanisms have been described, which may regulate the cellular response to TRAIL, and lead to escape from apoptosis induction, accounting for both intrinsic and acquired resistance to TRAIL. Several examples in the literature and in our hands demonstrate that the regulation of the TRAIL-induced apoptosis can occur by directly inhibiting apoptosis either at the receptor level or further downstream, or by promoting survival pathways.

Surface expression of agonistic TRAIL receptors - The level of expression of TRAIL-R1 and TRAIL-R2 at the plasma membrane can contribute to modulation of the cellular response to TRAIL. Indeed, downregulation or loss of TRAIL agonistic receptors expression on the cell surface has been observed in several cancers, and can be correlated with cellular resistance to TRAIL-induced apoptosis (Horak et al, 2005; Hopkins-Donaldson et al, 2003; Jin et al, 2004). Deletions or mutations in the genes coding for the TRAIL receptors have been frequently found in many cancers, including non-Hodgkin's lymphoma, lung, colon, breast, prostate, hepatocellular, ovarian, and head and neck cancer (el Naggar et al, 1998; Emi et al, 1992; Fujiwara et al, 1994; Kagan et al, 1995; MacGrogan et al, 1994; Mitelman et al, 1997; Monni et al, 1996; Wistuba et al, 1999; Wright et al, 1998; Yaremko et al, 1995). Epigenetic changes have also been described in neuroblastoma, melanoma, lung and ovarian cancers (Van Noesel et al, 2003; Bae et al, 2007; Hopkins-Donaldson et al, 2003; Horak et al, 2005), which led to a reduced TRAIL agonistic receptor expression on the cell surface. Reestablishing the TRAIL-R1 and/or TRAIL-R2 cell surface expression was reported to be able to re-sensitize cancer cells to TRAIL-induced apoptosis (Ozoren et al, 2000; Horak et al, 2005).

Mutations in the TRAIL agonistic receptor gene sequence can also be responsible for the regulation of the cellular sensitivity to TRAIL. Indeed, mutations within the extracellular cysteine-rich domain, or the cytoplasmic death domain of TRAIL-R1 or TRAIL-R2 have been observed in breast, head and neck cancers and non-Hodgkin's lymphoma, and correlated with a loss of receptor functionality, due to inefficient transduction of the apoptotic signal, which leads to hampering the effect of TRAIL-induced cell death (Fisher et al, 2001; Lee et al, 1999b, 2001; Pai et al, 1998; Shin et al, 2001; Bin et al, 2007).

Surface expression of antagonistic TRAIL receptors -. Besides the presence of two TRAIL agonistic receptors able to transmit apoptotic signals within the cells, TRAIL has also two antagonistic receptors, also called decoy receptors, which either possess a nonfunctional death domain, or lack the death domain altogether. These receptors therefore cannot transduce the death signal within cells, and fail to induce apoptosis. Therefore, their presence on the cell surface is correlated with cellular resistance to TRAIL-induced apoptosis (Pan et al, 1997b; Sheridan et al, 1997; Degli-Esposti et al, 1997a,b), and was initially thought to be a prerequisite of normal cells (Spierings et al, 2004). However, subsequent studies demonstrated that the decoy receptors are also expressed in many cancers, including prostate, lung and acute myeloid leukemia cancer cells, where they are responsible for the acquired resistance of these cells to TRAIL stimuli (Sanlioglu et al, 2007; Aydin et al, 2007; Riccioni et al, 2005). In addition, over-expression of decoy receptors in cells sensitive to TRAIL was reported to attenuate TRAIL-induced apoptosis (Sheridan et al, 1997; Mérino et al, 2006; Marsters et al, 1997), and their downregulation with small interfering RNA, or the inhibition of TRAIL binding using specific blocking antibodies, restored the cell sensitivity to TRAIL stimulation (Zhang et al, 2000; Bouralexis et al, 2003; Sanlioglu et al, 2005; Sanlioglu et al,

2007; Aydin et al, 2007). Sensitivity of cells to TRAIL-mediated apoptosis can then be considered a function of the ratio of TRAIL decoy to agonistic death receptors at the cell surface. However, several studies failed to show the correlation between decoy receptor expression and resistance to TRAIL (Zhang et al, 1999; Leverkus et al, 2000; Daniels et al, 2005). Therefore, the role of decoy receptors in mediating resistance to TRAIL is still controversial and needs further investigation.

c-FLIP - Activation of caspase-8 and -10 can be inhibited by the recruitment of the antiapoptotic protein c-FLIP (cellular FLICE-like inhibitory protein) at the DISC level (**Figure 1.11**). Three different forms of c-FLIP are expressed in humans: one long variant, c-FLIP$_L$, and two short, c-FLIP$_R$, first isolated from the human Burkitt lymphoma B-cell line Raji, and c-FLIP$_S$ (Irmler et al, 1997; Thome et al, 1997; Djerbi et al, 2001; Golks et al, 2005). c-FLIP is highly homologous to the caspases. It has two DEDs in its amino-terminal region through which it can interact with FADD, and one caspase-like domain at its carboxy-terminal, which does not possess enzymatic activity because it lacks the cysteine residue 297 in the catalytic domain (Irmler et al, 1997). The small variants differ from the long form due to the presence of a short carboxy-terminal region (Irmler et al, 1997). Usually all the three FLIP variants associate with FADD within the DISC, competing with caspase-8, and leading to inhibition of caspase-8 activation and consequently of the apoptotic cascade (**Figure 1.11**). However, the precise physiological role of c-FLIP$_L$ is still not completely elucidated. Although, several data describes that the presence of c-FLIP$_L$ protects cells from death receptor-induced apoptosis, c-FLIP$_L$ sometimes displays pro-apoptotic activity. Indeed, overexpression of FLIP in HEK 293T cells was reported by several groups to cause efficient cell death (Shu et al, 1997; Goltsev et al, 1997; Inohara et al, 1997). Moreover, c-FLIP$_L$ was described to promote caspase-8 activation, due to its capacity to induce the first cleavage steps of caspase-8, but

the caspase-8 activity remained highly impared and limited, leading to cell resistance to Fas-mediated apoptosis (Krueger et al, 2001; Chang et al, 2002; Micheau et al, 2002). A possible molecular explanation for this phenomenon has been reported. Once c-FLIP$_L$ is recruited within the DISC, it associates with proforms of caspase-8, and c-FLIP$_L$-caspase-8 heterodimers are formed, which induce activation and processing of caspase-8. However, caspase-8 is only partially processed and the caspase-8 cleaved products are not released into the cytosol, but remain associated with the DISC, preventing activation of the effector caspases, and the further transduction of the apoptotic signal. Nevertheless, the active caspase-8 bound to the DISC can activate other substrates, such as RIP, which favors the activation of nonapoptotic signaling pathway (Micheau et al, 2002).

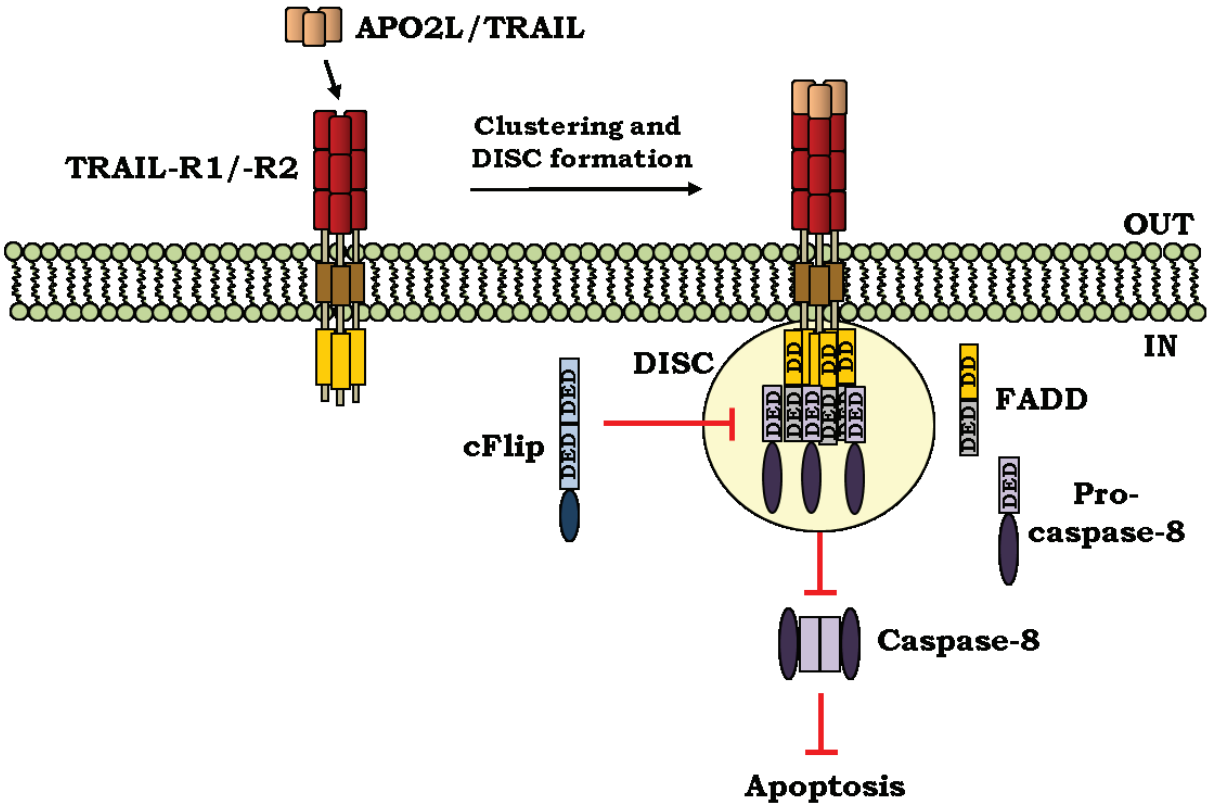

Figure 1.11. cFlip can block the apoptotic pathway. Binding of cFlip to the TRAIL DISC prevents the recruitment of caspase-8 to the DISC, leading to the inhibition of TRAIL-induced cell death.

The Bcl-2 proteins - The balance between pro- versus anti-apoptotic Bcl-2 members is an important determinant for transducing the apoptotic signal in type II cells, which require an amplification loop through the mitochondria in order to efficiently undergo TRAIL-induced apoptosis. Alteration in the expression of Bcl-2 members has been often observed in many cancers, where these proteins interfere with the correct propagation of the apoptotic signal within the cells, leading to the acquisition of a TRAIL-resistant phenotype. For instance, over-expression of Bcl-2, Bcl-X_L or Mcl-1 inhibits TRAIL-induced apoptosis in many cancers, including lung, prostate and pancreatic cancer cells (Fulda et al, 2004; Song et al, 2007a; Wang et al, 2008; Taniai et al, 2004; Zhan and Fang et al, 2005). In contrast, TRAIL-induced apoptosis is impaired in HCT116 colorectal cancer cells that are deficient in pro-apoptotic Bax (Ravi and Bedi, 2002; Deng et al, 2002; LeBlanc et al, 2002). In these cells, the absence of Bax accounted for the cellular resistance to TRAIL, which inhibits the activation of caspase-9, and thus prevents the activation of the effector caspases. Moreover, a deficiency in Bax could be responsible for the inefficiency in the release of cytochrome c after TRAIL stimulation in mouse embryonic fibroblasts depleted in Bax, leading to inhibition of TRAIL-induced apoptosis (Kandasamy et al, 2003).

IAPs proteins - The activation of caspases can be neutralized and then inhibited by the IAPs (inhibitors of apoptosis proteins) proteins, which bind to and inactivate caspases (**Figure 1.12**). Members of this family, which includes cellular IAP-1 and -2, and XIAP, possess three baculovirus IAP repeat (BIR) domains and, in some cases, a carboxy-terminal RING domain with E3 ubiquitin ligase activity through which they can ubiquitinate themselves and other interacting proteins, targeting them for proteosomal degradation (Vaux and Silke, 2005). XIAP is the most potent inhibitor of caspase-3, -7, and -9 (Deveraux et al, 1997), which directly binds to

caspases at the level of their catalytic domain, impairing their catalytic activity and their activation induced by formation of dimers, and favoring their ubiquitination leading to their degradation. All these events block the caspase cascade and ultimately result in the inhibition of cell death. Furthermore, over-expression of XIAP has been correlated with inhibition of TRAIL-induced apoptosis (Chawla-Sarkar et al, 2004; Ndozangue-Touriguine et al, 2008; Lee et al, 2006b; Lippa et al, 2007). On the other hand, inhibition or downregulation of IAP proteins restored sensitivity of cells to TRAIL-induced apoptosis (Mori et al, 2007; Gill et al, 2009; Shrader et al, 2007; Symanowski et al, 2009; Lee et al, 2006b). IAPs are neutralized by Smac/DIABLO, which is released from the mitochondria during TRAIL-induced apoptosis. Smac/DIABLO directly binds to and inhibits IAPs, competing with them for binding to caspases (Deng et al, 2002; Zhang et al, 2001; Ng and Bonavida, 2002).

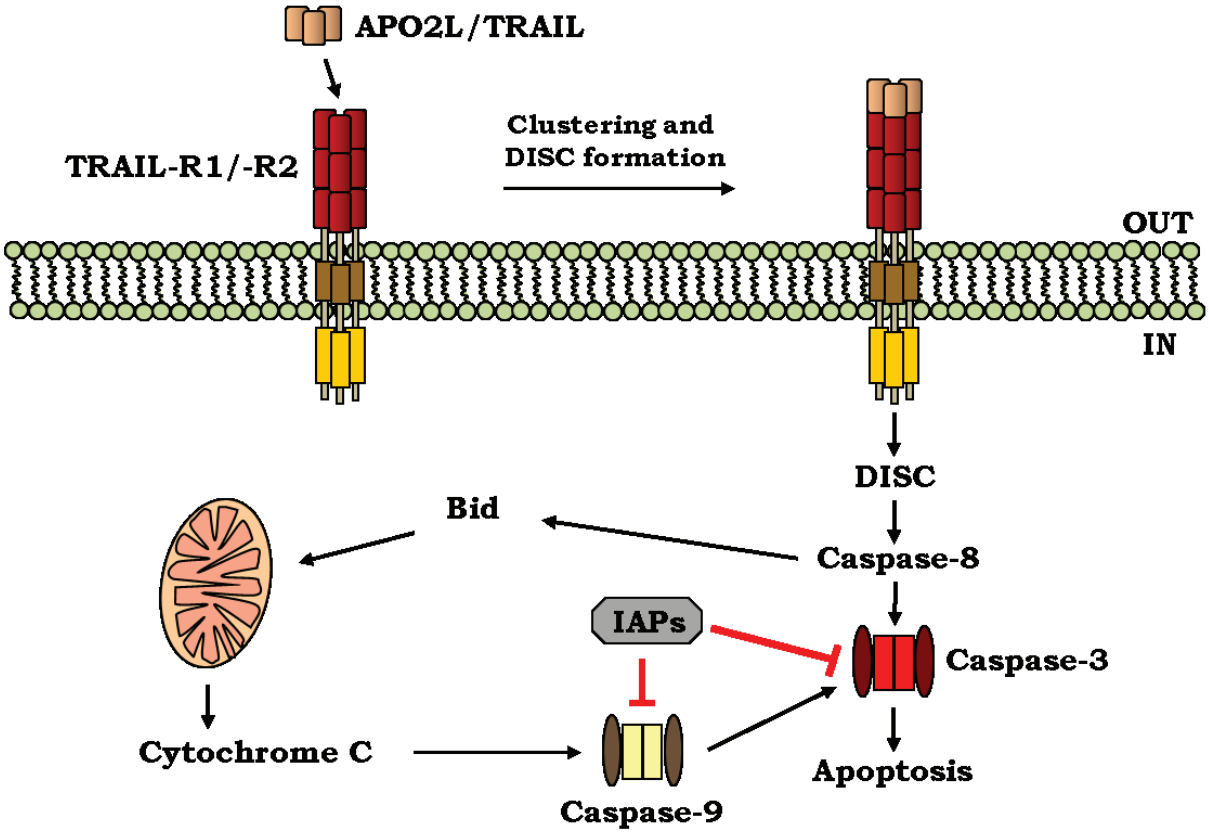

Figure 1.12. IAPs proteins can block the apoptotic pathway. IAP proteins can associate with and attenuate the activation of caspase-9 and -3, leading to the inhibition of TRAIL-induced cell death.

1.3.7. Biological role of TRAIL

In order to investigate the physiological role of TRAIL a large number of research groups have been using mouse models. These experiments confirmed the essential and crucial role of TRAIL for triggering apoptosis, and ruled out any possible involvement of the TRAIL pathway in normal mouse embryonic development. Indeed, TRAIL or TRAIL-R knockout mice were reported to be viable and develop normally, and they did not display any apparent haematological or reproductive defects (Cretney et al, 2002; Sedger et al, 2002; Diehl et al, 2004; Finnberg et al, 2005).

TRAIL is not present at the plasma membrane of T-cells, B-cells, monocytes, dendritic cells, and natural killer cells, but its expression can be induced by type I interferons. This peculiarity of TRAIL has suggested that TRAIL could be involved in the regulation of immune responses. For instance, stimulation of monocytes with type I or II interferons or LPS induces the expression of TRAIL, and these cells acquire the ability to kill tumour cells (Ehrlich et al, 2003; Halaas et al, 2000). LPS and type I interferons are also able to induce the expression of TRAIL in T cells (Ehrlich et al, 2003; Kayagaki et al, 1999a,b; Arbour et al, 2005). Moreover, dendritic cells express TRAIL at their cell surface following stimulation with IFN-α or IFN-β, which enhance their cytotoxic activity against tumour cells (Kemp et al, 2003; Liu et al, 2001). TRAIL is also expressed on the surface of natural killer cells after IFN-γ stimulation (Kayagaki et al, 1999a; Takeda et al, 2001a).

The TRAIL system may have a role in the immune response against infectious pathogens. Indeed, it has been reported that viral infections can sensitise cells to TRAIL-induced apoptosis. For instance, lymphocytes and monocyte-derived macrophages from uninfected donors are normally resistant to TRAIL-induced apoptosis, but they can become sensitive following a human immunodeficiency virus infection (Lum et al, 2001). TRAIL can kill HEK293 cells infected with

reoviruses, which induce an increase in the expression of TRAIL-R1 and TRAIL-R2 (Clarke et al, 2000). The cytotoxicity activity of natural killer cells is enhanced following viral infection, and it is correlated with an increase in the TRAIL surface expression mediated by IFN-α and -β (Sato et al, 2001). TRAIL can also be considered a negative regulator of the immune response against viral infections. TRAIL-R knockout mice are more resistant to murine cytomegalovirus then other pathogens (Diehl et al, 2004). In TRAIL-R knockout mice infected with murine cytomegalovirus, an increase in the production of IL-12, IFN-α and IFN-γ by dendritic cells, macrophages and natural killer cells is observed, and is correlated with an enhanced innate immune response against viruses (Diehl et al, 2004). Consistent with this hypothesis, TRAIL knockout mice display enhanced resistance to Listeria monocytogenes infection (Zheng et al, 2004).

Divergent roles of TRAIL are described in autoimmunity. TRAIL is upregulated in certain autoimmune disorders. In fact, studies report the presence of elevated levels of soluble TRAIL in the serum of patients with systemic lupus erythematosus or human multiple sclerosis (Lub-de Hooge et al, 2005; Wandinger et al, 2003; Cretney et al, 2006). Increased levels of soluble TRAIL have also been found in a human endotoxemia model after administration of endotoxins (Lub-de Hooge et al, 2004). All these events have led to the assumption that TRAIL could be involved in the acceleration of autoimmune diseases. However, TRAIL may also play an inhibitory role in the development of autoimmune diseases. Treatments of wild-type mice with TRAIL blocking antibodies increase the development of autoimmune disease (Hilliard et al, 2001; Cretney et al, 2005). TRAIL knockout in mice enhances autoimmune disease progression (Lamhamedi-Cherradi et al, 2003) and increased defects in negative selection of thymocytes (Cretney et al, 2003).

Finally, TRAIL may also participate in immune surveillance mechanisms acting against the development of primary and metastatic tumours. The first

evidence for this role arose from studies using mice in which treatments with a soluble recombinant form of TRAIL reduced growth of human tumour xenografts (Walczak et al, 1999; Ashkenazi et al, 1999). Mice inoculated with neutralizing anti-TRAIL antibodies or mice in which TRAIL is knocked out present increased tumour growth and enhanced propensity to metastatize as compared to control mice (Cretney et al, 2002; Takeda et al, 2001a). Similarly, TRAIL-R knockout mice display an increased development of metastasis of lymphoma and skin carcinoma (Finnberg et al, 2008; Grosse-Wilde et al, 2008). Recently, gene expression analysis of several human breast tumours showed a correlation between the downregulation of TRAIL and incidence of breast cancer metastasis to the brain (Bos et al, 2009). However, some reports have not confirmed a role of TRAIL in controlling initiation and growth of primary tumours. For example, loss of TRAIL-R does not modify tumour development in p53 null or adenomatous polyposis coli (APC) mutant mice (Yue et al, 2005). Thus, the physiological role of the TRAIL system during tumourigenesis remains controversial. A definitive role for TRAIL in this field has yet to be fully established.

1.3.8. TRAIL as anti-cancer drug

During the past decade, TRAIL has raised a growing interest in oncology due to its ability to selectively trigger cancer cell death, while sparing normal cells. Thanks to this peculiarity of TRAIL, and in light of the use of TRAIL as therapeutic agent against malignant deseases, several recombinant molecules of TRAIL have been generated.

Soluble recombinant forms of TRAIL - To date, different forms of soluble recombinant TRAIL have been generated in order to utilize TRAIL in anti-cancer therapies. Most of them are fused at their amino terminal region to specific tags, which facilitate the production of the ligand and favor its aggregation. These recombinant molecules include histidine (His)-tagged TRAIL (Pitti et al, 1996), leucine zipper (LZ)-TRAIL (Rozanov et al, 2009), and Flag-tagged TRAIL (Schneider, 2000). Only the non-tagged soluble recombinant human (rh) version of TRAIL, also called dulanermine, that exhibits full apoptotic activity has been selected for clinical trials, as some other recombinant forms of TRAIL have been reported to induce toxicity in normal cells, such as human hepatocytes and human brain cells (Jo et al, 2000; Nitsch et al, 2000; Lawrence et al, 2001; Ganten et al, 2006). Toxicity towards normal human hepatocytes or human brain cells was observed only after treatments with the histidine- or Flag-tagged recombinant forms of TRAIL, whereas treatments with non-tagged or leucine zipper-tagged recombinant TRAIL were not toxic. In vitro and in vivo experiments with leucine zipper-TRAIL and recombinant human TRAIL demonstrated that administration of these molecules did not cause apoptosis in normal cells, and that they can be used safely (Ashkenazi et al, 1999; Lawrence et al, 2001; Walczak et al, 1999; Hao et al, 2004). Thus, the toxicity observed in normal cells could be attributed to the presence of the tag in the recombinant ligand. Furthermore, the tagged versions of TRAIL were reported to have unusual

tertiary structures, which could induce formations of multi-molecular aggregates of the death receptors, and could result in induction of apoptosis in hepatocytes (Lawrence et al, 2001). These variants of TRAIL also contained a lower level of zinc compared to the native ligand (Lawrence et al, 2001), that was shown to be crucial for the stability and function of the trimer (Hymowitz et al, 2006). Therefore, the method of preparation of recombinant tagged ligands could be responsible for the side effects observed in normal cells. The recombinant human variant of TRAIL has proceeded to the clinic, and phase I and II clinical trials have been initiated and are still in progress (Herbst et al, 2006: Ling et al, 2006; Yee et al, 2007). Preliminary results indicated that the non-tagged variant of TRAIL is well tolerated in patients, and the side effects are mild. However, the half-life of TRAIL is very short, and its administration rarely leads to a complete eradication of the tumour. Indeed, most of the patients included in the trial experienced a stable disease and just one had a partial response (Herbst et al, 2006).

Other recombinant variants of TRAIL - Other recombinant variants of TRAIL that have higher affinity for TRAIL-R1 and/or TRAIL-R2, and are able to bind selectively to a specific TRAIL death receptor have also been generated in order to enhance the efficacy and specificity of the TRAIL apoptotic signalling for the better use of TRAIL in anti-cancer therapies. These variants were developed using either a computational method, or by a phage display approach. In both cases, a point mutation leading to a single amino acid change was introduced in the receptor binding region, which induced the generation of TRAIL variants selective for TRAIL-R1 or TRAIL-R2. Using the computational method, Tur and co-authors generated TRAIL-R1 selective forms of TRAIL with reduced binding affinity for TRAIL-R2 and decoy receptors, and enhanced affinity for TRAIL-R1. These TRAIL variants were able to signal for apoptosis in cell lines which undergo cell death through TRAIL-R1,

and not in TRAIL-R2 responsive cells, but the variants were less efficient in inducing apoptosis compared to native TRAIL (Tur et al, 2008). Therefore, other TRAIL variants able to bind both TRAIL-R1 and TRAIL-R2 with increased affinity were generated, and were shown to be more effective in inducing cell death in cells responsive to only TRAIL-R1, or to both death receptors than native TRAIL (Reis et al, 2009). Using the same method, van der Sloot and co-authors developed TRAIL-R2 selective TRAIL variants which present higher binding affinity for TRAIL-R2, and decreased affinity for TRAIL-R1 and decoy receptors (van der Sloot et al, 2006). The phage display approach was used by Kelley and co-authors to generate other TRAIL variants selective for both death receptors (Kelley et al, 2005). These death receptors variants showed different efficiency in inducing apoptosis, suggesting that TRAIL-induced cell death could involve TRAIL-R2 more than TRAIL-R1. Indeed, the TRAIL-R1 selective variants were less potent in triggering apoptosis as compared to Flag-tagged TRAIL, whereas the TRAIL-R2 selective variants had unchanged apoptosis-inducing ability (Kelley et al, 2005). The new TRAIL variants specific for TRAIL-R1 or TRAIL-R2 synthetized at a later point demonstrated that leukemia or lymphoma cells undergo apoptosis almost exclusively through TRAIL-R1 (MacFarlane et al, 2005).

TRAIL agonistic antibodies - Specific agonistic antibodies against TRAIL-R1 and TRAIL-R2 have also been generated. The use of these antibodies presents several advantages. They have a half life of around 2-3 weeks, which is longer than that of recombinant human TRAIL (Tolcher et al, 2007; Plummer et al, 2007). Moreover, they are able to bind specifically and with high affinity to death receptors present at the surface of various tumour cell lines, activating the apoptotic pathway, and without inducing toxicity in normal cells. These antibodies also inhibit tumour growth in mouse xenograft models (Pukac et al, 2005; Zeng et al, 2006; Smith et al,

2007; Adams et al, 2008; Ichikawa et al, 2001; Li et al, 2008a). Six human monoclonal antibodies have been developed, one directed against TRAIL-R1, the HGS-ETR1 or mapatumumab, and the other directed against TRAIL-R2, namely HGS-ETR2 or lexatumumab, Apomab or PRO95780, TRA-8 or CS-1008, AMG 655 and LBY135. The TRAIL agonistic antibodies have been studied or are currently in use in several phase I and II clinical trials, either as a single agent or in combination with chemotherapeutic drugs (Tolcher et al, 2007; Plummer et al, 2007; Greco et al, 2008; Leong et al, 2009). The antibodies are well tolerated, and the response usually observed is the establishment of a stable disease. 3 out of 40 tumour responses, one complete and two partial, were also observed using mapatumumab (Younes et al, 2005). Alternatively, multivalent small peptido-mimetics targeting TRAIL-R2 have also been generated and shown to be functional both *in vitro* and *in vivo* (Pavet et al, 2010).

Combination therapies - Recombinant human TRAIL is not only used as a single agent in anti-cancer therapy, but also in combination with chemotherapeutic drugs. Combination therapies have been demonstrated to enhance the anti-tumour effect of TRAIL. Indeed, TRAIL seems to synergize with chemotherapeutic agents, leading to increased induction of apoptosis. Chemotherapy usually targets the intrinsic apoptotic pathway, whereas TRAIL activates the extrinsic apoptotic pathway. Thus, the use of combination therapies results in the activation of both apoptotic pathways within cells, and cross-talk between the two pathways can also be initiated through the cleavage of Bid, leading to enhanced transduction of the apoptotic signal and an increased level of TRAIL-induced apoptosis. Furthermore, combinations with chemotherapeutic drugs, such as fluorouracil (5-FU), cisplatin and etoposide, was demonstrated to restore tumour cells sensitivity to TRAIL-induced apoptosis. Indeed, studies reported that combination therapies results not

only in increased expression of TRAIL-R1 and TRAIL-R2, but also in enhanced DISC formation, increasing the recruitment and activation of caspase-8 and decreasing the level of c-Flip (Kondo et al, 2006; Lacour et al, 2003; Ganten et al, 2004; Song et al, 2007b; Morizot et al, 2011).

Other compounds that target proteins that are involved in the TRAIL signalling pathway are also currently used in combination therapies.

The histone-deacetylase (HDAC) inhibitors belong to this group. These proteins can induce cell cycle arrest, promote differentiation, and stimulate apoptosis in cancer cells (Johnstone et al, 2002; Singh et al, 2005). They can activate both the extrinsic and intrinsic apoptotic pathways. Like chemotherapeutic agents, they synergize with recombinant human TRAIL to induce apoptosis, enhancing the expression of TRAIL-R1, TRAIL-R2 and pro-apoptotic Bcl-2 proteins, decreasing the anti-apoptotic Bcl-2 members, and activating caspase-8, -9, -3 (Singh et al, 2005). The histone-deacetylase inhibitors are currently used in phase I, II and III clinical trials (reviewed in Ma et al, 2009).

Another example of a therapeutic agent is represented by the proteasome inhibitors (Sayers et al, 2006), which shows synergistic effects on apoptosis in various tumour cells. Surface expression of TRAIL-R1 and TRAIL-R2 are enhanced following bortezomib treatments in hepatocellular, colonic, and pancreatic cancer cell lines (Koschny et al, 2007 a,b). The sensitization of tumour cells to recombinant human TRAIL was also attributed to a downregulation of the anti-apoptotic protein c-Flip and inhibition of AKT, induced by the proteasome inhibitor (Kyritsis et al, 2007).

The inhibitor of the heat shock protein 90, 17-AAG, also enhances apoptosis when combined with recombinant human TRAIL or antagonistic antibodies in phase I studies (Georgakis et al, 2006; Ma et al, 2006).

In the clinic, recombinant human TRAIL has also been combined with rituximab, a CD20-targeting antibody, in patients with relapsed low-grade non Hodgkin's lymphoma (NHL) (Fanale et al, 2008). Preliminary data have shown three complete responses and three partial responses out of twelve patients (Fanale et al, 2008). A phase II clinical trial with rituximab plus recombinant human TRAIL has been started and it is still ongoing.

Another trial, where TRAIL was combined with carboplatin, paclitaxel and bevacizumab, an anti-VEGF antibody, was also performed in patients with non-small cell lung cancer (NSCLC) (Soria et al, 2010). The combination was well tolerated. One complete tumour response and thirteen partial responses out of twenty-four patients were obtained (Soria et al, 2010). This combination has being studying in a phase II clinical trials.

1.4. Ezrin

Ezrin is a member of the Ezrin, Radixin, Moesin (ERM) family of proteins, that links various integral membrane proteins to the actin cytoskeleton (Bretscher et al, 2002), and that participates in several cortical actin-based processes, including membrane projections (Berryman et al, 1995; Lamb et al, 1997; Mackay et al, 1997), cell adhesion (Takeuchi et al, 1994; Hiscox and Jiang, 1999; Pujuguet et al, 2003), cell motility (Crepaldi et al, 1997; Ng et al, 2001), cytokinesis, phagocytosis (Lugini et al, 2003), and signalling for cell survival (Gautreau et al, 1999).

ERM proteins are members of the erythrocyte protein 4.1 superfamily and share a high sequence amino acid homology to each other (70-85%). They are encoded by three different genes, localized in humans on chromosomes 6, 11 and X, respectively, whereas the lower eukaryotes possess only one ERM gene (Miller, 2003). Thus, it is likely that the ezrin, radixin, and moesin genes have diverged from a common ancestral gene, through gene duplication events. Moreover, ERM proteins are not present in yeast; only multicellular organisms express these proteins. The ERM proteins have high similarity at the amino acid level also with the protein merlin, also known as neurofibromatosis type 2 (NF2), a tumour suppressor gene that is mutated in neurofibromatosis. Genomic studies from diverse group of animals indicated that the merlin and ERM genes are highly conserved between species, and originated from a common ancestral gene during the metazoan period (McClatchey and Fehon, 2009).

Ezrin was originally characterized as a substrate of protein tyrosine kinases in the human A431carcinoma cell line, in response to the addition of epidermal growth factor (EGF) (Cooper and Hunter, 1981; Gould et al, 1986), and as a component of microvillar structures of the chicken intestinal epithelial cell brush borders (Bretscher, 1983), and of membrane ruffles of choriocarcinoma cells (Pakkaner et al, 1987).

Although most cells in culture co-express the three ERM proteins (Franck et al, 1993; Sato et al, 1992; Louvet-Vallèe, 2000), they exhibit a cell-type and tissue-specific pattern of expression (Berryman et al, 1993, reviewed in Fehon et al, 2010). Indeed, ezrin is predominantly enriched in microvilli on the apical sides of epithelial cells, while moesin is concentrated in the microvilli of endothelial and hematopoietic cells (Berryman et al, 1993; Schwartz-Albiez et al, 1995; reviewed Fehon et al, 2010) and radixin in microvilli and adherent junctions of hepatocytes (Amieva et al, 1994; Tsukita et al, 1989; reviewed Fehon et al, 2010). Other regions of ezrin expression are along the basolateral surfaces of ductal epithelial cells, renal podocytes, and retinal epithelial cells (Berryman et al, 1993; Bonilha et al, 1999).

1.4.1. Biochemical structure

Ezrin contains 586 amino acid residues, it has a molecular mass of 80 kDa, and it is encoded at the end of the long arm of chromosome 6 (6q22-q27) (Majander-Nordenswan et al, 1998; Turunen et al, 1989). Ezrin, like radixin and moesin, consists of a ~ 300-residue globular amino-terminal domain which is highly conserved in the ERM family, the FERM domain, also know as N-ERMAD (N-terminal ERM associated domain), followed by a long region with a high α-helical propensity and terminating in a carboxy-terminal domain, known as the C-terminal ERM associated domain (C-ERMAD) (Gary et al, 1995; Chshti et al, 1998, Yonemura et al, 1999) (**Figure 1.13**).

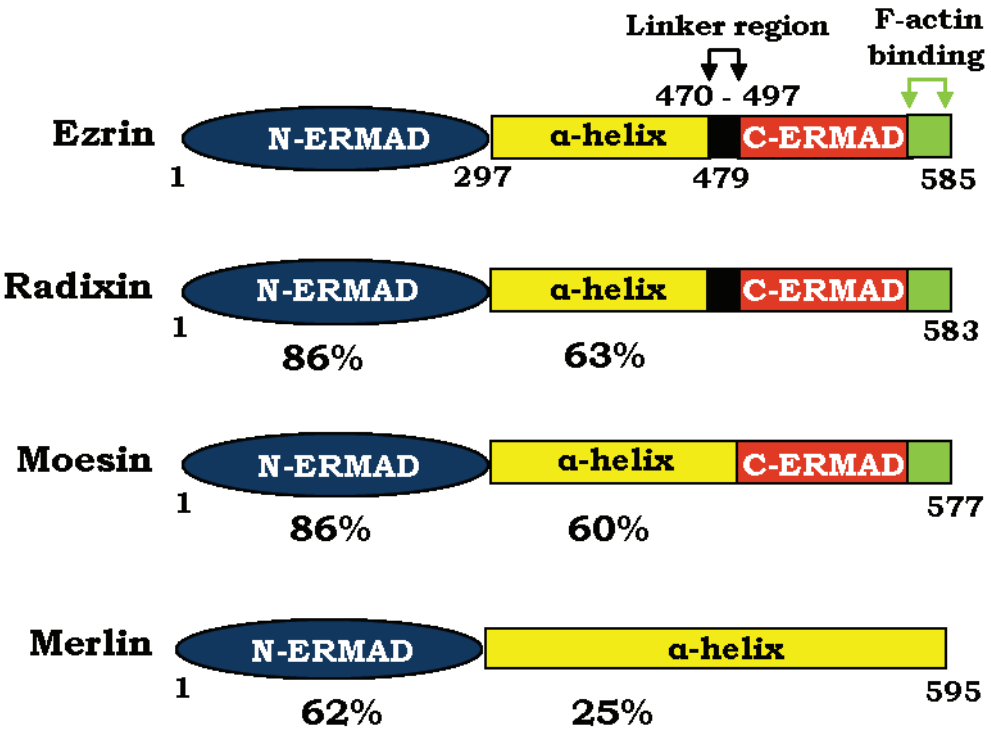

Figure 1.13. Domain organization of ERM proteins. Comparison of the domain organization between ERM and merlin (members of the 4.1 superfamily). The ERM proteins (Ezrin, Radixin, Moesin) have a similar domain structure and show high sequence homology, divergent from that of merlin. ERM consist of an amino-terminal FERM or N-ERMAD domain (blue) organized in three subdomains (F1, F2 and F3), a central α-helical region (yellow) terminating in ezrin and radixin, but not in moesin, with a proline rich linker region, and a carboxy-terminal C-ERMAD domain (red), which contain the F-actin-binding site. (Figure adapted from Bretscher et al, 2002).

The FERM domain is responsible for membrane binding, and it is arranged in a cloverleaf structure composed of three subdomains, called lobes F1, F2, and F3 (Pearson et al, 2000, Smith et al, 2003) (**Figure 1.14**), that possess structural homology to well characterised protein folds. F1 seems to be structurally similar to ubiquitin, F2 to acyl CoA-binding protein and F3 to a PTB domain.

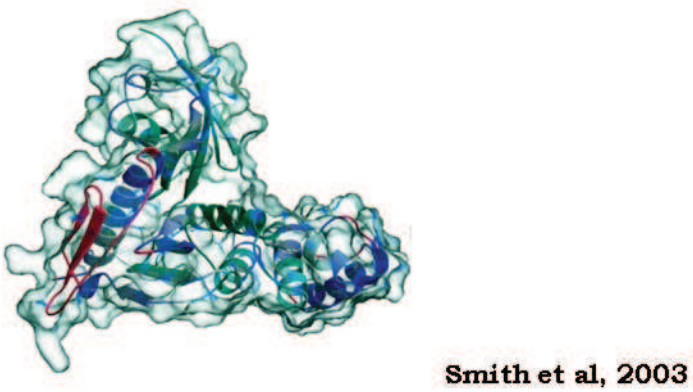

Figure 1.14. Three dimensional structure of the ezrin FERM domain. (Figure adapted from Smith et al, 2003).

The FERM domain can bind directly to the cytoplasmic tails of various membrane proteins, including ICAM-1, -2, -3, CD43, CD44, NHE-1 and Syndecan-2 (Helander et al, 1996; Yonemura et al, 1993; Yonemura et al, 1998; Tsukita et al, 1994; Denker et al, 2000; Granés et al, 2000) (**Figure 1.15**). Alternatively, the association with integral membrane proteins, such as NHE-3, CFTR, β2A, PDGF-4 and podocalyxin (Yun et al, 1997; Short et al, 1998; Wang et al, 1998b; Hall et al, 1998; Maudsley et al, 2000; Takeda et al, 2001b; Orlando et al, 2001) can occur through adaptor molecules known as EBP50 (ERM binding protein 50) (Reczek et al, 1997) or E3KARP (NHE type 3 kinase A regulatory protein) (Yun et al, 1998) (**Figure 1.15**).

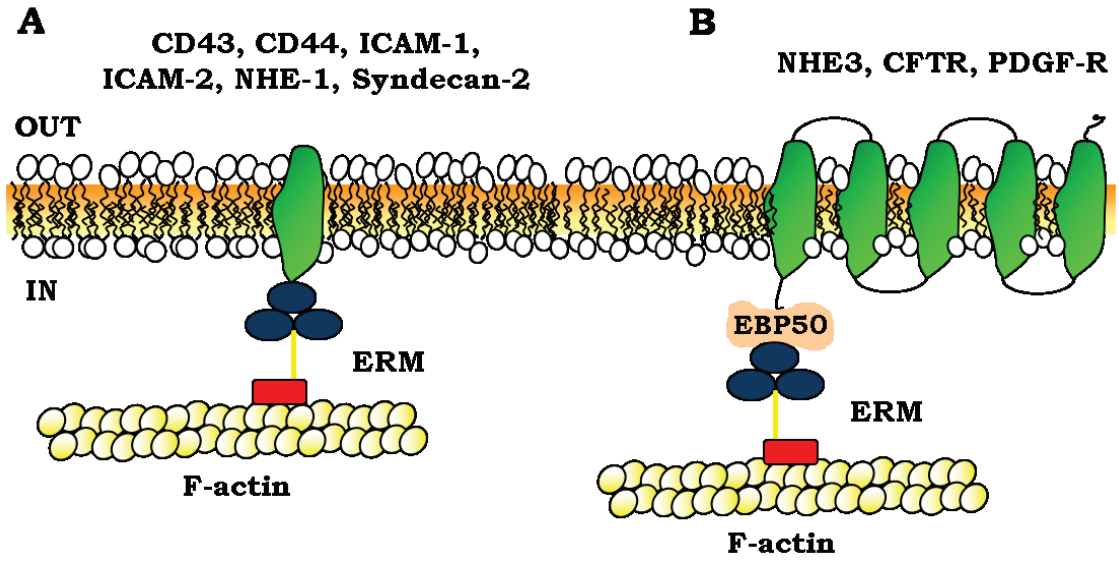

Figure 1.15. Model for ERM association to membrane proteins. Ezrin, Radixin and Moesin can direct bind to transmembrane proteins (A) or indirectly through adaptor proteins, such as EBP50 and E3KARP (B). (Figure adapted from Bretscher et al, 2002).

The C-ERMAD domain consists of ~ 80 residues and it is composed of one β strand and six helical regions (Pearson et al, 2000). It has the ability to bind the filamentous actin (F-actin) through the F-actin binding site, located in the last 34 residues of ezrin, which correspond to the last three helices of the carboxy-terminal tail, and are strongly conserved in the ERM family (Turunen et al, 1994). The C-ERMAD domain is also able to bind to and cover a large surface of the lobes F2 and F3 of the globular FERM domain, as shown by the crystal structure of the moesin N-ERMAD/C-ERMAD complex (Pearson et al, 2000).

1.4.2. Mechanism of activation

ERM proteins are usually present in the cytoplasm of cells as monomers, due to intramolecular association between the FERM and C-ERMAD domains (Berryman et al, 1995; Bretscher et al, 1995; reviewed Fehon et al, 2010) (**Figure 1.16**). In this state, the F-actin binding site (Gary et al, 1995) and the FERM domain (Reczek and Bretscher, 1998) are masked, and proteins adopt a dormant, closed, inactive conformations (**Figure 1.16**). Thus, conformational changes leading to the release of the C-ERMAD from the FERM domain are required to fully activate the molecule (Gary et al, 1995). The first evidence for the ERM head-to-tail interaction model came about from the discovery that ezrin and moesin can form homo- and hetero-dimers in cultured cells (Gary et al, 1993), and from the finding that the N-ERMAD domain can bind with high affinity to approximately 90 carboxy-terminal residues in the C-ERMAD domain of any ERM member (Gary et al, 1995; Magendantz et al, 1995). Further studies supported this model. Indeed, it was shown that cells expressing a high level of the carboxy-terminal domain of ezrin or radixin present abundant membrane-surface extensions (Martin et al, 1995). Interestingly, subsequent over-expression of the amino-terminal fragments of ezrin is able to revert this phenotype (Martin et al, 1995). Moreover, this model was confirmed by the crystal structure of the moesin N-ERMAD/C-ERMAD complex, which revealed that the C-ERMAD has an elongated structure that masks and blocks a large surface of the globular FERM domain (Pearson et al, 2000).

Therefore, in their inactive state, ERM are in a closed conformation maintained through the association between the FERM and C-ERMAD domains (**Figure 1.16**). Once the affinity between the FERM and the C-ERMAD domains is reduced, the C-ERMAD releases from the FERM domain, and the protein exposes membrane binding sites in the FERM domain and the F-actin-binding site of the C-ERMAD.

Ezrin activation, leading to the release of the FERM/C-ERMAD interaction, is proposed to occur in two steps through phosphorylation on a specific threonine in the carboxy-terminal domain (Matsui et al, 1998) and/or binding of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Fievet et al, 2004) (**Figure 1.16**).

Phosphorylation of ezrin on the conserved threonine 567 residue in the carboxy-terminal domain, of moesin on threonine 558, and of radixin on threonine 564 was demonstrated to reduce the affinity of the C-ERMAD for the FERM domain and to induce a transition to the open/active form (Matsui et al, 1998; Nakamura et al, 1995; Hayashi et al, 1999). Moreover, this threonine residue was predicted to be localized in the FERM/C-ERMAD interface (Pearson et al, 2000). Then, addition of a phosphate group, negatively charged, would induce electrostatic and stearic effects between the negatively charged FERM domain and the C-ERMAD that weakens their interaction, and induce conformational changes resulting in an open conformation of the protein (Pearson et al, 2000). In this state, ERM expose binding sites for membrane proteins in the FERM domain and F-actin in the C-ERMAD. Phosphorylation on threonine residues was also correlated with the recruitment of ERM proteins to the plasma membrane, where they bind membrane molecules. Indeed, studies reported that actin-rich cell-surface structures contain phosphorylated ERM proteins (Oshiro et al, 1998; Hayashi et al, 1999). Further studies also showed that expression of a mutant form of ezrin in which the threonine 567 was replaced with an aspartic acid that mimics the constitutively phosphorylated protein (T567D Ezrin), induced formation of abundant actin-rich cell-surface structures, including lamellipodia, membrane ruffles, and microvilli (Gautreau et al, 2000). To date, the serine/threonine kinases responsible for phosphorylation in vivo has not yet identified. However, potential candidates include protein kinase Cα (PKCα) (Ng et al, 2001), and PKCθ, (Pietromonaco et al, 1998; Simons et al, 1998), PIP_2-dependent kinase (Matsui et al, 1999), ROCK (Rho-

associated coiled coil-containing protein kinase) kinase (Matsui et al, 1998; Oshiro et al, 1998; Tran et al, 2000), Cdc 42 (Nakamura et al, 2000), G protein-coupled receptor kinase 2 (GRK2) (Cant et al, 2005), MST4 and lymphocyte-oriented kinase (LOK, also known as STK10) (ten Klooster et al, 2009; Belkina et al, 2009).

Binding of phosphatidylinositol 4,5-bisphosphate (PIP_2) seems to be also required for ERM activation. Indeed, it has been shown that PIP_2 binds to three clusters of lysines that are part of a basic groove located between lobes F1 and F3 in the FERM domain (Barret et al, 2000; Hamada et al, 2000). Mutagenesis of the PIP_2 binding site on ezrin was reported to inhibit the interaction of ezrin with PIP_2 and to alter its localization to the plasma membrane, suggesting that PIP_2 is an important determinant for ezrin localization and function at the plasma membrane (Barret et al, 2000). Moreover, over-expression of phosphatidylinositol 4-phosphate 5-kinase, that produces PIP_2, enhanced the level of ERM phosphorylation and induced formation of microvilli (Matsui et al, 1999). Interestingly, microinjection of neomycin into L cells, that disrupts the PIP_2 interaction with ERM proteins, induced ERM dephosphorylation and their subsequent inactivation, resulting in ERM translocation from microvilli to the cytoplasm and in loss of microvilli, indicating that PIP_2 is necessary for ERM activation (Yonemura er at, 2002). Lastly, crystal structure analysis of the radixin FERM domain with bound inositol 1,4,5-triphosphate (IP_3) revealed that binding of PIP_2 to the FERM domain might induce a conformational change in the lobe F3 that is transmitted through the α helix to the C-ERMAD and that render the carboxy-terminal threonine more accessible for phosphorylation (Hamada et al, 2000; Fievet et al, 2004). Recent finding also suggested that lipid binding is a prerequisite for threonine phosphorylation in ERM regulation (Janke et al, 2008). Thus, it is still controversial whether PIP_2 binding to the FERM domain of ERM proteins is involved in the opening of closed ERM proteins or in the stabilization of threonine phosphorylated ERM.

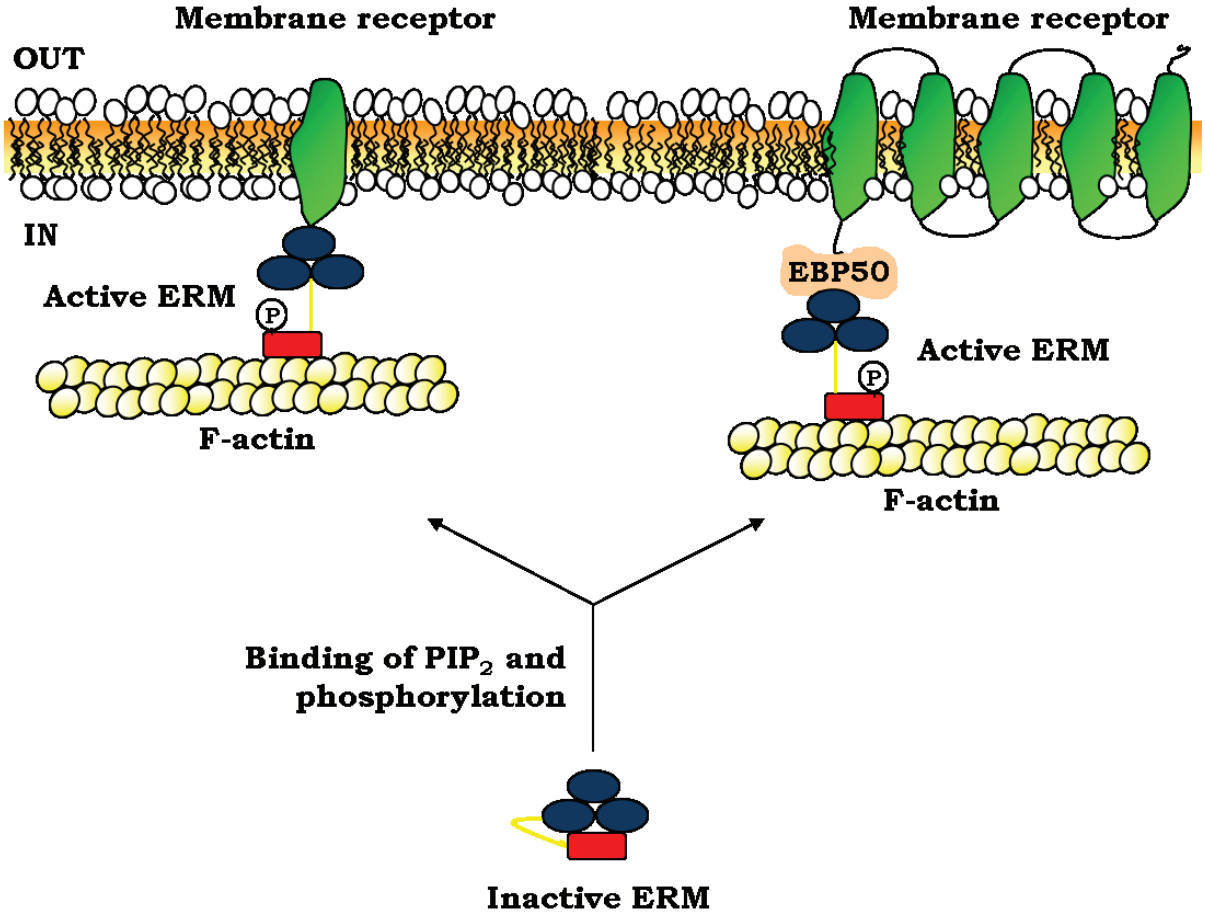

Figure 1.16. Model for ERM activation. Binding of PIP_2 to ERM proteins and phosphorylation of Thr567 reduce the affinity of the FERM domain for the C-ERMAD, and activates the protein. After these events ERM result in the open conformation, and expose binding sites for F-actin and the cytoplasmic tails of membrane proteins, such as CD43, CD44, ICAM1, ICAM2, NHE 1. (Figure adapted from Bretscher et al, 2002).

1.4.3. Mechanisms of regulation

The small GTPase RhoA, an organizator of stress fibers (Tapon and Hall, 1997), was shown to be the principal regulator of ERM proteins activation. Indeed, evidence in the literature reported that, following RhoA activation, ERM associates with plasma membranes (Hirao et al, 1996). Moreover, ERM are phosphorylated on threonine 567 and localize to the microvilli after activation of RhoA (Shaw et al, 1998; Matsui et al, 1998; Matsui et al, 1999). Enhanced levels of phosphorylated ERM and microvilli were observed following over-expression of phosphatidylinositol 4-phospate 5-kinase type Iα, an effector of Rho, and which produces PIP_2 (Matsui et al, 1999). Whereas, cellular treatments with the toxin C3 transferase, an inhibitor of RhoA function, resulted in microvilli breakdown and ERM inactivation (Yonemura et al, 2002). Interestingly, studies also indicate that ERMs may positively regulate RhoA activity, due to their interaction with proteins that can regulate Rho GTPases, including the Rho guanine nucleotide exchange factors (RhoGEFs), Rho GTPase-activating proteins (RhoGAPs), and Rho GDP-dissociation inhibitors (RhoGDIs) (Hatzoglou et al, 2007; Reczek and Bretscher, 2001; Hamada et al, 2001; D'Angelo et al, 2007).

The threonine 567 is not the only phosphorylation site present on ERM proteins. Other phosphorylation sites have been described in vertebrates. In particular, ezrin was reported to be phosphorylated on other serine/threonine and tyrosine residues in response to different stimuli, and these phosphorylation events were associated to specific functions.

For instance, during pRb-induced cell senescence cyclin-dependent kinase 5 (cdk5) can phosphorylate ezrin on the threonine residue 235, which is conserved in all the ERM members and lies on the FERM–C-ERMAD interface directly opposite threonine 567 (Yang and Hinds, 2003).

Zhou et al. described another phosphorylation site, serine 66, a substrate of protein kinase A (PKA). PKA-mediated ezrin phosphorylation on serine 66 was shown to be associated with hydrochloric acid secretion in gastric cells upon histamine stimulation (Zhou et al, 2003).

ERM proteins are also regulated by tyrosine phosphorylation in response to growth factor. It has been reported that epidermal growth factor (EGF) (Gould et al, 1986; Gould et al, 1989; Bretscher et al, 1989), platelet-derived growth factor (PDGF) (Fazioli et al, 1993) and hepatocyte growth factor (HGF) (Crepaldi et al, 1997) can stimulate ezrin phosphorylation on tyrosine residues. The major phosphorylated sites by the EGF and HGF have been mapped to tyrosine 145 in the amino-terminal domain and tyrosine 353 in the α-helical region of ezrin (Crepaldi et al, 1997; Krieg and Hunter 1992). Ezrin was reported to be phosphorylated on tyrosine residues 145 and 353 in A-431 cells in response to epidermal growth factor concomitantly to promote dimer formation (Krieg and Hunter, 1992; Berryman et al, 1995). However, this type of phosphorylation was mainly associated with survival pathways. In fact, phosphorylation on tyrosine 353, which is not conserved in the other ERM proteins, has been described to signal for survival in LLC-PK1 cells through activation of the phosphatidylinositol 3-kinase pathway during epithelial differentiation (Gautreau et al, 1999). Moreover, phosphorylation on tyrosine 353 was proposed to be required for the binding of the carboxy-terminal SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase to ezrin and its subsequent activation (Gautreau et al, 1999). Indeed, impairing a proper activation of the phosphatidylinositol 3-kinase pathway through expression of a mutated ezrin, in which the tyrosine 353 was changed to phenylalanine, and that mimics a nonphosphorylable protein, was reported to sensitize cells to apoptosis (Gautreau et al, 1999). In addition, mutagenesis of both, tyrosine 145 and tyrosine 353 in ezrin

was shown to decrease the morphogenic responses of epithelial cells to HGF (Crepaldi et al, 1997).

The tyrosine 145 residue of ezrin was demonstrated to be a target for phosphorylation by the tyrosine kinase Src in epithelial cells. Indeed, following the binding of Src SH2 domain to the phosphorylated tyrosine 190 of ezrin, Src phosphorylate ezrin on the tyrosine residue 145 (Srivastava et al, 2005). This phosphorylation event has been observed to promote and stabilize Src activity (Srivastava et al, 2005). Src phosphorylated tyrosine 145 ezrin was proposed to participate in cell-cell and cell-matrix adhesion signaling events leading to cell spreading and proliferation in epithelial cells (Srivastava et al, 2005). Indeed, expression of a mutant ezrin, in which tyrosine 145 was substituted with phenylalanine, abolishing the phosphorylation on this residue, caused delayed cell spreading, and inhibition of cell proliferation in LLC-PK1 cells (Srivastava et al, 2005).

Different members of Src family tyrosine kinases can phosphorylate ezrin at tyrosine residues. For istance, the Src tyrosine kinase $p56^{lck}$ catalyzes the phosphorylation of ezrin on tyrosine 145 during T activation (Autero et al, 2003). Cross-linking of intercellular adhesion molecule-1 (ICAM-1) activates the Src kinase $p53/56^{Lyn}$ leading to tyrosine phosphorylation of ezrin (Wang et al, 2002), whereas ezrin tyrosine phosphorylation event requires the Src tyrosine kinase $p62^{yes}$ after ICAM-2 cross-linking in T cells (Perez et al, 2002). Engagement of CD81 on the surface of B-lymphocytes induces ezrin phosphorylation on tyrosine residues by the spleen tyrosine kinase Syk (Coffey et al, 2009).

Furthermore, there has also been described a new phosphorylation site target of the tyrosine kinase Src. Src has been found to phosphorylate ezrin at tyrosine 477, located between the stretch of seven proline in the α-helical region and the C-ERMAD domain, and not conserved in other ERM members (Heiska and Carpén,

2005). Phosphorylation of ezrin on tyrosine 477 by Src induces the ezrin association with KBTBD2 (Kelch-repeat and BTB/POZ domain containing 2), a member of the kelch-repeat superfamily of proteins involved in regulation of cell adhesion and cell morphology (Heiska and Carpén, 2005). However, the functional significance of the KBTBD2/ezrin interaction remains to be fully elucidated.

It has been found that the kinase Fes can interact directly, through its SH2 domain, with ezrin phosphorylated at tyrosine 477 in response to HGF stimulation in epithelial cells (Naba et al, 2008). The Fes interaction with ezrin was reported to be required for the recruitment of Fes to cell-cell contacts and for its activation (Naba et al, 2008). Indeed, impairment of the Fes/ezrin interaction was reported to induce a defective epithelial response to HGF stimulation (Naba et al, 2008). However, the functional significance of this interaction in cancer cells still remains to be elucidated.

In addition, ERM proteins can be regulated by proteolic cleavage. Calpain is able to cleave ezrin, but not moesin, to form 55 kDa and 30 kDa fragments in response to the stimulation of leucocytes with phorbol 12-myristate 13-acetate (PMA) (Shcherbina et al, 1999). However, the functional significance of this proteolytic cleavage is still unclear. A related study on talin, another member of the FERM superfamily, showed that the amino-terminal part of talin, containing the FERM domain, dissociates from the carboxy-terminal tail after cleavage by calpain (Yan et al, 2001). Thus, the cleaved amino-terminal domain acquires an increased affinity for the cytoplasmic tail of β3 integrin, facilitating clustering and activation of integrins (Yan et al, 2001). Furthermore, calpain I can cleave ezrin to form 55 kDa fragments in response to addition of Ca^{2+} in resting parietal gastric cells. The calpain I-mediated ezrin proteolysis leads to the disappearance of ezrin from the apical surface of epithelial gastric cells, and prevents the acid secretory response of these cells to histamine stimulation (Yao et al, 1993). Once the gastric epithelium is

activated by histamine, ezrin seems to be protected against calpain I cleavage (Wang et al, 2005). In addition, activation of gastric parietal cell secretion by histamine was shown to be mediated by phosphorylation of ezrin at serine 66 by PKA (Zhou et al, 2003). Thus, PKA-mediated ezrin phosphorylation at serine 66 would induce a conformational change in the protein that protects ezrin from calpain I cleavage (Wang et al, 2005).

Table 1-1. Physiological role of the ezrin phosphorylation sites.

Phosphorylation site	Kinase involved	Function	Reference
Ser 66	PKA	Hydrocloric acid secretion	Zhou et al, 2003
Tyr 145	Src	Cell spreading and proliferation	Srivastava et al, 2005
Thr 235	CDK5	pRb-induced senescence	Yang and Hinds, 2003
Tyr 353	PI3K	Cell survival	Gautreau et al, 1999
Tyr 477	Src	Cell adhesion and morphology	Heiska and Carpén, 2005
Thr 567	ROCK/PIP2K/ PKC/Cdc42	Microvillar localization	Matsui et al, 1998

1.4.4. Physiological roles of ezrin in normal development

ERM were first described as structural linkers between the plasma membrane and the actin cytoskeleton, and organizers of complex and specialized domains of the plasma membrane, thanks to their ability to interact with transmembrane proteins, phospholipids, membrane-associated cytoplasmic proteins and the cytoskeleton.

Ezrin is primarily expressed in epithelial cells of the intestine, stomach, lungs and kidneys (Tsukita and Hieda, 1989; Berryman et al, 1993), and is mainly involved in the biogenesis of microvilli and the formation of functional epithelium during normal development (Crepaldi et al, 1997; Yonemura et al, 1999; Bonilha et al, 1999; Dard et al, 2001). For instance, microvilli disappeared from the cell surface after treatment of primary retinal pigment epithelial cells with ezrin antisense oligonucleotides (Bonilha et al, 1999). Transfection with ezrin cDNA in a primary retinal epithelial cell line, which express low level of ezrin and moesin, induced formation of microvilli (Bonilha et al, 1999). Ezrin was found in the lateral domain of epithelial cells instead of the apical membrane upon expression of a mutant ezrin, containing only the amino-terminal domain, and this event led to an alteration in the microvilli formation (Crepaldi et al, 1997). Ezrin was also found in gastric parietal cells where it contributes to acid gastric secretion (Hanzel et al, 1991; Urushidani et al, 1989). Within the gastric epithelium, ezrin has been localized exclusively to the apical canalicular membrane of parietal cells (Urushidani et al, 1989; Yao et al, 2003). Stimulation of gastric acid secretion induced phosphorylation of ezrin and its translocation to microvilli (Hanzel et al, 1991; Urushidani et al, 1989). This phosphorylation event was reported to involve the serine 66 residue of ezrin and the protein kinase A (PKA) (Zhou et al, 2003), and was required for the interaction of ezrin with WWOX, a WW-domain containing oxidoreductase (Jin et al, 2006), and ACAP4, an ADP-ribosylation factor (ARF)

GTPase-activating protein (Ding et al, 2010). In addition, in rabbit renal proximal tubules, anoxia may cause microvillar breakdown by dephosphorylating ezrin and dissociating the brush border membrane from the cytoskeleton (Chen et al, 1995). In those cells, energy depletion was also reported to weaken the interaction between the cytoskeleton and the apical membrane, and was correlated with blebbing from the renal brush-border membrane (Chen and Wagner, 2001).

In addition to its function in supporting microvillar structures, ezrin plays an important role in maintaining cellular polarity. A classical example is represented by the immunological synapse (IS) formation. When T cells recognize an antigen bound to major histocompatibily complex (MHC) molecules on the surface of dendritic or B cells (antigen-presenting cells or APCs), they polarize towards those cells, forming the IS. Formation of IS requires the remodelling of the actin cytoskeleton (Miletic et al, 2003). First, the microvilli, covering the surface of spheric non stimulated T cells, disassembled at the site of interaction through ERM dephosphorylation (Faure et al, 2004). In addition, T-cell receptors (TCR) aggregate at the site of IS, and proteins such as CD43 are removed, whereas adhesion molecules are recruited within the IS because they need to bring the two cells together. Ezrin and moesin are believed to be necessary in these processes and to play different functions (Delon et al, 2001; Allenspach et al, 2001; Roumier et al, 2001). In particular, when T cells are in a non stimulated condition, moesin is associated with CD43 at the plasma membrane, whereas ezrin is in the cytoplasm and in an inactive state. After TCR triggering, moesin is dephosphorylated and dissociates from CD43, which is excluded from the IS, whereas ezrin associates with and recruites Zap70 (ζ-chain associated protein kinase of 70 kDa) to the synapse (Ilani et al, 2007; Shaffer et al, 2009). Other examples of polarization events directed by ezrin have been reported. For instance, when T lymphocytes are susceptible to Fas-mediated cell death, Fas is polarized in uropodal structures, and

is linked to the actin cytoskeleton through ezrin (Parlato et al, 2000). Efficient cell killing by cytotoxic T lymphocytes and natural killer cells (NK) requires ezrin and ICAM-2 localization to the uropod of the target cell (Helander et al, 1996). Uropods are also observed in migrating lymphocytes (Sanchez-Madrid and del Pozo, 1999) or following chemokine stimulation (del Pozo et al, 1996).

Ezrin is the only ERM expressed in the gut, where it seems to be involved in the villar organization and stabilization (Saotome et al, 2004). Ezrin-deficient mice present a disturbed villus morphogenesis, in which the intestinal microvilli aggregate abnormally. These mice have defects in the apical terminal web of the gut epithelium, resulting from an incomplete transition from stratified to columnar epithelium, and incorrect formation and extension of secondary lamina during embryonic development (Saotome et al, 2004). Thus, ezrin knockout mice die within three weeks after birth due to defects in the gastrointestinal tract, leading to inefficiency in the absorption of nutrients (Saotome et al, 2004).

Ezrin has also been described to be important for the development of gastric parietal and retinal pigment epithelial cells (Tamura et al, 2005; Bonilha et al, 2006). Inactivation of ezrin in mice through ezrin knockdown generated mice with growth retardation and high mortality, due to a reduction in acid secretion by gastric parietal cells caused by defects in the formation and function of apical canaliculi (Tamura et al, 2005). In addition, mice deficient in ezrin have reductions in the apical microvilli and basal infoldings in retinal epithelial cells, and retardation in the development of photoreceptors (Bonilha et al, 2006).

1.4.5. Role of ezrin in cancer

Increasing evidence suggests that ezrin has a role in cancer. In fact, a great deal of data is available in the literature, supporting the hypothesis that ezrin participates in tumour progression and promotes tumour metastasis. Ezrin is highly expressed in almost all types of cancers, and its level of expression is reported to increase with the progression of cancer cells versus the malignant phenotype, when these cells acquire the capacity to migrate within interstitial stroma and blood vessels, and adhere to distant organs. Indeed, many studies documented a large increase in ezrin expression in metastatic cells from different origins compared to their non-metastatic counterparts (Akisawa et al, 1999; Ohtani et al, 1999; Khanna et al, 2001), and in invasive cells (Nestl et al, 2001; Geiger et al, 2000; Tokunou et al, 2000). Moreover, most of these studies described a unique role for ezrin in the metastatic process. The two pioneering studies about the ezrin's role in cancer identified ezrin as a key component in the metastasis of two pediatric solid tumours of mesenchymal origin, osteosarcama and rhabdomyosarcoma (Khanna et al, 2004; Yu et al, 2004). These studies described the involvement of ezrin in the formation of tumour metastasis, and they correlated the level of ezrin expression with the metastatic behavior of those tumours. Indeed, inhibition of ezrin's expression abrogated the capability of tumour cells to metastasize, whereas inhibition of moesin and radixin did not show any functional role in the metastatic process, excluding the involvement of the other ERM proteins in this event (Khanna et al, 2004; Yu et al, 2004). Further experiments performed in adult tumours supported the unique and central role of ezrin in the acquisition of the metastatic phenotype.

Several studies have demonstrated that ezrin is transversally involved in the multiple and different events which culminate in the acquisition of the metastatic phenotype by cancer cells.

One function of ezrin that is directly related to the invasion and metastatic behavior of tumour cells is the capacity of ezrin to connect the actin cytoskeleton with certain proteins that have been implicated in tumour invasion and metastasis, such as CD44, podoplanin, podocalyxin, and LAMP-1 (Martin et al, 2003; Martin-Villar et al, 2006; Sizemore et al, 2007; Sarafian et al, 1998). CD44 is an adhesion molecule that function as a receptor for the hyaluronic acid and a co-receptor for MET, a tyrosine kinase transmembrane protein which is known to promote tumour invasion and cell motility. It has been shown that the association of CD44 with ezrin is required for CD44 to carry out both those functions, and it has been correlated with the capacity of tumours to invade and adhere to distant organs (Ponta et al, 2003; Orian-Rousseau et al, 2008). In a similar way, podoplanin and podocalyxin, two transmembrane sialoproteins found in the kidney podocyte, promote tumour cell migration and invasion in a manner that is dependent on their association with ezrin (Martin-Villar et al, 2006; Sizemore et al, 2007). Ezrin associates also with LAMP-1, a lysosomal antigen that is involved in adhesion of cancer cells to the extracellular matrix (Sarafian et al, 1998). Ezrin interacts with both CD44 and Lamp-1 in metastatic melanoma cells, and this linkage was described to be required for the acquisition of the metastatic phenotype by human melanoma cells (Federici et al, 2009). Expression of a deletion mutant of ezrin, which includes the first 146 amino acids, and lacks the actin-binding region, inhibits the metastatic behaviour and reduces the invasiveness of human metastatic melanoma cells by abrogating the surface expression of LAMP-1 and resulting in a loss of CD44 functionality (Federici et al, 2009).

Ezrin exerts a key role in regulating the correct localization to the plasma membrane of E-cadherin, a cell adhesion molecule also involved in tumorigenesis. Interestingly, ezrin over-expression results in loss of E-cadherin surface expression and in an aberrant intracellular cytoplasmic accumulation of E-cadherin, which

has been correlated with formation of breast cancer metastasis (Elliot et al, 2005). Furthermore, over-expression of the amino-terminal fragments of ezrin or ezrin silencing by small hairpin RNA led to membrane relocalization of E-cadherin, increased cell–cell contact and decreased cell motility and invasion (Elliot et al, 2005; Li et al, 2008b).

Binding of the cell neural adhesion molecule L1 to ezrin is also necessary for colon metastasis formation (Gavert et al, 2010). Metastasis did not develop when the association between ezrin and L1 was disrupted or the ezrin expression was suppressed using small hairpin RNA (Gavert et al, 2010).

Another function that has been correlated with the acquisition of the metastatic phenotype by cancer cells, and which requires ezrin, is the phagocytic and cannibalistic activity presented by metastatic melanoma cells. Recently a study demonstrated that cell lines derived exclusively from metastatic tumours showed strong phagocytic activity against latex beads, yeast and apoptotic cells of different origins in conditions of nutrients and oxygen depletions (Lugini et al, 2003). Moreover, these cell lines where also able to endocytose and digest live T cells (Lugini et al, 2006). Ezrin was shown to be necessary in both cases. In fact, ezrin was found either in phagocytic vacuoles of melanoma cells or in the endolysosomal compartment of cannibal tumour cells, where it drives the complex phagocytic/cannibalistic framework. Treatments with ezrin antisense oligonucleotides or ezrin knockdown using siRNAs were able to abrogate the phagocytic or cannibalistic activity of metastatic melanoma cells (Lugini et al, 2003; Lugini et al, 2006).

During the transformation of primary tumour cells into metastatic tumour cells, cells can become resistant to treatments with chemotherapeutic agents following various cycles of drug administration, leading to a phenomenon called multidrug resistance (MDR). The multidrug resistance phenotype is often associated

with an increased expression and functionality at the plasma membrane of tumour cells of the P-glycoprotein (Pgp), an ATP-dependent efflux pump which transports a variety of structurally and functionally unrelated anticancer drugs out of the cells (Lavie et al, 1998; Luciani et al, 2002). Recent evidence suggested that Pgp function may depend on its binding to actin cytoskeleton through ezrin, and treatments with ERM antisense oligonucleotides induce the entire abrogation of the linkage between P-glycoprotein and the actin cytoskeleton, together with inhibition of drug efflux, thus restoring sensitivity to chemotherapeutics (Luciani et al, 2002).

Together, these findings demonstrated that ezrin is a key and peculiar regulator for the development of malignant deseases and a good possible prognostic indicator of invasiveness and metastasis in human cancer cells.

Table 1-2. Ezrin-associated membrane receptors related to the formation of tumour metastasis.

Membrane receptor	Type of protein	Function	References
CD44	Adhesion molecule	Tumour cell migration and invasion	Orian-Rousseau et al, 2008
Podoplanin	Sialoprotein	Tumour cell migration and invasion	Martin-Villar et al, 2006
Podocalyxin	Sialoprotein	Tumour cell migration and invasion	Sizemore et al, 2007
LAMP-1	Lysosomal antigen	Tumour cell adhesion to the extracellur matrix	Sarafian et al, 1998
E-cadherine	Adhesion molecule	E-cadherine localization	Li et al, 2008
Caveolin-1	Component of caveolae	Phagocytosis	Lugini et al, 2003
Pgp	Efflux pump	Multidrug resistance phenotype	Luciani et al, 2002

1.5. Role of ezrin in the Fas-mediated cell death

The receptor Fas (CD95 or APO-1) is another proapoptotic member of the tumor necrosis factor (TNF) receptor family, and it is the most intensively studied protein of this family (Peter et al, 1999). Fas is a type I transmembrane protein of 319 amino acids, which shares high similarity in its structure with the TRAIL agonistic receptors, TRAIL-R1 and TRAIL-R2. Similar to those receptors, Fas consists of an amino-terminal extracellular domain containing three cysteine-rich domains, a transmembrane domain and a carboxy-terminal cytoplasmic domain. The intracellular domain is characterized by a Death Domain (DD) of 80 amino acid residues that is essential for triggering apoptosis. The engagement of Fas by its cognate ligand induces the activation of the apoptotic signalling pathway. The Fas signalling pathway is very similar to TRAIL signalling. For instance, activation of Fas drives the recruitment of the adaptor FADD and the initiator caspase-8 to form the DISC, that activates the apoptotic cascade.

Recent evidence indicates that the actin cytoskeleton and the ERM proteins are implicated in Fas ligand-induced apoptosis. Indeed, data have shown that the Fas linkage to the actin cytoskeleton through ezrin primes human CD4+ T lymphocytes to Fas-mediated apoptosis and drives receptors polarization into uropods (Parlato et al, 2000; Fais et al, 2003). In fact, both treatments with non toxic doses of cytochalasin D, an actin-perturbing agent, and treatments with ezrin antisense oligonucleotides induced: i) loss of Fas polarization; ii) inhibition of human CD4+ T cell lines susceptibility to Fas-mediated apoptosis (Parlato et al, 2000). Moreover, treatments with latrunculin, which also induces the disruption of the actin cytoskeleton, inhibits DISC formation and Fas internalization upon its engagement by Fas ligand (Algeciras-Schimnich et al, 2002). In Jurkat cells, ezrin and moesin were also shown to be pre-associated with Fas and required for cell death triggering (Hébert et al, 2008). Ezrin was demonstrated to associate directly

with Fas (**Figure 1.17**), and the ezrin region implicated in the Fas/actin association was mapped between the amino-acids 149-168, in the middle lobe of the ezrin FERM domain (Lozupone et al, 2004). Mutations of this region, which was replaced with the corresponding sequence of moesin, that was shown to be unable to bind Fas (Parlato et al, 2000), resulted in the loss of Fas/ezrin association, which led to protection of T cells from Fas-mediated cell death (Lozupone et al, 2004).

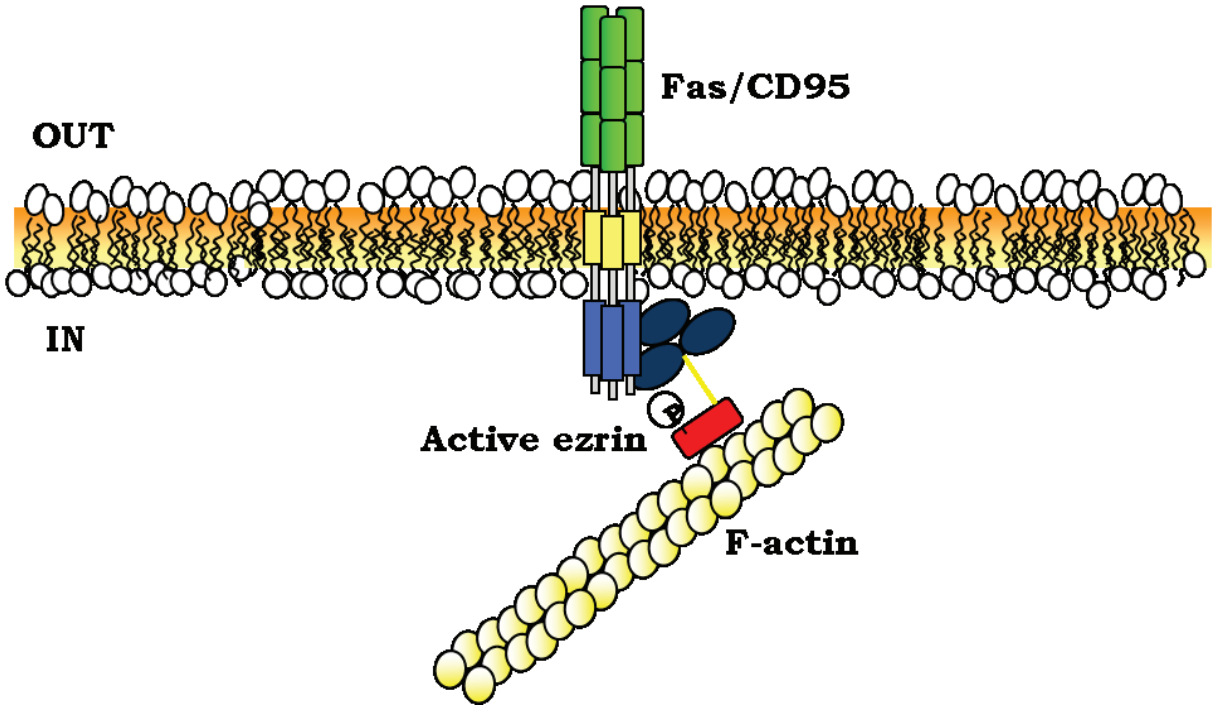

Figure 1.17. Fas linkage to F-actin through ezrin. Ezrin associates with the intracellular part of Fas in cells sensitive to Fas-mediated cell death. (Figure adapted from Lozupone et al, 2004).

2. Aim of the thesis

TRAIL is currently drawing attention in the field of cancer therapy because of its ability to induce specific tumour cell apoptosis, while normal cells are resistant to TRAIL. Indeed, several phase I-II clinical trials with recombinant human TRAIL or agonistic TRAIL-R1 or TRAIL-R2 antibodies have been initiated in patients suffering from different types of malignant disease. Nevertheless, the signalling transduction pathway of TRAIL is not yet completely elucidated. For a deeper understanding and a more effective use of TRAIL in a clinical setting, further studies are necessary to dissect out the molecular mechanisms leading to TRAIL-induced apoptosis.

TRAIL triggers cell death upon binding to the agonistic receptors TRAIL-R1 and/or TRAIL-R2, which drives the recruitment of the adaptor protein FADD and the initiator caspase-8 to form the macromolecular complex called DISC (Death-Inducing Signalling Complex). Within this complex, caspase-8 is activated and starts the apoptotic cascade.

Fas is, like TRAIL, a member of the TNF receptor family that also signals apoptosis through DISC formation. Recent evidence indicates that ERM (Ezrin-Radixin-Moesin) proteins are implicated in the regulation of the early steps of Fas-mediated apoptosis.

At the beginning of this project the contribution of ezrin to Fas signalling was extensively studied, while little was known regarding TRAIL, with the exception of a recent study in which ezrin was proposed to impair both Fas ligand and TRAIL induced cell death in the tumour T cell line H9 (Kuo et al, 2010).

In light of these considerations, we investigated the initial steps of the signalling pathway induced by the TRAIL agonistic receptors (TRAIL-R1/TRAIL-R2). Since the Fas-DISC closely resembles that of TRAIL, and since the Fas/actin linkage through ezrin was demonstrated to be essential in priming CD4+ T cells to

Fas-mediated apoptosis, we hypothesised that TRAIL-R1/TRAIL-R2 might induce cell death through an ezrin/actin dependent process.

Therefore, the aim of this thesis was to determine whether ezrin is involved in TRAIL signalling, and consequently to define its role in TRAIL signalling. During this study the main questions addressed were:

1. Could the ezrin/TRAIL receptors/actin association be involved in cancer cells responsiveness to TRAIL-mediated apoptosis, and participate in TRAIL DISC formation?
2. Could ezrin activation, through phosphorylation, modulate cancer cell sensitivity to TRAIL-induced cell death?
3. In which molecular mechanisms does ezrin play a role in the TRAIL signalling pathway?

3. Materials and methods

3.1. Material

3.1.1. Chemicals

Table 3-1. Chemicals

Reagent	Provider
Cisplatin	Sigma Aldrich
8-bromo-cyclic AMP	Sigma Aldrich
Orthovanadate	Sigma Aldrich
H89	Cayman
Puromicin	InvivoGen
Staurosporine	Sigma Aldrich
EGF	Sigma Aldrich
ZVAD	Tocris
Folskolin	Ozyme

3.1.2. Buffers and solutions

Protein lysis buffer

20 mM Tris-HCl pH 7.5

150 mM NaCl

10 % (w/v) glycerol

1 % (w/v) NP40

1 x complete proteinase inhibitor cocktail (Roche)

Protein buffer for total lysates

20 mM Tris-HCl pH 7.5

150 mM NaCl

10 % (w/v) glycerol

1 % (w/v) SDS

1 x complete proteinase inhibitor cocktail (Roche)

SDS blotting buffer

50 mM Tris-base

50 mM Boric acid

pH 8.5

10 % SDS resolving gel

For 1 1.5 mm Gel of the Mini-PROTEAN®3 System:

H_2O: 10 ml

1 M Tris-HCl plus 20% SDS pH 8.8: 5 ml

40 % Acrylamide/Bis solution 37.5:1: 5 ml

10 % APS: 100 µl

TEMED: 20 µl

4 % SDS stacking gel

For 1 Gel of the Mini-PROTEAN®3 System:

H2O: 7.8 ml

1 M Tris-HCl plus 20 % SDS pH 6.8: 3 ml

40 % Acrylamide/Bis solution 37.5:1: 1.2 ml

10 % APS: 100 µl

TEMED: 20 µl

3 x SDS gel loading buffer

187.5 mM Tris-HCl

6 % (w/v) SDS

0.03 % phenol red

30% (w/v) glycerol

100 mM DTT

pH 6.8

SDS gel running buffer

200 mM Glycine

25 mM Tris-base

0,1% SDS

TBS (Tris buffered saline)

10 mM Tris-HCl pH 7.4

150 mM NaCl

PBS (Phosphate buffered saline)

137 mM NaCl

2,7 mM KCl

8,1 mM Na_2HPO_4

1,7 mM KH_2PO_4

pH 7.4

TBE (Tris Borate EDTA)

89 mM Tris base

89 mM Boric acid

2 mM EDTA

pH 8.3

FACS buffer

1 x PBS

0,5% BSA

0,1% Sodium azide

FACS buffer 2

1 X PBS

2,5% FCS

Permeabilization buffer for FACS analysis

1 x PBS

1% BSA

0,1% Saponin

3.1.3. Culture Media

3.1.3.1. Media for culturing bacteria: LB

10 g/l peptone

5 g/l NaCl

5 g/l yeast extract

pH 7.4

LB-agar plates were prepared by addition of 12 g/l SELECT-agar before heating.

3.1.3.2. Media for culturing eukaryotic cells

All culture media were from Invitrogen/Lonza. RPMI contained L-glutamine, and DMEM 4.5 g/L glucose and L-glutamine. All media were supplied with 10 % FBS (Lonza) and penicillin/streptomycin (Lonza) (100 µg/ml of each) before use.

For selection of stably transfected cells 2.5 µg/ml Puromicin (InvivoGen) was used.

3.1.4. Biological material

Table 3-2. Bacterial strains.

Strain designation	Purpose	Provider

TOP10	Propagation of plasmids	Invitrogen
XL-1 blue	Propagation of plasmids	Stratagene

Table 3-3. Eukaryotic cell lines and growth media.

Name	Tissue	Growth medium	Property, morphology
SKW 6.4	human lymphoblats	RPMI 1640 10 % FBS	suspension
HeLa WT	human cervix adenocarcinoma	DMEM 10 % FBS	adherent epithelial
HCT116	human colorectal carcinoma	DMEM 10 % FBS	adherent epithelial
SW480	human colorectal adenocarcinoma	DMEM 10 % FBS	adherent epithelial
293T	kidney	DMEM 10 % FBS	adherent epithelial
MDA-MB468	human breast adenocarcinoma	Leibovitz L-15 10 % FBS	adherent epithelial
SK-BR-3	human breast adenocarcinoma	McCoy 5a 10 % FBS	adherent epithelial

3.1.5. Antibodies

Table 3-4. Primary antibodies used for immunoblotting

Protein	Size MW (kDa)	Clone	Company	Blocking Buffer	Dilution
β-Actin	42	AC-40	Sigma	5% MP	1:4000
Caspase-2	51, 13, 12	C-20	Santa Cruz	5% MP	1:1000
Cleaved Caspase-3	17, 19	#9661	Cell Signaling	5% MP	1:1000*
Caspase-9 (p35)	46, 35	H-170	Santa Cruz	5% MP	1:1000
Cleaved Caspase-9	35	#9505	Cell Signaling	5% MP	1:1000*
Caspase-8	55, 43, 18	5F7	MBL	5% MP	1:1000
Caspase-10	57, 43	4C1	MBL	5% MP	1:1000
Ezrin	80	3C12	Sigma	5% MP	1:1000
Ezrin	80	18/Ezrin	Trasduction Laboratory	5% MP	1:1000

Ezrin (Tyr145)-R	80	sc-12941-R	Santa Cruz	5% MP	1:1000
Ezrin (Tyr353)	80	#3144	Cell Signaling	5% MP	1:1000*
Ezrin (pT567)	80	J37-954.281.307	BD Pharmingen	5% MP	1:1000
ERM	75, 80	#3142	Cell Signaling	5% MP	1:1000*
FADD	24	1/FADD	Trasduction Laboratory	5% MP	1:1000
Fas	48	C-20	Santa Cruz	5% MP	1:1000
Flag		M2	Sigma	5% MP	1:1000
GAPDH	37	0411	Santa Cruz	5% MP	1:1000
HSC70	70	B-6	Santa Cruz	5% MP	1:1000
Moesin	80	38/Moesin	BD Pharmingen	5% MP	1:1000
pERM	75, 80	#3141	Cell Signaling	5% MP	1:1000*
pSerine		PSR-45	Sigma	5% MP	1:1000
Radixin	80	C-15	Santa Cruz	5% MP	1:1000
TNFR1	55	H-5	Santa Cruz	5% MP	1:1000
TRAIL-R1	57	AB16955	Chemicon	5% MP	1:1000
TRAIL-R2	57	AB16942	Chemicon	5% MP	1:1000
TRAIL-R2	57	B-K29	Diaclone	5% MP	1:1000
TRAIL-R2	57	B-D37	Diaclone	5% MP	1:1000
VSV		P5D4	Sigma	5% MP	1:1000
EGFR	175	D38B1	Cell Signaling	5% MP	1:1000*
EGFR	175	1F4	Cell Signaling	5% MP	1:1000*
p-EGFR (Tyr1068)	175	D7A5	Cell Signaling	5% MP	1:1000*
HER2	185	D8F12	Cell Signaling	5% MP	1:1000*
p-STAT3 (Tyr705)	79, 86	D3A7	Cell Signaling	5% MP	1:1000*
p44/42 MAPK (ERK 1/2) (Thr202/Tyr204)	44, 42	D13.14.4E	Cell Signaling	5% MP	1:1000*
S6 ribosomal protein	32	5G10	Cell Signaling	5% MP	1:1000*
p70 S6 kinase	70, 85		Cell Signaling	5% MP	1:1000*
phospho- p70 S6 kinase (Thr389)	70, 85		Cell Signaling	5% MP	1:1000*
phospho-CREB (Ser133)	43	87G3	Cell Signaling	5% MP	1:1000*
phospho-Bad (Ser112)	23	7E11	Cell Signaling	5% MP	1:1000*

* antibodies were diluted in 5 % BSA; remaining antibodies were diluted in blocking buffer

Table 3-5. Primary antibodies used for flow cytometry

Protein	Size MW (kDa)	Clone	Company	FACS Buffer	Dilution
TRAIL-R1	57	wB-K32	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R1	57	wB-N36	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R2	57	B-K29	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R2	57	B-L27	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R2	57	B-B42	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R3	57	wB-K35	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R3	57	B-H47	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R4	57	wB-P30	Diaclone	PBS-BSA-NaN3	1:100
TRAIL-R4	57	wB-R27	Diaclone	PBS-BSA-NaN3	1:100
Bax	22	6A7	BD Pharmingen	PBS-BSA-Saponin	1:200

Table 3-6. HRP conjugated secondary antibodies

Antibody	Company	Dilution
Donkey anti-goat IgG, HRP conjugate	Santa Cruz	1:10000
Goat anti-mouse IgG1, HRP conjugate	SouthernBiotech	1:10000
Goat anti-mouse IgG2a, HRP conjugate	SouthernBiotech	1:10000
Rat anti-mouse IgG2b, HRP conjugate	SouthernBiotech	1:10000
Goat anti-rabbit IgG, HRP conjugate	Santa Cruz	1:10000
Goat anti-mouse, Alexa-488 conjugate	Invitrogen	1:1000

Table 3-7. Antibodies used for immunoprecipitation

Protein	Size MW (kDa)	Clone	Company	Quantity

Caspase-8 (p18)	55, 18, 10	C-20	Santa Cruz	5 µg
Ezrin	80	H-276	Santa Cruz	5 µg
Flag		M2	Sigma	5 µg
GAPDH	37	0411	Santa Cruz	5 µg
p-Tyrosine		# 9411	Cell Signaling	5 µg
TRAIL-R1	57	wB-S26	Diaclone	5 µg
TRAIL-R2	57	B-D37	Diaclone	5 µg
VSV		P5D4	Sigma	3 µg

Table 3-8. TNF-superfamily ligands

Ligands	Provider
Flag-TRAIL	Schneider, 2000
His-TRAIL	Schneider, 2000
FasL	Schneider, 2000

3.1.6. Materials for molecular biology

Table 3-9. Vectors

Vector name	Purpose	Provider
pCR3-VSV	Vector for eukaryotic expression	Invitrogen
pCR3-VSV-Ezrin WT	Eukariotic expression of Ezrin WT	
pCR3-VSV-Ezrin mutant	Eukariotic expression of Ezrin mutant	
pCR3-VSV-Ezrin S66A	Eukariotic expression of Ezrin S66A	
pCR3-VSV-Ezrin S66D	Eukariotic expression of Ezrin S66D	
pCR3-VSV-Ezrin Y145F	Eukariotic expression of Ezrin Y145F	
pCR3-VSV-Ezrin Y145D	Eukariotic expression of Ezrin Y145D	
pCR3-VSV-Ezrin Y353F	Eukariotic expression of Ezrin Y353F	
pCR3-VSV-Ezrin Y353D	Eukariotic expression of Ezrin Y353D	
pCR3-VSV-Ezrin T567A	Eukariotic expression of Ezrin T567A	

pCR3-VSV-Ezrin T567D	Eukariotic expression of Ezrin T567D	
pCR3-VSV-Ezrin R579A	Eukariotic expression of Ezrin R579A	
pEGFP-N1	Vector for eukaryotic expression	Clontech
pEGFP-N1-Ezrin WT	Eukariotic expression of Ezrin WT	
pMSCV-puro	Vector for stable expression	Invitrogen
pMSCV-puro-Ezrin WT	Vector for stable expression of Ezrin WT	
pMSCV-puro-Ezrin mutant	Vector for stable expression of Ezrin mutant	
pMSCV-puro-Ezrin S66A	Vector for stable expression of Ezrin S66A	
pMSCV-puro-Ezrin S66D	Vector for stable expression of Ezrin S66D	
pMSCV-puro-Ezrin Y145F	Vector for stable expression of Ezrin Y145F	
pMSCV-puro-Ezrin Y145D	Vector for stable expression of Ezrin Y145D	
pMSCV-puro-Ezrin Y353F	Vector for stable expression of Ezrin Y353F	
pMSCV-puro-Ezrin Y353D	Vector for stable expression of Ezrin Y353D	
pMSCV-puro-Ezrin T567A	Vector for stable expression of Ezrin T567A	
pMSCV-puro-Ezrin T567D	Vector for stable expression of Ezrin T567D	
pMSCV-puro-Ezrin R579A	Vector for stable expression of Ezrin R579A	
pUC57-Ezrin mutant	Vector for bacterial expression	Genscript

Table 3-10. Oligonucleotides

Name	Sequence (5’ - 3’)
Ezrin S66A - FOR	ctggataagaaggtggctgcccaggaggtca
Ezrin S66A - REV	tgacctcctgggcagccaccttcttatccag
Ezrin S66D - FOR	gctggataagaaggtggatgcccaggaggtcagg
Ezrin S66D - REV	cctgacctcctgggcatccaccttcttatccagc
Ezrin Y145F - FOR	gtgcacaagtctgggttcctcagctctgagc
Ezrin Y145F - REV	gctcagagctgaggaacccagacttgtgcac
Ezrin Y145D - FOR	gtgcacaagtctggggacctcagctctgagc

Ezrin Y145D - REV	gctcagagctgaggtccccagacttgtgcac
Ezrin Y353F - FOR	tgctgcggctgcaggactttgaggagaag
Ezrin Y353F - REV	cttctcctcaaagtcctgcagccgcagca
Ezrin Y353D - FOR	ctgcggctgcaggacgatgaggagaagacaa
Ezrin Y353D - REV	ttgtcttctcctcatcgtcctgcagccgcag
Ezrin T567A - FOR	gggacaagtacaaggccctgcggcagatccg
Ezrin T567A - REV	cggatctgccgcagggccttgtacttgtccc
Ezrin T567D - FOR	ccgggacaagtacaaggacctgcggcagatccggc
Ezrin T567D - REV	gccggatctgccgcaggtccttgtacttgtcccgg
Ezrin R579A - FOR	tcgaactcgtcgatggcctgcttggtgttgcc
Ezrin R579A - REV	ggcaacaccaagcaggccatcgacgagttcga

Table 3-11. Enzymes and kits

Enzyme/KIT	Supplier
Restriction enzymes	Promega
NucleoSpin Extract II	Macherey - Nagel
T4 DNA ligase	Invitrogen
NucleoBond Xtra MIDI KIT	Macherey - Nagel
QuickChange II Site-directed mutagenesis KIT	Stratagene

3.2. Methods

3.2.1. Cell biological methods

3.2.1.1. Cell culture conditions

Cells were cultured in the appropriate culture medium supplemented with 10 % fetal bovine serum (Lonza) and penicillin/streptomycin (100 µg/ml of each). All cell lines were grown in 5 % CO_2 at 37°C.

3.2.1.2. Preparation of frozen stocks

Cell lines were trypsinized, washed in medium and centrifuged for 5 min at 500 g. The pellet was resuspended in FBS supplemented with 10 % DMSO. The cells were

slow frozen at – 80 ºC for 24 hours, and were then transferred into liquid nitrogen for long-time storage.

3.2.1.3. Starting cultures from frozen stocks

Vials with frozen cells were transferred to a 37 ºC water bath. After thawing, the cells were transferred to tubes containing prewarmed medium, then washed twice with medium and seeded into cell culture flasks.

3.2.1.4. Passaging of cells

Cells were washed with PBS, trypsinized until cells detached from the plastic, harvested in medium, diluted at an appropriate ratio and seeded into cell culture flasks.

3.2.1.5. Transient transfection of 293T cells

$1.5x10^6$ HEK293T cells were transfected for 8 hours with the appropriate plasmids using 2 M calcium phosphate ($CaCl_2$) and warm HEBS 2X solution (8 g NaCl, 0.1 g Na_2HPO_4 anhydrous, 6 g HEPES pH 7.0) in 5 % CO_2 at 37 °C. Cells were then washed once with warm PBS and cultured for an additional 24 hours in fresh complete medium prior to assay for protein expression and protein-protein interactions.

3.2.1.6. Retrovirus production and cell transduction

The retroviral vector pMSCV-puro expression and the generation of viruses have been previously described (Micheau et al, 2001). Each of the cell lines was infected for 16 hours with viral supernatants containing 8 µg/ml polybrene (Hexadimethrin Bromide from Sigma Aldrich), washed in phosphate-buffered saline from Lonza

(PBS), and cultured in complete medium containing 2.5 µg/ml puromycin from InvivoGen.

3.2.1.7. Analysis of TRAIL receptor expression by FACS

$5x10^5$ cells were incubated with the indicated antibodies or control mouse IgG1 at 10 µg/ml for 1 hour at 4°C, followed by Alexa-488 secondary goat anti-mouse antibody (Invitrogen, Cergy Pontoise Cedex, France) for 30 minutes at 4°C. Antibodies and conjugates were diluted in FACS buffer (PBS containing 0.5% BSA and 0.1% Sodium azide). After each incubation, the cells were washed once with PBS. Surface staining was analysed on a FACScalibur flow cytometer (BD Biosciences).

3.2.1.8. Analysis of TRAIL receptor internalization by FACS

$5x10^5$ cells were treated or not with 1 µg/ml His-tagged TRAIL and then incubated with the indicated antibodies or control mouse IgG1 at 10 µg/ml for 1 hour at 4°C, followed by Alexa-488 secondary goat anti-mouse antibody (Invitrogen, Cergy Pontoise Cedex, France) for 30 minutes at 4°C. Antibodies and conjugates were diluted in FACS buffer (PBS containing 0.5% BSA and 0.1% Sodium azide). After each incubation, the cells were washed once with PBS. Surface staining was analysed on a FACScalibur flow cytometer (BD Biosciences).

3.2.1.9. Measurement of cell viability

In 96-well plates, 50 000 cells were incubated at 37°C for 24 hours with increasing concentrations of Fas ligand or His-TRAIL (from 0 to 100 000 ng/ml) or for 48 hours with increasing concentration of CDDP (from 1 to 1000 µM). For sequential treatments, cells were treated for 48 hours with three different concentrations of CDDP from Sigma Aldrich (1.25 – 2.5 - 20 µM) in complete medium and then

washed before being treated for 16 hours with increasing concentrations of His-TRAIL (from 0 to 1000 ng/ml). Cell viability was determined by methylene blue (Micheau et al, 2001).

3.2.1.10. Hoechst analysis

Cells, treated or untreated with 500 ng/ml His-TRAIL or Fas ligand, were incubated at 37°C for 6 hours, or were treated with 1 µM Staurosporine (Sigma) for 72 hours at 37°C. Alternatively, cells pre-treated or not for 30 minutes with 20 µM H89 or for 20 minutes with 1 mM 8-bromo-cyclic AMP (8B), followed by TRAIL (50 - 100 or 500 ng/ml) or Fas ligand (100 or 500 ng/ml), were incubated at 37°C for 6 hours. Apoptosis was assessed by Hoechst staining by determining the percentage of condensed and fragmented nuclei from at least 300 cells per condition.

3.2.1.11. Apo 2.7 staining

Cells, treated or untreated with 500 ng/ml His-TRAIL or Fas ligand for 6 hours, or pre-treated with 10 µM H89 for 48 hours, were permeabilized (PBS, FCS 2.5 % and digitonin 100 µg/ml) for 10 minutes at 4°C and stained with a PE-conjugated 2.7A6A3 antibody (Beckman Coulter) which recognizes the apo 2.7 mitochondrial membrane protein exposed at an early stage on cells undergoing apoptosis. In all, 10 000 events were analysed using a FACScalibur flow cytometer (BD Biosciences).

3.2.1.12. Analysis of Bax activation by flow cytometry

Cells, treated or untreated with 20 or 200 ng/ml His-TRAIL for 6 or 16 hours, were fixed with 4 % PFA, permeabilized (PBS, BSA 1 % and saponin 0.1 %) for 10 minutes at room temperature and stained with an anti-Bax antibody which recognizes the active N-terminal form of Bax (clone 6A7, Tebu-bio). 10 000 events were analysed using a FACScalibur flow cytometer (BD Biosciences).

3.2.1.13. Gene silencing using small interfering RNA

Ezrin and Moesin SiGenome SMART pool siRNAs (set of 4) were purchased from Thermo Scientific (Dharmacon Division). For siRNA-mediated ezrin or moesin downregulation, 500 000 cells were transfected with scramble, ezrin or moesin targeting siRNAs using Dharmafect-4 reagent (Dharmacon Division) according to the manufacturer's specifications. 48 hours after transfection, ezrin and moesin expression levels were determined by western blot and sensitivity to TRAIL or Fas ligand was assessed by apo 2.7 staining.

3.2.2. Molecular biological methods

3.2.2.1. Cloning of ezrin wyld-type and mutant

VSV-tagged ezrin WT was subcloned from pEGFP-N1 vector (Clontech) to pCR-3 (Invitrogen) as EcoRI fragment and then into the pMSCV-puro expression vector as HindIII/XhoI fragment. VSV-tagged ezrin mutant was subcloned from pUC57 vector (Genscript) to pCR-3 (Invitrogen) as NotI/EcoRV fragment and then into the pMSCV-puro expression vector as HindIII/NotI fragment. All constructs were confirmed by sequencing.

3.2.2.2. Site-directed mutagenesis

Site-directed mutagenesis was performed to introduce errors in the ezrin wild-type construct to generate the following point mutations: S66A, S66D, Y145F, Y145D, Y353F, Y353D, T567A, T567D, R579A. Mutagenesis was performed using the QuickChange Kit (Stratagene, La Jolla, CA), which employs a PCR-based method amplifying the whole initial plasmid which contains the sequence to be mutated. The procedures were performed according to the manifacturer's protocol. The S66D, Y145D, Y353D, T567D were created to mimic phosphorylated ezrin, whereas S66A, Y145F, Y353F, T567A were generated as nonphosphorylable ezrin. All constructs

were confirmed by sequencing. The VSV-tagged ezrin mutants were subcloned into the pMSCV-puro expression vector as HindIII/XhoI fragments. All constructs were confirmed by sequencing.

3.2.3. Protein biochemical methods

3.2.3.1. Preparation of cell lysates

Cells were harvested by centrifugation at 500 g for 5 minutes at 4°C, washed once with ice cold PBS and lysates were prepared by resuspending the resulting cell pellets in 50 µl lysis buffer per $5x10^6$ cells (1% of NP40, 20mM Tris-HCl pH 7.5, 150mM NaCl and 10% glycerol) supplemented with complete protease inhibitor cocktail (Roche Diagnostic) according to the manufacturer's instructions. After 20 minutes incubation on ice, the lysates were centrifugated at 20000 g for 15 minutes at 4°C to remove cell debris and transferred to a fresh tube.

3.2.3.2. Immunoprecipitations

For TRAIL DISC analysis, 10^8 cells were stimulated with 5 µg of Flag-TRAIL cross-linked by 10 µg anti-Flag M2 in 1ml of medium for the indicated times at 37°C. Cells were then washed with cold phosphate saline buffer (PBS) and lysed in 1ml of lysis buffer containing 1% NP40, 20mM Tris-HCl pH 7.5, 150mM NaCl, 10% glycerol and proteinase inhibitor cocktail. Lysates were pre-cleared with Sepharose 6B (Sigma-Aldrich), and immunoprecipitated overnight at 4°C with protein G-Sepharose beads (Amersham Biosciences, Les Ullis, France). For TRAIL receptor, caspase-8, GAPDH, and VSV immunoprecipitations, cells were stimulated as described above with 5 µg/ml His-TRAIL. For the ezrin immunoprecipitates, cells were stimulated with 5 µg/ml His-TRAIL or FasL and 0.1 mM orthovanadate. In the phospho-tyrosine immunoprecipitates, cells were stimulated with 0.1 mM orthovanadate. In each case, cells were lysed in NP-40-lysis buffer and lysates were

precleared before 5 μg of immunoprecipitating antibody was added. Beads were then washed four times with detergent, and immunoprecipitates were eluted in lysis buffer (Tris-HCl 63 mM, SDS 2%, phenol red 0.03%, glycerol 10% and DTT 100mM pH 6.8), boiled for 5 min, and processed for immunoblotting.

3.2.3.3. Western blot analysis

Immunoprecipitates or cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in PBS containing 0.05% Tween 20 and 5% milk powder for at least 1 hour. Membranes were then incubated with a specific primary antibody overnight at 4°C by mixing, followed by horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, and were developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

3.2.4. Statistical analysis

For in vitro studies, differences were determined either with two-way repeated-measures analysis of variance (ANOVA) with Bonferroni's multiple comparison test, or by student's t test, using Prism 5.0a software (GraphPad Software, San Diego, CA, USA). A significance level of *P<0.05, **P<0.01 or ***P<0.001 was assumed for all tests.

4. Results

4.1. TRAIL-R2-ezrin association in the TRAIL pathway in SKW6.4 cells

Ezrin has been shown to connect the Fas receptor to the actin cytoskeleton (Parlato et al, 2000; Lozupone et al, 2004). Fas linkage to the actin cytoskeleton through ezrin primes human CD4+ T lymphocytes for Fas-mediated apoptosis (Parlato et al, 2000; Lozupone et al, 2004). In Jurkat cells, ezrin and moesin are associated with Fas and required for cell death triggering (Hébert et al, 2008).

There are many similarities between the Fas and TRAIL signalling pathways. Therefore the initial aim of this study was to determine whether ezrin is involved in TRAIL signalling, and if it is able to associate with the TRAIL agonistic receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5).

B lymphoma cells (SKW6.4), colon carcinoma cancer cells (HCT116), colon adenocarcinoma cells (SW480) and cervical cells (HeLa) were first characterized by determination of TRAIL sensitivity and their TRAIL receptor expression profile. All of them were susceptible to TRAIL-induced cell death (**Figure 4.1.a**), and the agonistic receptors were well expressed on the cell surface (**Figure 4.1.b**). TRAIL-R3 and TRAIL-R4, however, were not detectable in HCT116, SW480 and HeLa cells (**Figure 4.1.b**). Only SKW6.4 cells expressed TRAIL-R4 at the membrane level (**Figure 4.1.b**).

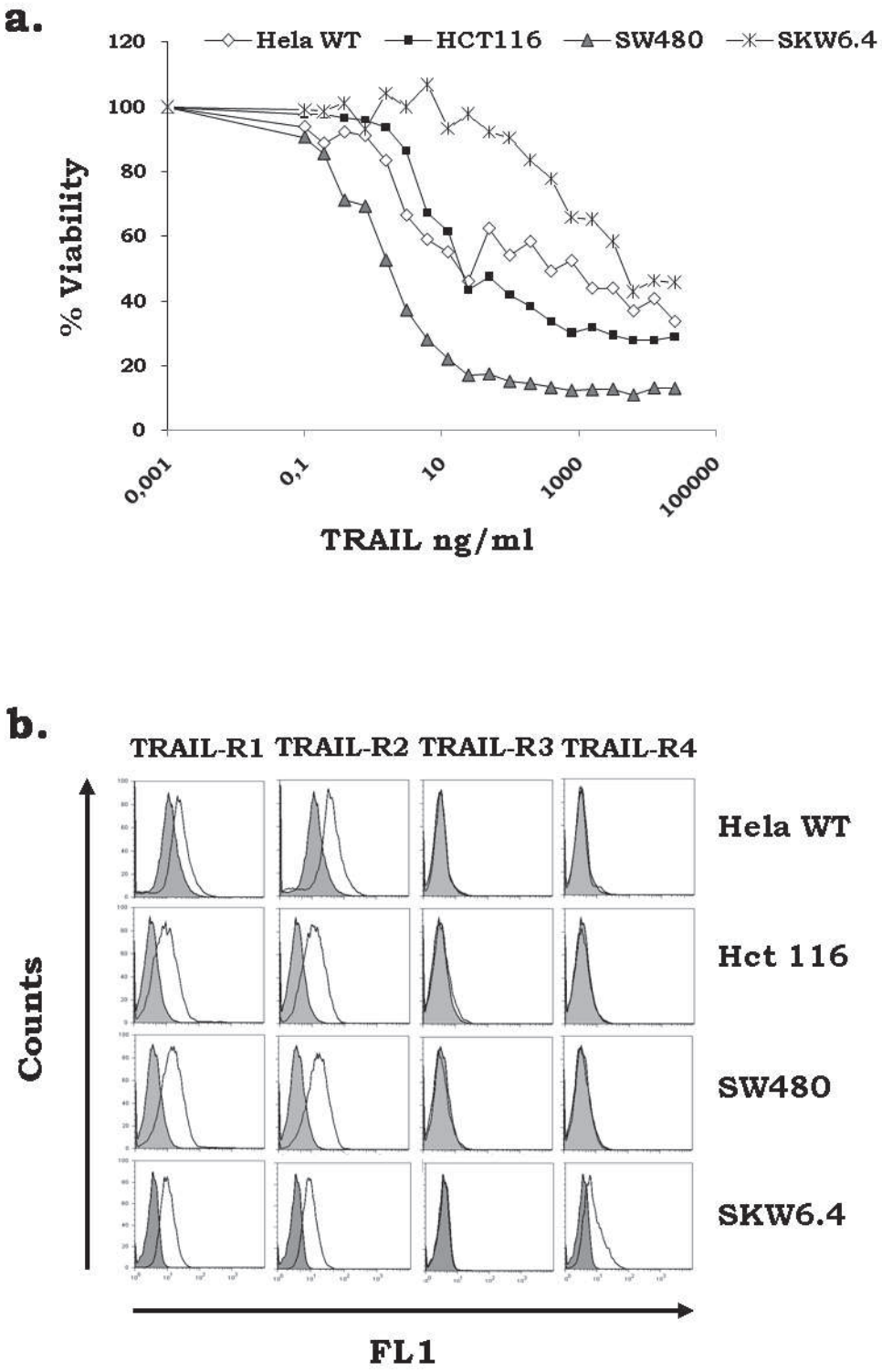

Figure 4.1. HeLa WT, HCT116, SW480 and SKW6.4 cells are sensitive to TRAIL and express TRAIL-R1 and TRAIL-R2 on the cell surface. (a) Cells were treated with increasing concentrations of His-TRAIL for 24 hours and cellular viability was evaluated by alamar blue coloration for SKW6.4 cells, and methylene blue coloration for the others. (b) Expression of TRAIL receptors at the membrane level was measured by flow cytometry (unfilled peaks). Filled peaks correspond to the isotype control antibody staining.

In order to determine whether ezrin could be involved in TRAIL signalling, we analysed the TRAIL DISC composition in the B lymphoma cell line, SKW6.4, using Flag tagged-TRAIL ligand immunoprecipitations. Stimulation with Flag tagged-TRAIL ligand induced DISC formation, where TRAIL-R2 was found to be associated with FADD and proforms of caspase-8 (**Figure 4.2**), which were processed after treatment with TRAIL. When we analysed the precipitated complex for the presence of ezrin, we found ezrin pre-associated with TRAIL-R2, but we observed inconsistencies in ezrin recruitment (**Figure 4.2**). Addition of TRAIL increased ezrin levels in the first experiment, **panel a of Figure 4.2**, whereas they did not change in the other two experiments (**Figure 4.2, panels b and c**), except when we incubated the cells with the ligand for 1 hour at 4°C (**Figure 4.2, panel c**).

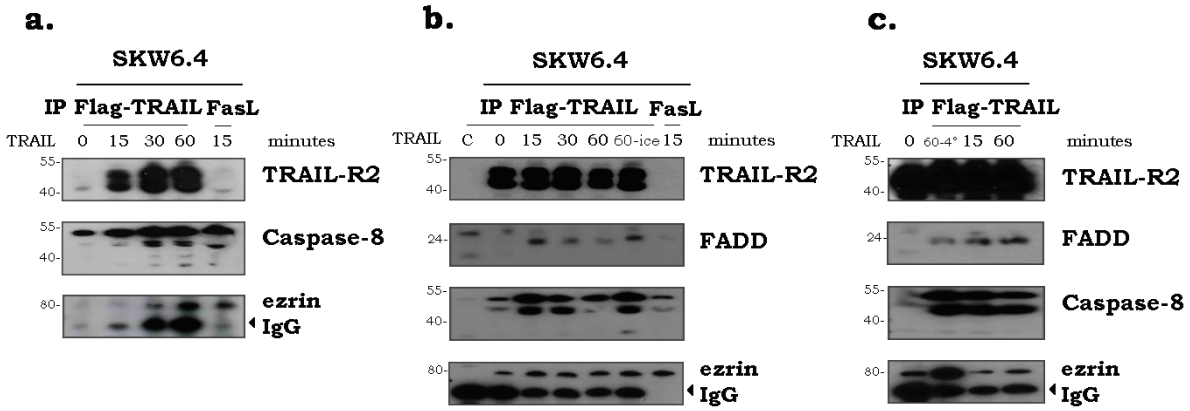

Figure 4.2. Ezrin is present in TRAIL immunoprecipitates of SKW6.4 cells. SKW6.4 cells were stimulated with 5 µg/ml Flag-TRAIL cross-linked to 10 µg/ml of anti-Flag (M2) antibody or Fas ligand for the indicated time. After cell lysis, the DISC was immunoprecipitated and analyzed by western blot for the presence of DISC components and ezrin. C corresponds to the anti-Flag antibody in the whole cell lysates plus protein G-sepharose. Three independent experiments are shown.

Flag tagged-TRAIL ligand immunoprecipitations were also performed in SKW6.4 cells in the presence or absence of ZVAD, the pan-caspase inhibitor, in order to evaluate whether ZVAD could affect the recruitment of ezrin to TRAIL-R2. Activation of TRAIL-R2 by Flag tagged-TRAIL ligand cross-linked by anti-Flag (M2) antibody led to the recruitment of FADD and proforms of caspase-8 together with TRAIL-R2 (**Figure 4.3**). Within this complex, the pro-caspases-8 were well processed, as indicated by the presence of the cleavage products p43/41 in the DISC immunoprecipitations (**Figure 4.3**). We found that ezrin was immunoprecipitated with TRAIL-R2, and addition of TRAIL did not change significantly the ezrin levels (**Figure 4.3**). Moreover, we observed no difference in ezrin recruitment, regardless of whether ZVAD was added or not to the cell lysates (**Figure 4.3**), and the FADD immunoblot was inconclusive, a possible consequence of technical problems (**Figure 4.3**).

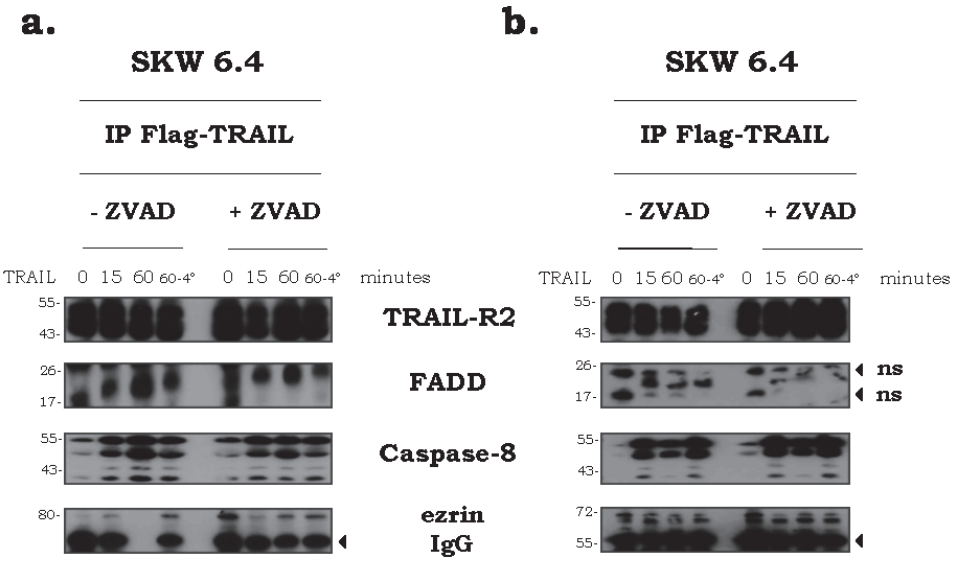

Figure 4.3. ZVAD doesn't interfere with the TRAIL-R2 association with ezrin. SKW6.4 cells were stimulated with 5 μg/ml Flag-TRAIL cross-linked to 10 μg/ml of anti-Flag (M2) antibody for the indicated time. After cell lysis, the DISC was immunoprecipitated and analyzed by western blot for the presence of DISC components and ezrin. 60-4 corresponds to SKW6.4 cells incubated with Flag-tagged TRAIL ligand for 1 hour at 4 C. Two independent experiments are shown.

To further examine the association of ezrin with TRAIL-R2, TRAIL DISC immunoprecipitations were performed in SKW6.4 and HeLa WT cell lines. The TRAIL ligand protein complex precipitated from SKW6.4 cells was compared with that of HeLa cells. Cells were then stimulated with Flag tagged-TRAIL ligand cross-linked by anti-Flag (M2) antibody and lysates were prepared, followed by SDS-PAGE and western blot analysis. As shown in **Figure 4.4**, ezrin was present in TRAIL immunoprecipitations from both SKW6.4 and HeLa WT cell lysates. Moreover, no change in ezrin recruitment to TRAIL-R2 could be observed after TRAIL stimulation in B lymphoma cells, whereas there may be a slight increase in the amount of ezrin recruited with TRAIL treatment in HeLa WT cells.

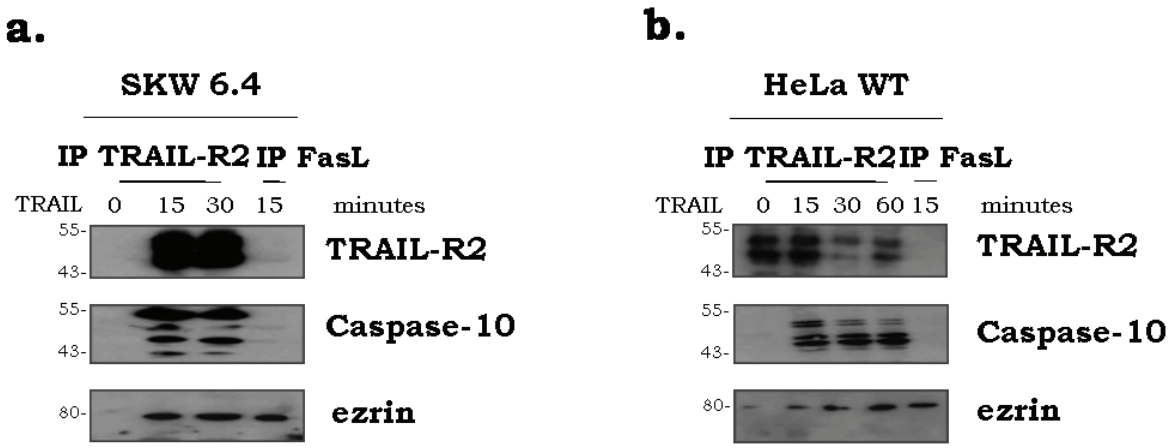

Figure 4.4. Ezrin recruitment to TRAIL-R2 in SKW6.4 and HeLa cells. SKW6.4 and HeLa cells were stimulated with 5 μg/ml Flag-TRAIL cross-linked by 10 μg/ml of anti-Flag (M2) antibody or Fas ligand for the indicated time. After cell lysis, the DISC was immunoprecipitated and analyzed by western blot for the presence of TRAIL-R2, caspase-10 and ezrin.

A previous study demonstrated that ezrin was phosphorylated on tyrosine residues in human CD4+ T lymphocytes, prone to Fas-mediated cell death (Luciani et al, 2004b). So, we addressed the question whether the ezrin present in the TRAIL DISC complex was phosphorylated on tyrosine residues. We also investigated the effects that ezrin post-translational modifications may have regarding its binding to the complex. We performed double immunoprecipitation experiments, pulling down

TRAIL ligand followed by phospho-tyrosine. This double precipitate protein complex was compared with that of TRAIL ligand or phospho-tyrosine. SKW6.4 cells were left untreated or stimulated for 15 and 60 minutes with 5 μg/ml Flag tagged-TRAIL ligand cross-linked by 10 μg/ml anti-Flag (M2) antibody before being lysed. Cell extracts were precipitated overnight with mixing followed by a second precipitation for 4 hours with the phospho-tyrosine antibody on protein G beads which were then processed for western blot analysis. For a positive control of the tyrosine immunoprecipitation, cells were stimulated with 0.1 mM orthovanadate for the same time points. We found that orthovanadate, as expected, was able to induce ezrin phosphorylation on tyrosine residues, and, surprisingly, FADD phosphorylation as well (**Figure 4.5**). TRAIL stimulation induced DISC formation, where TRAIL-R2 was found associated with FADD and proforms of caspase-8 (**Figure 4.5**), which were processed after treatment with TRAIL. We also observed that ezrin immunoprecipitated with TRAIL-R2 (**Figure 4.5**). However, when we analysed the double precipitate complexes we did not observe ezrin or FADD phosphorylated on tyrosine residues (**Figure 4.5**).

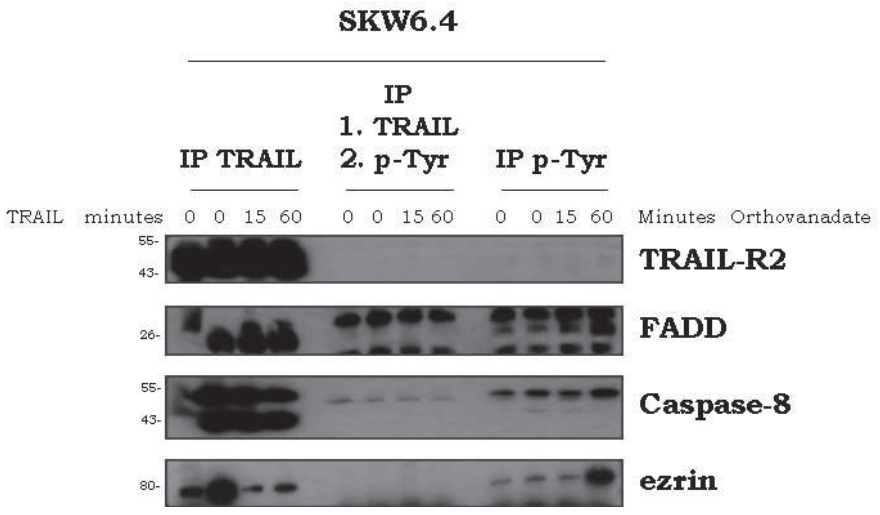

Figure 4.5. Analysis of ezrin tyrosine phosphorylation in the TRAIL DISC. SKW6.4 cells were stimulated with 5 μg/ml Flag-TRAIL cross-linked by 10 μg/ml anti-Flag (M2) antibody for 15 and 60 minutes at 37 C. After cell lysis, the DISC was immunoprecipitated and followed by p-tyrosine immunoprecipitation. The protein complexes were analyzed by western blot. TRAIL ligand and p-tyrosine immunoprecipitations were used as positive controls. The second point 0 corresponds to cells left at 4 C instead of ice.

4.2. Ezrin over-expression in tumour cells

In order to define the role of ezrin in the TRAIL signalling pathway, we took advantage of the finding that the ezrin region implicated in the Fas/actin association was previously mapped (between the amino-acids 149-168) and shown to be required to protect T cells from Fas-mediated cell death (Lozupone et al, 2004). We hypothesised that the same region could be required to bind TRAIL-R1 and TRAIL-R2. We constructed plasmid vectors to express two chimeric ezrin molecules, one resembling the endogenous wild-type ezrin, and the second a mutant form of ezrin, that we called ezrin-moesin (**Figure 4.6**). The mutant ezrin lacked the Fas-binding region because this region was replaced with the corresponding sequence of moesin, which was shown to be unable to bind Fas (Parlato et al, 2000). Since the chimeric mutant ezrin remains able to bind actin, its over-expression behaves as a dominant negative construct by preventing the actin linkage to Fas.

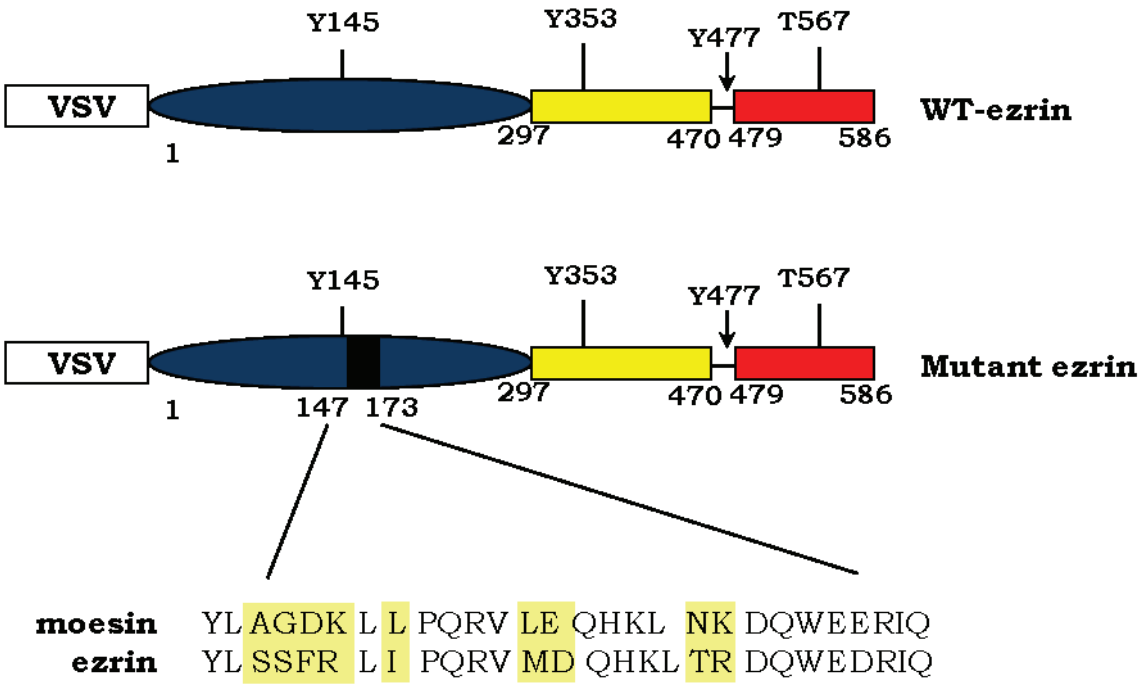

Figure 4.6. Schematic representation of the two chimeric ezrins.

We have ectopically expressed these two forms of ezrin using a retroviral approach in HeLa WT, HCT116 and SW480 cancer cells. Once the populations were stable following selection, we checked by western blot analysis for the presence of each construct in the corresponding different cell lines. Following this, we evaluated the possible modulation of the sensitivity to TRAIL compared with the sensitivity to Fas ligand and staurosporine.

HeLa WT, HCT116, and SW480 cells, stably infected with retroviruses encoding for ezrin wild-type, ezrin mutant or the empty vector, were stimulated for 6 hours with Fas ligand or His-TRAIL (500 ng/ml) and for 72 hours with staurosporine (1 μM). Apoptosis was determined by Hoechst staining. We also evaluated the expression of the TRAIL agonistic receptors TRAIL-R1 and TRAIL-R2 on the cell surface by fluorescence-activated cell sorter staining (FACS).

The first population generated was HeLa cells expressing an ezrin mutant. We observed that stable over-expression of the chimeric mutant ezrin in these cells induced a marked inhibition of TRAIL- and Fas ligand-induced cell death (**Figure 4.7.c**). The ezrin mutant over-expression also led to the inhibition of staurosporine-induced apoptosis in these cells, an agent known to induce apoptosis in a death receptor-independent manner (**Figure 4.7.c**). This mutant appeared also to downregulate TRAIL-R1 as measured by FACS analysis, and to enhance TRAIL-R2 cell surface expression (**Figure 4.7.b**).

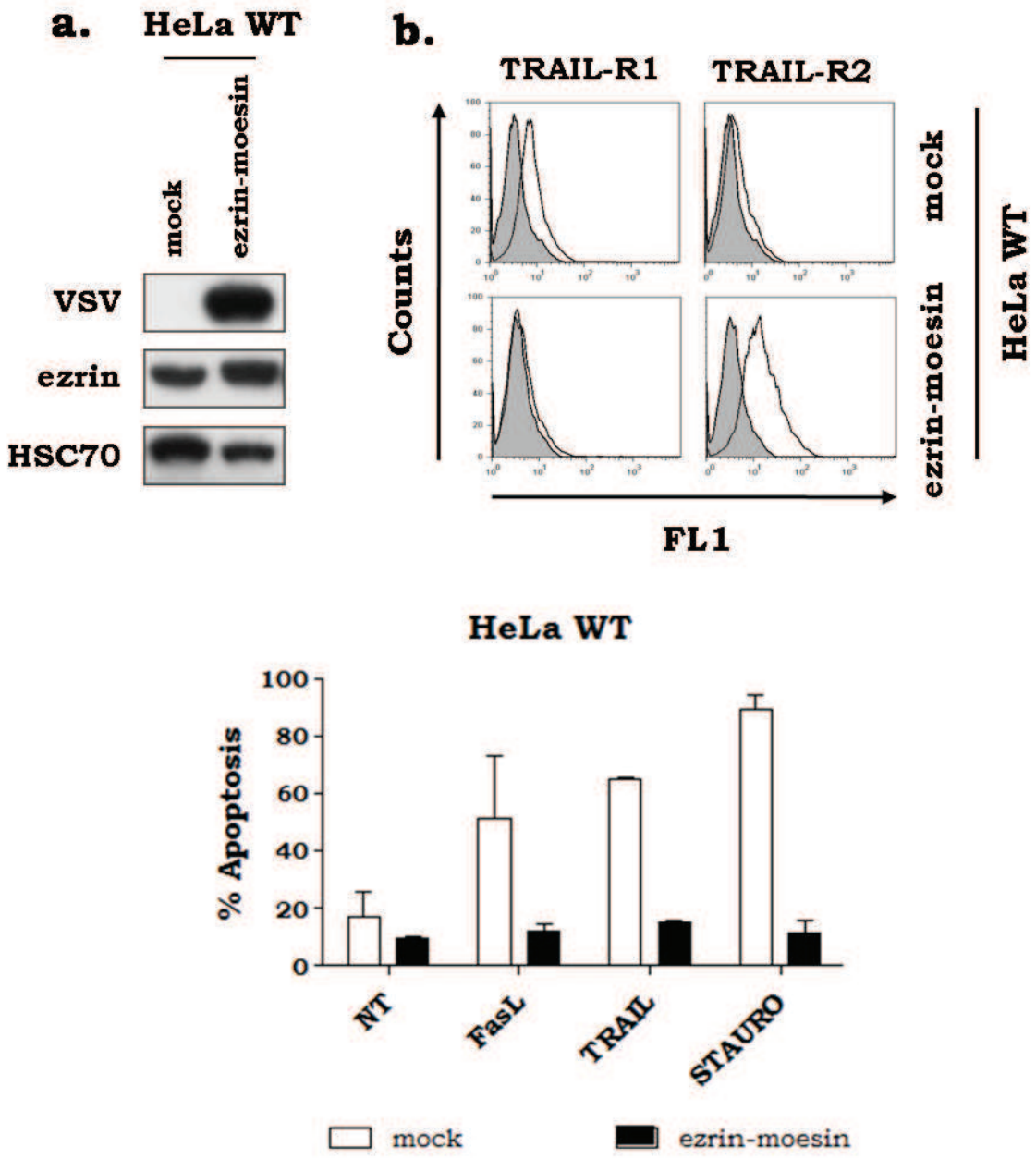

Figure 4.7. Protection against TRAIL and Fas ligand-induced cell death in HeLa cells over-expressing the mutant ezrin. (a) Western blot analysis of chimeric ezrin expression in mock and ezrin mutant over-expressing HeLa cells. The HSC70 was probed for normalization. (b) Cell surface expression of TRAIL-R1 and TRAIL-R2 was measured by flow cytometry (unfilled peaks). Filled peaks correspond to the isotype control antibody staining. (c) Control cells or ezrin mutant-expressing HeLa cells were treated with Fas ligand or His-TRAIL (500 ng/ml) for 6 h, or staurosporine (1 μM) for 72 h. Apoptosis was quantified by Hoechst staining. The mean and standard deviation of two independent experiments are shown.

We also evaluated whether the mutant form of ezrin could associate with TRAIL-R2 and TRAIL-R1, and whether addition of TRAIL would induce differences in ezrin recruitment. Therefore, the TRAIL ligand precipitate protein complex was

compared with that of TRAIL-R2, ezrin, or VSV. Wild-type or ezrin mutant-expressing HeLa cells were then left untreated or stimulated for 60 minutes with 5 μg/ml His tagged, or Flag tagged-TRAIL ligand cross-linked by anti-Flag (M2) antibody before being lysed. Cell extracts were precipitated with the corresponding antibody (TRAIL-R2, ezrin or VSV antibody) and processed for western blot. We found endogenous ezrin, but not moesin, associated with the TRAIL receptors in the TRAIL ligand and TRAIL-R2 but not in the ezrin immunoprecipitates of Hela wild-type and over-expressing the mutant ezrin (**Figure 4.8**). We also observed that the mutant ezrin was not associated with neither TRAIL-R1 nor TRAIL-R2 in any of the immunoprecipitated complexes assessed.

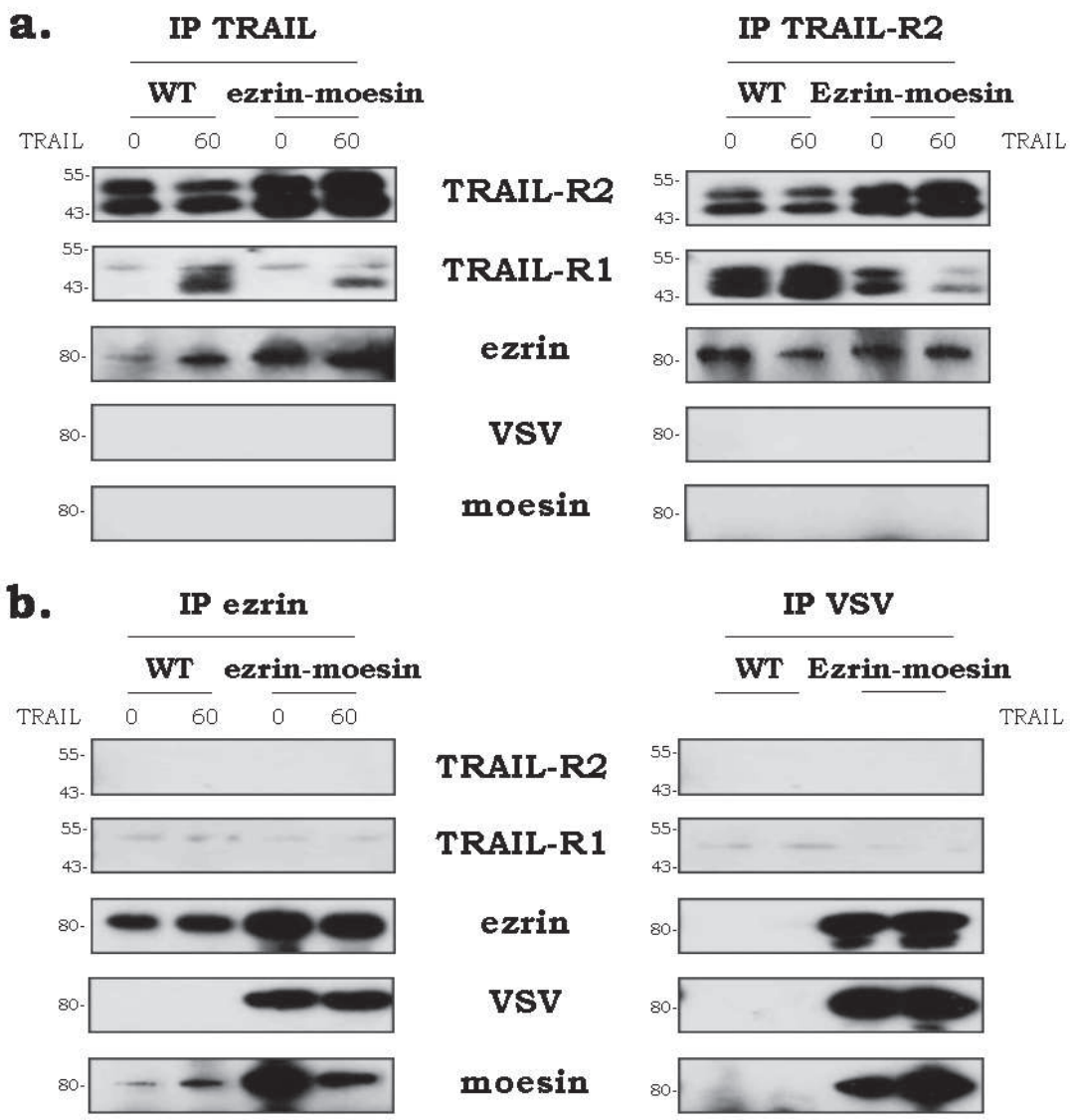

Figure 4.8. The mutant ezrin is not associated with TRAIL-R2. Western blotting for TRAIL-R2, ezrin, VSV and moesin in TRAIL ligand, TRAIL-R2, ezrin and VSV immunoprecipitates of control or ezrin mutant-expressing HeLa cells stimulated or not with 5 μg/ml His-TRAIL or with Flag-TRAIL cross-linked by 10 μg/ml anti-Flag (M2) antibody for 60 minutes. One of two independent experiments is shown.

We next generated and characterized populations of HCT116 and SW480 cells stably expressing chimeric wild-type or mutant ezrin. First, cell lysates were prepared for analysis by western blot to determine the expression level of each of the constructs in the corresponding cell lines. As shown in **Figure 4.9.a and 4.10.a**, the two chimeric forms of ezrin were well expressed in both cell lines.

Control, wild-type, or mutant ezrin-expressing HCT116 or SW480 cells were then stimulated for 6 hours with 500 ng/ml His-TRAIL or Fas ligand, or for 72 hours with 1 μM staurosporine. Apoptosis was quantified by Hoechst staining. We found that expression of the mutant ezrin in HCT116 cells appears to inhibit TRAIL-induced apoptosis (**Figure 4.9.c**). However, its expression in SW480 cells had no effect on TRAIL and Fas sensitivity (**Figure 4.10.c**). Surprisingly, expression of the chimeric wild-type ezrin in the same cells induced a marked inhibition of Fas ligand- and TRAIL-induced cell death (**Figure 4.9.c; 4.10.c**).

At the same time we evaluated the expression of TRAIL-R1 and TRAIL-R2 at the membrane level. No changes in TRAIL-R1 or TRAIL-R2 cell surface expression could be measured by FACS analysis (**Figure 4.9.b; 4.10.b**).

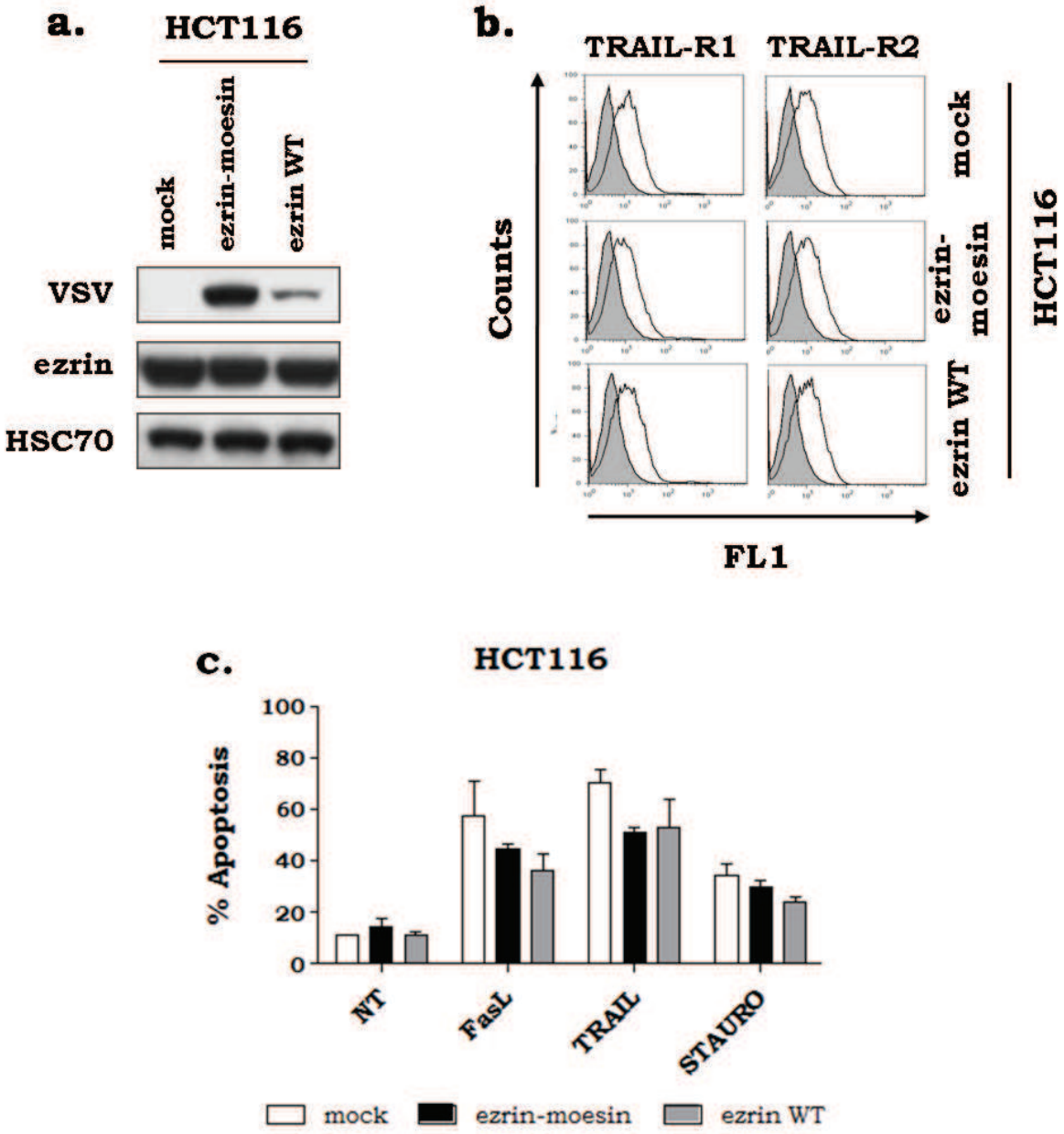

Figure 4.9. Protection against TRAIL and Fas ligand-induced cell death in HCT116 cells over-expressing wild-type ezrin. (a) Western blot analysis of chimeric ezrin expression in mock, wild-type or mutant ezrin over-expressing HCT116 cells. The HSC70 was probed for normalization. (b) Cell surface expression of TRAIL-R1 and TRAIL-R2 was measured by flow cytometry (unfilled peaks). Filled peaks correspond to the isotype control antibody staining. (c) Control cells, wild-type or mutant ezrin-expressing HCT116 cells were treated with Fas ligand or His-TRAIL (500 ng/ml) for 6 h, or staurosporine (1 μM) for 72 h. Apoptosis was quantified by Hoechst staining. The mean and standard deviation of three independent experiments are shown.

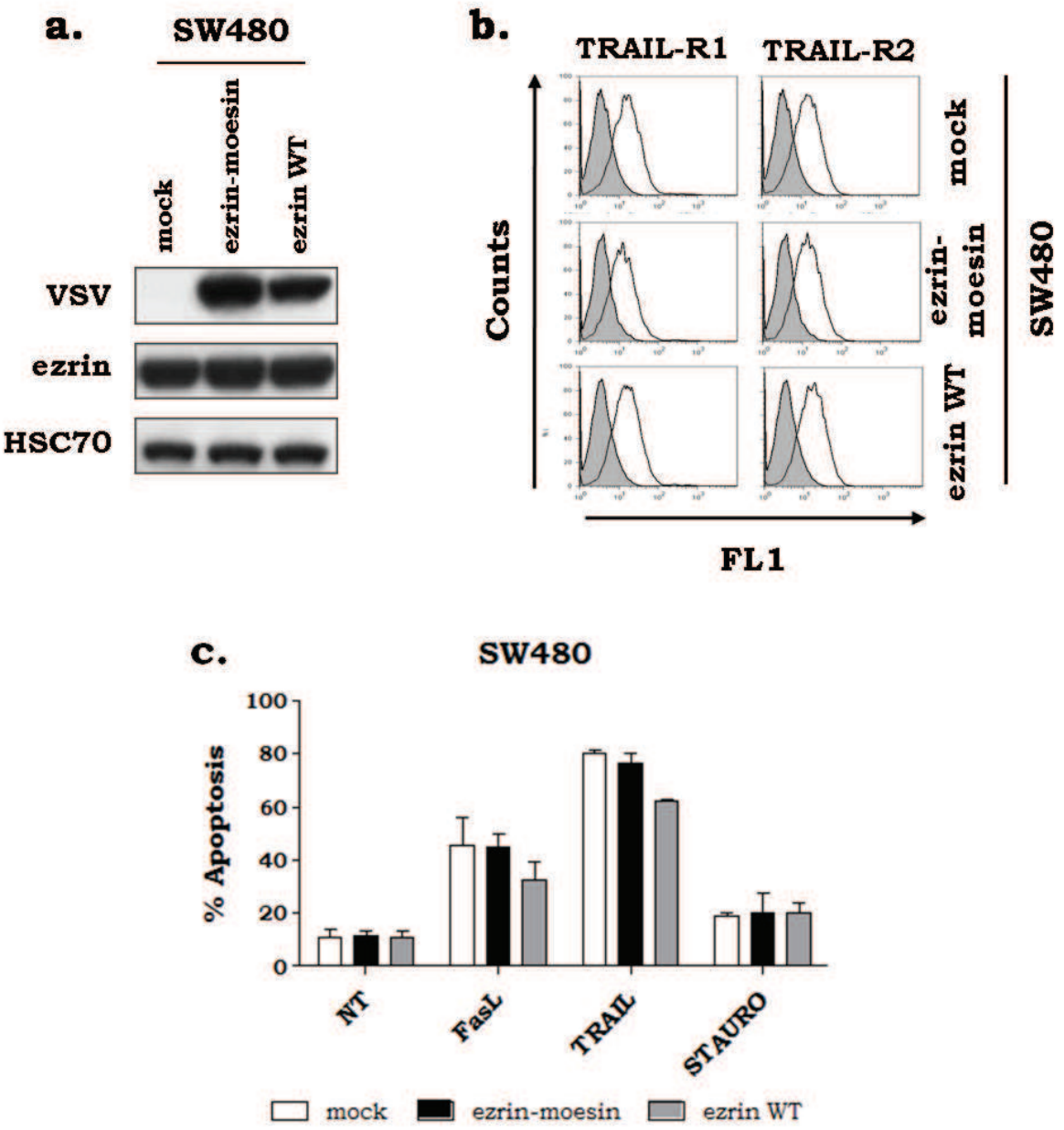

Figure 4.10. Protection against TRAIL and Fas ligand-induced cell death in SW480 cells over-expressing wild-type ezrin. (a) Western blot analysis of chimeric ezrin expression in mock, wild-type or mutant ezrin over-expressing SW480 cells. The HSC70 was probed for normalization. (b) Cell surface expression of TRAIL-R1 and TRAIL-R2 was measured by flow cytometry (unfilled peaks). Filled peaks correspond to the isotype control antibody staining. (c) Control cells, wild-type or mutant ezrin-expressing SW480 cells were treated with Fas ligand or His-TRAIL (500 ng/ml) for 6 h, or staurosporine (1 μM) for 72 h. Apoptosis was quantified by Hoechst staining. The mean and standard deviation of three independent experiments are shown.

In the first infection performed, we obtained only a population of HeLa cells expressing the mutant form of ezrin. The characterization of this initial population eventually revealed that these cells were phenotypically different from the

corresponding populations generated subsequently in the HCT116 and SW480 cell lines. In these HeLa cells, the expression of the dominant negative form of ezrin, was found to modulate either the sensitivity to Fas ligand- and TRAIL-induced apoptosis and the TRAIL agonistic receptors expression on the cell surface. Unfortunately, these data could not be repeated in the two colorectal carcinoma cell lines tested.

In order to understand if these findings in HeLa cells were reproducible and restricted to this peculiar cell line, we repeated the experiment, generating a second set of stable HeLa populations over-expressing the two chimeric forms of ezrin. We then compared the sensitivity of ezrin mutant-expressing HeLa cells with that of wild-type ezrin-expressing HeLa cells. Hoechst staining performed after addition of Fas ligand or TRAIL for 6 hours showed that the new HeLa populations differed from those obtained in the initial experiment, and were similar to the corresponding populations generated using HCT116 and SW480 cells (**Figure 4.11**). Moreover, the ezrin mutant failed to afford protection against Fas ligand- and TRAIL- as well as staurosporine-induced cell death, and the wild-type ezrin expressing population was not significantly protected from these cell death inducers (**Figure 4.11.c**). The viability assays also showed the same trend that the Hoechst staining (**Figure 4.11.d**). Again no change in TRAIL-R1 and TRAIL-R2 expression at the membrane level could be measured by flow cytometry (**Figure 4.11.b**).

The finding that the new HeLa populations differed from those obtained in the initial experiment induced us to hypothesize that the HeLa WT cells were not the good cellular model for this project. We then decided to focus our attention mainly on the HCT116 and SW480 cell lines. We also excluded the SKW6.4 cells because they cannot be easily transfected.

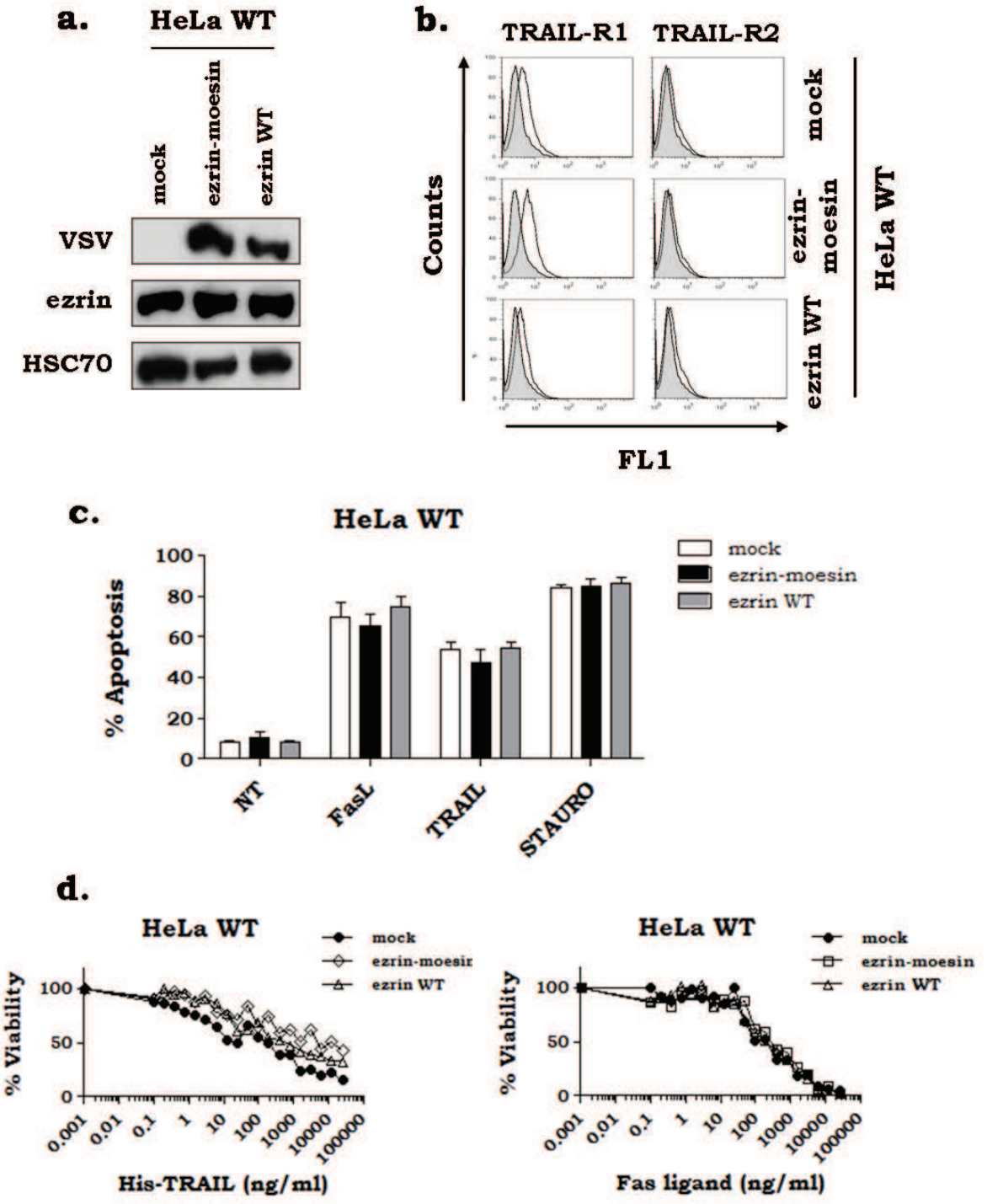

Figure 4.11. The new HeLa cells over-expressing the mutant ezrin are not protecting against TRAIL and Fas ligand-induced cell death. (a) Western blot analysis of chimeric ezrin expression in mock, wild-type or mutant ezrin over-expressing HeLa cells. The HSC70 was probed for normalization. (b) Cell surface expression of TRAIL-R1 and TRAIL-R2 was measured by flow cytometry (unfilled peaks). Filled peaks correspond to the isotype control antibody staining. (c) Control cells, wild-type or mutant ezrin-expressing HeLa cells were treated with Fas ligand or His-TRAIL (500 ng/ml) for 6 h, or staurosporine (1 μM) for 72 h. Apoptosis was quantified by Hoechst staining. The mean and standard deviation of three independent experiments are shown. (d) Cell viability was evaluated by methylene blue assay after 24 hours treatment with increasing concentrations of His-TRAIL or Fas ligand.

4.3. Ezrin depletion by siRNAs had no significant effect in the TRAIL pathway

Previous research has demonstrated that ezrin knockdown by small interfering RNAs, but not of moesin, was able to enhance TRAIL- and Fas ligand-induced apoptosis in normal T cells and type I cells, highlighting ezrin as a negative regulator in the death pathway (Kuo et al, 2010). Alternatively, the downregulation of ezrin or moesin in Jurkat cells decreased Fas-triggered apoptosis (Hébert et al, 2008) suggesting that the interaction of Fas with ezrin and the actin cytoskeleton is required for the initiation of the Fas-mediated apoptosis process (Hébert et al, 2008).

To investigate whether ezrin is involved in regulating TRAIL-induced cell death in colon carcinoma cells, an siRNA approach was applied to knockdown either ezrin or moesin. Ezrin or moesin were downregulated in HCT116 and SW480 cells by transfection with targeted siRNAs. 48 hours after transfection with the respective siRNAs, cell lysates were prepared to determine the total amounts of ezrin and moesin in the corresponding two cell lines by western blot. As shown in **Figure 4.12.a**, expression of ezrin or moesin was knocked down by their respective siRNAs, as compared with cells transfected with control siRNAs.

HCT116 and SW480 cells, deficient in ezrin or moesin, were then stimulated for 6 hours with Fas ligand or His-TRAIL (500 ng/ml) and apoptosis was evaluated by apo 2.7 staining followed by flow cytometry (**Figure 4.12.b-c**). The knockdown of ezrin or moesin, however, led to a slight but not significant increase in Fas ligand- and TRAIL-induced cell death, probably due to the fact that we could not obtain a complete ezrin or moesin downregulation. Ezrin has a low-turn-over rate, and it is possible that a longer incubation time with the siRNAs was needed to obtain a better depletion.

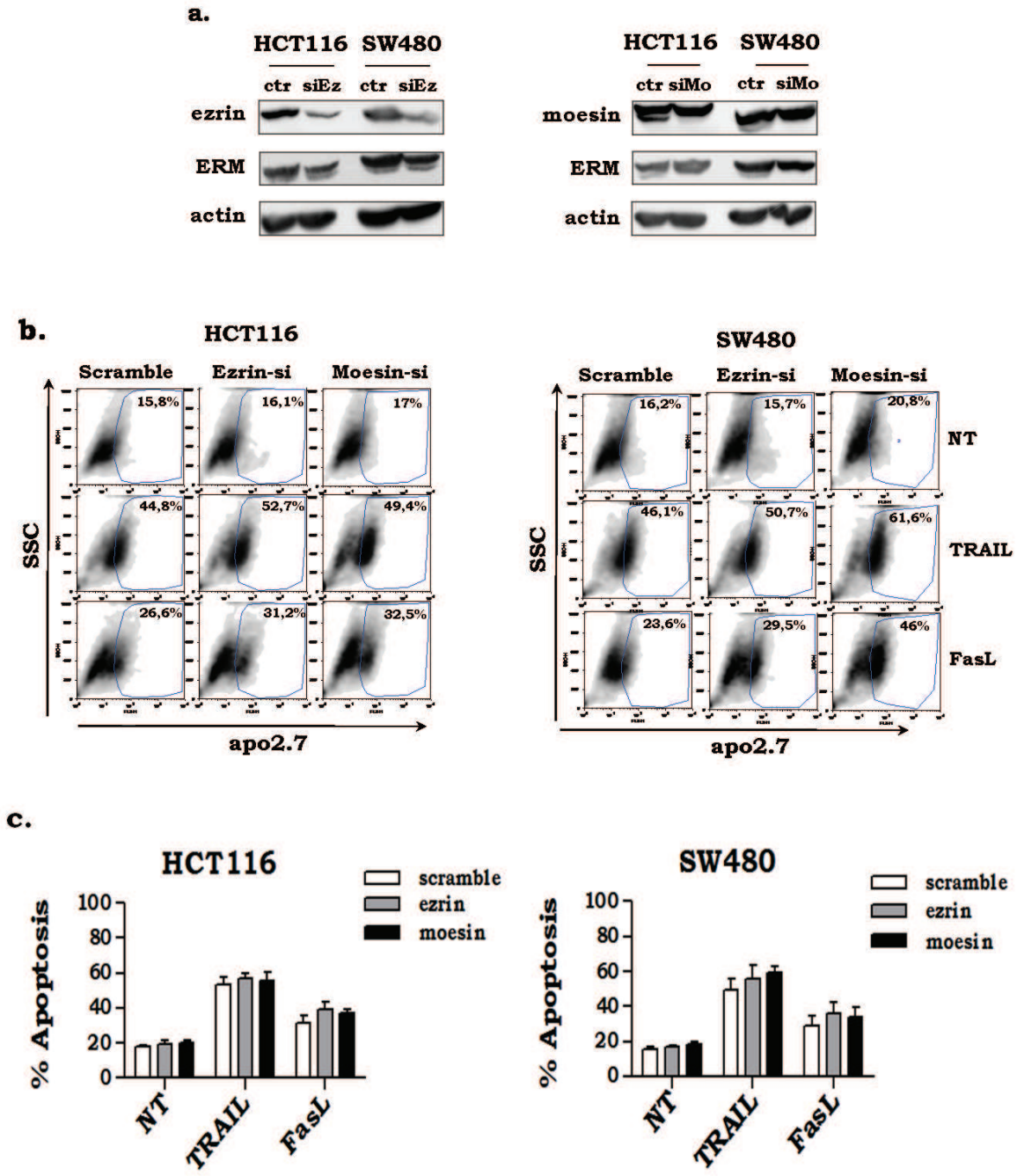

Figure 4.12. Ezrin depletion by siRNA slightly enhances TRAIL and Fas ligand-induced cell death in HCT116 and SW480 cells. (a) HCT116 and SW480 cells were trasfected with control, ezrin or moesin siRNA. 48 hours after transfection, the ezrin and moesin levels were determined by western blot. Anti-ERM antibody recognizes ezrin, moesin and radixin. (b-c) 48 hours after transfection, cells were treated with 500 ng/ml of His-TRAIL or FasL for 6 h, stained with apo 2.7 antibody and analyzed by flow cytometry. Density blots are shown for each experiments (b). The mean and standard deviation of three independent experiments are shown (c).

4.4. TRAIL-R1/R2-ezrin association in the TRAIL pathway in HCT116 and SW480 cells

We assessed for the second time TRAIL DISC composition in order to further analyse and characterize the receptor linkage to the actin cytoskeleton through the ERM proteins, and to definitively elucidate whether ezrin was truly involved in TRAIL signalling. The TRAIL DISC composition was analysed in HCT116, SW480 and HeLa WT cells, using TRAIL ligand, TRAIL-R1 and TRAIL-R2 immunoprecipitations. The TRAIL ligand precipitate protein complex from these cells was compared with that of TRAIL-R1 and TRAIL-R2.

Activation of TRAIL-R1/R2 by Flag tagged-TRAIL ligand cross-linked by anti-Flag (M2) antibody or His tagged-TRAIL ligand led to the recruitment of FADD and proforms of caspase-8 together with TRAIL-R1 and TRAIL-R2 (**Figure 4.13**). The pro-caspases-8/10 were well processed within this complex, as indicated by the presence of the cleavage products p43/41 in the DISC immunoprecipitations (**Figure 4.13**).

When we analysed the TRAIL ligand precipitate complex we found that ezrin, moesin, and actin, were pre-associated with TRAIL-R1 and TRAIL-R2. We also recovered bands corresponding to ezrin, moesin, and actin when we used a non relevant antibody (anti-Flag) to immunoprecipitate, strongly supporting the idea that these proteins nonspecifically bound to the protein G-sepharose that is used to perform the immunoprecipitations (**Figure 4.13.a, isotype control line**). However, radixin, another member of the ERM family, was not immunoprecipitated with TRAIL. We also found that these nonspecific interactions were progressively lost upon TRAIL stimulation.

In TRAIL-R1 and TRAIL-R2 immunoprecipiatations we found ezrin, but not radixin or moesin, pre-associated with TRAIL-R1/R2 in non stimulated cells, but we observed inconsistencies in ezrin recruitment (**Figure 4.13. panels b and c**).

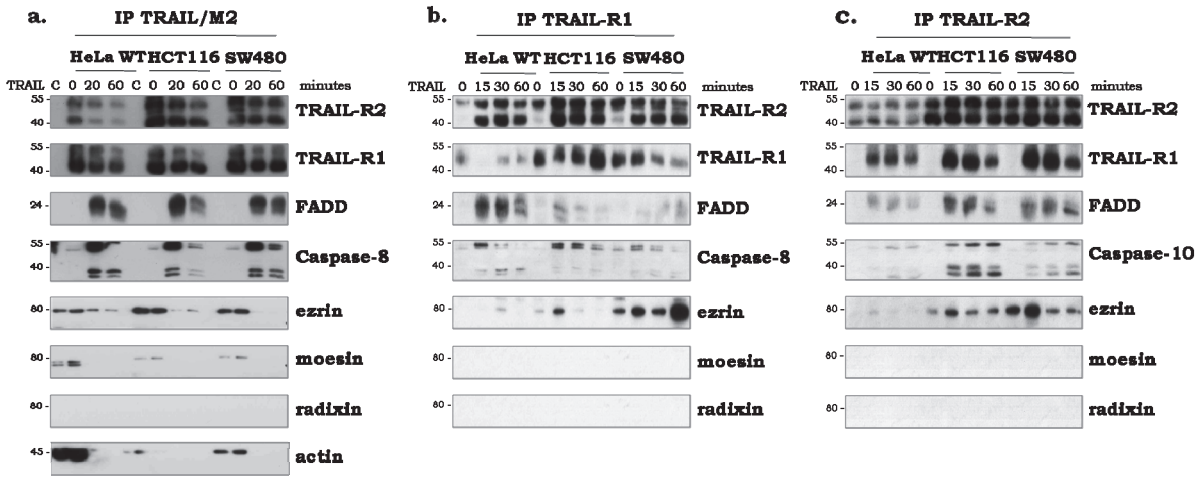

Figure 4.13. Ezrin is present in a nonspecific manner in TRAIL immunoprecipitates. (a) HeLa, HCT116 and SW480 cells were stimulated with 5 µg/ml Flag-TRAIL cross-linked by 10 µg/ml of anti-Flag (M2) antibody for the indicated time. After cell lysis, the DISC was immunoprecipitated and analyzed by western blot for the presence of DISC components, ERM and actin proteins. C corresponds to the anti-Flag antibody in the whole cell lysates plus protein G-sepharose. One of three independent experiments is shown. (b, c) HeLa, HCT116 and SW480 cells were stimulated with 5 µg/ml His-TRAIL for the indicated time. After cell lysis, the DISC was immunoprecipitated using an antibody against TRAIL-R1 (**panel b**) or TRAIL-R2 (**panel c**) and analyzed by western blot. One of three independent experiments is shown.

Co-immunoprecipitation experiments were performed to further analyse a possible interaction between ezrin and the TRAIL receptors. 293-T cells were transfected with a plasmid expressing wild-type ezrin together with another plasmid expressing TRAIL-R1 or TRAIL-R2. Wild-type ezrin was also co-expressed with a plasmid encoding TNFR1 or Fas. TRAIL-R1 has been shown to associate with TRAIL-R2. Therefore, plasmids encoding full-length TRAIL-R1 and TRAIL-R2 were used as positive controls for the pulldown. 24 hours after transfection, the ezrin wild-type or the TRAIL receptors were immunoprecipitated using an anti-Flag antibody. The western blot analysis revealed that ezrin was not associated with TRAIL-R1 or TRAIL-R2 (**Figure 4.14**).

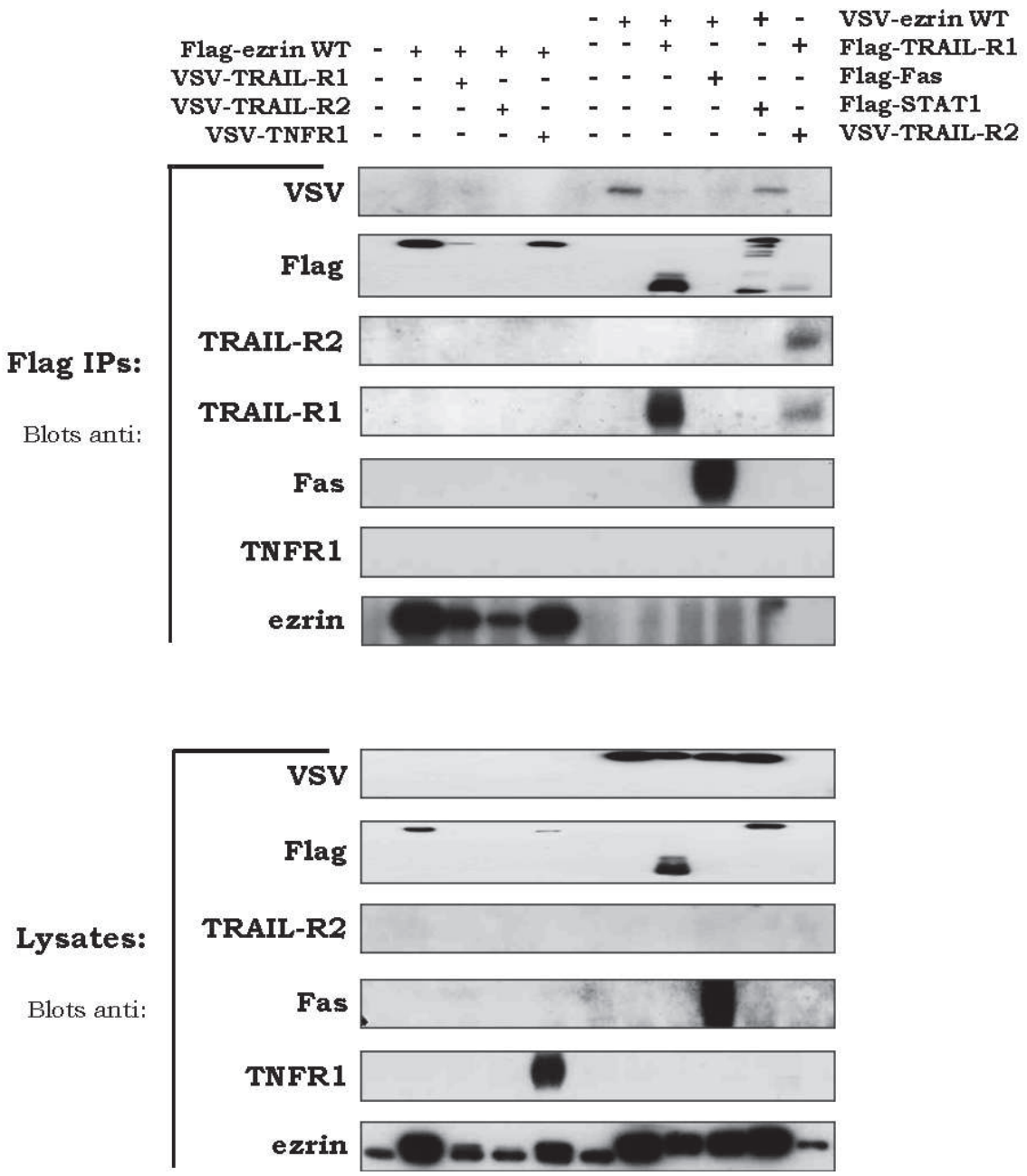

Figure 4.14. Ezrin was not found associated with TRAIL-R1 and TRAIL-R2 in co-expression experiments. 293-T cells were transfected with pCR3-VSV vectors encoding TRAIL-R1, TRAIL-R2 or TNFR1 together with a pCR3-Flag-ezrin WT vector or with pCR3-Flag vectors encoding TRAIL-R1, Fas, STAT1 together with a pCR3-VSV-ezrin WT vector. After 24h, the cells were collected and lysed in NP40-containing buffer. Flag-tagged proteins were immunoprecipitated with an anti-Flag (M2) monoclonal antibody.

To determine whether the association we observed between ezrin and TRAIL receptors was nonspecific, we performed caspase-8 and GADPH

immunoprecipitations. Ezrin should not associate with GADPH because GAPDH is an enzyme that is usually located in the cytosol of cells, whereas ezrin is a membrane cytoskeleton protein. Therefore, the caspase-8 precipitate protein complex was compared with that of GAPDH. Cells were then stimulated with 5 µg/ml His-tagged TRAIL ligand for 20 or 60 minutes before being lysed and immunoprecipitated with caspase-8 or GAPDH antibody, then processed for western blot analysis. We found that TRAIL-R1 and TRAIL-R2 were not associated with caspase-8 in non stimulated cells, and ligand-induced receptor cross-linking led to the recruitment of FADD and caspase-8 to the TRAIL receptors (**Figure 4.15**). We also observed that actin, ezrin, and moesin, but not radixin, associated with caspase-8 in non stimulated conditions (**Figure 4.15**), and the amount of protein recruited decreased upon TRAIL stimulation (**Figure 4.15**). When we analysed the proteins complexed with GAPDH, we again found bands corresponding to ezrin and actin in the GAPDH immunoprecipitates of non stimulated cells.

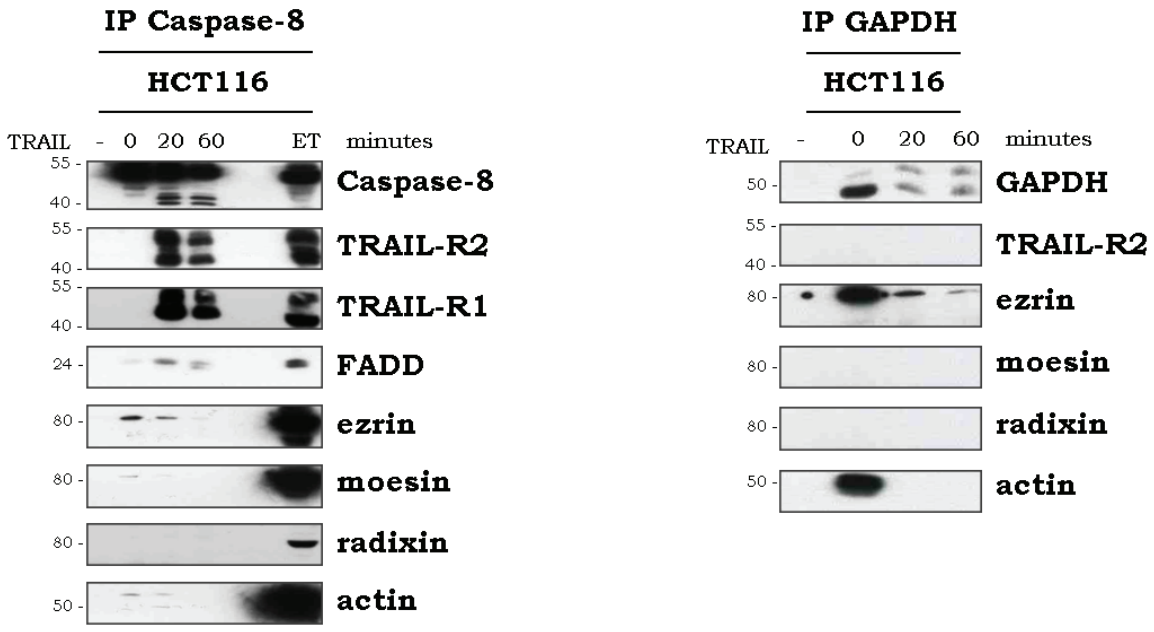

Figure 4.15. Analysis of ERM association with caspase-8 and GAPDH. HCT116 cells were stimulated with 5 µg/ml His-TRAIL for 20 and 60 minutes at 37 C. After cell lysis, caspase-8 or GAPDH antibody was added to cell lysates and the protein complexes were analyzed by western blot. - corresponds to protein G-sepharose into lysis buffer plus anti-caspase-8 or GAPDH antibody.

In this first set of immunoprecipitation experiments, we found ezrin, moesin, and actin present in TRAIL ligand, TRAIL-R1, TRAIL-R2, caspase-8 and GAPDH immunoprecipitates, strongly suggesting a nonspecific binding of these proteins with polymers of sepharose cross-linked to the protein G used to perform the immunoprecipitations. To test this hypothesis, HCT116 cells were stimulated with 5 µg/ml His tagged-TRAIL ligand for 20 minutes, or left untreated. Cells were then lysed in NP40-containing buffer and whole cell lysates were incubated with protein G or A cross-linked to polymers of sepharose or agarose. As additional negative controls, the protein G and protein A sepharoses were also incubated with lysis buffer. The western blot analysis revealed that ezrin, moesin, and actin were associated with either polymers of sepharose or agarose cross-linked to protein G and to a lesser extent with polymers of agarose cross-linked to protein A (**Figure 4.16**). We also observed that these nonspecific interactions were decreased upon addition of TRAIL (**Figure 4.16**).

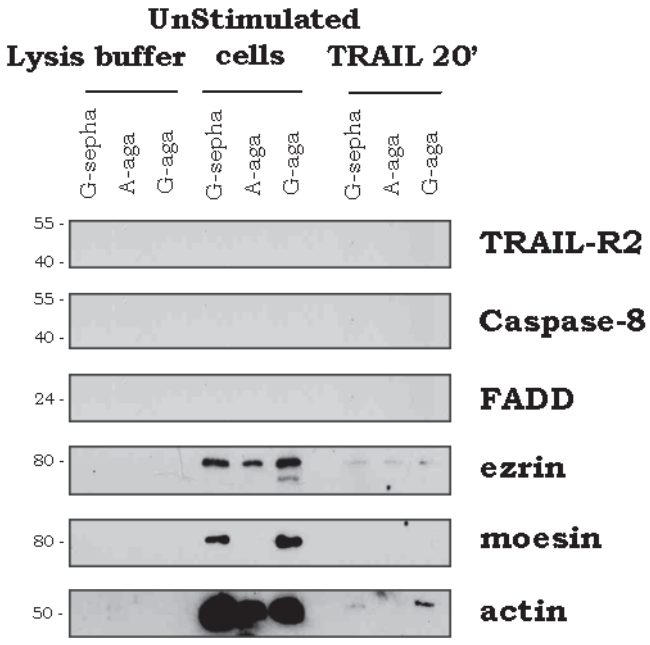

Figure 4.16. Ezrin binds the sepharose and agarose polimers crosslinked to proteins G or A. HCT116 cells were stimulated or not with 5 µg/ml His-TRAIL for 20 minutes. Cells were then lysed and incubated with protein G sepharose, protein G agarose and protein A agarose and analyzed by Western blotting.

To increase the specificity of binding, and avoid the nonspecific interactions of ezrin, moesin, and actin to the protein G-sepharose beads, and then to definitively elucidate whether ezrin was truly recruited within the TRAIL DISC, we performed a TRAIL DISC composition analysis using protein G-sepharose beads saturated with BSA. Protein G-sepharose beads were incubated overnight with PBS-BSA 2% with mixing in order to saturate all the unoccupied protein binding sites on the sepharose, with BSA as a competitive protein. The day after, beads were washed three times with NP40-containing buffer, and used for immunoprecipitation experiments. The precipitated TRAIL ligand protein complex was compared with that of TRAIL-R1 and TRAIL-R2. Flag tagged-TRAIL ligand immunoprecipitations again showed bands corresponding to ezrin and actin when we used sepharose beads that had been cross-linked by protein G and saturated with BSA, with whole cell lysates of SW480 cells, suggesting that saturation with BSA was still not enough to abrogate of the nonspecific binding of ezrin, moesin, and actin to protein G-sepharose beads (**Figure 4.17, panel a**). TRAIL-R1 and TRAIL-R2 immunoprecipitations confirmed this idea, as shown in **Figure 4.17, panel b and c, c line**. We also found ezrin pre-associated with TRAIL-R2 in all three experiments, but we observed inconsistencies in ezrin recruitment. Addition of TRAIL increased ezrin levels in TRAIL ligand immunoprecipitations, and decreased them in TRAIL-R1 and TRAIL-R2 immunoprecipitations. Moesin was not associated with TRAIL-R2 in non stimulated cells, and it was not present in the TRAIL DISC.

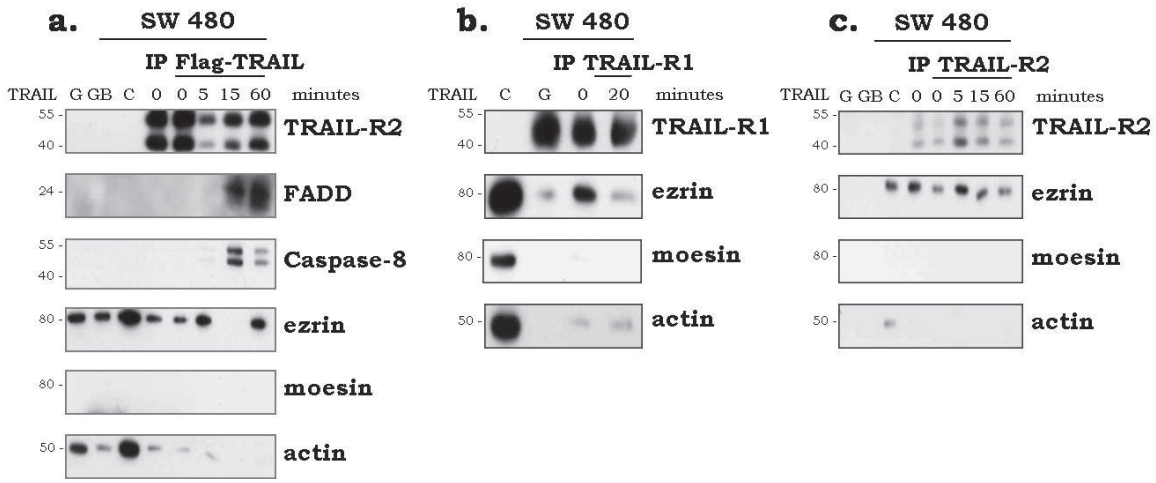

Figure 4.17. Nonspecific binding of ezrin to the proteins G-sepharose saturated with BSA that are used to immunoprecipitate. SW480 cells were stimulated for the indicated time with 5 μg/ml Flag-TRAIL cross-linked by 10 μg/ml anti-Flag (M2) antibody (a) or with 5 μg/ml His-TRAIL (b,c). After cell lysis, the DISC was immunoprecipitated and analyzed by western blot for the presence of DISC components and ERM proteins. C, G, GB correspond to an IgG control antibody in whole cell lysates plus protein G-sepharose, to proteinG-sepharose into lysates, and to proteinG-sepharose saturated with BSA into lysates, respectively.

This data shows, for the first time, that cytoskeletal proteins such as ezrin, moesin, and actin, are able to bind polymers in a nonspecific manner, like the sepharose coupled to protein G that are commonly used to perform the immunoprecipitations. Moreover, we never found ezrin associated with TRAIL-R1 or TRAIL-R2 in ezrin immunoprecipitates and in co-expression experiments. These results clearly indicate that ezrin recruitment to TRAIL-R1 and TRAIL-R2 observed in TRAIL DISC analysis by immunoprecipitation is not specific, and strongly support the idea that there is no direct interaction between ezrin and either of the TRAIL agonistic receptors. Ezrin cannot be considered a component of the TRAIL DISC.

4.5. TRAIL induces ezrin phosphorylation

The ERM are usually present in the cytoplasm of cells in an inactive, closed conformation, where the amino- and carboxy-terminal domains are masked. These proteins need to be activated in order to be able to switch to the open, active form and to translocate from the cytoplasm to the membrane cytoskeleton fraction, where they associate with membrane receptors. Activation involves binding of PIP_2, usually a Rho-dependent process, and phosphorylation at a specific, conserved threonine residue: the Thr567 of ezrin, the Thr564 of radixin, and the Thr558 of moesin (Matsui et al, 1998). ERM can also be phosphorylated at serine 66, and tyrosines 145 and 353, sites that are known to be required in signalling pathways (Zhou et al, 2003; Srivastava et al, 2005; Gautreau et al, 1999).

Moreover, recent findings demonstrate that phosphorylation or dephosphorylation events can regulate cell sensitivity to Fas ligand-induced cell death. In fact, ERM inactivation through dephosphorylation was associated with cell sensitivity to Fas-mediated cell death in activated T cells (Ramaswamy et al, 2007). Whereas ROCK I-mediated ezrin phosphorylation on Thr567 induced by an anti-Fas antibody was shown to facilitate Fas aggregation, caspase-8 activation and apoptotic signalling in Jurkat cells (Hébert et al, 2008).

Therefore, in this part of the study we investigated the possible involvement of ezrin activation, through phosphorylation, in cell sensitivity to TRAIL-induced apoptosis. To this end, we analysed ezrin's phosphorylation/dephosphorylation status following TRAIL stimulation.

We then investigated whether TRAIL and Fas ligand stimulation can induce ezrin phosphorylation at previously identified phosphorylation target residues of the protein. To this aim, SW480 cells were left untreated or were stimulated with 500 ng/ml His-TRAIL or Fas ligand for 5, 15 or 30 minutes at 37°C. As a positive control for tyrosine phosphorylation, cells were stimulated with 0.1 mM orthovanadate for

15 and 30 minutes, or were starved overnight in medium containing 0.1% serum, then stimulated with fresh complete medium for 15 minutes. Lysates were prepared and processed for western blot analysis with antibodies specific for each of the ezrin phosphorylation sites, except for the serine residue. As shown in **panel a of Figure 4.18**, and consistent with the report of Hébert et al. (2008), both TRAIL and Fas ligand induced ezrin phosphorylation on threonine 567 (thr567). We did not find any differences in tyrosine 145 phosphorylation, and the serine and phospho-ezrin (Y353) immunoblots were not conclusive.

The literature also demonstrates that growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), and platelet derived growth factor (PDGF), can stimulate ezrin phosphorylation on tyrosine residues 145 and 353. We then used EGF stimulation as an additional positive control for ezrin tyrosine phosphorylation. To this aim, cells were stimulated with 20 or 100 ng/ml EGF for 5 and 30 minutes, or were left untreated. The western blot analysis shown in **panel b of Figure 4.18** illustrates that EGF induces phosphorylation of ezrin on tyrosine residue 353 either in HeLa WT cells, or in MDA-MB468 cells which over-express the EGF receptor on the cell surface. Moreover, in cells expressing low membrane levels of EGF receptor, such as the SKBR3 cells, ezrin is not phosphorylated on the residue 353 following EGF stimulation but it can still be phosphorylated on the threonine residue 567.

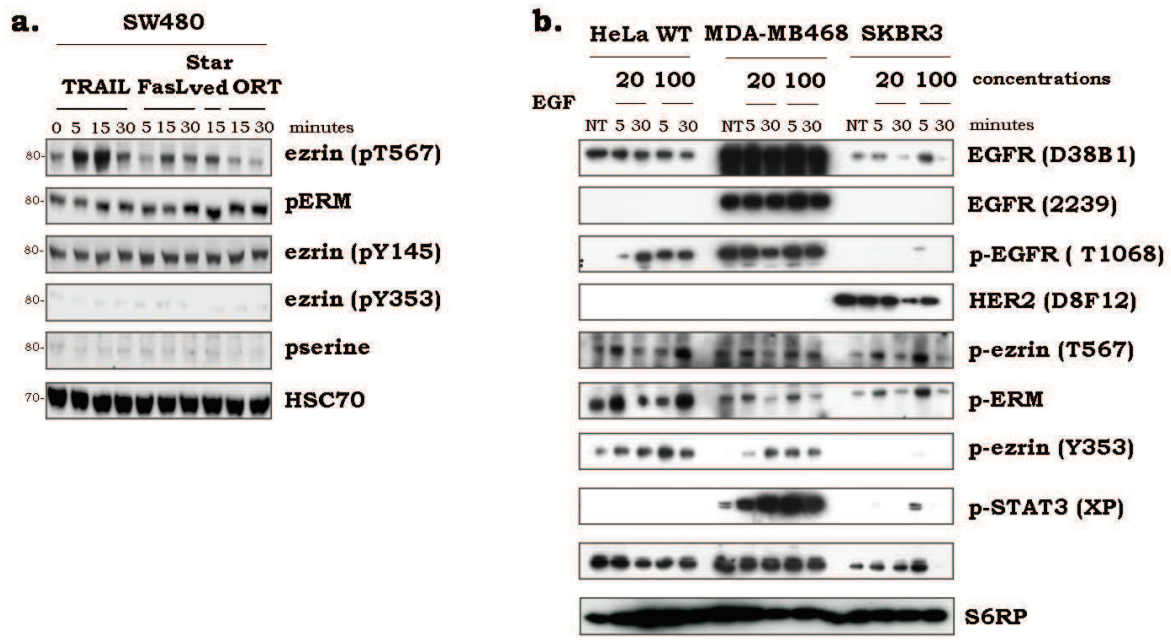

Figure 4.18. Phosphorylation of ezrin after TRAIL, Fas ligand and EGF stimulation. (a) SW480 cells were treated with 500 ng/ml His-TRAIL or Fas ligand, or 0.1 mM orthovanadate for the indicated times. After cell lysis, the levels of phospho-ezrin were determined by western blot using the appropriate antibodies. (b) HeLa WT, MDA-MB468 and SKBR3 cells were treated with 20 or 100 ng/ml EGF for 5 and 30 munutes. After cell lysis, the levels of phospho-ezrin were determined by western blot using the appropriate antibodies. Anti-phospho-ERM antibody recognizes p-ezrin, p-moesin, and p-radixin, respectively. One of two independent experiments is shown.

Ezrin immunoprecipitations were performed in the same cell lines in order to further analyse and characterize the phosphorylation status of ezrin after TRAIL or Fas ligand stimulation. SW480 cells were either left untreated or stimulated for 15 minutes with 500 ng/ml His tagged-TRAIL ligand or Fas ligand, or 0.1 mM orthovanadate before being lysed and precipitated with ezrin antibody and processed for western blot. The analysis revealed that the ezrin immunoprecipitated from these lysates was phosphorylated at each of the residues in non stimulated conditions, and the level of ezrin phosphorylation on threonine, serine and tyrosine 145 were increased upon stimulation with TRAIL and, in a lesser extent, Fas ligand (**Figure 4.19**). We also observed the disappearance of a higher band in the serine immunoblot after Fas ligand stimulation (**Figure 4.19**).

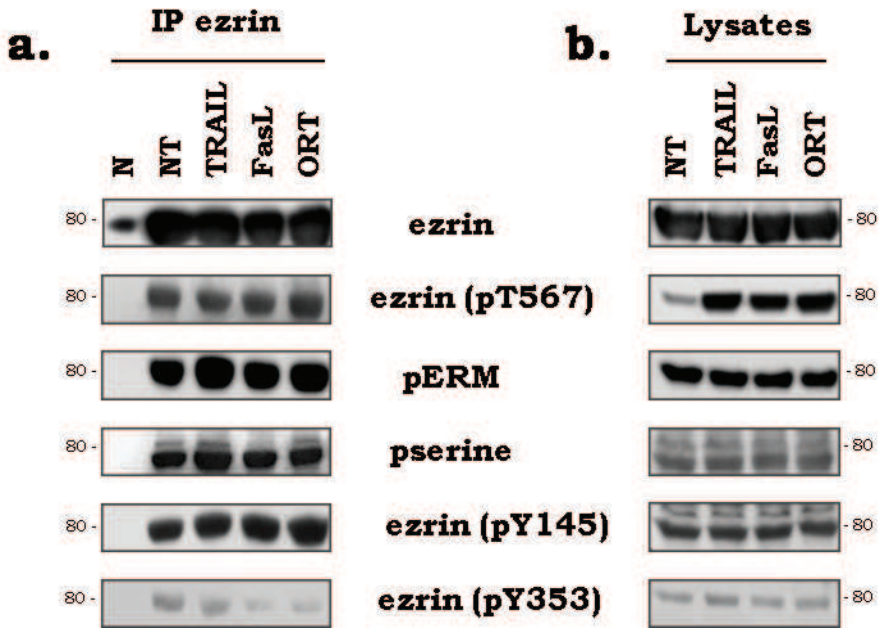

Figure 4.19. Analysis of ezrin phosphorylation in ezrin immunoprecipitates. (a) SW480 cells were treated with 500 ng/ml His-TRAIL or Fas ligand, or 0.1 mM orthovanadate for 15 minutes at 37 C. After cell lysis, ezrin antibody was added to cell lysates and the protein complexes were analyzed by western blot using the appropriate antibodies. N corresponds to an IgG control antibody in whole cell lysates plus protein G-sepharose. (b) The levels of phospho-ezrin on cell lysates were also determined by western blots. Anti-phospho-ERM antibody recognizes p-ezrin, p-moesin, and p-radixin.

4.6. Characterization of ezrin phosphorylation mutants

In order to understand the impact of ezrin's phosphorylation status in regulating the TRAIL signalling pathway, we generated several ezrin phosphorylation mutants by site directed mutagenesis, resembling nonphosphorylable or phosphomimetic variants of the protein on serine 66 (S66), tyrosines 145 (Y145) and 353 (Y353), and threonine 567 (T567) (**Figure 4.20.b**). The ezrin constructs S66D, Y145D, Y353D, T567D were created to mimic phosphorylated ezrin, whereas ezrins S66A, Y145F, Y353F, T567A were generated as nonphosphorylable ezrin. We also generated a mutant form of ezrin to be defective in F-actin binding: ezrin R579A, which was shown to be unable to bind actin (Saleh et al, 2009). Its over-expression behaves as a dominant negative construct by preventing the actin linkage.

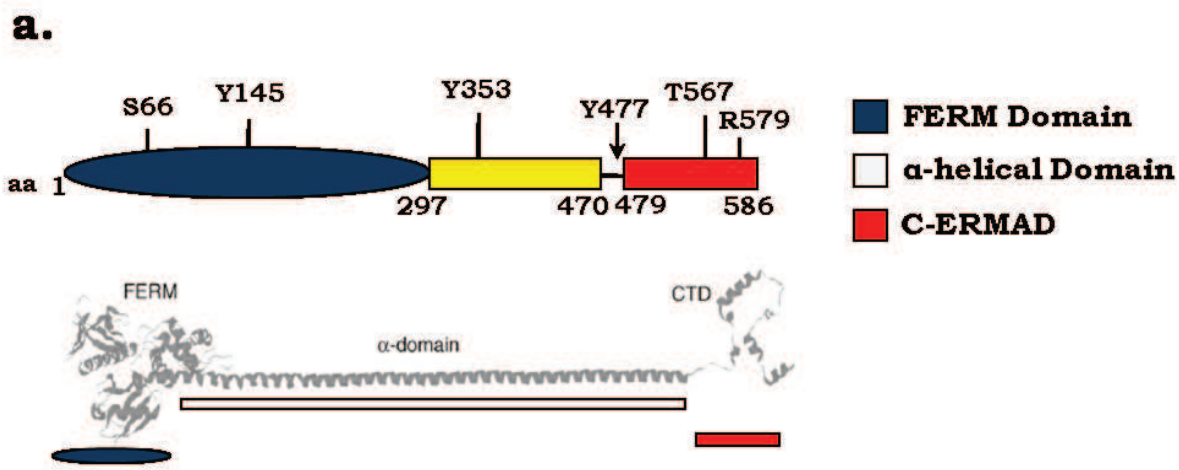

Figure 4.20. Ezrin phosphorylation sites. (a). Schematic representation of ezrin phosphorylation sites within the protein. (b) Three-dimensional structure of the ezrin phosphorylation mutants obtained with PHYRE2.

We ectopically expressed these variants of ezrin using a retroviral approach in HCT116 and SW480 cells. Once the populations were stable, we checked by western blot analysis for the presence of each construct in the corresponding two cell lines. As shown in **Figure 4.21**, each of the ezrin mutants, except the nonphosphorylable variant Y145F, were well expressed in SW480 cells, whereas their expression level in HCT116 cells was not appreciable (data not shown). We then focused on SW480 cells expressing the different ezrin phosphorylation variants.

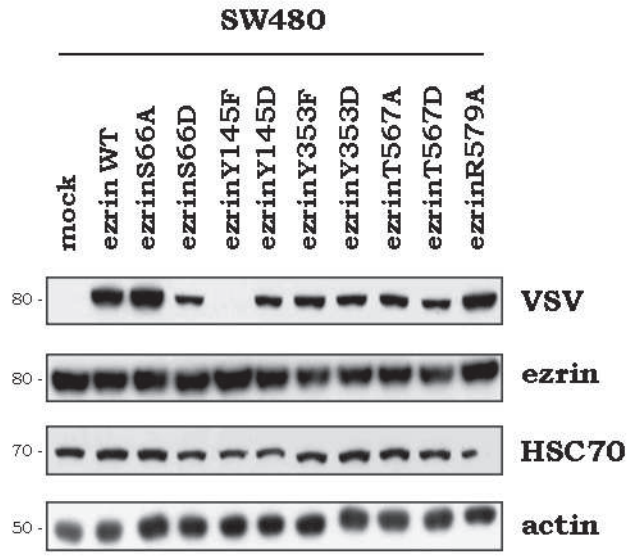

Figure 4.21. The ezrin phosphorylation variants are well expressed in SW480 cells except the Y145F mutants. Western blot analysis of ezrin phosphorylation mutants expression in mock, wild-type or mutants ezrins-expressing SW480 cells. The HSC70 and actin were probed for normalization. One of three independent experiment is shown.

We checked whether the nonphosphorylable variant Y145F was localized in the NP40-insoluble fraction. Total and NP40-soluble lysates were prepared from the ezrin S66A, Y145F, Y353F, Y353D and R579A-expressing SW480 cells, and compared after western blot analysis. As demonstrated in the total lysates panel of **Figure 4.22**, the ezrin Y145F was expressed in the cells to a lesser extent then the other mutants. We also found that most of the chimeric ezrin wild-type and the nonphosphorylable variant Y353F remained in the insoluble fractions, represented by the pellet, whereas there was no ezrin S66A or actin in this fraction (**Figure 4.22**).

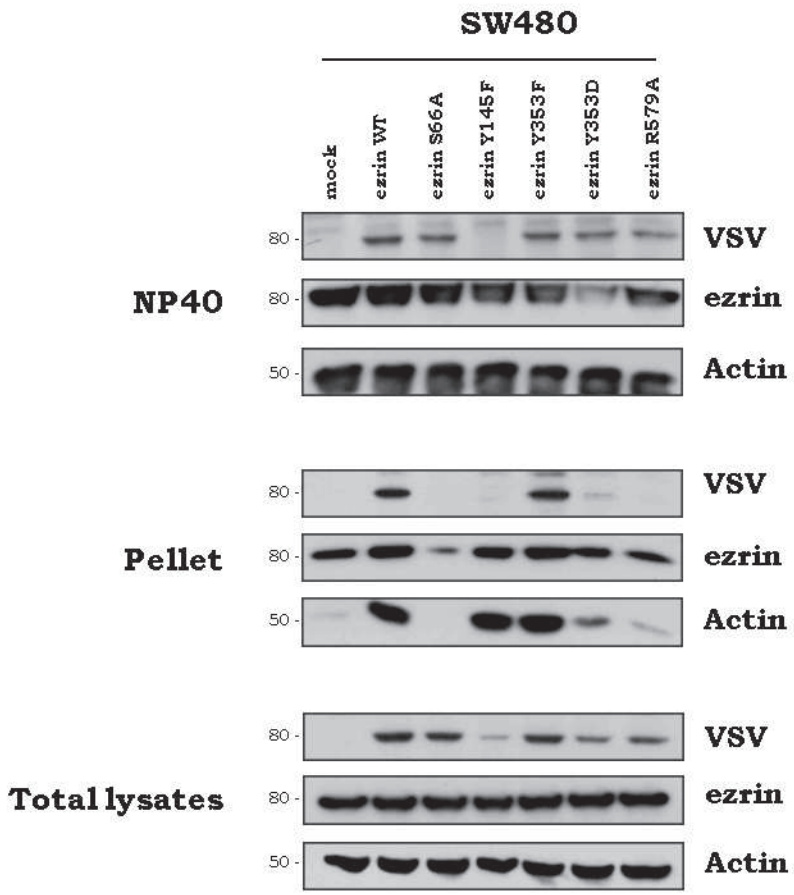

Figure 4.22. Ezrin Y145F is expressed in a lesser extent than the other mutants. Western blot analysis of ezrin wild-type, S66A, Y145F, Y353F, Y353D, R579A mutants expression in mock, wild-type or mutants ezrins-expressing SW480 cells. The actin was probed for normalization. Results shown are from one representative experiment.

These SW480 populations expressing the phosphorylation variants of ezrin were next characterized by their TRAIL agonistic receptor expression profile and endocytosis, and sensitivity to TRAIL.

First, we evaluated the membrane levels of TRAIL-R1 and TRAIL-R2 by flow cytometry to understand whether ezrin phosphorylation variants could somehow modulate their surface expression. Cells were then collected and stained with antibodies recognizing membrane forms of TRAIL-R1 and TRAIL-R2. As shown in

Figure 4.23, no changes in TRAIL-R1 or TRAIL-R2 cell surface expression could be measured by FACS analysis.

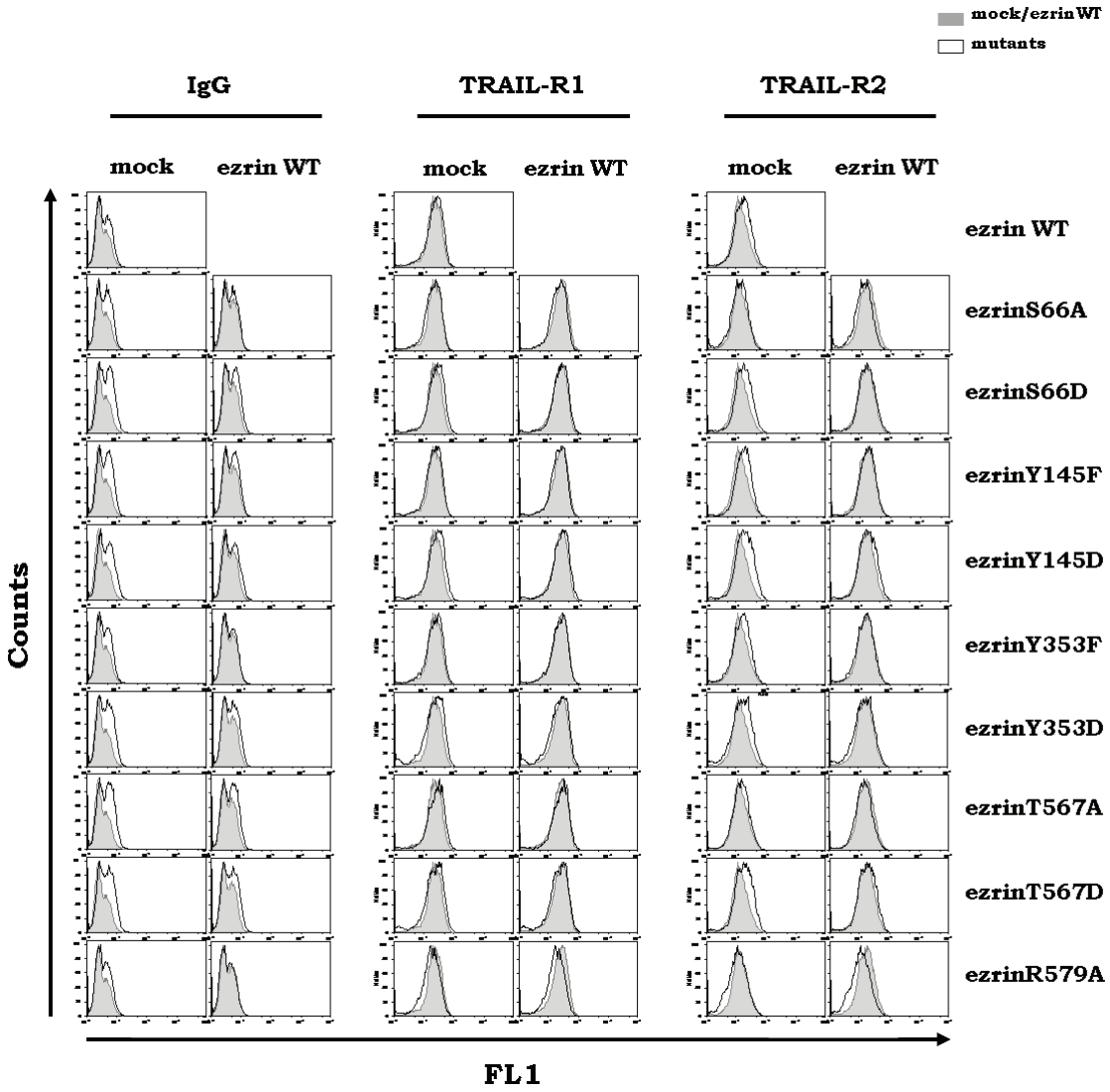

Figure 4.23. The phosphorylation variants of ezrin did not change the TRAIL-R1 and TRAIL-R2 expression at the membrane level. Cell surface expression of TRAIL-R1 and TRAIL-R2 was measured by flow cytometry in mock, ezrin WT or phosphorylation mutants-expressing SW480 cells. Unfilled peaks correspond to the staining in ezrin phosphorylation mutants-expressing SW480 cells and filled peaks is the staining in mock or ezrin wild-type expressing-SW480 cells. One of three independent experiments is shown.

We next analysed whether the internalization of TRAIL-R1 and TRAIL-R2 was affected by ezrin phosphorylation variants expression in SW480 cells. The surface TRAIL-R1 and TRAIL-R2 levels in SW480 cells expressing the wild-type or ezrin phosphorylation variants after receptor engagement were measured by flow

cytometry. Cells were then left untreated or stimulated with 1 μg/ml TRAIL for 30 minutes and stained with antibodies recognizing membrane forms of TRAIL-R1 and TRAIL-R2. **Figure 4.24** illustrates that TRAIL stimulation led to significant reduction of TRAIL-R1 and TRAIL-R2 from the cell surface in SW480 wild-type cells.

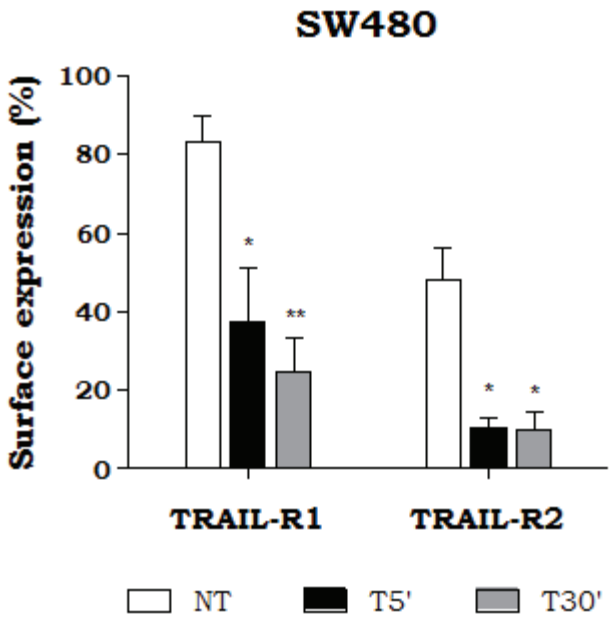

Figure 4.24. TRAIL-R1 and TRAIL-R2 internalization in SW480 cells. Cell surface expression of TRAIL-R1 and TRAIL-R2 was measured by flow cytometry in SW480 cells after stimulation with 1 μg/ml TRAIL for 5 and 30 minutes. The mean and standard deviation of three independent experiments are shown. (*P<0.05; **P<0.01 respective to untreated cells).

When we analysed the SW480 cells expressing the ezrin phosphorylation variants, we found that the TRAIL-R expression seems to be reduced in some mutant expressing cells as compared to mock transfected cells (for example the SW480 cells expressing the ezrin Y353F) (**Figure 4.25**). In addition, TRAIL-R1 internalization seems to be affected in some mutant expressing cells (Y353D, T567A, T567D) (**Figure 4.25**).

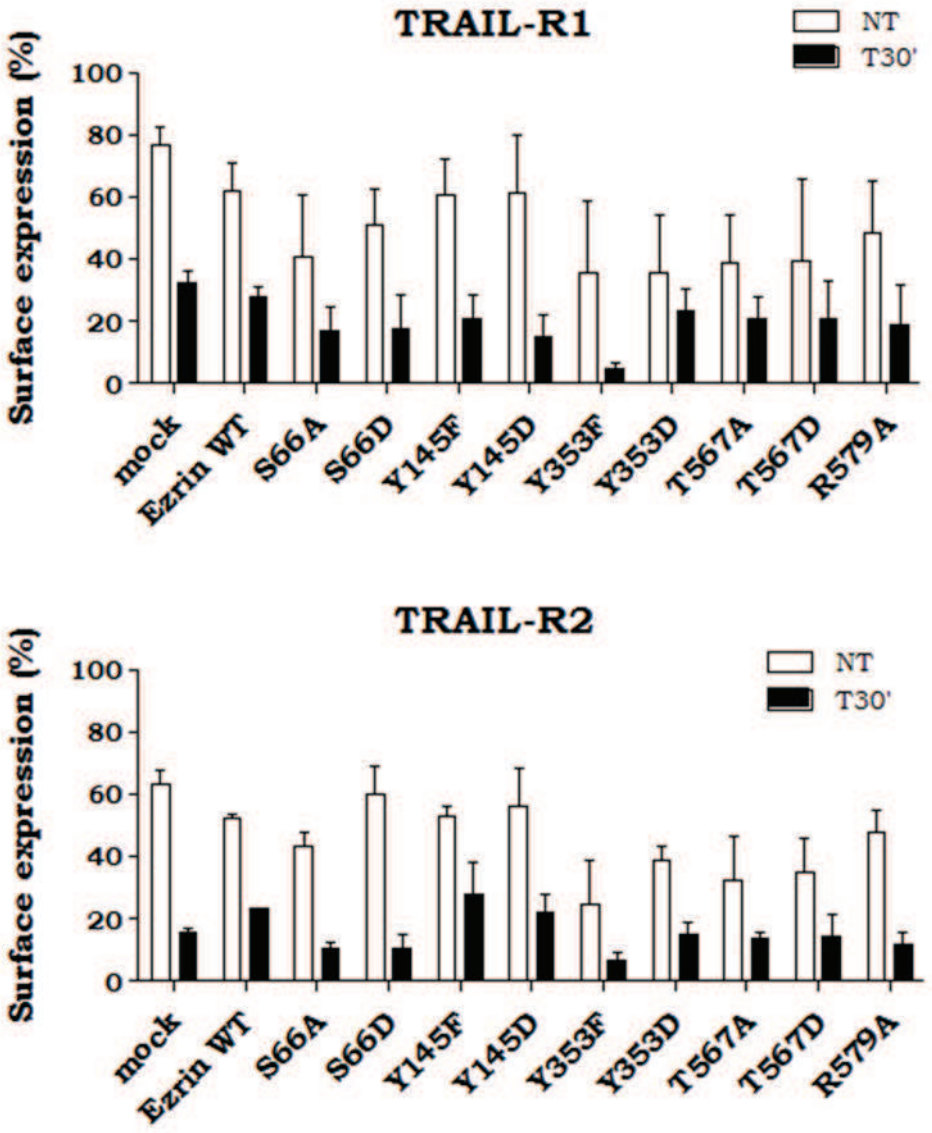

Figure 4.25. TRAIL-R1 and TRAIL-R2 internalization in ezrin phosphorylation mutants-expressing SW480 cells. Cell surface expression of TRAIL-R1 and TRAIL-R2 was measured by flow cytometry in mock, ezrin WT or phosphorylation mutants-expressing SW480 cells after stimulation with 1 μg/ml TRAIL for 30 minutes. The mean and standard deviation of three independent experiments are shown.

Following this, we evaluated the possible modulation of the sensitivity to TRAIL compared to the sensitivity to Fas ligand. SW480 cells expressing the ezrin phosphorylation variants were stimulated for 6 hours with Fas ligand or His-TRAIL (500 ng/ml) and apoptosis was evaluated by apo 2.7 staining followed by flow cytometry (**Figure 4.26**). We observed that stable expression of the nonphosphorylable variant S66A in SW480 cells induced a marked and significant

enhancement of apoptosis induced by TRAIL as compared to ezrin wild-type expressing cells. On the other hand, expression of its corresponding phosphomimetic variant, ezrin S66D, led to protection against TRAIL-mediated cell death. This phenomenon seemed to be selective for TRAIL, since the expression of those mutants did not significantly change the sensitivity of SW480 cells against Fas ligand-induced cell death (**Figure 4.26**) as compared to ezrin wild-type expressing cells.

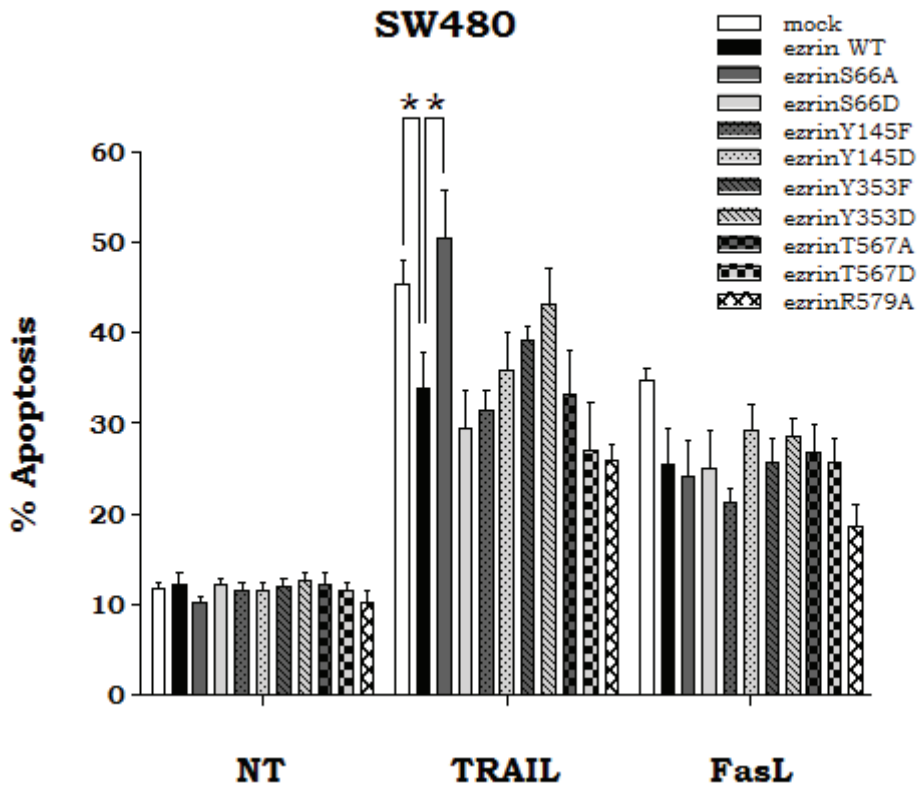

Figure 4.26. Expression of ezrin S66A variant in SW480 cells enhanced TRAIL but not Fas ligand-induced cell death. Mock, ezrin WT or phosphorylation mutants-expressing SW480 cells were treated with 500 ng/ml of His-TRAIL or FasL for 6 h, stained with apo 2.7 antibody and analyzed by flow cytometry. The mean and standard deviation of three independent experiments are shown.

Finally, we determined the sensitivity of these cell lines to TRAIL. Cells were treated for 16 hours with increasing concentrations of TRAIL and cell viability was assessed by methylene blue coloration (Micheau et al, 2001). As shown in **Figure 4.27**, TRAIL-induced cell death was also increased by ectopic expression of ezrin phosphomimetic Y353D while its nonphosphorylable version, ezrin Y353F, protected cells against TRAIL apoptosis. Regulation of ezrin tyrosine 353 and serine 66 phosphorylation modulated TRAIL-induced cell death by an order of magnitude ranging from 3 to 6 fold as compared to parental cells, while the remaining phosphorylation mutants only achieved a 1.2 to 2 fold increase resistance, as extrapolated from the IC50 scores (**Figure 4.27**). The amount of TRAIL ligand required to induce cell death in 50% and 90% of the cellular population was 20 to 30 fold higher in S66D as compared to S66A expressing SW480 cells, and 10 fold higher in cells expressing ezrin Y353F as compared to Y353D, respectively (**Figure 4.28**).

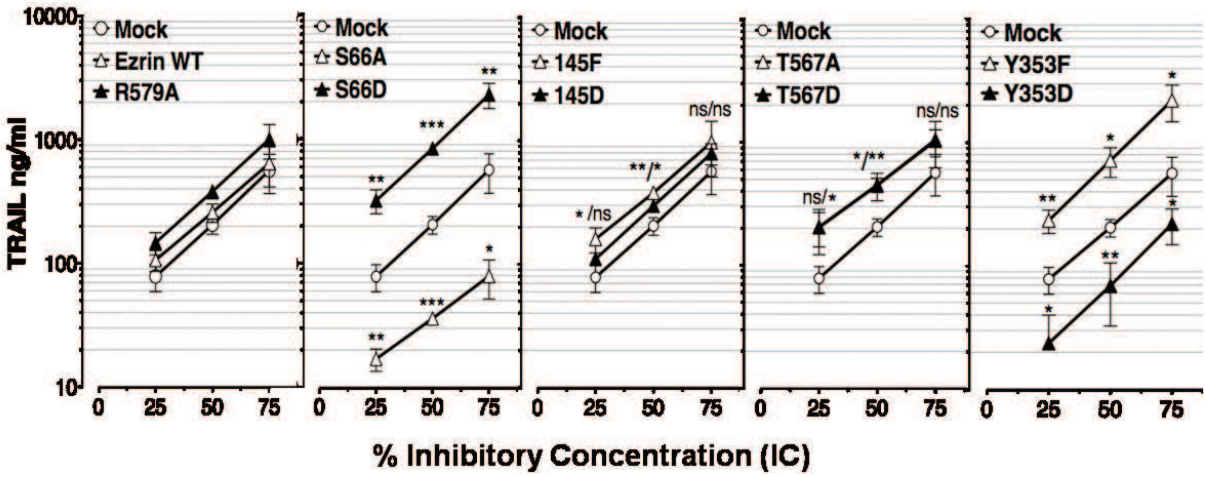

Figure 4.27. TRAIL inhibitory concentration curves. Percent TRAIL inhibitory concentration curves from SW480 cells expressing the indicated ezrin mutants were obtained by methylene blue coloration 16 hours after treatment with increasing concentrations of His-TRAIL, using CompuSyn. The mean and standard deviation of at least three independent experiments are shown. *P<0.05; **P<0.01; ***P<0.001 respective to mock control cells.

TRAIL Inhibitory Concentrations (ng/ml)

	IC 25	SD	IC 50	SD	IC 75	SD
Mock	**78**	19	**205**	33	**565**	198
ezrin WT	**106**	20	**258**	44	**644**	229
ezrin S66A	**17**	3	**36**	3	**79**	27
ezrin S66D	**320**	70	**841**	14	**2288**	525
ezrinY145F	**160**	37	**380**	42	**978**	465
ezrin Y145D	**110**	9	**297**	23	**795**	155
ezrin Y353F	**234**	50	**717**	194	**2206**	726
ezrin Y353D	**24**	16	**69**	37	**220**	71
ezrin T567A	**203**	81	**447**	113	**1051**	430
ezrin T567D	**205**	64	**450**	60	**1031**	236
ezrin R579A	**147**	30	**382**	50	**1009**	313

Figure 4.28. Table of TRAIL inhibitory concentration. The TRAIL inhibitory concentrations in ezrin phosphorylation mutants-expressing SW480 cells were calculated using CompuSyn. IC25, IC50 and IC75 percent values correspond to the mean of four independent experiments. Mean and SD shown in this table were used to plot figure 4.27.

4.7. Ezrin inhibition could act downstream of the TRAIL DISC

The results obtained with the ezrin phosphorylation variants suggested that post-translational modifications are required for ezrin to be able to perform its modulatory action in the TRAIL signalling pathway. Moreover, the results from these experiments demonstrated that the ezrin phosphorylation site serine 66 plays a particular and selective role in regulating TRAIL signalling. We next attempted to elucidate the molecular mechanisms underlying the inhibitory role of ezrin in TRAIL-induced apoptosis. To this end, we monitored cell viability, TRAIL DISC formation and activation of caspases in control, ezrin WT-, S66A-, and S66D-expressing SW480 cells.

First, we determined the sensitivity of these cell lines to TRAIL, and compared this with the sensitivity to CDDP, an alkaline agent known to induce cell death in a receptor independent manner. Cells were then treated for 24 hours with increasing concentrations of TRAIL or for 48 hours with increasing concentrations of CDDP. Cell viability was assessed by methylene blue coloration (Micheau et al, 2001). We observed that ezrin S66A-expressing SW480 cells were more sensitive to TRAIL-induced cell death than mock-infected cells, whereas expression of its phosphomimetic variant, the ezrin S66D, protected SW480 cells from TRAIL-induced cell death (**Figure 4.29.a**). We also observed that the presence of the mutant form of ezrin defective in F-actin binding, the ezrin R579A mutant, did not significantly modulate the sensitivity of SW480 cells to TRAIL-induced cell death compared to mock-infected cells (**Figure 4.29.a**). **Figure 4.29.b**, also illustrates that the effect of those mutants was restricted to TRAIL because none of these ezrin phosphorylation variants affected the efficacy of CDDP in inducing cell death.

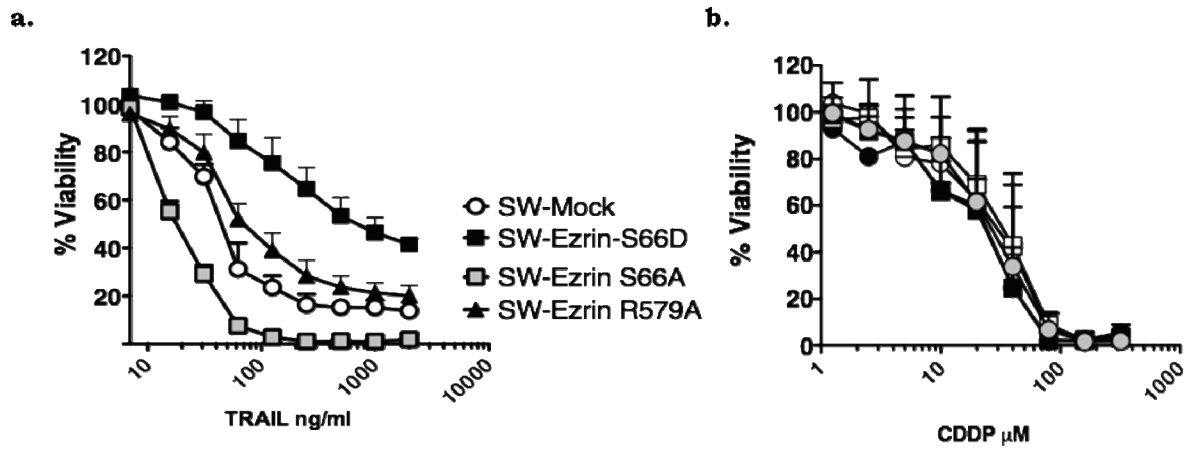

Figure 4.29. Ezrin S66A sensitize SW480 cells to TRAIL- but not CDDP-induced cell death. Cell viability in mock-, ezrin WT-, S66A-, and S66D-expressing SW480 cells was evaluated by methylene blue assay after 24 hours treatment with increasing concentrations of His-TRAIL or after 48 hours treatment with increasing concentrations of CDDP. The mean and standard deviation of three independent experiments are shown.

We next analysed the caspase-activation profile by western blot. Each of the cell populations were left untreated or stimulated with 100 ng/ml His-TRAIL for 30 minutes, 4 or 6 hours. Lysates were then prepared to determine the activation levels of caspases in the corresponding cell lines by western blot. **Figure 4.30** illustrated that caspases were activated after the addition of TRAIL, as indicated by the presence of cleavage products. Moreover, we observed no differences in caspase-8 and -10 activation profile in SW480 cells expressing ezrin WT or S66A or S66D variant when compared with mock-infected cells (**Figure 4.30**). When we looked at caspase-2 and -3 activation the situation was not the same. Their activation was enhanced in ezrin S66A-expressing SW480 cells compared to mock-infected cells, and decreased in SW480 cells expressing its phosphomimetic variant, the ezrin S66D (**Figure 4.30**). The amounts of active caspase-2 and -3 recovered in this case were comparable to ezrin WT- or R579A-expressing SW480 cells. Unfortunately, the blot for caspase-9 could not give conclusive results (**Figure 4.30**).

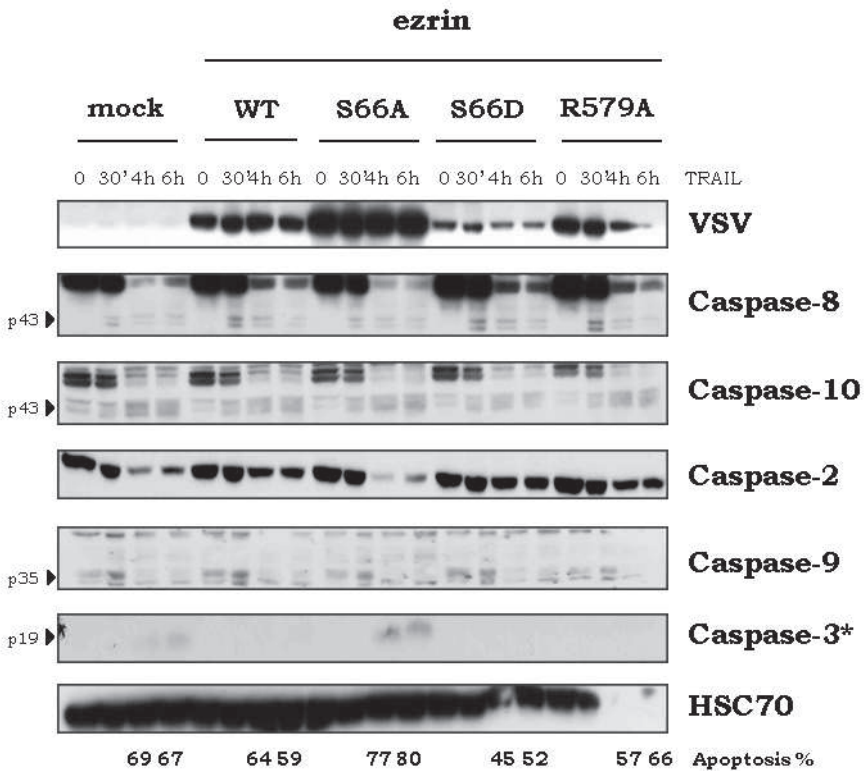

Figure 4.30. Caspase 2 and 3 are more activated in ezrin S66A-expressing SW480 cells. Mock-, ezrin WT-, S66A-, S66D-expressing cells were stimulated with 100 ng/ml His-TRAIL for the indicated time points. Lysates were prepared and the caspase levels were determined by western blot. One of two independent experiments is shown.

We also analysed the effect of two different concentrations of TRAIL regarding the caspase activation profile. To this aim, each cell line was stimulated with 200 ng/ml His-TRAIL for 6 hours or with 20 ng/ml His-TRAIL for 16 hours. The analysis by western blot confirmed the results obtained in the previous experiment. As shown in **Figure 4.31**, proforms of caspase-8 and -10 were well processed after treatment with TRAIL and the presence of the ezrin phosphorylation variants did not modulate their pattern of activation. In ezrin S66A-expressing SW480 cells we observed lower levels of the proform of caspase-2, suggesting an increase in its activation, and more cleavage products of caspase-3 and -9 (**Figure 4.31**) compared to mock-infected cells. Activation of caspase-3 and -9 were reduced in ezrin S66D-expressing SW480 cells as well as ezrin WT- and R579A-expressing cells.

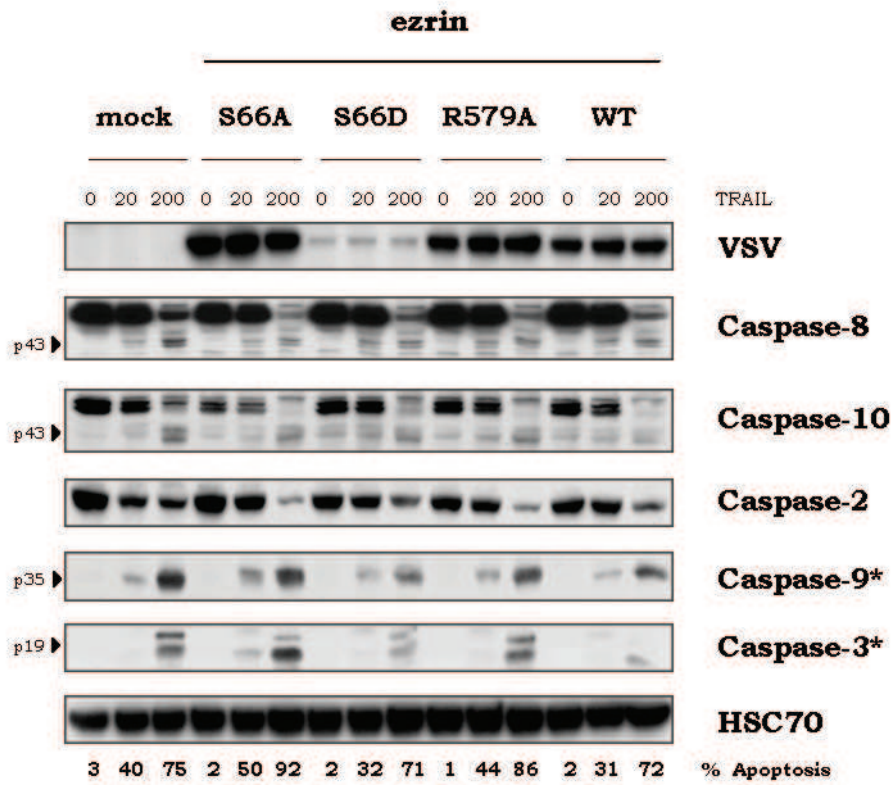

Figure 4.31. Caspase 2, 9 and 3 are more activated in ezrin S66A-expressing SW480 cells. Mock-, ezrin WT-, S66A-, S66D-expressing cells were stimulated with 20 ng/ml His-TRAIL for 16 hours and with 200 ng/ml His-TRAIL for 6 hours. Lysates were prepared and the caspase levels were determined by western blot. One of two independent experiments is shown.

We then evaluated whether the mutations at serine 66 site could affect TRAIL DISC formation. TRAIL ligand immunoprecipitations were performed to assess the TRAIL DISC composition. As shown in **Figure 4.32**, the levels of receptors and caspase-8 recruited to the DISC after Flag-tagged TRAIL ligand stimulation in ezrin S66A- or S66D-expressing SW480 cells were comparable to SW480 cells expressing ezrin WT. Instead, the recruitment of FADD was slightly but not consistently enhanced in ezrin S66A-expressing SW480 cells, compared to cells expressing ezrin WT (**Figure 4.32**).

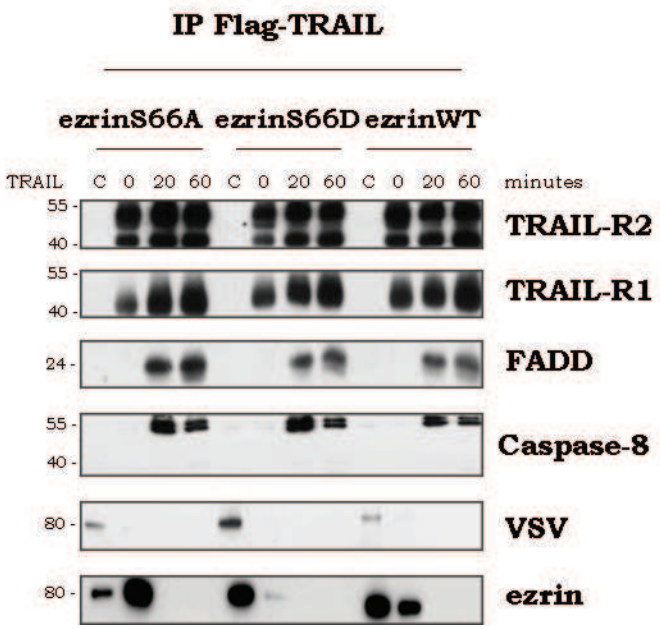

Figure 4.32. Mutations on serine 66 do not affect TRAIL DISC formation. Ezrin WT-, S66A-, S66D-expressing SW480 cells were stimulated with 5 µg/ml Flag-TRAIL cross-linked by 10 µg/ml of anti-Flag (M2) antibody for the indicated time. After cell lysis, the DISC was immunoprecipitated and analyzed by western blot. C corresponds to the anti-Flag antibody in the whole cell lysates plus protein G-sepharose. One of two independent experiments is shown.

We also performed immunoprecipitations with an anti-VSV antibody following TRAIL stimulation to pull-down the mutant forms of ezrin and analyse the TRAIL DISC. The western blot analysis of the protein complexes revealed that neither the chimeric ezrin WT nor the two phosphorylation ezrin variants were able to associate with TRAIL-R1 and TRAIL-R2 in non stimulated conditions (**Figure 4.34**). We also did not find any DISC formation in those immunocomplexes after addition of TRAIL (**Figure 4.34**), strongly suggesting that the ezrin phosphorylation on serine 66 did not play a role in this kind of event.

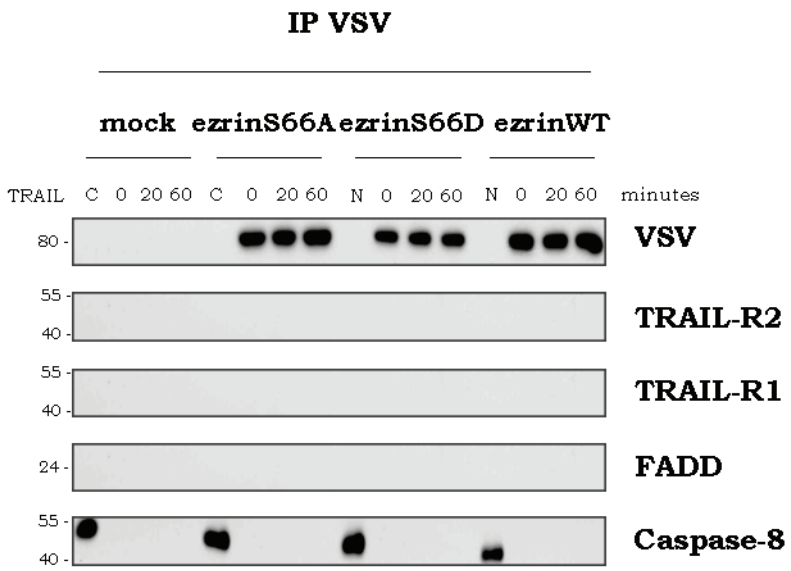

Figure 4.33. Phosphorylation variants of ezrin are not present in the DISC. Mock-, ezrin WT-, S66A-, S66D-expressing SW480 cells were stimulated with 5 µg/ml His-TRAIL for the indicated time. After cell lysis, VSV antibody was added to cell lysates and the protein complexes were analyzed by western blot. C corresponds to an IgG control antibody in whole cell lysates plus protein G-sepharose. One of two independent experiments is shown.

This set of experiments showed that substitution of serine with an alanine at position 66 on ezrin sensitized cancer cells to TRAIL-induced cell death. This phenomenon was neither due to alterations in DISC formation nor in caspase activation profile, and it did not modulate the TRAIL-R1 and TRAIL-R2 cell surface expression and internalization after TRAIL stimulation.

We then hypothesized that ezrin could play an inhibitory role at the mitochondrial level. To answer this question, we analysed activation of Bax in those three selected mutants following stimulation with TRAIL. Two different concentrations of TRAIL were used (20 and 200 ng/ml) with two time points for the highest concentration (6 and 16 hours). Flow cytometry analysis showed that addition of TRAIL induced activation of Bax in mock-infected cells as well as in ezrin phosphorylation variant expressing cells (**Figure 4.34**). Both TRAIL concentrations activated Bax to a similar level. In ezrin WT- or ezrin S66D- and

R579A-expressing SW480 cells, Bax activation increased levels comparable to mock infected cells, whereas the active Bax observed in SW480 cells expressing the nonphosphorylable mutant, ezrin S66A, was much higher than in the other cell lines (**Figure 4.34**).

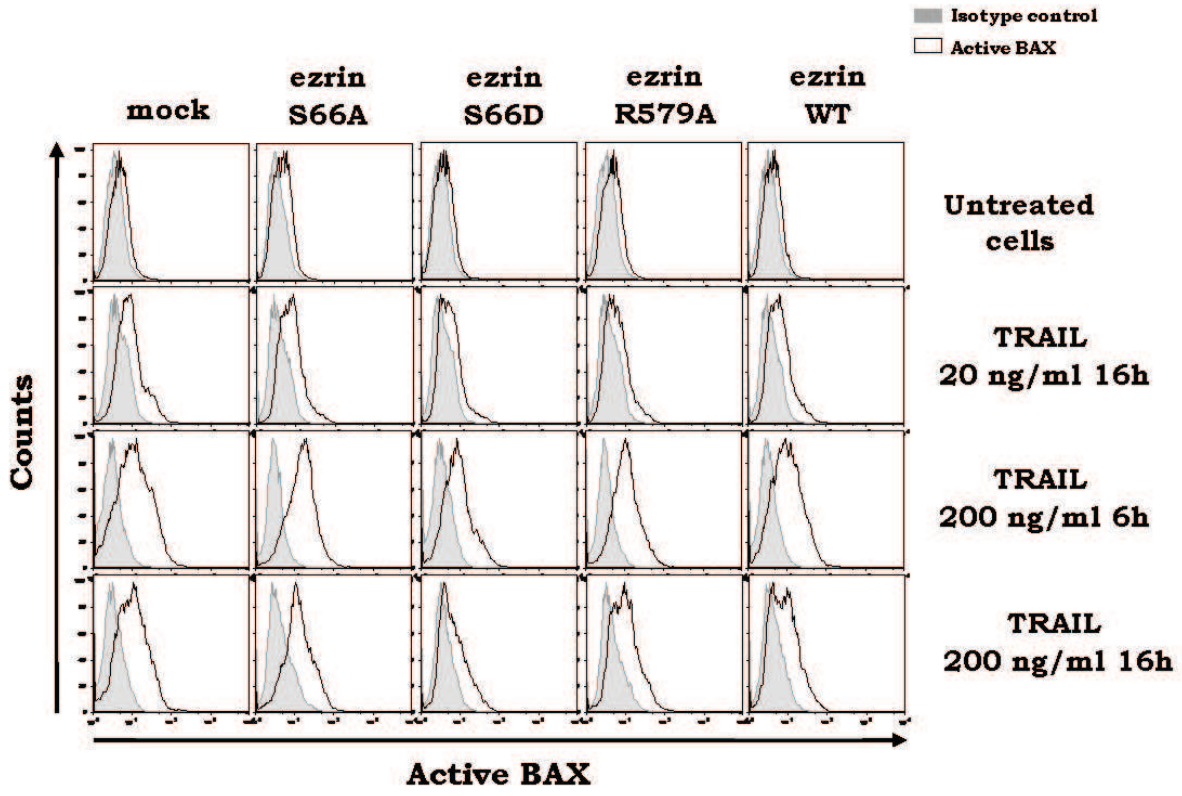

Figure 4.34. There is more active Bax in SW480 cells expressing ezrin S66A than in control or ezrin WT-expressing cells. Control SW480 cells and ezrin WT-, ezrin S66A-, S66D-, and R579A-expressing SW480 cells were stimulated or not with 200 ng/ml His-TRAIL for 6 and 16 hours or with 20 ng/ml His-TRAIL for 16 hours. Cells were then permeabilized and stained with an antibody recognizing active Bax and analysed by flow cytometry.

4.8. PKA inhibition enhanced TRAIL-induced apoptosis

Our data underlined the importance of ezrin phosphorylation on residue serine 66 in regulating the TRAIL pathway. Recently , Zhou et al, 2003 showed that in gastric parietal cells, protein kinase A (PKA) specifically targets this residue of the protein. We evaluated whether inhibition of this kinase could have an effect on TRAIL-induced cell death. To this aim, SW480 cells were treated for 48 hours with 10 μM H89, followed by six hours TRAIL treatment at 500 ng/ml, and then apoptosis was evaluated by apo 2.7 staining followed by flow cytometry. We observed that inhibition of PKA by H89 induced a slight increase in TRAIL-induced apoptosis in SW480 cells, as shown in **Figure 4.35**.

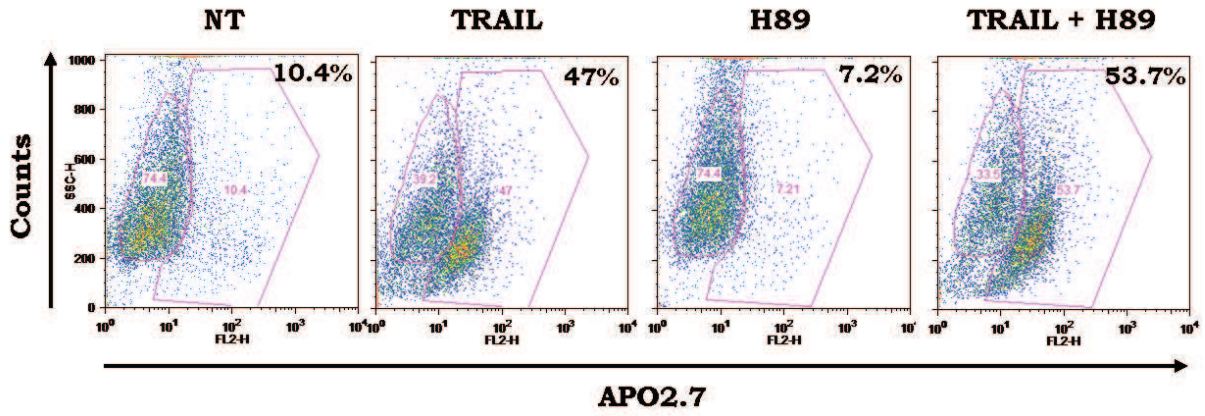

Figure 4.35. Effect of PKA inhibition on TRAIL-induced cell death. SW480 cells were pretreated with 10 μM H89 for 48 hours and then treated with 500 ng/ml TRAIL for 6 hours. Cells were then stained with apo 2.7 antibody and analyzed by flow cytometry. One representative experiment is shown.

We next compared the effect of PKA inhibition and activation in regulating TRAIL-induced cell death in SW480 and HCT116 cells. Apoptosis quantification by Hoechst staining showed that PKA inhibition by H89 led to a significant increase in TRAIL-induced apoptosis in both cell lines, as shown in **Figure 4.36**. However, Fas ligand induced cell death was not significantly affected by inhibition of this kinase

(**Figure 4.36**). Treatment with 8-Bromoadenosine 3′,5′-cyclic monophosphate (8B), a PKA activator, significantly reduced TRAIL- but not Fas ligand-induced apoptosis in SW480 cells (**Figure 4.36**). Similar results were obtained in HCT116 cells, albeit to a lesser extent (**Figure 4.36**).

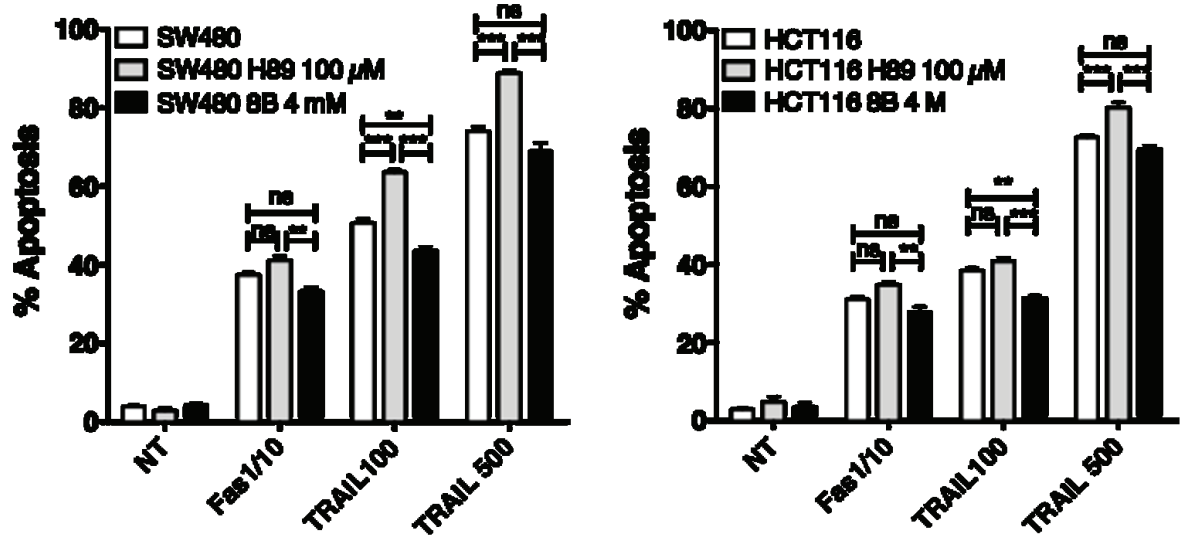

Figure 4.36. Effect of PKA inhibition or activation on TRAIL-induced cell death in SW480 and HCT116 cells. SW480 or HCT116 cells were pre-treated or not for 30 minutes with 100 μM H89 or 20 minutes with 4 mM 8B, followed by TRAIL (100 or 500 ng/ml) or Fas ligand (dilution 1/10) stimulation for 6 hours. Apoptosis was quantified by Hoechst staining. Data represent the mean plus or minus SD of at least three different experiments. (**P<0.01; ***P<0.001 respective to control or H89 stimulated cells; ns stands for not statistically relevant).

We also analysed the effect of treatment with two different concentrations of H89 on the TRAIL-induced cell death activation/inhibition profile in SW480 cells. In order to enhance the levels of PKA activation we used the compound Folskolin. Analysis by Hoechst staining confirmed the results obtained in the previous experiment with the H89. As shown in **Figure 4.37**, H89-mediated increase in TRAIL-induced cell death was concentration dependent and it was restricted to the TRAIL signalling pathway. Forskolin, such as 8B, was able to significantly inhibit TRAIL- but not Fas-mediated apoptosis (**Figure 4.37**).

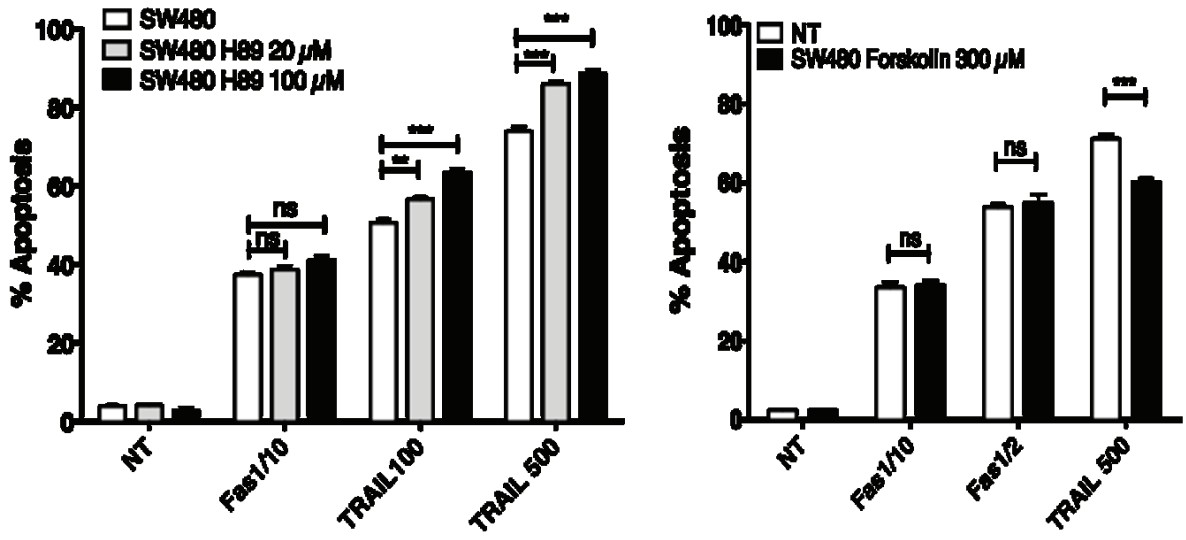

Figure 4.37. Effect of PKA inhibition or activation on TRAIL-induced cell death in SW480 cells. SW480 cells were pre-treated or not for 30 minutes with 20 or 100 μM H89 or 20 minutes with 300 μM Forskolin, followed by TRAIL (100 or 500 ng/ml) or Fas ligand (dilutions 1/10 or 1/2) stimulation for 6 hours. Apoptosis was quantified by Hoechst staining. Data represent the mean plus or minus SD of at least three different experiments. (**P<0.01; ***P<0.001 respective to control cells; ns stands for not statistically relevant).

Following, we evaluated whether TRAIL can induce the activation of PKA by immunoblots for specific downstream targets of PKA, such as phospho-p70. Preliminary results shown in **Figure 4.38** gave indications that p70 is activated by phosphorylation following TRAIL treatment.

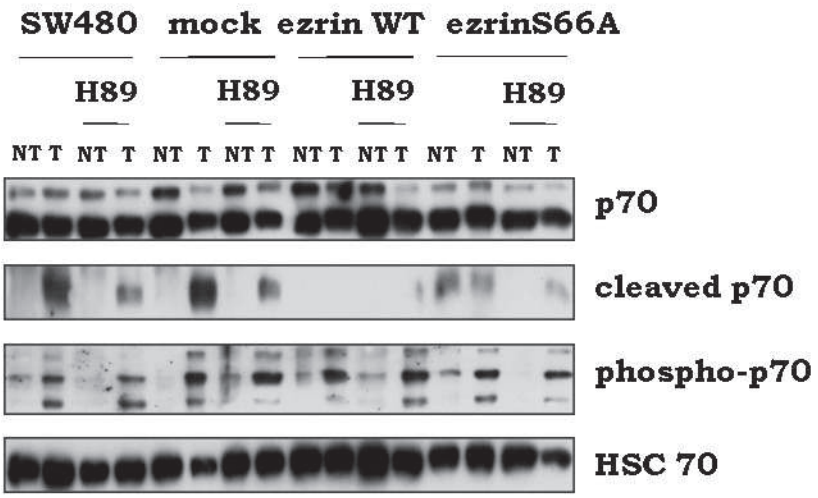

Figure 4.38. Phospho-p70 is phosphorylated upon TRAIL stimulation. SW480 cells or mock-infected, ezrin WT- and S66A-expressing SW480 cells were pre-treated or not with 10 μM H89 for 48 hours and then treated with 500 ng/ml TRAIL for 6 hours. After cell lysis, the levels of p70 and phospho-p70 were determined by western blot using the appropriate antibodies. One preliminary experiment is shown.

We also analysed the activation profile of other downstream targets of PKA, such as the transcriptor factor phospho-CREB. **Figure 4.39** illustrated that phospho-CREB is constitutively modified by phosphorylation upon TRAIL stimulation in SW480 cells expressing the ezrin S66A variant, whereas in cells expressing its phosphomimetic variant, the ezrin S66D, CREB remains in its inactive state. We also checked whether the presence of the ezrin phosphorylation variant on the serine 66 could somehow induce changes in the ezrin phosphorylation on the residues threonine 567 and tyrosine 353. We found that ezrin was highly phosphorylated on the threonine 567 in SW480 cells expressing the ezrin nonphosphorylable variant ezrin S66A, while the phosphorylation on the residue tyrosine 353 seems not to be significant changed (**Figure 4.39**).

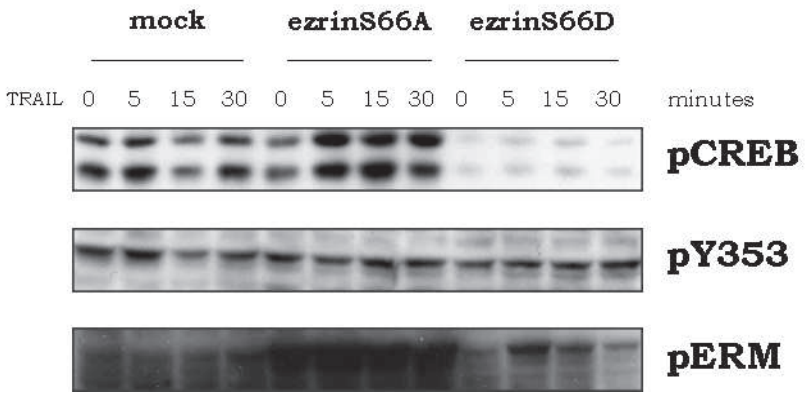

Figure 4.39. TRAIL induced activation by phosphorylation of CREB. Mock-infected SW480 cells, or ezrin S66A- and S66D-expressing SW480 cells were treated or not with 500 ng/ml TRAIL for the indicated time. After cell lysis, the levels of phospho-CREB and phospho-ezrin were determined by western blot using the appropriate antibodies. One preliminary experiment is shown.

Lastly, we checked the effect of CDDP treatment regarding the activation of PKA. Preliminary results, shown in **Figure 4.40**, gave indications that CDDP could impair the activation of CREB, Bad, phospho-p70, and indirectly inhibit the activation of PKA (**Figure 4.40**). Moreover, the results from this preliminary experiment may support the hypothesis that CDDP and TRAIL modulate the phosphorylation of ezrin differentially.

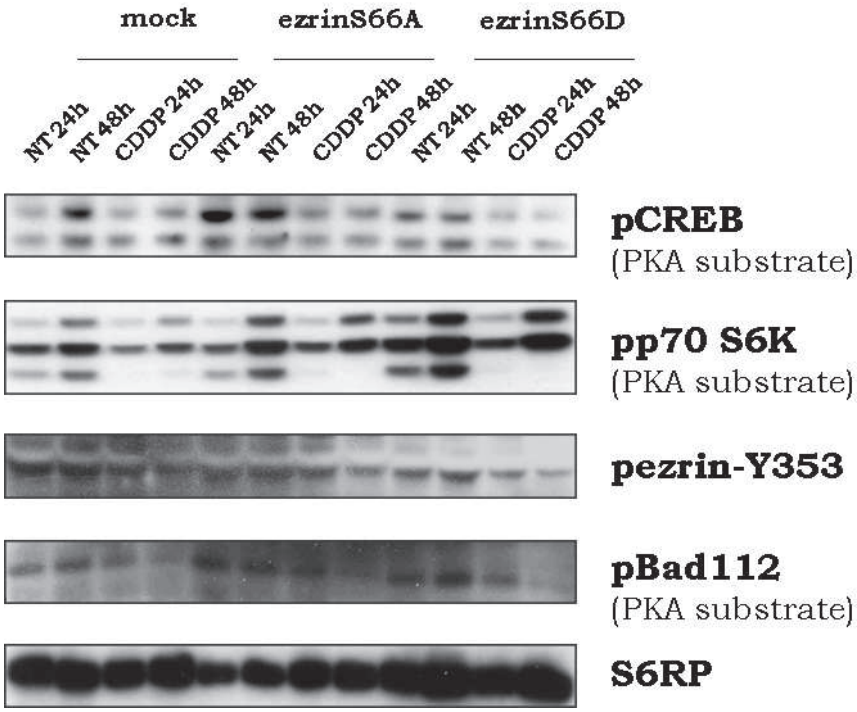

Figure 4.40. CDDP inhibits PKA by indirectly blocking the phosphorylation of CREB, p70 and Bad. Mock-infected SW480 cells, or ezrin S66A- and S66D-expressing SW480 cells were treated or not with 20 μM CDDP for 24 and 48 hours. After cell lysis, the levels of phospho-CREB, phospho-p70, phospho-Bad and phospho-ezrin were determined by western blot using the appropriate antibodies. One preliminary experiment is shown.

4.9. WWOX depletion protects against TRAIL-induced apoptosis

WWOX, also known as WOX-1 or FOR, is a WW domain-containing oxidoreductase. WWOX contains 414 amino acid residues, which gives it a molecular mass of 46 kDa, and it is encoded at chromosome 16q23.3–24.1, on the common fragile site FRA16D (Bednarek et al, 2000; Ried et al, 2000). It is composed of two amino-terminal WW domains, a nuclear localization sequence, and a short chain oxidoreductase domain containing a mitochondria-targeting sequence (Bednarek et al, 2000; Chang et al, 2001). WW domains of WWOX are involved in protein/protein interactions due to their ability to associate with proline-rich ligands (Hu et al, 2004), and can provide a platform for the assembly of multiprotein intracellular signalling (Sudol et al, 1995; Ingham et al, 2005). WWOX is regulated by post-translational modifications such as phosphorylation. It has been reported that Src kinase mediates phosphorylation of WWOX on tyrosine 33 (Y33) in the first WW domain, which in turn regulates the ability of WWOX to interact with other proteins (Aqeilan et al, 2004a; Chang et al, 2007). Numerous immunohistochemical studies have shown that WWOX is predominantly a cytoplasmic protein (Aqeilan et al, 2004b; Guler et al, 2004; Nunez et al, 2005a,b; Nunez et al, 2006; Aqeilan et al, 2007), but it can also reside in the Golgi apparatus (Ludes-Meyers et al, 2003), or in mitochondria or the nucleus (Chang et al, 2007).

Studies demonstrate that WWOX expression is lost or reduced in a variety of human malignancies (Ludes-Meyers et al, 2003; O'Keefe and Richards, 2006), as well as in tumour cells when compared to normal cells (Guler et al, 2004; Park et al, 2004; Aqeilan et al, 2004a; Nunez et al, 2005a,b; Pimenta et al, 2006; Donati et al, 2007). Hypermethylation of the WWOX gene can also occur, and may inactivate its expression (Iliopoulos et al, 2005).

Since WWOX is frequently inactivated in human malignancies, it has been hypothesized that it may function as a tumor suppressor. Ectopic WWOX expression is reported to strongly inhibit the growth of different types of cancer cells, such as pancreatic, prostate, lung and breast cancer cell lines (Bednarek et al, 2001; Kuroki et al, 2004; Fabbri et al, 2005; Qin et al, 2006; Iliopoulos et al, 2007). In addition, WWOX overexpression in these studies resulted in caspase-mediated apoptosis (Fabbri et al, 2005; Qin et al, 2006; Iliopoulos et al., 2007).

In mice, knockout of WWOX induces the development of cancer. Young postnatal mice carrying different deletions of the WWOX gene were shown to develop tumours in the absence of any carcinogenic treatment (Aqeilan et al, 2007). Moreover, $WWOX^{+/-}$ heterozygous mice also developed tumours, including lymphomas, lung papillary carcinomas, liver tumors, gastric squamous cell carcinomas (Aqeilan et al, 2007), suggesting that inactivation of one WWOX allele is sufficient for tumorigenesis.

Several partners of WWOX have been described. WWOX, via its first WW domain, can associate with some transcription factors such as p73, AP2g, and Jun in the cytoplasm, and prevents their translocation into the nucleus, thus regulating their transcriptional activity (Aqeilan et al, 2004a,c; Gaudio et al, 2006). WWOX also interacts with ErbB4, a protooncogene implicated in breast cancer (Junttila et al, 2005; Maatta et al, 2006). It has been reported that when WWOX is present, it retains the carboxy-terminal fragment of ErbB4 in the cytoplasm and presumably inhibits its transactivation function (Aqeilan et al, 2005). The murine WWOX protein has been described to interact with p53, JNK1, and MDM2 independently of its WW domain (Chang et al, 2007). Ezrin is another binding partner of WWOX. A recent study demonstrated that ezrin directly binds to the first WW domain of WWOX via its carboxy-terminal proline-rich region (Jin et al, 2006). The residue tyrosine 477 has been reported to play en essential role for the ezrin-WWOX

association (Jin et al, 2006). This association seems to be regulated by the PKA-mediated phosphorylation of ezrin at the serine 66 (Jin et al, 2006).

Previous research has demonstrated that in gastric parietal cells, PKA-mediated phosphorylation of ezrin on residue serine 66 regulates the interaction of ezrin with WWOX (Jin et al, 2006). This WWOX/ezrin association was also reported to be crucial for the retention of WWOX in the membrane/cytoskeleton area of gastric parietal cells (Jin et al, 2006).

To determine whether WWOX may be somehow involved in the ezrin-mediated inhibition of TRAIL-induced cell death in colon carcinoma cells, an siRNA approach was applied in order to knockdown WWOX. WWOX was downregulated in SW480 cells expressing ezrin phosphorylation variants S66A, S66D, Y353F, Y353D and in control cells by transfection with targeted siRNAs. 48 hours after transfection with the respective siRNAs, cells were stimulated for 6 hours with His-TRAIL (100 ng/ml) and apoptosis was quantified by Hoechst staining (**Figure 4.41**). Knockdown of WWOX led to a significant decrease in TRAIL-induced cell death in mock-infected cells, strongly supporting the hypothesis that WWOX can play a role in TRAIL signalling (**Figure 4.41**). When we analysed the effect of WWOX knockdown in SW480 cells expressing the nonphosphorylable ezrin variant, ezrin S66A, we also observed a decrease in TRAIL-induced cell death (**Figure 4.41**). In SW480 cells expressing the corresponding ezrin phosphomimetic variant, ezrin S66D, we did not recover any change regarding TRAIL-induced cell death following WWOX downregulation (**Figure 4.41**). Similar results were obtained in SW480 cells expressing the ezrin variants Y353F and Y353D. TRAIL-induced cell death was decreased by depletion of WWOX in SW480 cells expressing the ezrin phosphomimetic variant Y353D, while in cells expressing the nonphosphorylable version Y353F, silencing of WWOX did not change the sensitivity to TRAIL of these cells (**Figure 4.41**).

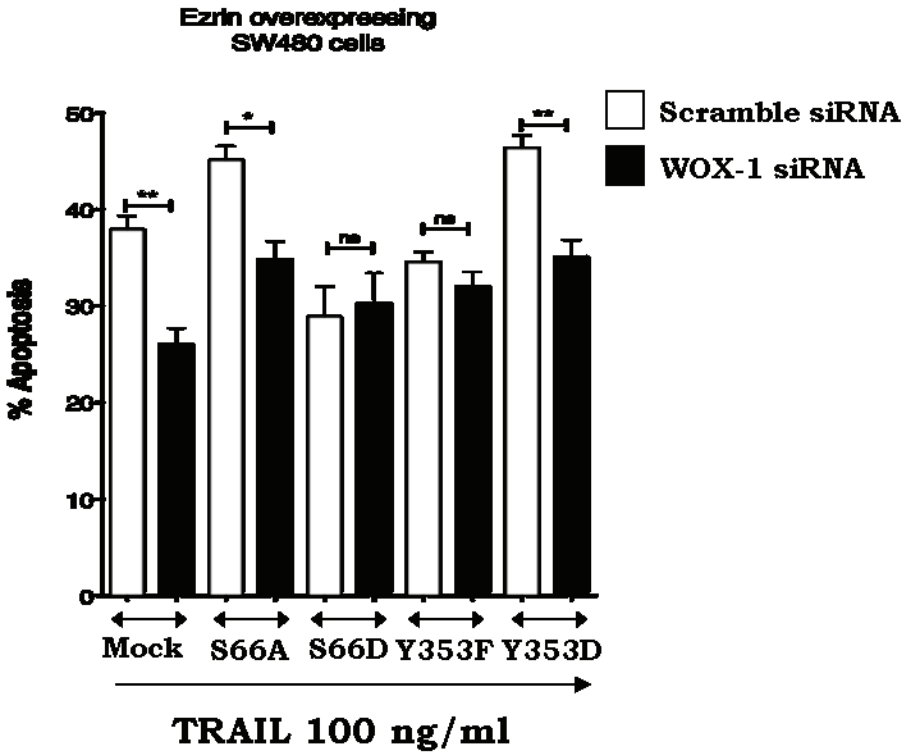

Figure 4.41. WWOX depletion by siRNA significant protects mock-infected and ezrin S66A- and Y353D-expressing SW480 cells against TRAIL-induced cell death. Mock-infected and ezrin S66A-, S66D-, Y353F- and Y353D-expressing SW480 cells were transfected with control or WWOX siRNA. 48 hours after transfection, cells were treated with 100 ng/ml His-TRAIL for 6 hours, and apoptosis was quantified by Hoechst staining. Data represent the mean plus or minus SD of at least four different experiments. (*P<0.05; **P<0.01 respective to cells infected with scramble siRNA; ns stands for not statistically relevant).

4.10. Effect of cisplatin and ezrin treatments in the pathway

Cisplatin (CDDP) is a potent drug commonly used in chemotherapy that is able to induce cell death in a wide variety of solid tumours. Recent findings have demonstrated that combined treatments of chemotherapeutic drugs plus TRAIL restores tumour cells's sensitivity to apoptosis due to either TRAIL-R4 or c-FLIP expression, or Bax deficiency (Mérino et al, 2006; Morizot et al, 2011). Our results strongly suggest that ezrin plays an inhibitory role in regulating cell sensitivity to TRAIL-induced apoptosis, consistent with the study of Kuo et al, 2010. We therefore checked whether treatment with cisplatin followed by TRAIL could somehow modulate tumour cell sensitivity to TRAIL-induced cell death, overcoming ezrin-mediated TRAIL inhibition in SW480 cells expressing the ezrin phosphorylation variants.

To this aim, we monitored cell viability in SW480 control cells, or populations expressing ezrin WT, or phosphorylation variants. Cells were treated first for 48 hours with 1.25 or 2.5 μM cisplatin and then for 16 hours with increasing concentrations of TRAIL. Cell viability was assessed by methylene blue coloration (Micheau et al, 2001). Interestingly, we observed that regardless of the ectopic expression levels of the ezrin mutants, cisplatin synergized with TRAIL in an additive and dose dependent manner (**Figure 4.43**), especially when cells exhibited cell resistance, as seen in the SW480 cells expressing ezrin S66D (**Figure 4.44**). The combination effect was not so evident when cells already exhibited high sensitivity to TRAIL-induced apoptosis, such as SW480 cells expressing ezrin S66A or ezrin Y353D (**Figure 4.44**). However, TRAIL resistance induced by the expression of the ezrin phosphorylation mutants S66D and Y353F could be circumvented by cisplatin (**Figure 4.44**). Moreover, isobolograms extrapolated from these cytotoxic assays clearly demonstrated that cisplatin and TRAIL can synergize when cells exhibit cell resistance, such as SW480 cells expressing ezrin S66D (**Figure 4.44**),

whereas the combination leads to antagonism in SW480 cells expressing ezrin S66A, which exhibit high sensitivity to TRAIL-induced apoptosis (**Figure 4.44**).

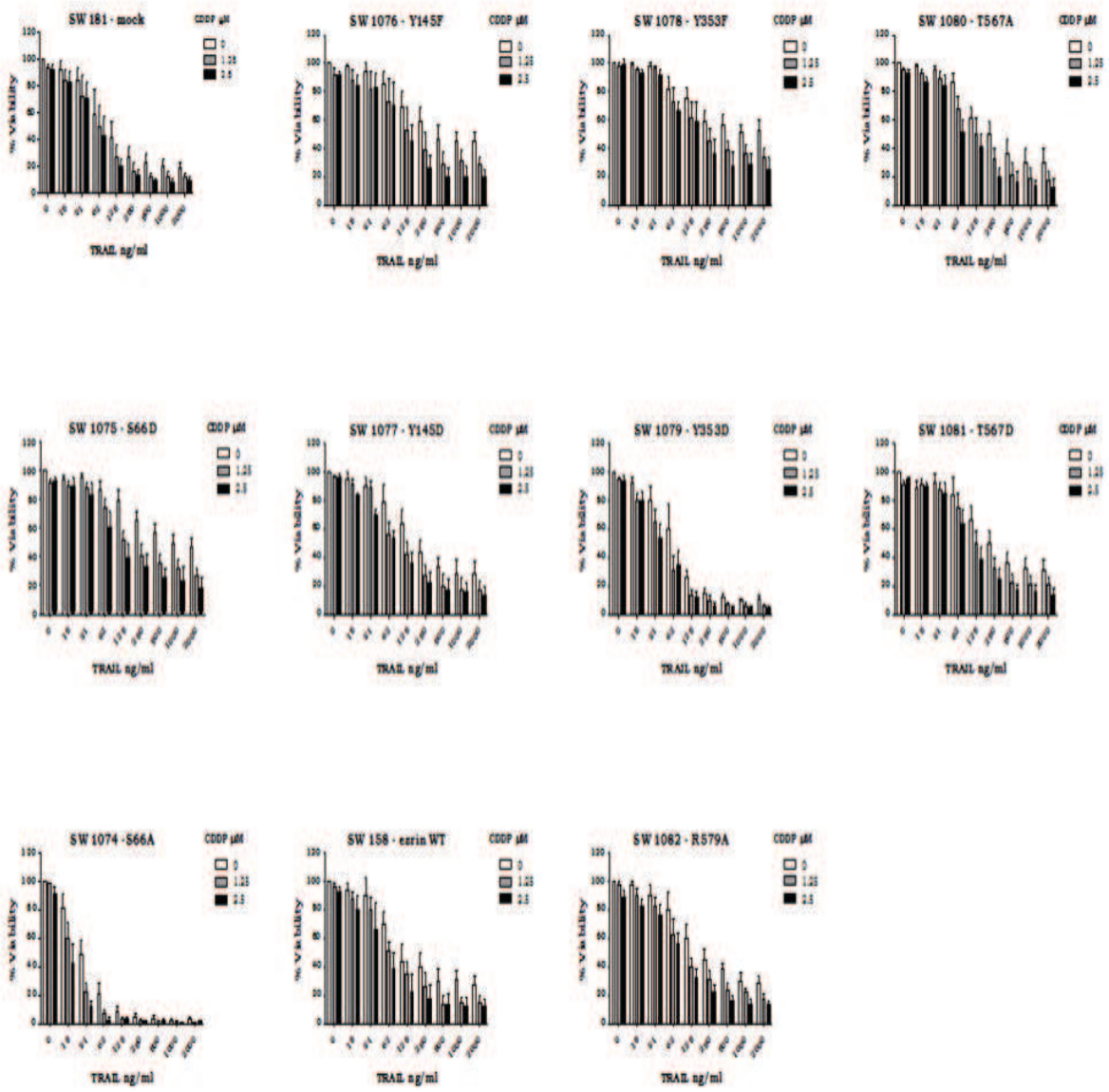

Figure 4.42. Effect of CDDP treatment followed by TRAIL stimulation on ezrin phosphorylation variants-expressing SW480 cells. Dose-response curves were obtained by treating SW-ezrin WT, or phosphorylation variants, and mock-infected cells for 48 hours with CDDP (1.25 – 2.5 µM) followed by a 16 hours treatment with increasing concentrations of His-TRAIL. The mean and standard deviation of three independent experiments are shown.

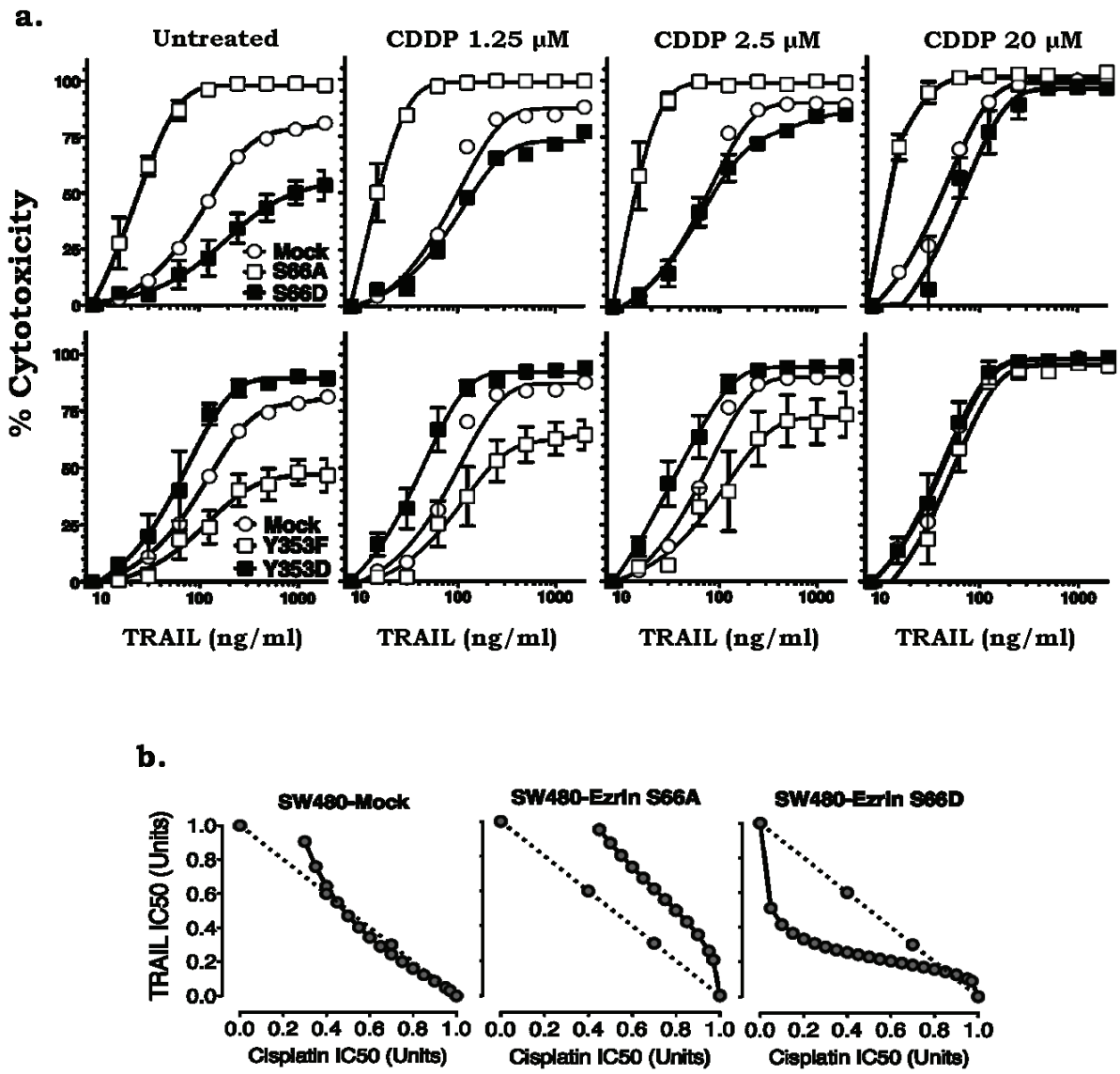

Figure 4.43. Cisplatin restores TRAIL sensitivity in ezrin S66D and Y353F mutants. (a) Dose-response curves were obtained by treating control SW480 cells and ezrin S66A-, S66D-, Y353F- and Y353D-expressing SW480 cells with CDDP (1.25 – 2.5 - 20 µM) followed by a 16 hours treatment with increasing concentrations of His-TRAIL. Cytotoxicity was assessed by methylene blue. (b) Isobologram of combined cisplatin (CDDP) and TRAIL combination indexes in control SW480 cells (mock) or SW480 cells expressing ezrin S66A or S66D. Combination indexes (CIs) values 1, <1 or >1 indicate an additive, synergistic or antagonistic effect, respectively. The dotted line in these isobolograms curves represents theoretical additivity CI values. Data points below or above this line indicate synergy or antagonism respectively.

4.11. Manuscript: Ezrin phosphorylation at serine 66 and tyrosine 353 regulates TRAIL-induced apoptosis in human colon cancer cells.

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Ezrin phosphorylation at serine 66 and tyrosine 353 regulates TRAIL-induced apoptosis in human colon cancer cells.

Short Title : Ezrin phosphorylation regulates TRAIL-induced cell death

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Abbreviations: DISC, Death-Inducing Signalling Complex; TRAIL, TNF-Related Apoptosis Inducing Ligand; ERM, Ezrin Moesin, Radixin;

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Disclosures: The authors declare no conflict of interest.

Writing Assistance : none

Author Contributions: E.I., Ay.M., G.J. and O.M. conceived and designed the experiments; E.I., Ay.M., G.J., A.M., S.S., N.L., A.E., and M.B. performed experiments and data analysis; S.F. material supporting and critical revision; E.I., C.G., E.S. and O.M. wrote the paper with all authors providing detailed comments and suggestions; O.M. directed the project.

Abstract :

Background and Aim: Ezrin belongs to the ERM (ezrin-radixin-moesin) protein family and has been demonstrated to regulate early steps of Fas receptor signalling. We address here the role of ezrin regarding TRAIL-induced cell death in colon cancer cells.

Methods: Molecular and biochemical approaches were employed to study the relevance of the ezrin and its phosphorylation status in TRAIL signalling.

Results: We demonstrate herein that ezrin-mediated TRAIL-induced cell death regulation is tightly controlled through phosphorylation events at serine 66 and tyrosine 353, but is mainly independent of TRAIL DISC (Death Inducing Signalling Complex) formation or activation. Mutations of these residues to alanine (S66A) or aspartic acid (Y353D) selectively enhanced TRAIL-induced cell death, whereas point mutations mimicking ezrin phosphorylation on S66 (S66D) or a nonphosphorylatable version on Y353 (Y353F) strongly protected colon cancer cells from apoptosis induced by TRAIL. Moreover, inhibition of ezrin serine 66 PKA target site, using the pharmacological inhibitor H89, increased colon cancer cell sensitivity to TRAIL, while activation of PKA, induced by cyclic AMP analogue (8-bromo-cyclic AMP), decreased TRAIL-induced cell death. Remarkably, however, combined TRAIL/cisplatin treatments completely abrogated ezrin-mediated inhibition of TRAIL-induced apopotsis.

Conclusions: Altogether our findings demonstrate that ezrin phosphorylation at serine 66 or tyrosine 353 differentially regulates TRAIL-induced cell death in colon carcinoma cells. Importantly, we also provide evidence that ezrin-mediated resistance to TRAIL can be overcome by combined chemotherapy.

Keywords : Cancer; Caspase-8; Bax; Chemotherapy

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Introduction

TNF-Related Apoptosis-Inducing Ligand (TRAIL or Apo2L) induces cell death in a wide variety of cancer cells, but not in normal cells. This peculiarity renders TRAIL and TRAIL derivatives innovative and promising therapeutic agents against malignant diseases. TRAIL triggers cell death upon binding to two transmembrane agonistic receptors: TRAIL-R1 (DR4) [1-3] and TRAIL-R2 (DR5) [1, 2, 4, 5], containing within their intracellular region a Death Domain (DD), which is essential for triggering apoptosis. Activation of TRAIL-R1/TRAIL-R2 drives the recruitment of the adaptor protein FADD and proforms of caspase-8 and -10 to form the macromolecular complex called DISC (Death-Inducing Signalling Complex)[6]. Within this complex, caspase-8 and -10 are activated by auto-proteolytic cleavage and released in the cytosol allowing activation of effector caspases[7].

Similar to TRAIL receptors, another member of TNF receptor family, the transmembrane protein Fas also coined CD95 or APO-1, signals apoptosis through DISC formation[8]. Experimental evidence indicates that Fas linkage to the actin cytoskeleton through ezrin primes human CD4+ T lymphocytes for Fas-mediated apoptosis[9, 10]. In T lymphomas such as Jurkat cells, ezrin and moesin were shown to pre-associate with Fas, and to be required for cell death triggering[11]. However, more recently ezrin was suggested to inhibit TRAIL- and Fas ligand-induced cell death[12]. While the role of the Fas-ezrin-actin connection regarding Fas ligand-mediated apoptosis has been well studied, the function of ezrin in the TRAIL pathway remains poorly described.

Ezrin is a member of the ezrin, radixin, moesin (ERM) family of proteins, that link various integral membrane proteins to the actin cytoskeleton[13]. ERM proteins are

usually present in the cytoplasm in an inactive/closed form, in which the amino-terminal membrane protein-binding domain (FERM or N-ERMAD domain) is masked due to its association with the carboxyl, actin-binding domain (C-ERMAD).

ERM activation is proposed to occur through phosphorylation and binding of phosphatidylinositol 4,5-bisphosphate (PIP_2)[14]. Phosphorylation of ezrin on the conserved threonine 567 residue in the carboxyl-terminal domain, of moesin on threonine 558, and of radixin on threonine 564 was demonstrated to induce a transition to the open/active form, and to correlate with the recruitment of ERM proteins to the plasma membrane, where they bind membrane molecules.

Other phosphorylation sites on ezrin have been described. After epidermal growth factor stimulation, ezrin is phosphorylated on tyrosine residues 145 and 353 in A431 cells concomitantly to promote dimer formation[15]. This type of phosphorylation was mainly associated with survival pathways. In fact, phosphorylation on tyrosine 353 has been described to signal for survival in LLC-PK1 cells through activation of the phosphatidylinositol 3-kinase pathway during epithelial differentiation[16]. Moreover, src-mediated ezrin phosphorylation on tyrosine 145 represents a key input to signal adhesion between cell surface and extracellular matrix in epithelial cells[17]. Zhou et al. described a new phosphorylation site, serine 66, a substrate of protein kinase A (PKA). PKA-mediated ezrin phosphorylation on serine 66 was shown to be associated with acid secretion in gastric cells[18].

We demonstrate in this study that ezrin phosphorylation at serine 66 and tyrosine 353, selectively contributes to TRAIL-induced cell death regulation downstream of the TRAIL DISC in colon cancer cells.

Materials and Methods

Ligand production and antibodies.

Flag-tagged recombinant soluble human TRAIL, His-tagged TRAIL and Fas ligand were produced and used as described previously[19]. Anti-Flag (M2), 8-bromo-cyclic AMP and orthovanadate were purchased from Sigma-Aldrich (Lyon, France). PKA inhibitor, H89 was from Cayman (Interchim, Montluçon, France). For western blot analysis, anti-TRAIL-R1 and anti-TRAIL-R2 antibodies were purchased from Chemicon (Millipore, Molsheim, France), anti-FADD, anti-phospho-ezrin (Thr567) and anti-moesin were obtained from Transduction Laboratories (BD biosciences, Le Pont de Claix, France), anti-caspase-8 and anti-caspase-10 were from Medical & Biological Laboratories (Clinisciences, Montrouge, France). Antibodies against phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr 558) and the active cleaved fragment of caspase-3 and caspase-9 were from Cell Signaling (Ozyme). Anti-radixin, caspase-2, GAPDH and HSC-70 were from Santa Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France). Anti-actin, anti-ezrin and anti-VSV glycoprotein antibodies were purchased from Sigma-Aldrich (Lyon, France). For flow cytometry experiments, anti-Bax was obtained from BD biosciences. The secondary antibody was an Alexa-488-coupled goat anti-mouse from Molecular Probes (Invitrogen, Cergy Pontoise, France). For immunoprecipitation, the anti-ezrin (clone 3C12), anti-Flag (M2) and anti-VSV glycoprotein (P5D4) antibodies were purchased from Sigma-Aldrich Anti-TRAIL-R1 (wB-S26) and anti-TRAIL-R2 (B-D37) antibodies were provided by Gen-Probe (Diaclone, Besançon, France).

Cell culture.

The HCT116 (human colon carcinoma) and SW480 (human colon adenocarcinoma) cell lines were cultured with high-glucose Dulbecco's modified Eagle's medium (Lonza, Levallois-Perret, France) supplemented with 10 % fetal bovine serum (Lonza) and penicillin/streptomycin (100 µg/ml of each). All cell lines were grown in 5 % CO_2 at 37°C.

Plasmid construction.

VSV-tagged ezrin WT was subcloned from pEGFP-N1 vector (Invitrogen) to pCR-3 (Invitrogen). Mutations S66A, S66D, Y145F, Y145D, Y353F, Y353D, T567A, T567D, R579A were created by standard PCR methods and a site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's manual (see supplemental Table I for primer description). The S66D, Y145D, Y353D, T567D were created to mimic phosphorylated ezrin, whereas S66A, Y145F, Y353F, T567A were generated as nonphosphorylatable ezrin. The VSV-tagged ezrin mutants were subcloned into the pMSCV-puro expression vector as HindIII/XhoI fragments. All constructs were confirmed by sequencing.

Retrovirus production and cell transduction.

The retroviral vector pMSCV-puro expression and the generation of viruses have been previously described[20]. HCT116 and SW480 cells were infected for 16 hours with viral supernatants containing 8 µg/ml polybrene (Hexadimethrin Bromide from Sigma

Aldrich), washed in phosphate-buffered saline from Lonza (PBS), and cultured in complete medium containing 2.5 µg/ml puromycin from InvivoGen.

Treatments with CDDP and TRAIL.

For sequential treatments, cells were treated for 48 hours with three different concentrations of CDDP from Sigma Aldrich (1.25 – 2.5 - 20 µM) in complete medium and then washed before being treated for 16 hours with increasing concentrations of His-TRAIL (from 0 to 1000 ng/ml).

Measurement of cell viability.

In 96-well plates, 50 000 cells were incubated at 37°C for 24 hours with increasing concentration of his-TRAIL (from 0 to 10 000 ng/ml) or for 48 hours with increasing concentration of CDDP (from 1 to 1000 µM). Cell viability was determined by methylene blue[20].

Hoechst analysis.

Cells, treated or untreated with His-TRAIL or FasL, were incubated at 37°C for 6 hours. Alternatively, cells pre-treated or not for 30 minutes with 100 µM H89 or for 20 minutes with 4 mM 8-bromo-cyclic AMP (8B), followed by TRAIL (100 or 500 ng/ml for 6 hours) or Fas ligand (100 ng/ml for 6 hours), were incubated at 37°C for 6 hours. Apoptosis was assessed by Hoechst staining by determining the percentage of condensed and fragmented nuclei from at least 300 cells per conditions. Experiments were repeated at least three times.

APO 2.7 staining.

Cells, treated or untreated with His-TRAIL, FasL or pre-treated with 10 µM H89 for 48 hours, were permeabilized (PBS, FCS 2,5 % and digitonin 100 µg/ml) for 10 min at 4°C and stained with a PE-conjugated 2.7A6A3 antibody (Beckman Coulter) which recognizes the APO2.7 mitochondrial membrane protein exposed at an early stage on cells undergoing apoptosis. In all, 10 000 events were analyzed using a FACScalibur flow cytometer (BD Biosciences).

Immunoprecipitations.

For TRAIL DISC analysis, 10^8 cells were stimulated with 5 µg of Flag-TRAIL cross-linked with 10 µg of anti-Flag M2 in 1ml of medium for the indicated times at 37 °C. Cells were then washed with cold phosphate saline buffer (PBS) and lysed in 1ml of lysis buffer containing 1 % of NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 10 % glycerol and proteinase inhibitor cocktail. Lysates were pre-cleared with Sepharose 6B (Sigma-Aldrich), and immunoprecipitated overnight at 4 °C with protein G-Sepharose beads (Amersham Biosciences, Les Ullis, France). For TRAIL receptor or GAPDH immunoprecipitations, cells were stimulated as described above with 5 µg/ml of His-TRAIL. In both cases, cells were lysed in NP-40-containing lysis buffer. Cell extracts were precleared and immunoprecipitated using 5 µg of corresponding antibodies. Beads were then washed four times with lysis buffer, and immunoprecipitates were eluted in loading buffer (Tris-HCl 63 mM, SDS 2 %, phenol red 0.03 %, glycerol 10% and DTT 100 mM of pH 6.8), boiled for 5 min and processed for immunoblotting.

Western blotting.

Immunoprecipitates or cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in PBS containing 0.05 % Tween 20 and 5 % milk powder. Membranes were then incubated with a specific primary antibody followed by horseradish peroxidase-conjugated secondary antibody, and were developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

Analysis of Bax activation by flow cytometry.

Cells, treated or untreated with His-TRAIL, were fixed with 4 % PFA, permeabilized (PBS, BSA 1 % and saponin 0.1 %) for 10 min at room temperature and stained with an anti-Bax antibody which recognizes the active N-terminal form of Bax (clone 6A7, BD Biosciences). 10 000 events were analyzed using a FACScalibur flow cytometer (BD Biosciences).

Statistical Analysis.

For in vitro studies, differences were determined either with two-way repeated-measures analysis of variance (ANOVA) with Bonferroni's multiple comparison test, or by student's t test, using Prism 5.0a software (GraphPad Software, San Diego, CA, USA). A significance level of $*P<0.05$, $**P<0.01$ or $***P<0.001$ was assumed for all tests.

Results :

Ezrin is a negative regulator of TRAIL-induced cell death but is not a component of the TRAIL DISC

Ezrin was initially shown to be a positive regulator of Fas ligand-induced cell death through its ability to interact with Fas receptor [9, 11]. Subsequently, ezrin was found to inhibit both Fas ligand and TRAIL-induced cell death in some T lymphoma cell lines [12]. The present study aimed at evaluating the relevance of ezrin in regulating TRAIL-induced apoptosis in colon carcinoma cells. For that purpose, ezrin was either overexpressed (Figure 1A) or knocked-down using siRNA (Supplementary Figure 1) in HCT116 and SW480 cells and apoptosis-induced by TRAIL or Fas ligand, used here as a control, was assessed by Hoescht staining. Ezrin overexpression significantly attenuated Fas ligand- and TRAIL-induced cell death in both cell lines (Figure 1B), but ezrin partial knockdown did not reach significance, although the trend was towards a modest increase in ligand-induced apoptosis (Supplementary Figure 1). To explore whether ezrin may be a component of the TRAIL DISC, as observed in the Fas pathway[11, 12], HCT116 or SW480 cells were left unstimulated (C and 0) or stimulated with TRAIL for 15, 20, 30 or 60 minutes. DISC analysis was performed by immunoprecipitation in the presence of either an irrelevant antibody (C) or antibodies targeting the rh-FLAG-TRAIL, TRAIL-R1 and -TRAIL-R2, to pool-down TRAIL DISC components. As shown Figure 1C and 1D, TRAIL stimulation induces DISC formation as evidenced by the recruitment to the TRAIL receptors of the adaptor protein FADD as well as the initiator caspases-8 and -10. In this assay, ezrin recruitment was rather unspecific (Figure 1C and D & Supplementary Figure 2), *i.e.* ezrin was pooled-down from non-stimulated cell lysates incubated with an

irrelevant antibody (C) and from non-stimulated or TRAIL-stimulated cell lysates after immunoprecipitation of the GAPDH (Figure 1E). Moesin and actin, albeit to a lower extent, were similarly detected in these immunoprecipitations (Figure 1C and E). These results suggest that TRAIL-induced apoptosis inhibition by ezrin very unlikely occurs at the TRAIL DISC level.

TRAIL and Fas induce Ezrin phosphorylation

Dephosphorylation of ERM sensitizes active T cells to Fas ligand-induced cell death[21], whereas Fas ligand-mediated ezrin phosphorylation at T567 promotes Fas receptor aggregation and apoptosis signalling in some lymphoma T-cell lines[11]. We show here that, similar to Fas ligand, TRAIL induces ezrin phosphorylation at threonine 567 in SW480 cells (Figure 2A). Phosphorylation of other residues including tyrosine 145, tyrosine 353 or serines[17, 18, 22] was evidenced by western blot after ezrin immunoprecipitation (Supplementary Figure 3). Since serine 66 is selectively phosphorylated by protein kinase A[18], we evaluated whether its inhibition, by use of the pharmacological inhibitor H89, might have an effect on TRAIL-induced cell death. Although no commercial antibodies allowing the detection of ezrin phosphorylation on serine 66 exist, we could show that while H89 slightly, but significantly increased TRAIL-induced cell death in SW480 cells as measured by APO2.7 staining or Hoechst staining (Figure 2B and C). On the contrary, PKA activation using 8-Bromoadenosine 3′,5′-cyclic monophosphate (8B), reduced TRAIL-induced apoptosis in both SW480 and HCT116 cells (Figure 2C and D). However, apoptosis induced by Fas ligand was neither affected by PKA inhibition nor activation (Figure 2C). Altogether these results suggest

that TRAIL signalling may be regulated by ezrin through PKA-mediated phosphorylation events.

Ezrin phosphorylation modulates TRAIL-induced cell death

In order to determine whether ezrin phosphorylation affects TRAIL signalling, we next generated several phosphorylation mutants encoding nonphosphorylatable variants or pseudophosphorylated variants of the ezrin, at serine 66, threonine 567 and tyrosines 145 and 353 sites, by site directed mutagenesis (Figure 3A). In addition to these phosphorylation mutants, an ezrin mutant defective in F-actin binding, ezrin R579A was generated to determine the role of actin cytoskeleton in ezrin-mediated TRAIL inhibition [23]. Infection of SW480 cells with a retroviral vector encoding these constructs led to variable but appreciable expression levels of ezrin mutants (Figure 3B), with the exception of the nonphosphorylatable variant Y145F, that was mostly expressed in the insoluble fraction (Supplementary Figure 4A). Of note, expression levels of exogenous ezrin mutants were low as compared to endogenous ezrin (Supplementary Figure 4B). Yet most ezrin mutants impaired TRAIL- (Figure 3C) and Fas ligand-induced apoptosis (Supplementary Figure 4C). Remarkably, the nonphosphorylatable variant S66A significantly and selectively enhanced apoptosis induced by TRAIL whereas the pseudophosphorylated variant S66D demonstrated a protective effect (Figure 3C). TRAIL regulatory properties of ezrin phospho-serine 66 mutants were even more pronounced when cell viability was assessed by methylene blue assay (Figure 3D and E). TRAIL-induced cell death was also increased by ectopic expression of ezrin phosphomimetic Y353D while the nonphosphorylatable version Y353F mutant protected

cells against TRAIL (Figure 3F). Regulation of ezrin tyrosine 353 and serine 66 phosphorylation modulated TRAIL-induced cell death by an order of magnitude ranging from 3 to 6 fold as compared to parental cells, while the remaining phosphorylation mutants only achieve a 1.2 to 2 fold increase resistance, as extrapolated from IC50 (Figure 3E and F). The amount of TRAIL ligand required to induce cell death in 50 and 90% of the cellular population is 20 to 30 fold higher in S66D as compared to S66A expressing SW480 cells, and 10 fold higher in cells expressing ezrin Y353F as compared to Y353D, respectively (Supplementary Table 1). With the exception of serine 66 or tyrosine 353, none of the remaining ezrin mutants, however, were able to enhance TRAIL-induced cell death, irrespective of their phosphorylation status (Figure 3F).

Ezrin inhibits TRAIL-induced cell death downstream of the TRAIL DISC

Activation of caspase-8 and caspase-10 after TRAIL stimulation was both concentration (Figure 4A) and time dependent (Figure 4B), as analyzed by immunoblotting, but their activation neither correlated with ezrin expression levels nor ezrin mutations, including those targeting serine 66 and the F-actin binding domain (Figure 4A and B). Activation of caspase-9, -2 and -3, on the other hand, was slightly enhanced in ezrin S66A SW480 cells and substantially reduced in SW480 cells expressing ezrin WT, ezrin S66D and ezrin R579A (Figure 4A and B). Ezrin S66A or S66D mutants had no impact on TRAIL DISC formation (Figure 4C), caspase-8 activation (Figure 4A-C, white arrow), nonspecific ezrin binding to the TRAIL DISC (Figure 4C), TRAIL receptor expression (Supplementary Figure 5A and B) or internalization, after TRAIL stimulation (Supplementary Figure 6). We thus reasoned that ezrin might play a role downstream of

the TRAIL DISC. Supporting this assumption was the finding that Bax activation dropped from 33.8% and 45% to 17 and 30% of the population, in cells expressing ezrin WT or ezrin S66D as compared to mock-transfected cells, in response to TRAIL stimulation at 20 and 200 ng/ml, respectively (Figure 4D). Whereas, Bax activation in S66A ezrin expressing cells remained elevated and rose up to 57 % after stimulation (Figure 4D). These data indicate that regulation of TRAIL-induced cell death by the ezrin occurs downstream of the TRAIL DISC.

Cisplatin overcomes ezrin-mediated TRAIL resistance

Conventional chemotherapeutic drugs, e.g. cisplatin, can restore TRAIL-induced cell death and overcome resistance induced either by the overexpression of TRAIL-R4 or c-FLIP or by a deficiency in Bax [24, 25]. Interestingly, albeit neither ezrin WT nor ezrin phosphorylations mutants inhibited cisplatin-induced cell death (Supplementary Figure 7A), TRAIL resistance, induced by ectopic expression of ezrin phosphorylation mutants targeting serine 66 and tyrosine 353 could be circumvented by cisplatin (Figure 5A and Supplementary Figure 7B). Combined cisplatin treatments abrogated ezrin-mediated TRAIL-induced cell death inhibition irrespective of ezrin expression levels or mutants (Supplementary Figure 7C). Moreover, isobolograms extrapolated from these cytotoxic assays, clearly demonstrated that cisplatin and TRAIL can synergize, when cells exhibit cell resistance, such as SW480 cells expressing ezrin S66D (Figure 5B), whereas the combination leads to antagonism in SW480 cells expressing ezrin S66A, which exhibit high sensitivity to TRAIL-induced apoptosis (Figure 5B).

Discussion :

While the contribution of ezrin in Fas signalling has been extensively studied, little is known regarding TRAIL, with the exception of a recent study in which ezrin was proposed to impair both Fas ligand- and TRAIL-induced cell death in the tumor T-cell lymphoma cell line H9[12]. Ezrin was suggested to negatively regulate death receptor-mediated cell death in type I cells, which are independent of the mitochondrial pathway, while not affecting death receptor-mediated cell death in type II cells[12] that rely on the mitochondria[26].

We demonstrate here that ezrin regulates TRAIL signalling both negatively and positively, depending on its phosphorylation status. Ezrin's negative regulatory function was not restricted to type I cells, since Fas ligand- and TRAIL-induced cell death in both SW480 and HCT116 colon carcinoma cell lines, type I and type II, respectively, were inhibited by ezrin overexpression. In addition, our results demonstrate that ezrin-mediated TRAIL-induced cell death regulation is neither related to changes in TRAIL DISC component recruitment nor to differential activation of initiator caspases within the DISC. Moreover, and contrary to Fas itself [27, 28], TRAIL signalling regulation by ezrin was neither associated with variations in receptor steady state membrane expression levels nor with changes in receptor internalization after TRAIL stimulation. Rather, ezrin appeared to target the apoptotic machinery downstream of the TRAIL DISC as demonstrated by its ability to regulate Bax activation.

The pleiotropic regulatory function of ezrin was uncovered by the analysis of ezrin phosphorylation mutants targeting several major phosphorylation residues including serine 66, threonine 567, tyrosine 145 or tyrosine 353. Ectopic expression of these ezrin

mutants in SW480 cells allowed us to demonstrate that phosphorylation or dephosphorylation of ezrin at serine 66 or tyrosine 353 selectively contributes to the TRAIL-induced cell death regulation but not apoptosis triggered by Fas ligand or cisplatin. Remarkably, while S66D phosphomimetic ezrin mutant induced strong protection against TRAIL-induced cell death, the corresponding nonphosphorylatable mutant S66A increased cell sensitivity to TRAIL. Albeit to a lesser extent, a reverse phenotype was obtained with ezrin phosphorylation mutants targeting tyrosine 353. Ezrin phosphorylation on threonine 567, although reported as a prerequisite for Fas aggregation and caspase-8 activation [11], did not enhance Fas ligand- nor TRAIL-induced cell death in SW480 cells. Since expression levels of ezrin mutants in SW480 cells were much lower than endogenous ezrin, we cannot exclude the possibility that phosphorylation of ezrin on threonine 567, which is induced by TRAIL and Fas ligand in SW480 cells, may nevertheless contribute to the regulation of TRAIL or Fas signalling. Nevertheless, at comparable expression levels, TRAIL-induced cell death in cells expressing ezrin S66A, S66D, Y353F or Y353D mutants was strongly altered as compared to parental or ezrin WT expressing cells. Importantly, although the execution of the apoptotic machinery was differentially enhanced or impaired in these cells, changes in caspase-3 and Bax activation were neither associated with differential caspase-8 or caspase-10 recruitment nor with changes in initiator caspase activation within TRAIL DISC. These results indicate that ezrin may regulate TRAIL- or Fas ligand-induced cell death irrespective of its ability to interact with the corresponding death receptors in colon cancer cells. In line with this hypothesis, expression of ezrin R579A, an ezrin mutant defective in F-actin binding, inhibited TRAIL- and Fas ligand-induced apoptosis as efficiently as ezrin WT or

ezrin mutants targeting threonine 567 and tyrosine 145.

As demonstrated recently in the type I T-cell lymphoma cell line H9 [12], our results indicate that ezrin solely displayed a negative regulatory function towards Fas ligand-induced apoptosis in the colon cancer cell lines HCT116 and SW480. We and other have previously described a positive regulation of Fas signalling in primary lymphocytes, fibroblasts or cancer T-cell lines, including Jurkat and CEM cells [9-11, 21, 27]. Ezrin connection to Fas and the actin cytoskeleton was associated with Fas polarization and priming of human CD4+ T lymphocytes as well as T-cell lymphoma cell lines to Fas ligand-induced apoptosis[9, 10]. In the murine fibrosarcoma cell line L929, ezrin has also been shown to play an essential role in redistributing Fas to lipid rafts, contributing to receptor internalization and apoptosis engagement upon Fas ligand stimulation[28]. Fas addressing, receptor aggregation and apoptosis triggering, in activated human peripheral blood T cells, Jurkat T lymphoma cell line[11] and embryonic fibroblasts[27] were proposed to be regulated by Rho-associated, coiled-coil containing protein kinases through ezrin phosphorylation. Therefore, how can these discrepancies be therefore reconciled? The most probable explanation would be that positive regulation of Fas signalling by ezrin is tightly connected to the cell type, for instance those cells which are prone to microvilli and filopodia formation, including activated primary T cells[9], cells which are prone to motility or metastatic cells[29]. By contrast, ezrin might display a negative function in cells lacking these structures or characteristics. Alternatively, differential kinase cascade activation, e.g. Rho-associated, coiled-coil containing protein kinases, induced by Fas ligand or TRAIL stimulation may regulate ezrin's function, including its ability to interact

with death receptors. Whether ezrin specifically interacts with TRAIL receptors remains an open question, the answer of which may help explain the contrasting regulatory function of ezrin regarding TRAIL signalling. We have, so far, failed to demonstrate a direct interaction between ezrin and TRAIL-R1 or TRAIL-R2 (not shown), and from our experiments, ezrin recruitment to the TRAIL DISC appeared to be neither specific, nor correlated to the differential activation of caspase-8 or caspase-10 within the DISC. However, since ezrin knock-down in HCT116 and SW480 cells was incomplete, probably due to high steady state expression levels of ezrin, we cannot definitively rule out this possibility.

Nonetheless, our results clearly demonstrate that regulation of ezrin phosphorylation at serine 66 and tyrosine 353 selectively regulates cell death induced by TRAIL, both negatively and positively, highlighting novel and unexpected ezrin regulatory mechanisms. ERM proteins, including ezrin, predominantly exist in a "dormant" cytosolic form, folded by intramolecular association of their N and C termini, but are activated or unfolded through phosphatidylinositol 4,5-bisphosphate (PIP_2) binding and phosphorylation[14]. It is therefore tempting to speculate that ezrin phosphorylation on serine 66 and/or dephosphorylation of tyrosine 353 gives rise to similar ezrin conformational changes inducing specific inhibition of TRAIL signalling, while dephosphorylation of serine 66 and/or tyrosine 353 would lead to another conformation enhancing execution of the apoptotic program. Phosphorylation or dephosphorylation of these residues is likely to disrupt or favour interactions with unknown or known ezrin protein partners. For example, PKA-mediated ezrin phosphorylation at serine 66 has been demonstrated to be required for ezrin to interact[30]

with the tumor supressor gene WWOX[31]. Whether this interaction contributes to TRAIL signalling inhibition remains to be determined. Interestingly, in colon cancer cells, pharmacological agents inhibiting or activating PKA activity, resulted in a moderate increase or decrease in TRAIL-induced cell death respectively. These changes were not as marked as that observed with the ezrin phosphorylation mutants, but were similar to that of ezrin WT. Lastly, our results demonstrate that cisplatin, a conventional chemotherapeutic drug, completely abrogates ezrin-mediated TRAIL-inhibition, even in the highly resistant cell populations expressing ezrin S66D and Y353F.

The molecular mechanisms involved in TRAIL-induced apoptosis regulation by ezrin remain to be determined. It would be of interest to understand how TRAIL and Fas signalling are differentially regulated by ezrin, despite the fact that these TNF family members share many DISC components including the adaptor protein FADD and initiator caspases-8 and -10. Addressing this question may in addition provide some clues to the understanding of the mechanisms underlying TRAIL tumor selectivity.

Altogether our results demonstrate for the first time that TRAIL-induced cell death regulation by ezrin in colon carcinoma cells is tightly tuned by ezrin phosphorylation at serine 66 and tyrosine 353, and occurs downstream of the TRAIL DISC, most probably at the level of Bax. Keeping in mind that ezrin is often highly expressed in colorectal cancer tissues and that its expression is associated with tumor malignancy and poor prognosis [32-34], our findings suggest that combinatorial treatments [35] associating TRAIL with chemotherapy or kinase inhibitors may prove useful to abrogate ezrin-mediated TRAIL-inhibition in colon carcinoma patients.

FIGURE LEGENDS

Figure 1. Ezrin inhibits TRAIL-induced apoptosis downstream of the DISC. (A) Western blot analysis for ezrin ectopic expression levels using anti-VSV antibody in control or ezrin WT-expressing HCT116 and SW480 cells. HSC70 was used as a loading control. (B) Cells were treated with Fas ligand (100 ng/ml) or His-TRAIL (500 ng/ml) for 6 hours. Apoptosis was quantified by Hoechst staining. Data represent the mean plus or minus SD of at least three different experiments. (*P<0.05; **P<0.01 respective to control cells). (C) Analysis of TRAIL DISC formation. HCT116 and SW480 cells were stimulated or not with 5 µg/ml Flag-TRAIL, cross-linked with 10 µg/ml anti-Flag (M2) antibody. Cells were lysed, and the DISC was immunoprecipitated and analyzed by western blot. One of three independent experiments is shown. (D) HCT116 and SW480 cells were stimulated or not with His-TRAIL (5 µg/ml) and lysed. Cell lysates were immunoprecipitated with an anti-TRAIL-R1 or TRAIL-R2 antibody and analyzed by western blot. One of three independent experiments is shown. (E) HCT116 cells were stimulated or not with 5 µg/ml His-TRAIL for 20 and 60 minutes. After cell lysis, GAPDH antibody was added to the cell lysates and immunoprecipitates were analyzed by western blot.

Figure 2. TRAIL and Fas induce ezrin phosphorylation. (A) Western blot analysis of phospho-ERM and phospho-ezrin (Thr567) expression levels in SW480 cells after stimulation with His-TRAIL, Fas ligand or orthovanadate. Percentage of relative phospho-ERM or phospho-ezrin (Thr567) intensities were determined as follows: intensity of specific band in stimulated cells divided by the normalized intensity of unstimulated cells, normalized to HSC70. (B) Effect of PKA inhibition on TRAIL sensitivity. SW480 cells were pre-treated or not with 10 µM H89 for 48 hours, followed by TRAIL (500 ng/ml for 6 hours). Apoptosis was then assessed by APO2.7 staining by flow cytometry. (C) and (D) Cells were pre-treated or not for 30 minutes with 100 µM H89 or 20 minutes with 4 mM 8B, followed by TRAIL (100 or 500 ng/ml) or Fas ligand (100 ng/ml) stimulation for 6 hours. Apoptosis was quantified by Hoechst staining. Data represent the mean plus or minus SD of at least three different experiments. (**P<0.01; ***P<0.001 respective to control or H89 stimulated cells; ns stands for not statistically relevant).

Figure 3. Regulation of ezrin serine 66 and tyrosine 353 phosphorylation selectively enhances TRAIL-induced cell death. (A) Schematic representation of ezrin domains and phosphorylation sites within the protein. (B) SW480 cells were infected with an empty pMSCV retroviral vector (Mock) or with a pMSCV vector encoding ezrin WT, ezrin S66A, ezrin S66D, ezrin Y145F, ezrin Y145D, ezrin Y353F, ezrin Y353D, ezrin T567A, ezrin T567D and ezrin R579A. Expression levels of ezrin constructs was determined by western blot using anti-VSV, anti-ezrin and anti-actin antibodies (loading control). Data shown is representative of three independent experiments. (C) Effect of ezrin WT and ezrin phosphomutants ectopic expression on TRAIL-induced cell death in SW480 cells. Cells were stimulated with TRAIL 500 ng/ml for 6 hours. Apoptosis was measured by APO2.7 staining by flow cytometry. (D) Cell viability in the SW480 cells expressing ezrin S66A, ezrin S66D or ezrin R579A as compared to Mock infected cells

was evaluated by methylene blue assay 24 h after treatment using increasing concentrations of His-TRAIL. (E) SW-ezrinS66A, -ezrinS66D, - and mock-infected cells inhibitory TRAIL concentrations curves, in ng/ml, were obtained by methylen blue, 16h after increasing His-TRAIL concentrations, using CompuSyn. (F) Percent inhibitory TRAIL concentration curves from SW480 cells expressing ectopically the indicated ezrin mutants were obtained as above. With IC25, IC50 and IC90 corresponding to TRAIL concentrations, in ng/ml, inducing 25, 50 and 90% cell death, respectively. Data represent mean plus or minus SD of at least 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001 respective to Mock control cells.

Figure 4. Ezrin inhibits TRAIL-induced cell death at the mitochondria level. (A) Western blot analysis of caspase activation, in SW480 cells expressing either ezrin S66A, S66D, R579A or WT, 6 or 16 hours after His-TRAIL (20 or 200 ng/ml) stimulation, respectively. (B) Western blot analysis after His-TRAIL (100 ng/ml) stimulation at the indicated period of time in ezrin S66A, S66D, R579A and mock infected SW480 cells. (C) Analysis of TRAIL DISC formation. Cells were stimulated with 5 µg/ml Flag-TRAIL cross-linked with 10 µg/ml anti-Flag (M2) antibody. After cell lysis, the DISC was immunoprecipitated and analyzed by western blot. (D) Analysis of Bax activation. Ezrin S66A, S66D, WT and Mock-infected SW480 cells were left untreated or stimulated with His-TRAIL (20 or 200 ng/ml) for 16 h, then permeabilized and stained with an antibody recognizing active Bax before analysis by flow cytometry. The effect of two different concentrations of TRAIL (20 or 200 ng/ml, gray and black lines, respectively) were compared to unstimulated cells (gray filled curve). The percentage of cells containing active Bax after TRAIL stimulation is shown (upper and lower value, respectively).

Figure 5. Cisplatin restores TRAIL sensitivity in ezrin S66D and Y353F mutants. (A) Dose-response curves were obtained from SW480 cells expressing ezrin phosphorylation mutants S66A, S66D, Y353F, Y353D or from Mock parental cells stimulated for 48 h with CDDP (1.25 – 2.5 – 20 µM) followed by a 16 hours treatment with increasing concentrations of His-TRAIL. Cytotoxicity was assessed by methylene blue. (B) Isobologram of combined cisplatin (CDDP) and TRAIL combination indexes in control SW480 cells (Mock) or SW480 cells expressing ezrin S66A or S66D. Combination indexes (CIs) values 1, <1 or >1 indicate an additive, synergistic or antagonistic effect, respectively. The dotted line in these isobolograms curves represents theoretical additivity CI values. Data points below or above this line indicate synergy or antagonism.

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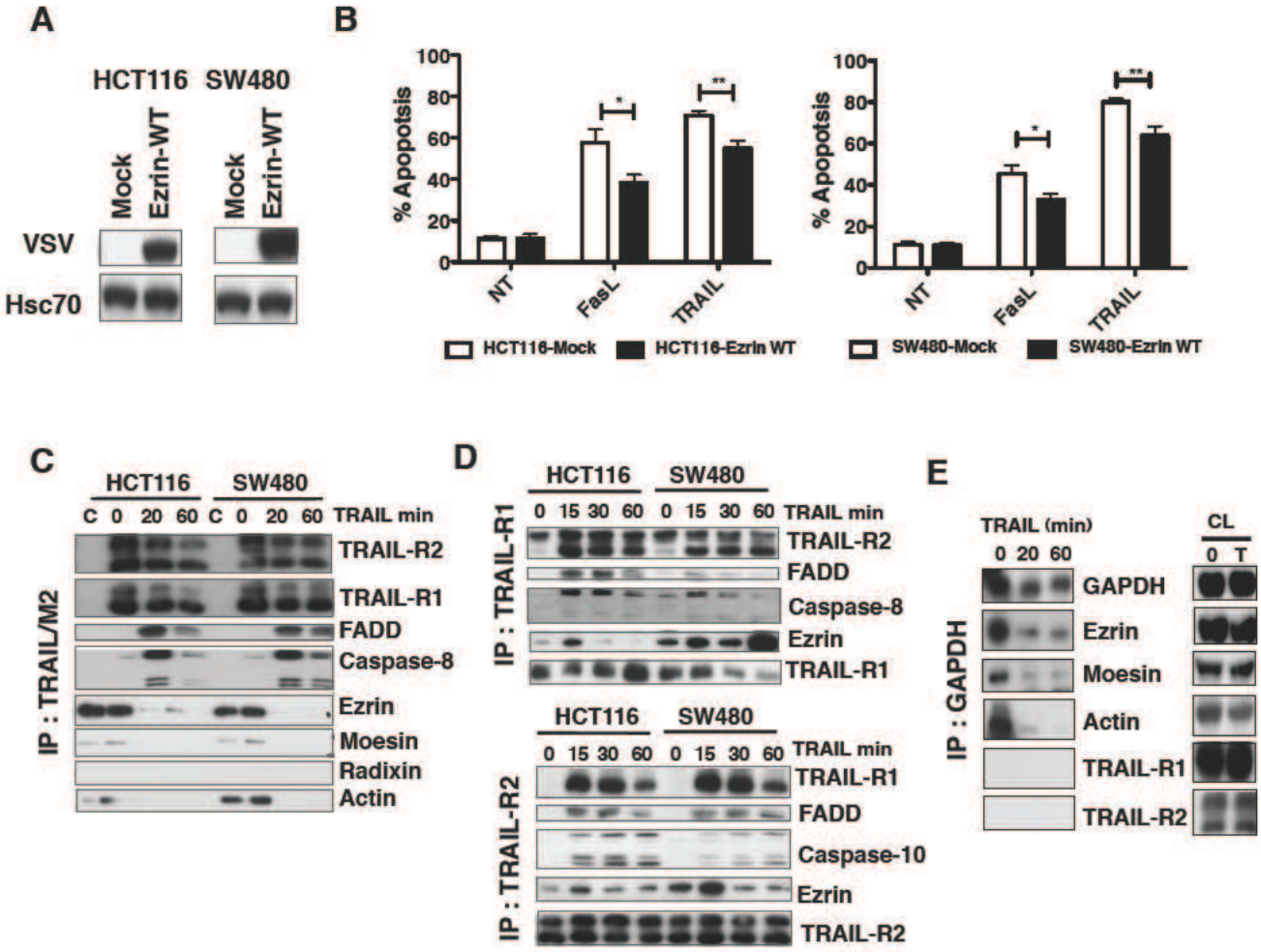

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Figure 1

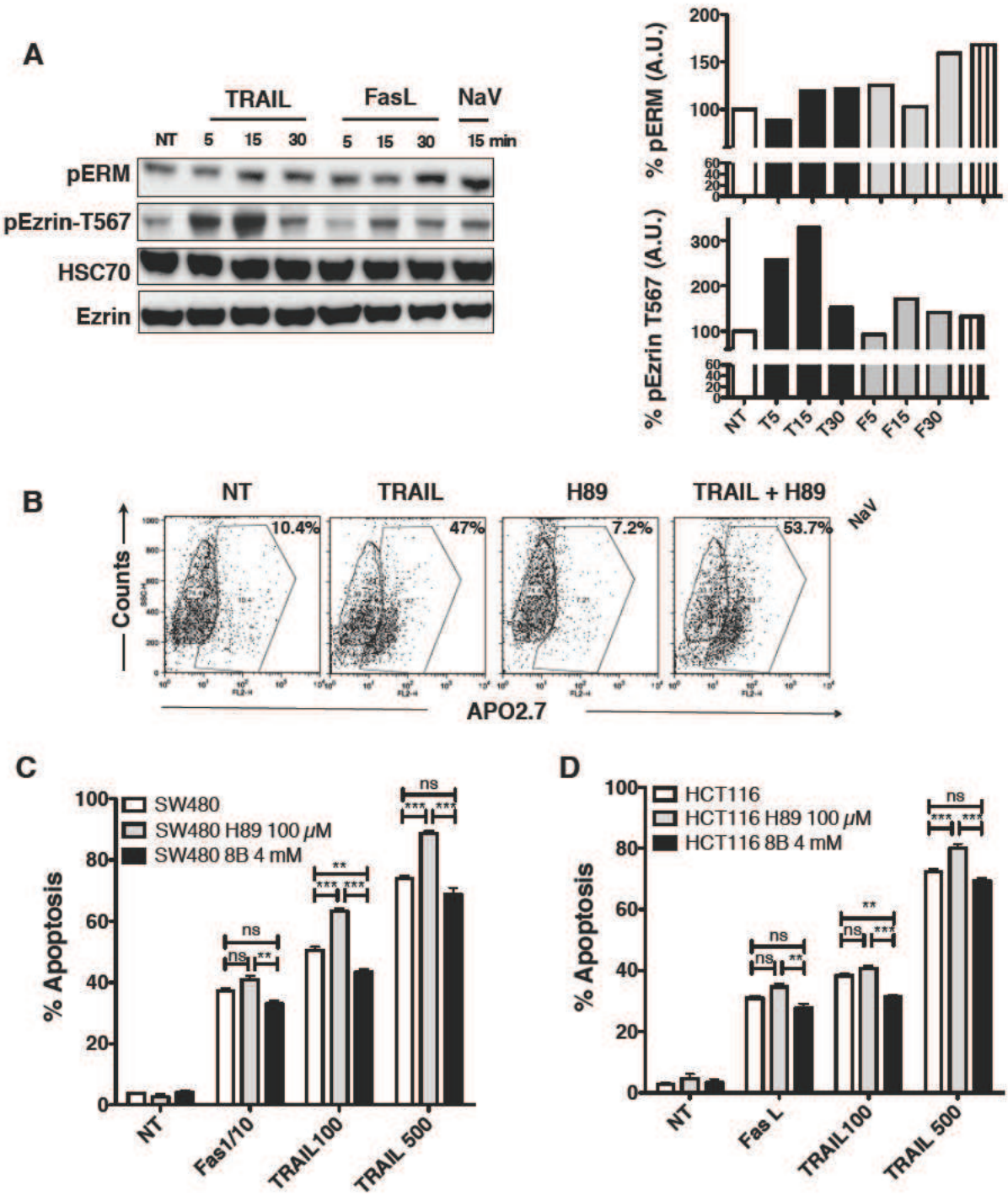

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Figure 2

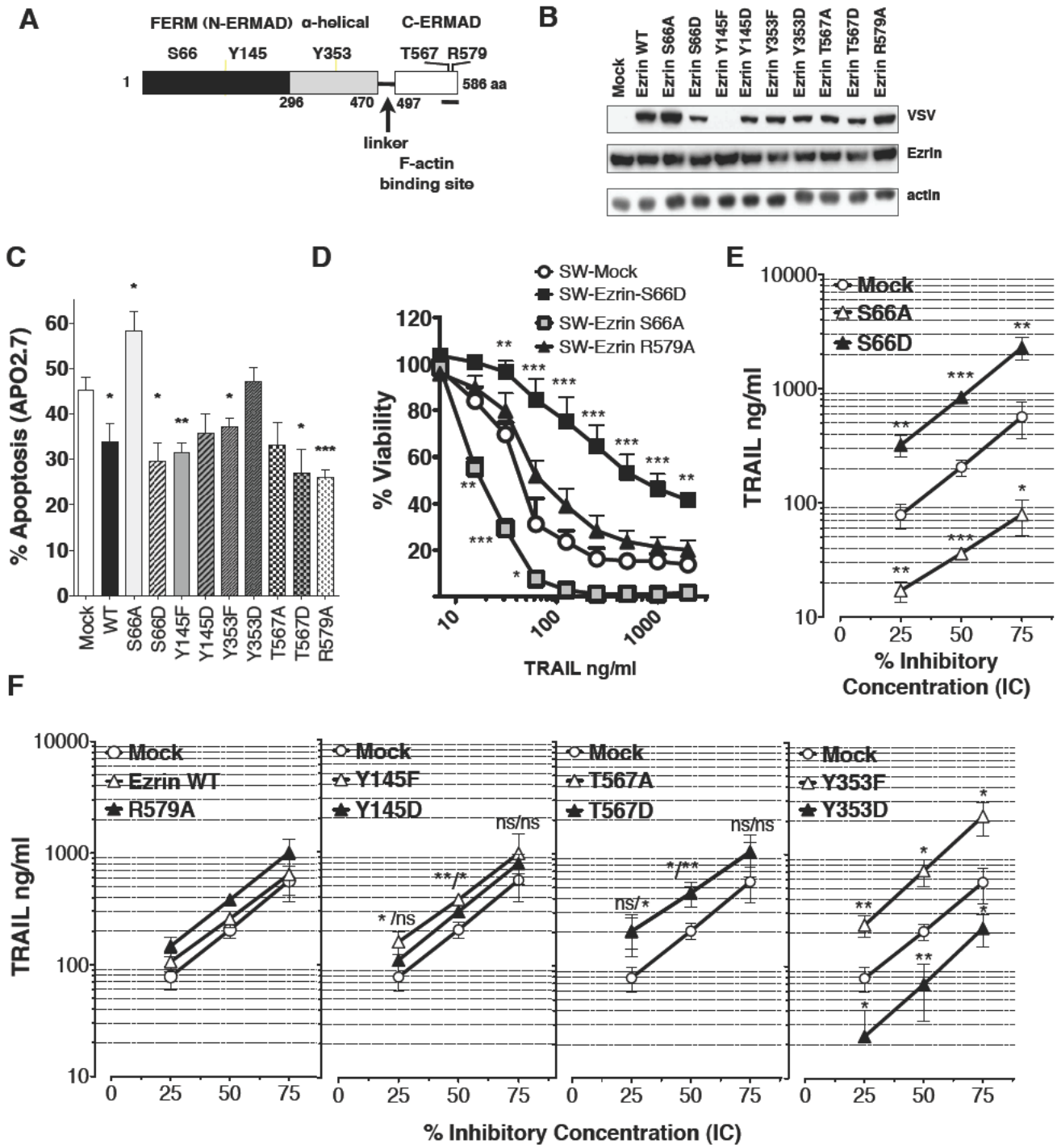

A
FERM (N-ERMAD)
α-helical
C-ERMAD
S66
Y145
Y353
T567
R579
1
586 aa
296
470
497
linker
F-actin
binding site
B
Mock
Ezrin WT
Ezrin S66A
Ezrin S66D
Ezrin Y145F
Ezrin Y145D
Ezrin Y353F
Ezrin Y353D
Ezrin T567A
Ezrin T567D
Ezrin R579A
VSV
Ezrin
actin
C
% Apoptosis (APO2.7)
Mock
WT
S66A
S66D
Y145F
Y145D
Y353F
Y353D
T567A
T567D
R579A
D
SW-Mock
SW-Ezrin-S66D
SW-Ezrin S66A
SW-Ezrin R579A
% Viability
TRAIL ng/ml
E
Mock
S66A
S66D
TRAIL ng/ml
% Inhibitory
Concentration (IC)
F
Mock
Ezrin WT
R579A
Y145F
Y145D
T567A
T567D
Y353F
Y353D
TRAIL ng/ml
ns/ns
% Inhibitory Concentration (IC)

Figure 3

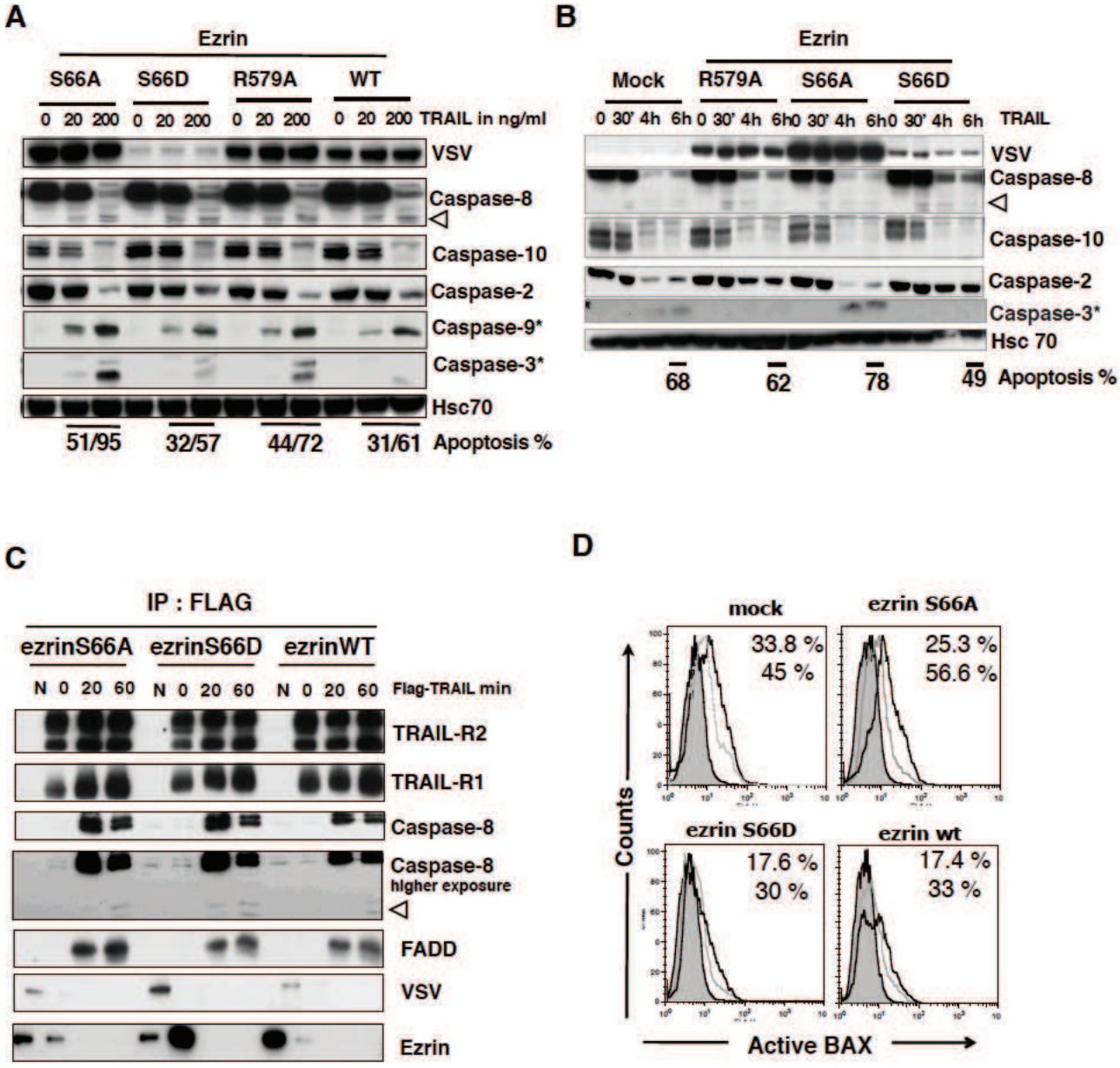
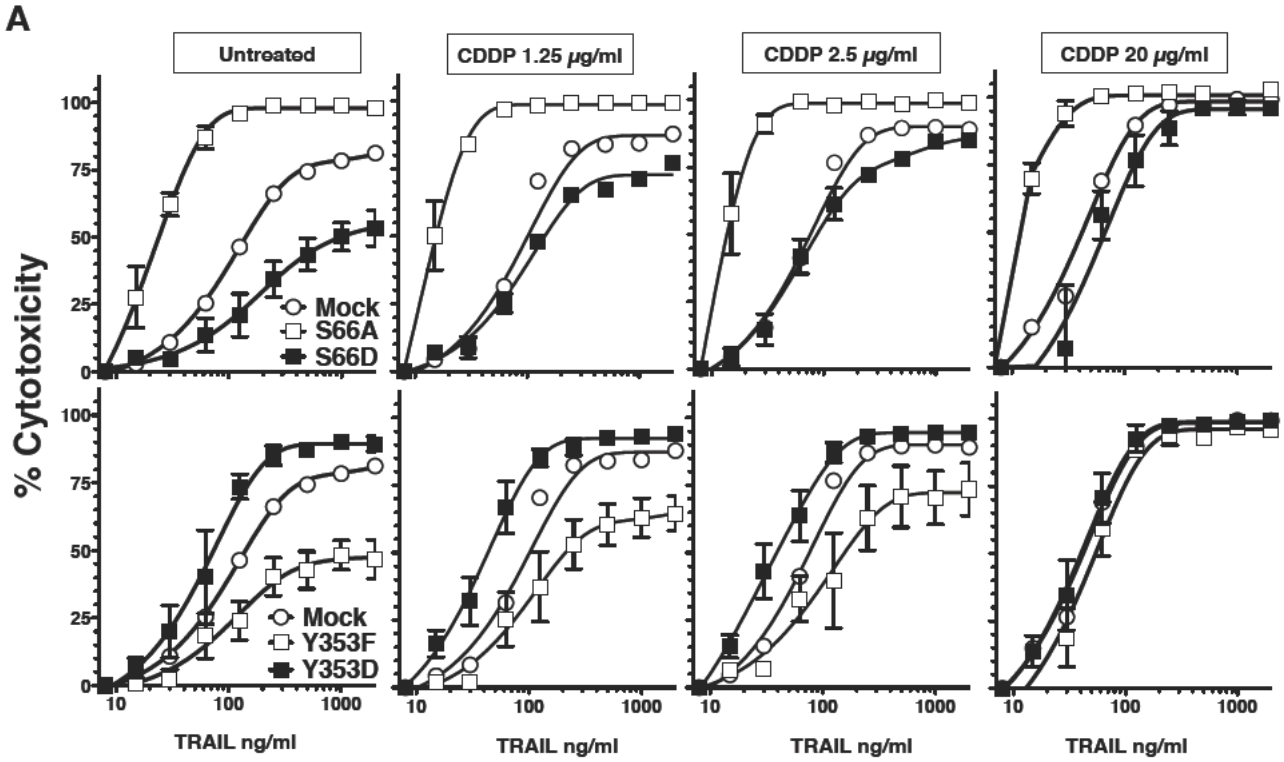

A
Ezrin
S66A
S66D
R579A
WT
0 20 200 0 20 200 0 20 200 0 20 200 TRAIL in ng/ml
VSV
Caspase-8
Caspase-10
Caspase-2
Caspase-9*
Caspase-3*
Hsc70
51/95
32/57
44/72
31/61 Apoptosis %
B
Ezrin
Mock
R579A
S66A
S66D
0 30' 4h 6h 0 30' 4h 6h 0 30' 4h 6h 0 30' 4h 6h TRAIL
VSV
Caspase-8
Caspase-10
Caspase-2
Caspase-3*
Hsc 70
68
62
78
49 Apoptosis %
C
IP : FLAG
ezrinS66A
ezrinS66D
ezrinWT
N 0 20 60 N 0 20 60 N 0 20 60 Flag-TRAIL min
TRAIL-R2
TRAIL-R1
Caspase-8
Caspase-8
higher exposure
FADD
VSV
Ezrin
D
mock
ezrin S66A
33.8 %
45 %
25.3 %
56.6 %
ezrin S66D
ezrin wt
17.6 %
30 %
17.4 %
33 %
Counts
Active BAX

Figure 4


A
Untreated
CDDP 1.25 μg/ml
CDDP 2.5 μg/ml
CDDP 20 μg/ml
% Cytotoxicity
Mock
S66A
S66D
Mock
Y353F
Y353D
TRAIL ng/ml
TRAIL ng/ml
TRAIL ng/ml
TRAIL ng/ml

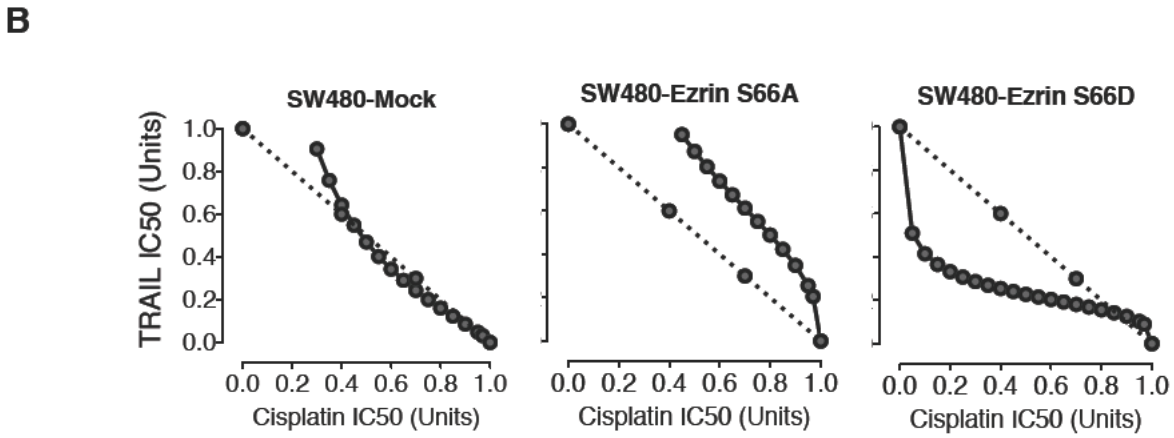

B
SW480-Mock
SW480-Ezrin S66A
SW480-Ezrin S66D
TRAIL IC50 (Units)
Cisplatin IC50 (Units)
Cisplatin IC50 (Units)
Cisplatin IC50 (Units)

Figure 5

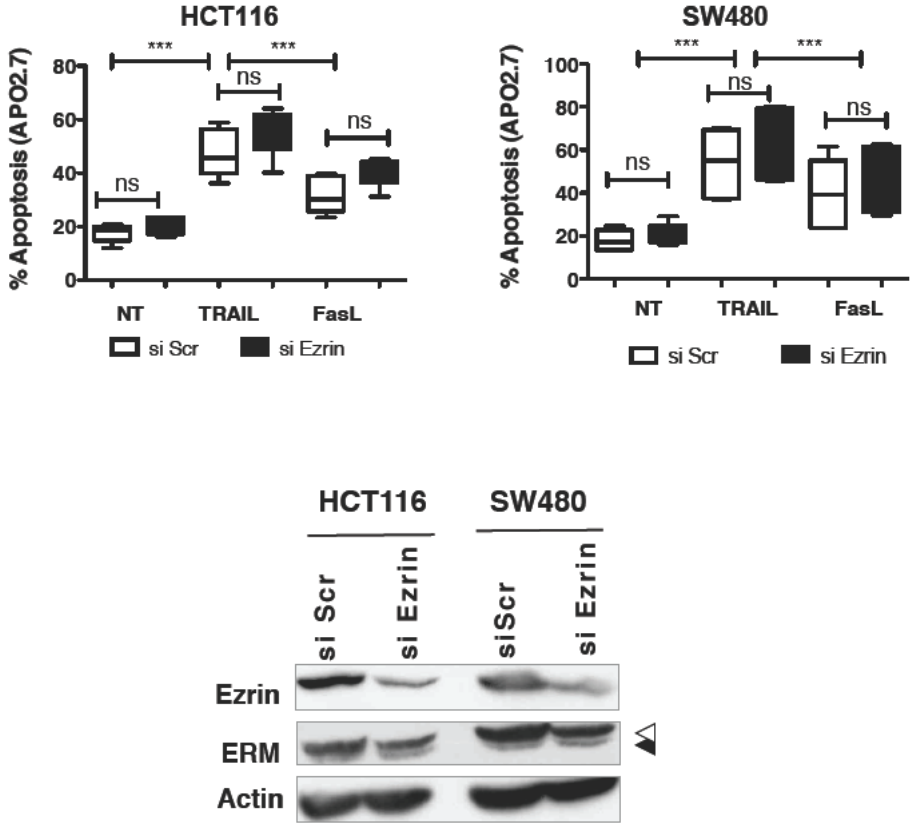
Supporting Table I

List of primers used to generate ezrin phosphorylation mutants

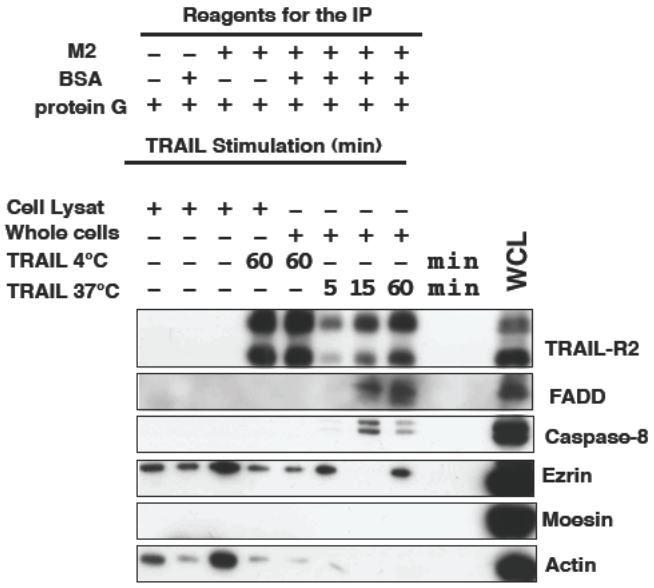
Ezrin S66A	**Forward**	5'-CTG GAT AAG AAG GTG GCT GCC CAG GAG GTC A-3'
	Reverse	5'-TGA CCT CCT GGG CAG CCA CCT TCT TAT CCA G-3'
Ezrin S66D	**Forward**	5'-GCT GGA TAA GAA GGT GGA TGC CCA GGA GGT CAG G-3'
	Reverse	5'-CCT GAC CTC CTG GGC ATC CAC CTT CTT ATC CAG C-3
Ezrin Y145F	**Forward**	5'-GTG CAC AAG TCT GGG TTC CTC AGC TCT GAG C-3'
	Reverse	5'-GCT CAG AGC TGA GGA ACC CAG ACT TGT GCA C-3'
Ezrin Y145D	**Forward**	5'-GTG CAC AAG TCT GGG GAC CTC AGC TCT GAG C-3'
	Reverse	5'-GCT CAG AGC TGA GGT CCC CAG ACT TGT GCA C-3'
Ezrin Y353F	**Forward**	5'-TGC TGC GGC TGC AGG ACT TTG AGG AGA AG-3'
	Reverse	5'-CTT CTC CTC AAA GTC CTG CAG CCG CAG CA-3'
Ezrin Y353D	**Forward**	5'-CTG CGG CTG CAG GAC GAT GAG GAG AAG ACA A-3'
	Reverse	5'-TTG TCT TCT CCT CAT CGT CCT GCA GCC GCA G-3'
Ezrin T567A	**Forward**	5'-GGG ACA AGT ACA AGG CCC TGC GGC AGA TCC G-3'
	Reverse	5'-CGG ATC TGC CGC AGG GCC TTG TAC TTG TCC C-3'
Ezrin T567D	**Forward**	5'-CCG GGA CAA GTA CAA GGA CCT GCG GCA GAT CCG GC-3'
	Reverse	5'-GCC GGA TCT GCC GCA GGT CCT TGT ACT TGT CCC GG-3'
Ezrin R579A	**Forward**	5'-TCG AAC TCG TCG ATG GCC TGC TTG GTG TTG CC-3'
	Reverse	5'-GGC AAC ACC AAG CAG GCC ATC GAC GAG TTC GA-3'

Supporting Table II

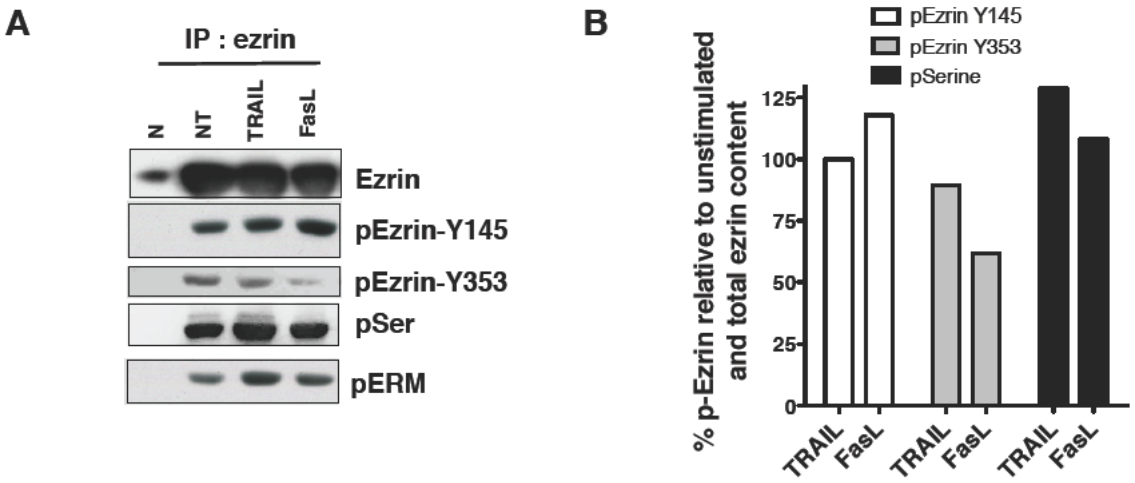
	IC 25	SD	IC 50	SD	IC 75	SD
Mock	**78**	19	**205**	33	**565**	198
Ezrin WT	**106**	20	**258**	44	**644**	229
Ezrin S66A	**17**	3	**36**	3	**79**	27
Ezrin S66D	**320**	70	**841**	14	**2288**	525
EzrinY145F	**160**	37	**380**	42	**978**	465
Ezrin Y145D	**110**	9	**297**	23	**795**	155
Ezrin Y353F	**234**	50	**717**	194	**2206**	726
Ezrin Y353D	**24**	16	**69**	37	**220**	71
Ezrin T567A	**203**	81	**447**	113	**1051**	430
Ezrin T567D	**205**	64	**450**	60	**1031**	236
Ezrin R579A	**147**	30	**382**	50	**1009**	313



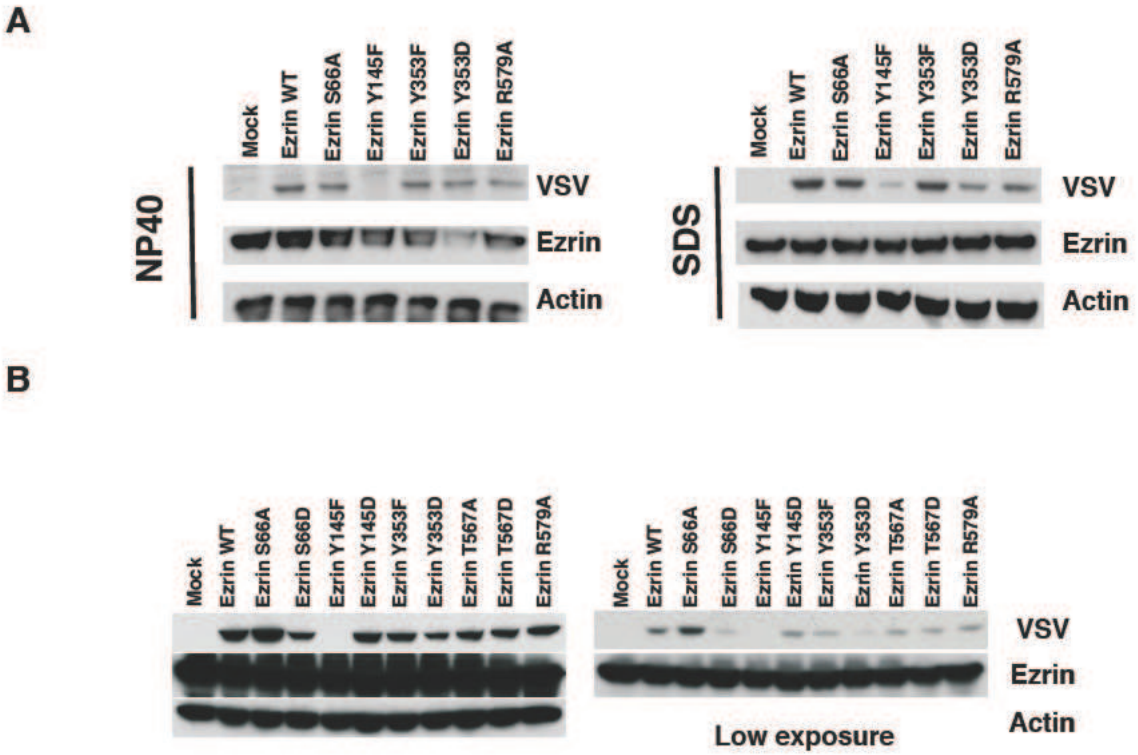
Supplemental Figure 1



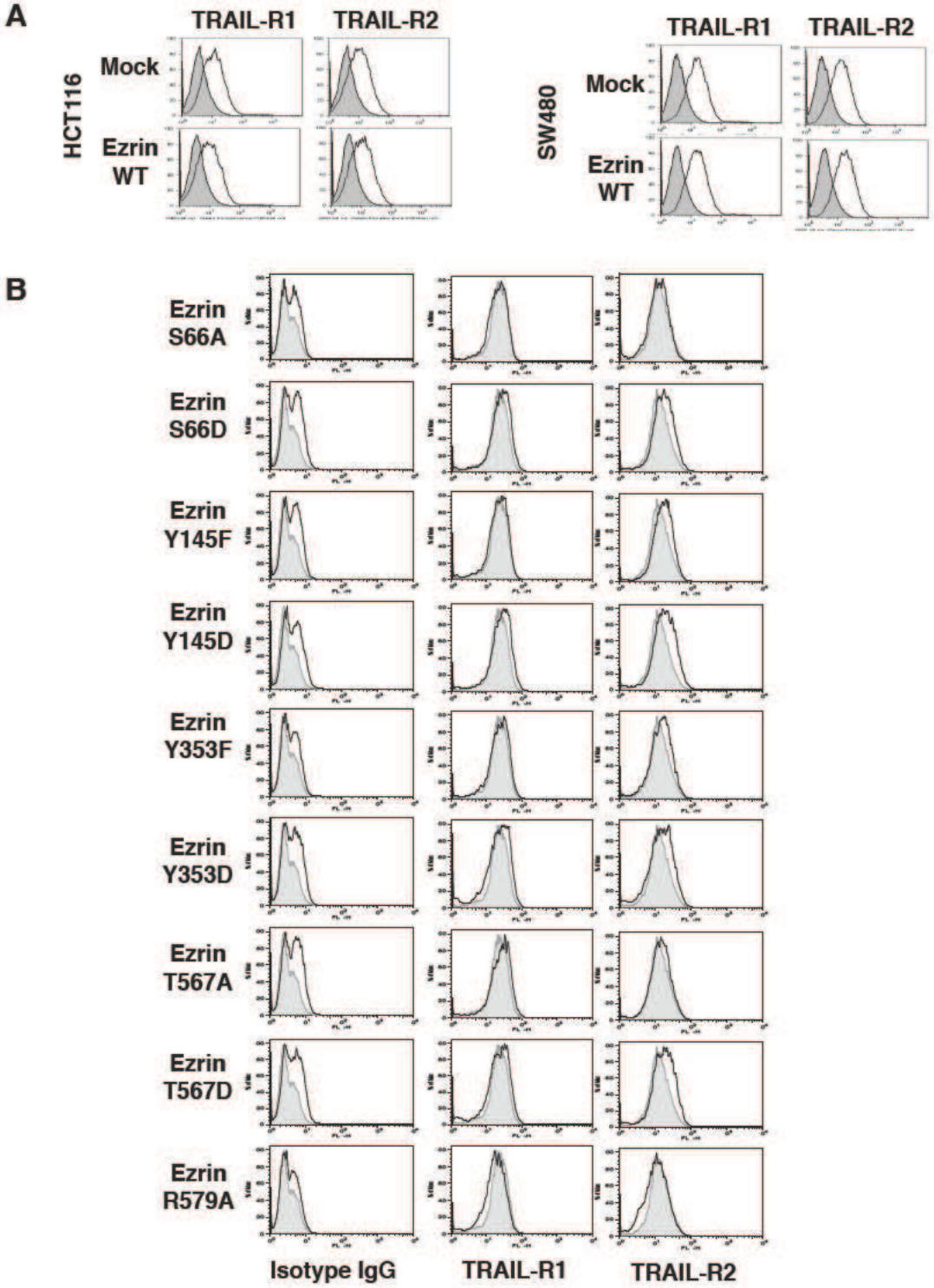
Supplemental Figure 2



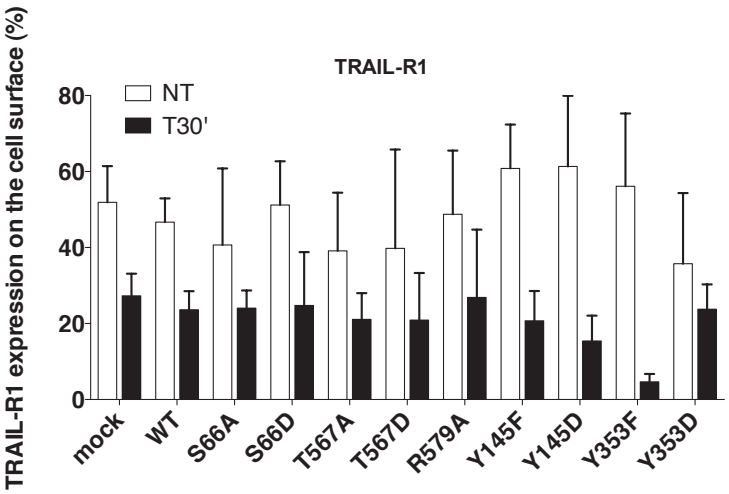
Supplemental Figure 3

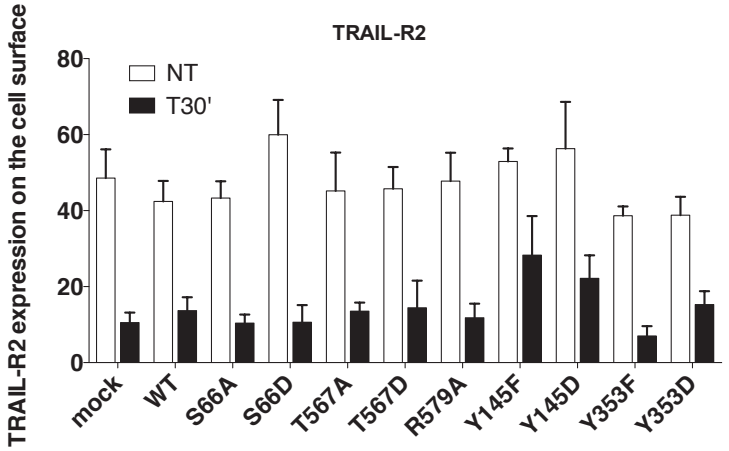


Supplemental Figure 4

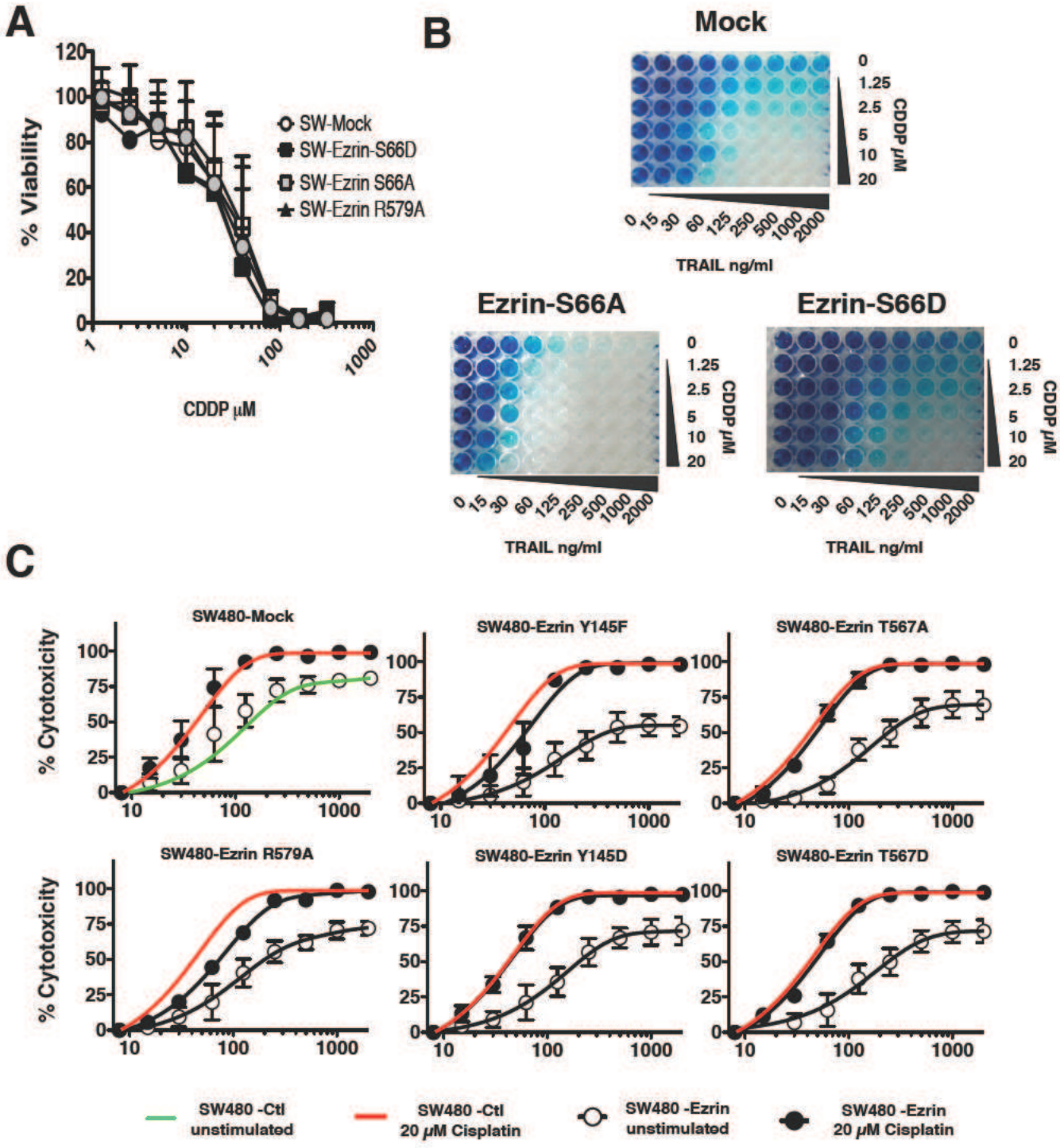


Supplemental Figure 5





Supplemental Figure 6



Supplemental Figure 7

Supplemental Materials and Methods Iessi et al.

Chemical. Reagents have been described in the manuscript.

Antibodies.

Anti-phospho-ezrin (Tyr 145) was obtained from Santa Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France). The anti-ERM (ezrin /radixin /moesin), phospho-ezrin (Tyr353), phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr 558) antibodies were from Cell Signaling (Ozyme, Saint Quentin en Yvelines, France). The anti-phosphoserine antibody was purchased from Sigma-Aldrich (Lyon, France). For flow cytometry experiments, anti-TRAIL-R1 (clones wB-K32 or wB-N36) and anti-TRAIL-R2 (clones B-K29 or B-B42) antibodies were kindly provided by Gen-Probe (Diaclone, Besançon, France). For immunoprecipitation, the anti-ezrin (clone 3C12) antibody was purchased from Sigma-Aldrich. Remaining antibodies were described in the manuscript.

Ezrin gene silencing using siRNAs.

Ezrin SiGenome SMART pool siRNAs (set of 4) were purchased from Thermo Scientific (Dharmacon Division). For siRNA-mediated ezrin downregulation, 500 000 cells were transfected with a scramble or ezrin targeting siRNAs using Dharmafect-4 reagent (Dharmacon Division) according to the manufacturer's specifications. 48 hours after transfection, the ezrin expression level was determined by western blot and sensitivity to TRAIL or Fas ligand was assessed by APO2.7 staining.

Receptor internalization.

500 000 cells, treated or not with 1 µg/ml His-TRAIL for 30 minutes at 37°C, were harvested and stained with an anti-TRAIL-R1 or TRAIL-R2 antibody (Diaclone) before being analyzed by flow cytometry. 10 000 events were analyzed using a Becton Dickinson FACScan machine (BD Biosciences).

Remaining methods are described in the manuscript.

SUPPLEMENTAL FIGURE LEGENDS Iessi et al.

Supplemental figure 1. Small interfering RNA-mediated ezrin downregulation. 48 hours after transfection with control or ezrin siRNA, HCT116 or SW480 cells were treated with 500 ng/ml His-TRAIL or 100 ng/ml Fas ligand for 6 hours, stained with APO2.7 antibody and analyzed by flow cytometry. Data represents mean plus or minus SD of three different experiments. (B) 48 hours after transfection, cells were lysed and analysed by western blot. Anti-ERM antibody recognizes ezrin (white arrow), moesin (black arrow) and radixin.

Supplemental figure 2. TRAIL DISC immunoprecipitation with protein G beads saturated or not with BSA. SW480 cells were stimulated with 5 µg/ml Flag-TRAIL cross-linked with 10 µg/ml anti-Flag (M2) antibody, then lysed. The DISC was immunoprecipitated using protein G beads saturated or not overnight at 4°C with BSA and analyzed by western blot for TRAIL DISC components, moesin, actin and ezrin.

Supplemental figure 3. TRAIL induces ezrin phosphorylation on Tyr 145 and serine 66. (A) SW480 cells were stimulated with 500 ng/ml His-TRAIL or 100 ng/ml Fas ligand for 15 minutes or left untreated. After cell lysis in NP-40-containing buffer, ezrin was immunoprecipitated with an anti-ezrin antibody (clone 3C12). The level of ezrin phosphorylation was determined by western blot using anti-phospho-ezrin targeting tyrosines 353 and 145, anti-phospho-ERM recognizing phosphorylated-ezrin on threonin 567, -moesin on threonine 558 and -radixin on threonin 564 and an anti-pan phosphoserine. (B) % of ezrin phosphorylation relative to total ezrin immunoprecipitated was determined using ImageJ.

Supplemental figure 4. Expression of ezrin phosphorylation mutants and effect on FasL-induced cell death in SW480 cells. (A) Expression levels of ezrin constructs were determined by western blot using an anti-VSV, anti-ezrin and anti-actin antibodies (loading control). Data shown is representative of three independent experiments in NP40-soluble fraction as compared to whome cell lysats obtained using SDS. (B) Representative chemoluminescent high or low exposure time, allowing comparison of expression levels of ezrin phosphomutants to endogenous ezrin. (C) Effect of ezrin WT or ezrin phosphomutants expression on Fas ligand-induced cell death in SW480 cells. Each cell population was left untreated or stimulated with Fas ligand 100 ng/ml for 6 hours. Apoptosis was measured by APO2.7 staining by flow cytometry. Data represent mean plus or minus SD of at least 3 independent experiments (*$P<0.05$; **$P<0.01$; ***$P<0.001$ respective to Mock control cells).

Supplemental figure 5. TRAIL-R1 and TRAIL-R2 cell surface expression in ezrin WT or phosphomutants SW480 cells. (A) Expression of agonistic TRAIL receptors was analyzed both in HCT116 or SW480 cells expressing ezrin WT as compared to Mock-infected cells. (B) Flow cytometry analysis of TRAIL-R1 or TRAIL-R2 expression levels in SW480 cells expressing ezrin phosphomutants-expressing (unfilled peaks). Filled peaks correspond to matching isotype control antibody staining.

Supplemental figure 6. TRAIL receptor internalization in SW480 cells expressing ezrin phosphomutants. Expression of TRAIL-R1 and TRAIL-R2 at the cell surface of control or ezrin phosphomutants-expressing SW480 cells was analyzed by flow cytometry after stimulation with 1 µg/ml TRAIL for 30 minutes (T30', Black bars). Data represents mean plus or minus SD of at least three different experiments.

Supplemental figure 7. Cellular viability assays after CDDP and TRAIL treatments. (A) Cell Ezrin S66A, ezrin S66D, ezrin R579A or Mock infected SW480 cells were stimulated with increasing concentrations of cisplatin for 48 h and cell viability was determined by methylene blue assay. (B) Ezrin-S66A, -S66D or mock expressing SW480 cells were treated for 48 hours with increasing concentrations of CDDP and subsequently stimulated with increasing concentrations of His-TRAIL for 16 hours. Cell viability was assessed by methylen blue. One representative exemple of 96-wells plates used to quantify cell viability is shown here. (C) Percentage of TRAIL cytotoxicity was evaluated as described panel B, by methylene blue assay, for the indicated ezrin phosphomutants as compared to Mock-infected cells, after cisplatin pretreatment.

Supplemental Table I: List of primers used to generate ezrin phosphorylation mutants.

Supplemental Table II: Calculated TRAIL inhibitory concentrations in ezrin phosphomutants-expressing SW480 cells, using CompuSyn. IC25, IC50 and IC75 percent values correspond to the mean of 4 independent experiments. Mean and SD shown in this table were used to plot figure 3 panels E and F, respectively.

5. Discussion

Ezrin is a member of the Ezrin, Radixin, Moesin (ERM) family of proteins that link various integral membrane proteins to the actin cytoskeleton. There is much evidence in the literature highlighting the importance of the ezrin-mediated association of Fas with the actin cytoskeleton in the first steps of Fas-mediated cell death in T cells, leading to Fas aggregation, caspase-8 activation and receptor internalization, and demonstrating an active role of the actin cytoskeleton in the Fas-activated death signal (Hébert et al, 2008; Chakrabandhu et al, 2007).

The cytokine TRAIL is expressed on the surface of cells of the immune system where it is involved in innate immune responses, homeostasis of T cells, and antitumor responses. TRAIL is considered a new and interesting molecule in cancer therapy due to its ability to kill cancer cells with minimal toxicity against normal cells. Laboratory experimentation over the last few years has given a good understanding of the events occurring during the TRAIL signalling pathway, and of the factors involved in its regulation. However, very little is known about the association of TRAIL agonistic receptors with the actin cytoskeleton. In particular, the function of ezrin in the TRAIL pathway remains largely unstudied. In light of the current use of TRAIL as a therapeutic agent against malignant diseases, it is important to understand how TRAIL-induced cell death is regulated by ezrin. This knowledge will help to better characterize the molecular mechanisms responsible for cancer cells' responsiveness or unresponsiveness to death stimuli. It will also help to enhance our knowledge in this field in order to develop alternative ways to obtain an effective regulation of TRAIL receptor-induced cell death. In addition, better understanding of how TRAIL-induced cell death is regulated by ezrin could open the way for a more effective use of TRAIL in a clinical setting.

In light of these considerations, the main goal of the thesis was to establish and define the role of ezrin in the TRAIL pathway. During the study we sought to:

1. Characterize the association of ezrin with the agonistic TRAIL receptors and its possible participation in TRAIL DISC formation;
2. Investigate the involvement of ezrin phosphorylation in conferring cell sensitivity to TRAIL-induced apoptosis;
3. Define the molecular mechanisms underlying the role of ezrin in regulating TRAIL signalling;
4. Analyse combined treatments, chemotherapeutic drugs plus TRAIL, in restoring tumour cells sensitivity to apoptosis.

5.1. Ezrin, moesin and actin bind in a nonspecific manner protein G-sepharose used to perform immunoprecipitations

Several groups have been working on the role of ezrin in Fas-mediated cell death. Many studies have described a basal association of Fas with ezrin in T cells in non stimulated conditions (Parlato et al, 2000; Lozupone et al, 2004; Hébert et al, 2008; Kuo et al, 2010). This connection was reported to be an important feature during the first step of the Fas-mediated cell death in T cells (Parlato et al, 2000; Lozupone et al, 2004; Hébert et al, 2008; Chakrabandhu et al, 2007).

In this study we were not able to demonstrate a direct interaction between ezrin and any of the TRAIL agonistic receptors, contrary to what has been published with Fas. Recruitment of ezrin to the TRAIL-R1 and TRAIL-R2 receptors observed in TRAIL DISC analysis by immunoprecipitation appeared to be nonspecific. Contrary to most DISC components, ezrin staining was not associated in a time-dependent manner, but probably and more importantly occurred in a rather inconsistent

manner. Indeed, ezrin, moesin and actin were immunoprecipitated not only from non stimulated cell lysates incubated with a non relevant antibody, but also from non stimulated or TRAIL-stimulated cell lysates immunoprecipitated with TRAIL ligand, TRAIL-R1 and TRAIL-R2 antibodies. We also showed that this nonspecific binding of ezrin, moesin, and actin was due to association of these proteins with either polymers of sepharose or agarose crosslinked to protein G or A. Saturation with BSA was still not enough to abrogate the nonspecific binding of ezrin, moesin and actin to protein G-sepharose beads.

This data provided evidence, for the first time, that cytoskeletal proteins such as ezrin, moesin, and actin are able to bind polymers in a nonspecific manner, such as the sepharose coupled to protein G that is commonly used to perform immunoprecipitations. The nonspecific binding observed is probably due to high expression of these proteins in cancer cells and, at the same time, to the large amount of cells needed to perform the immunoprecipitations. Alternatively, post-translational modifications, including differential phosphorylation status of the globular domain of these proteins may account for nonspecific binding. It is well-known that phosphorylation of ezrin at specific sites is required for its activation and for its subsequent binding to proteins. Moreover, other phosphorylation sites within the protein have been described to be involved in its regulation. For instance, phosphorylation of serine 66 within the N-ERMAD globular domain of ezrin regulates its interaction with WWOX (Jin et al, 2006). Ezrin phosphorylation may thus in turn change its conformation (Bretscher et al, 1997) and similar to requirements for WWOX binding (Jin et al, 2006), allow interaction with protein A or G. In agreement with this hypothesis, and albeit no anti-phospho-ezrin at serine 66 antibodies are so far available, ezrin phosphorylation at tyrosine 353 has been proposed to be a potent prognostic predictor for pancreatic cancers (Cui et al, 2010). Furthermore, it should be stressed that the main function of these kind of

proteins is to bind to other proteins. Indeed, the most recognized function of ezrin is to connect membrane protein to the actin cytoskeleton. There is much data in the literature reporting that ERM proteins bind to several different types of membrane proteins involved in various functions, such as determination of cell shape, cell to cell adhesion, cell adhesion to the extracellular matrix, migration (Bretscher et al, 2002), phagocytosis (Lugini et al, 2003), cannibalism (Lugini et al, 2006), metastatic behaviour of cancer cells (Federici et al, 2009), and acquired multidrug resistance (Luciani et al, 2002). This well-know function of ezrin could also contribute for its binding to protein A or G.

The questions of whether ezrin specifically interacts with TRAIL receptors, or whether ezrin can be considered a new component of the TRAIL DISC remain open as these possibilities cannot completely be excluded from the experimental settings that we have gathered during the course of this study. Further experiments might be required to get the answer. One possible approach to avoid nonspecific binding of ezrin, that was not used in this thesis, could be an immunodepletion approach. This could possibly be a more effective method than using BSA, used herein, to lessen background. Alternatively, co-localization experiments may prove an answer. It could be interesting to use a duo-link or a FRET approach to determine whether ezrin is present within the TRAIL DISC at the steady state or is recruited in a ligand-dependent manner.

5.2. Is ezrin a positive or negative regulator in the TRAIL signalling pathway?

At the beginning of this project, the contribution of ezrin towards cell death induced by Fas ligand was extensively described, and a positive regulatory function was proposed for this ERM protein. However, very little was known about the role of ezrin in the TRAIL signalling pathway. Indeed, only recently the Kuo group highlighted the fact that ezrin can act as a negative regulator of TRAIL-induced cell death in the type I T cell line H9 (Kuo et al, 2010). It was therefore of interest to investigate, define, and characterize the exact involvement of this ERM protein in the TRAIL pathway.

In agreement with the study of Kuo et al, 2010, our results indicated that ezrin in fact displays a negative function towards Fas ligand- and TRAIL-induced apoptosis in the colon carcinoma cell lines HCT116 and SW480. Indeed, if we ectopically express a chimeric ezrin WT in colon carcinoma cancer cells, we significantly inhibit Fas ligand- and TRAIL-induced cell death, and we do not recover variations in receptor cell surface expression. These results are in discrepancy with many of the previous findings about the involvement of ezrin in the Fas pathway. Indeed, the linkage of Fas to the actin cytoskeleton through ezrin has been shown to be required for the Fas polarization in uropodal structures (Parlato et al, 2000), and is involved in the first steps of the Fas-mediated cell death in T cells, leading to Fas aggregation and caspase-8 activation (Hébert et al, 2008), or assembly of the DISC in type I cells (Algeciras-Schimnich et al, 2002). Ezrin has also shown to play an essential role in Fas internalization after receptor triggering, contributing to DISC assembly and caspase activation that lead to cell death in the mouse fibroblast cells L1210 (Chakrabandhu et al, 2007). All this evidence points to an active role of the actin cytoskeleton in Fas-activated death signalling, and

highlights the linkage of Fas to the actin cytoskeleton though ezrin as an important event in mediating different steps of the Fas pathway. Why then, in colon carcinoma cancer cells, would ezrin play a negative function in regulating the Fas and TRAIL pathways? First of all, one may distinguish lymphoid tumours from epithelial-derived tumours, and it cannot be excluded that cell specificities may relate to the differential behaviour of ezrin regarding the death receptor machinery. Differences may arise due to differential expression levels or post-translational modifications that may alter ezrin subcellular compartmentalisation. Although a comparison between epithelial-derived and blast-derived tumours regarding these points has not, to the best of our knowledge, been published so far, it has been demonstrated that colon carcinoma cells express high levels of ezrin (Wang et al, 2009; Patara et al, 2011) and that ezrin is typically redistributed to the cytoplasmic region in these tumours (Elzagheid et al, 2008). Thus it would be expected that this excess of ezrin, together with its cytoplasmic localization, may prevent actin cytoskeleton reorganization during apoptosis. Lack of ezrin reorganisation may be counterproductive for an efficient signalling for cell death. Moreover, a great deal of data is available in the literature, supporting a critical role for ezrin over-expression in tumour progression to malignancy. Thus cancer cells may activate specific signalling pathway in order to be protected against cell death. In this view, ezrin may indirectly maintain Fas and TRAIL-R in a condition that does not allow their complete triggering or their correct clustering, leading to decreased cancer cell responsiveness to TRAIL-induced apoptosis.

Furthermore, interfering with the ezrin to Fas association through the expression of a dominant negative form of ezrin (ezrin-moesin) missing the binding domain for Fas (Lozupone et al, 2004), had no impact regarding the sensitivity of colon cancer cells to TRAIL and Fas ligand-induced apoptosis. We also observed contradictory results in HeLa cervical cells following the generation of two different

stable populations expressing the mutant form of ezrin. In fact, the marked inhibition of cell death induced by TRAIL and Fas ligand observed in the first stable HeLa population generated was not reproducible and was restricted to the first infection performed only. The findings obtained from these experiments are therefore not conclusive, and cannot be used to draw any meaningful conclusions, especially because our positive control, the effect of mutant ezrin expression on Fas-mediated cell death, did not work. In our experimental conditions, the mutant did not show inhibition of Fas ligand-induced apoptosis. Indeed, it was demonstrated that impairing Fas linkage to the actin cytoskeleton through expression of a dominant negative form of ezrin, which prevents the actin linkage to Fas, protected T cells from Fas-mediated cell death (Lozupone et al, 2004).

We also found a slight increase in Fas ligand- and TRAIL-induced cell death in absence of ezrin or moesin. However the statistical analysis showed that the difference was not significant. More experiments may be required to strengthen the analysis. Even if the ezrin and moesin downregulation was not complete, our data are in agreement with the ezrin WT over-expression experiments and with the study of Kuo et al, 2010, where it was shown that ezrin depletion enhanced Fas ligand- as well as TRAIL-induced apoptosis in normal T lymphocytes and H9 cells (Kuo et al, 2010). Moreover, these results possibly support our hypothesis of a negative role of ezrin in receptor-mediated apoptosis in colon cancer cells. However, since ezrin and moesin downregulation was not complete and since the statistical analysis remains poorly significant, these findings remain uncertain.

We have no good explanation for the low effect observed after ezrin and moesin downregulation. We can only speculate that the incomplete ezrin or moesin knockdown was due to the relatively low turnover of the protein, or its high abundance in these colon carcinoma cell lines and that ezrin's regulatory potential only requires a small proportion of total ezrin. Moreover, upon careful examination

of films with a higher exposure from the western blots to determine ezrin and moesin levels (data not shown), no appreciable reduction in the levels of these proteins were apparent after transfection with their respective interfering RNAs, as compared to cells transfected with the appropriate control RNAs. In addition, it should be stressed that ERM share an high sequence homology to each other. So, repression of one ERM protein by RNA interference-induced gene knockdown may be compensated by another protein from this family.

5.3. Ezrin phosphorylation in the TRAIL pathway

It has been reported that ezrin phosphorylation or dephosphorylation status could regulate cell sensitivity to Fas ligand-induced cell death. Indeed, ERM inactivation through dephosphorylation was associated with cell sensitivity to Fas-mediated cell death in activated T cells (Ramaswamy et al, 2007). Whereas ROCK I-mediated ezrin phosphorylation on threonine 567 induced by an anti-Fas antibody was shown to facilitate Fas aggregation, caspase-8 activation and apoptotic signalling in Jurkat cells (Hébert et al, 2008). Another study showed that a tight control of ezrin activation through phosphorylation is necessary for correct Fas signalling (Piazzolla et al, 2005).

Taking these findings into account, together with the general view that kinase activation is generally dysregulated in tumour cells as compared to normal cells, we analysed: (i) ezrin phosphorylation status after triggering; and (ii) how ezrin phosphorylation could modulate the ezrin-mediated inhibition of TRAIL-induced cell death.

Activation of the ERM proteins has been described to occur through phosphorylation on a specific, conserved threonine residue: Thr567 of Ezrin,

Thr564 of Radixin, and Thr558 of Moesin (Matsui et al, 1998). In this study, and consistent with the report of Hébert et al (2008), we found that both TRAIL and, to a lesser extent, Fas ligand induced ezrin phosphorylation on threonine 567 (Thr567) as demonstrated by western blot analysis on cell lysates. We also observed through ezrin immunoprecipitation that TRAIL could induce ezrin phosphorylation not only on the threonine 567 residue but also on tyrosine and serine residues. Surprisingly, Fas ligand showed the same profile for the ezrin phosphorylation as TRAIL, with the exception of the serine site.

This data suggests, for the first time, that there are possible differences in the pattern of ezrin phosphorylation between the TRAIL and Fas pathway, and provide a possible explanation for the differential behaviour of ezrin as regards these signalling pathways. Likewise, ezrin phosphorylation on serine may account for this difference. This hypothesis is further sustained by experiments using protein kinase A (PKA) inhibitors or activators, as well as ezrin phosphorylation mutants. Likewise, we found that inhibition or activation of PKA activity using pharmacological inhibitors moderately increased or decreased TRAIL-induced apoptosis in colon cancer cells, respectively. Interestingly, Fas ligand-induced cell death was not significantly affected by inhibition or activation of this kinase.

The importance of ezrin phosphorylation on serine 66 in TRAIL-induced cell death was definitively uncovered through the analysis of ezrin phosphorylation mutants targeting several major phosphorylation residues, including serine 66, threonine 567, and tyrosines 145 and 353. Ectopic expression of these ezrin mutants in SW480 cells allowed us to demonstrate that phosphorylation or dephosphorylation of ezrin at serine 66 or tyrosine 353 selectively contributes to modulate the sensitivity of colon cancer cells to TRAIL-induced cell death, but not apoptosis triggered by Fas ligand or cisplatin (CDDP). Remarkably, whereas phosphorylation variants on tyrosine 145 or threonine 567, or in the F-actin

binding site did not significantly modulate the sensitivity to TRAIL- and Fas ligand-induced cell death, mutation of serine 66 to alanine induced a marked and significant increase in apoptosis induced by TRAIL, but not by Fas ligand, as compared to wild-type or mock-infected cells. On the other hand, mutation of this serine to aspartate led to protection against TRAIL- but not Fas ligand-mediated cell death. Albeit to a lesser extent, a reverse phenotype was obtained with ezrin phosphorylation mutants targeting tyrosine 353.

We observed enhanced activation of caspase-3 and much higher levels of active Bax in SW480 cells expressing the nonphosphorylable mutant, ezrin S66A, than in the SW480 cell lines expressing ezrin S66D or ezrin WT. Although the execution of the apoptotic machinery was differentially enhanced or impaired in these cells, changes in caspase-3 and Bax activation were not associated with differential caspase-8 or caspase-10 recruitment or with changes in initiator caspase activation within TRAIL DISC, or with changes in TRAIL DISC component recruitment. Ezrin appeared to target the apoptotic machinery downstream of the TRAIL DISC as demonstrated by the modulation of Bax activation. Moreover, the ezrin-mediated TRAIL-cell death inhibition was not associated with variations in receptor membrane expression levels, or with changes in receptor internalization after TRAIL stimulation.

Our results highlighted a recently identified phosphorylation site in the ezrin, serine 66, as a specific and selective target of the TRAIL pathway but not of that of Fas or cisplatin in human colon carcinoma cancer cells. In addition, these results showed that another phosphorylation site could contribute to the ezrin-mediated TRAIL-induced cell death inhibition, such as the tyrosine 353. Remarkably, these results support the hypothesis that tight control of ezrin activation through phosphorylation is necessary for efficient TRAIL signalling. Indeed, ezrin phosphorylation or dephosphorylation events were shown to be able to modulate,

positively or negatively, cell sensitivity to TRAIL- but not Fas ligand-induced cell death. We can also speculate that ezrin activation through phosphorylation on serine 66 and/or dephosphorylation on tyrosine 353 could mediate events leading to specific inhibition of TRAIL signalling, while dephosphorylation of serine 66 and/or phosphorylation on tyrosine 353 would lead to other events that enhance execution of the apoptotic program. Phosphorylation or dephosphorylation of these residues is likely to favour or disrupt interactions with unknown or known ezrin protein partners. For example, PKA-mediated ezrin phosphorylation at serine 66 has been demonstrated to be required for ezrin to interact with the tumour suppressor gene WWOX (Jin et al, 2006). Whether this interaction contributes to TRAIL signalling inhibition is still under investigation.

Alternatively, we may expect that ezrin activation through phosphorylation on serine 66 and/or dephosphorylation on tyrosine 353 might activate nonapoptotic signalling pathways, which might be responsible for decreased responsiveness to TRAIL-induced apoptosis in colon cancer cells. The nonapoptotic pathways activated by TRAIL might be different than that of Fas. Thus they may account for other possible differences in the ezrin pattern of phosphorylation between the TRAIL and Fas pathway and provide another possible explanation for the differential behaviour of ezrin as regards to these signalling pathways. This aspect may further support the hypothesis of an indirect implication of ezrin in regulating death receptor signalling in colon cancer cells. Whether these nonapoptotic pathways contribute to inhibition of TRAIL signalling remains to be determined.

Lastly, another aspect that was not taken into account in this thesis could be represented by the role that phosphatases might play regarding the activation of ezrin within the TRAIL pathway. Phosphatases, likely kinases, might also play a prominent role because they can compensate for the effect of kinase activity. Interestingly, treatment of colon cancer cells with pharmacological agents inhibiting

or activating PKA activity resulted in a moderate increase or decrease in TRAIL-induced cell death respectively. These changes were not as marked as those observed with the ezrin phosphorylation mutants, but were similar to that of ezrin WT. It would be reasonable to say that the weaker effect of PKA inhibitors or activators on TRAIL-induced cell death is probably due to compensatory events involving phosphatases, as compared to mutants which are not subject to phosphatase activity. Thus, the balance between phosphatases and kinases within the cells could be an important determinant for the activation/inactivation of the ezrin, which might lead to the modulation of the TRAIL pathway. We can then conclude that a tight control of the phosphatases/kinases activity might be necessary for a correct and complete TRAIL signalling.

5.4. WWOX in the TRAIL pathway

Previous research has demonstrated that PKA-mediated phosphorylation of ezrin on the residue serine 66 regulates the interaction of ezrin with the tumour suppressor gene WWOX (Jin et al, 2006). Interestingly, our results indicate that WWOX depletion by RNA-interference gene knockdown strongly protects colon cancer cells from apoptosis induced by TRAIL, suggesting that WWOX may be involved in the regulation of TRAIL signalling. Thus we may expect that the ezrin-mediated inhibition of TRAIL-induced apoptosis occurs through the direct association of ezrin with WWOX. The findings that WWOX may be involved in the TRAIL signalling, together with the assumption that the PKA-mediated ezrin phosphorylation on serine 66 specifically affects TRAIL-induced apoptosis, may lead to the following working model (**Figure 5.1**). Stimulation with TRAIL triggers cell death and may also activate intracellular signalling pathways, which leads to the

activation of the PKA kinase. PKA in turn phosphorylates ezrin on the residue serine 66. Serine 66 phosphorylated ezrin then interacts with and sequesters WWOX. The ezrin association with WWOX might then impair the activation of WWOX and its subsequent translocation to the mitochondria, leading to the inhibition of the TRAIL signalling pathway (**Figure 5.1**).

The molecular mechanisms leading to the inhibition of TRAIL-induced apoptosis by ezrin remain an open question and are still under investigation. Interestingly, it has been described that in response to TNF-α, staurosporine, and ultraviolet radiation, WWOX becomes activated via phosphorylation on tyrosine residue 33 (Chang et al, 2005) by the Src kinase (Aqeilan et al, 2004). The Src kinase is also known to phosphorylate ezrin at tyrosine 477 (Heiska and Carpén, 2005), and this residue of ezrin has been reported to be essential for the ezrin-WWOX interaction (Jin et al, 2006). Thus we may expect that TRAIL could trigger the activation of the PKA and Src kinases. Src in turn may induce the activation of WWOX, and at the same time the ezrin phosphorylation on tyrosine 477. Tyrosine 477 phosphorylated ezrin then interacts with WWOX, impairing the formation of p53-WWOX complexes and their translocation to the mitochondria to mediate apoptosis. Work is in progress to investigate this possibility and also to elucidate the molecular mechanisms of the ezrin-mediated inhibition of TRAIL-induced apoptosis based on the ezrin-WWOX interaction.

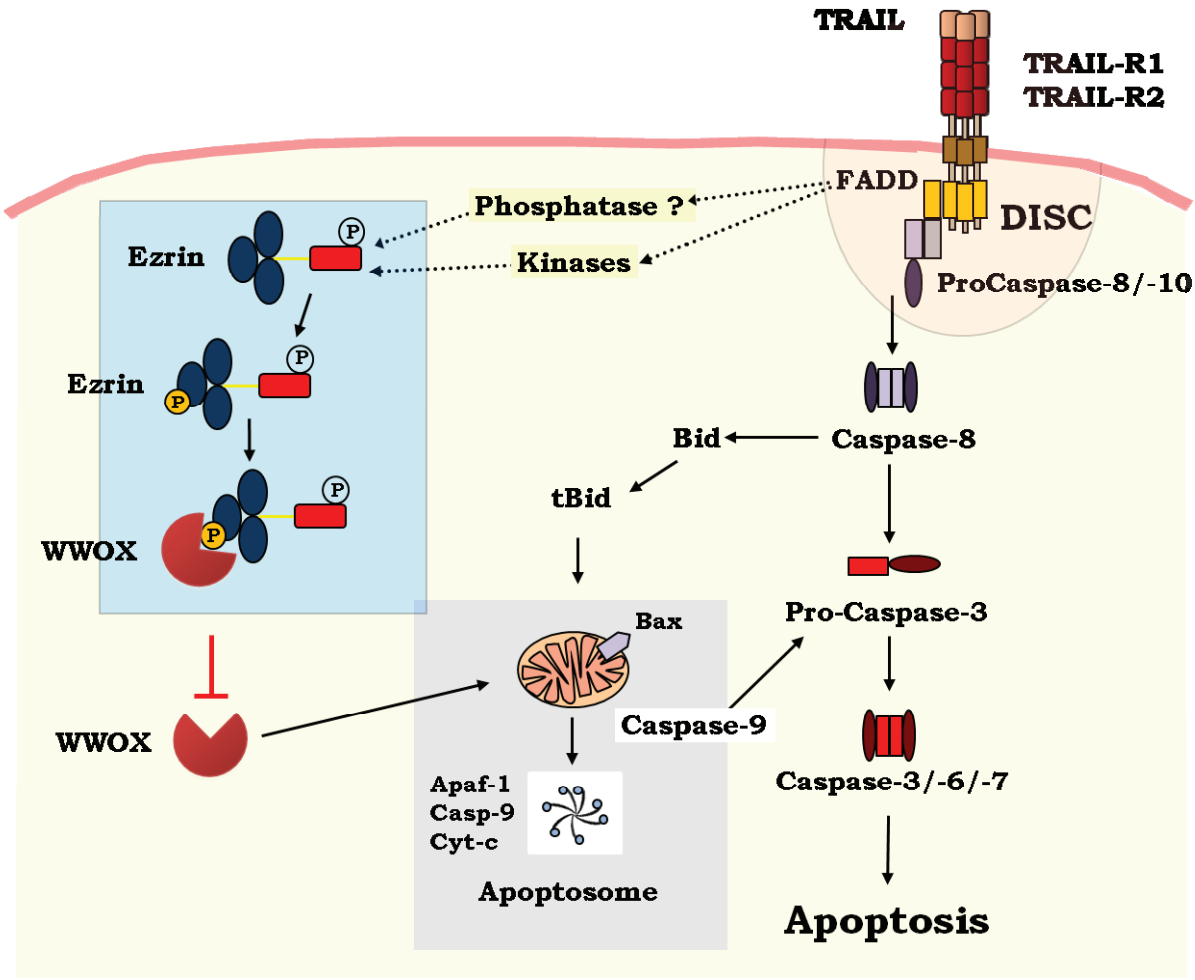


Figure 5.1. Proposed model of the ezrin-mediated inhibition of TRAIL-induced cell death. Binding of a TRAIL trimer to TRAIL-R1 and TRAIL-R2 induces apoptosis and phosphorylation of ezrin on the residue serine 66. Serine 66 phosphorylated ezrin interacts with and then sequesters WWOX, impairing its re-localization to the mitochondria. These events could lead to the inhibition of the TRAIL pathway at the mitochondria level.

5.5. The effect of combined treatments on TRAIL-induced cell death

In the last part of this study we demonstrated that cisplatin (CDDP), a conventional chemotherapeutic drug, is able to overcome ezrin-mediated TRAIL-inhibition. Indeed, we found that a double treatment of cisplatin followed by TRAIL, is more effective in inducing cell death than treatment with TRAIL alone on cells exhibiting partial resistance to TRAIL-induced apoptosis, such SW480 cells expressing the ezrin variants S66D or Y353F. This combined effect was not evident when cells already exhibited high sensitivity to TRAIL-induced apoptosis, such as SW480 cells expressing ezrin S66A or Y353D.

Cisplatin could sensitize tumour cells to TRAIL at the DISC level, regulating caspase-8 activation, as already seen in previous studies. Indeed, recent findings demonstrated that combined treatments of chemotherapeutic drugs plus TRAIL restored tumour cells' sensitivity to apoptosis due to either TRAIL-R4 or c-FLIP expression, or Bax deficiency (Mérino et al, 2006; Morizot et al, 2011). The sensitization was shown to occur at the TRAIL DISC level, and was correlated with enhanced caspase-8 recruitment and activation within the DISC (Morizot et al, 2011). In light of these findings, we can then propose the following model. Cancer cells over-expressing ezrin or engineered to express the phosphomimetic variant, ezrin S66D, or the nonphosphorylable variant, ezrin Y353F, are protected against TRAIL-induced cell death, and the ezrin regulation of the TRAIL signalling could occur at the mitochondria level (**Figure 5.2.a**). Cisplatin treatment could restore TRAIL sensitivity mainly enhancing caspase-8 recruitment to and activation at the DISC. Thus caspase-8 is activated and processed in the DISC in quantities that are sufficient to directly activate the effector caspases, and thereby to promote apoptosis, overcoming the ezrin-mediated inhibition of TRAIL-induced cell death

(**Figure 5.2.b**). Further TRAIL DISC analysis after cisplatin and TRAIL treatments are then required to establish whether cisplatin-mediated TRAIL sensitization occurs through an increased caspase-8 recruitment and activation at the DISC level in SW480 cells expressing ezrin phosphorylation variants.

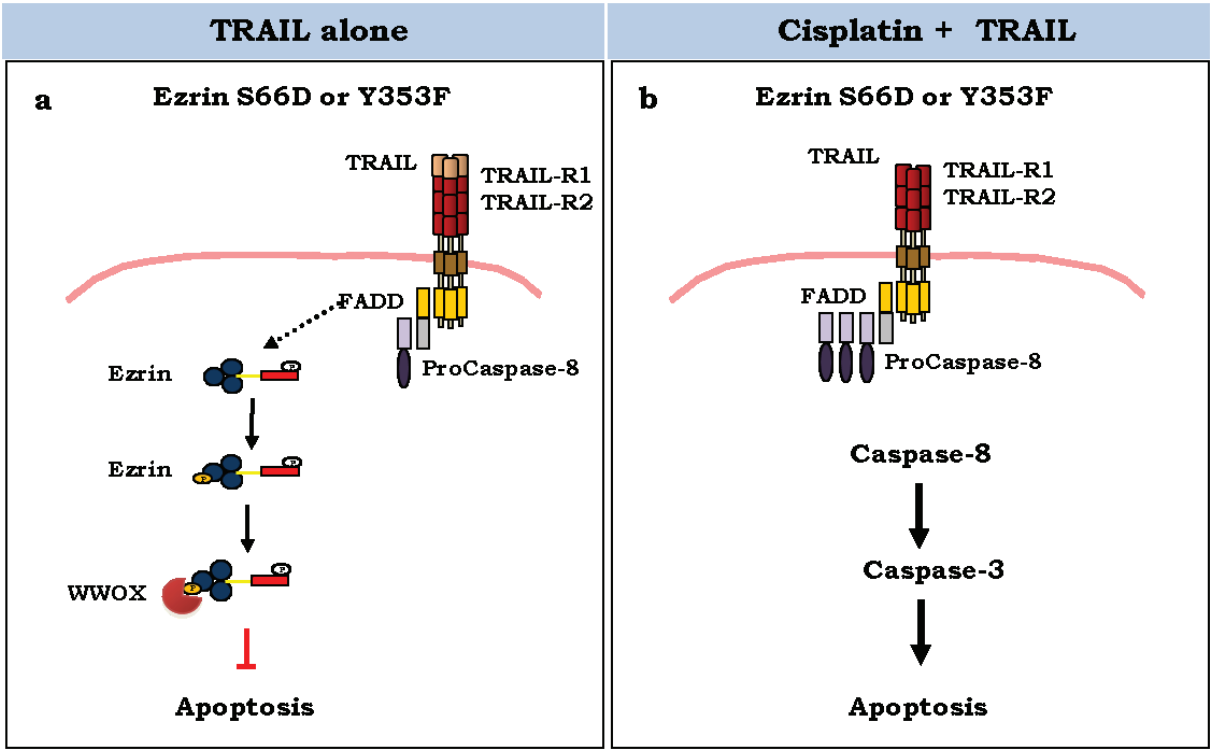


Figure 5.2. Proposed model of cisplatin-mediated sensitization to TRAIL-induced cell death. (a) Binding of a TRAIL trimer to TRAIL-R1 and TRAIL-R2 induces apoptosis and phosphorylation of ezrin on the residue serine 66. Serine 66 phosphorylated ezrin interacts with and then sequesters WWOX, leading to the inhibition of the TRAIL pathway at the mitochondrial level. (b) Cisplatin restores cell sensitivity to TRAIL, mainly through enhanced caspase-8 recruitment and activation at the DISC, which induces activation of caspase-3 and leads to cell death, overcoming the ezrin-mediated inhibition of TRAIL-induced cell death.

Alternatively cisplatin and TRAIL may alter PKA differentially. Preliminary results suggests that TRAIL could induce the activation of PKA whereas cisplatin inhibits this kinase. The hypothesis is supported by the finding that some substrates of PKA, such as p70 and the transcription factor CREB, are modified by phosphorylation following TRAIL treatment, while cisplatin impaired their activation. Moreover, stable over-expression of the ezrin phosphorylation variant

S66A induced a strong and time-independent activation of CREB upon TRAIL treatment. Cisplatin, instead, induced the reverse behaviour. Thus we may expect that the loss of function induced by the ezrin-mediate sequestering of WWOX could be overcome by cisplatin. Further experiments are required to get a definitive answer. One possible approach could be to monitor Bax and caspase-9 activation, but also PKA activation following cisplatin stimulation in cells exhibiting partial resistance to TRAIL-induced apoptosis, such SW480 cells expressing the ezrin variants S66D or Y353F, and compare all these events with TRAIL DISC IP and caspase-8 activation. The impairment in the PKA activation induced by cisplatin could represent an alternative mechanism through which cisplatin acts, which may account for restoration of tumour cell sensitivity to TRAIL, overcoming the ezrin-mediated inhibition of TRAIL-induced cell death.

In the third way, cisplatin could induce conformational changes in membrane receptors favouring their aggregations into particular parts of the membrane, that are rich in cholesterol, sphingolipids, and signalling molecules, which allow the complete activation of the death signalling pathway, overcoming the resistance to TRAIL induced by ezrin. This hypothesis is consistent with recently published reports showing that chemotherapeutic drugs enhance TRAIL receptor clustering at the cell surface through ceramide production and receptor partitioning into lipid rafts (Dumitru et al, 2007; Rebillard et al, 2010).

In line with these considerations, further experiments need to be performed in SW480 cells expressing ezrin phosphorylation variants in order to elucidate the events leading to the restoration of tumour cells sensitivity to apoptosis induced by combined treatments.

5.6. Conclusions

In this thesis we have shown that ezrin displays a negative function towards Fas ligand- and TRAIL-induced apoptosis in the human colon carcinoma cell lines HCT116 and SW480, and that the ezrin phosphorylation and/or dephosphorylation on serine 66 and/or tyrosine 353 specifically affects TRAIL-induced apoptosis. We also provide evidence that ezrin inhibits the TRAIL apoptotic machinery downstream of the TRAIL DISC, probably indirectly regulating the level of Bax. Our results strongly support the hypothesis that TRAIL, differently from Fas, induces PKA-mediated phosphorylation of ezrin on the serine 66, which could account for the subsequent association of ezrin with the tumour suppressor gene WWOX. Thus the WWOX association with ezrin impairs the activation of WWOX and its translocation to the mitochondria, leading to apoptosis inhibition. In addition, we found that the chemotherapeutic drug, cisplatin, is able to overcome the ezrin-mediated inhibition of TRAIL in cells resistant to TRAIL stimulation, such as SW480 cells expressing the ezrin variants, S66D or Y353F, restoring tumour cell sensitivity to TRAIL.

TRAIL is considered a new candidate in the field of cancer therapy because it triggers cell death in cancer cells but not in normal cells. However, cancer cells can sometimes become resistant to TRAIL or Fas stimulation, and then they do not respond to death receptor-mediated triggering for apoptosis. Our results provide the first evidence for an ezrin-mediated mechanism, possibly responsible for the lower responsiveness of colon carcinoma cells to TRAIL and Fas. Modulation of ezrin activation by phosphorylation could then be considered a new and alternative way to obtain an effective regulation of death receptor-induced apoptosis in colon carcinoma patients.

Our findings could help in the design of cancer treatments aimed to block the activation of ezrin, which might enhance induction of apoptosis. Moreover novel cancer treatments associating TRAIL with chemotherapy or kinase inhibitors could be useful in the future to abrogate ezrin-mediated TRAIL inhibition in colon carcinoma patients. These preclinical studies may lay the ground for the design of innovative therapeutic approaches which can be more effective in cure cancer.

6. Annexes

Cell Death and Differentiation (2011) 18, 700–711

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Chemotherapy overcomes TRAIL-R4-mediated TRAIL resistance at the DISC level

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TNF-related apoptosis-inducing ligand or Apo2L (Apo2L/TRAIL) is a promising anti-cancer drug owing to its ability to trigger apoptosis by binding to TRAIL-R1 or TRAIL-R2, two membrane-bound receptors that are often expressed by tumor cells. TRAIL can also bind non-functional receptors such as TRAIL-R4, but controversies still exist regarding their potential to inhibit TRAIL-induced apoptosis. We show here that TRAIL-R4, expressed either endogenously or ectopically, inhibits TRAIL-induced apoptosis. Interestingly, the combination of chemotherapeutic drugs with TRAIL restores tumor cell sensitivity to apoptosis in TRAIL-R4-expressing cells. This sensitization, which mainly occurs at the death-inducing signaling complex (DISC) level, through enhanced caspase-8 recruitment and activation, is compromised by c-FLIP expression and is independent of the mitochondria. Importantly, TRAIL-R4 expression prevents TRAIL-induced tumor regression in nude mice, but tumor regression induced by TRAIL can be restored with chemotherapy. Our results clearly support a negative regulatory function for TRAIL-R4 in controlling TRAIL signaling, and unveil the ability of TRAIL-R4 to cooperate with c-FLIP to inhibit TRAIL-induced cell death.
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TNF-related apoptosis-inducing ligand or Apo2L (TRAIL) is a promising tool for cancer therapy, owing to its ability to eradicate tumor cells while sparing normal cells.[1] TRAIL is a type II transmembrane protein, whose binding to its agonistic receptors, namely TRAIL-R1 (DR4) and TRAIL-R2 (DR5, TRICK2 or KILLER), triggers apoptosis in a p53-independent manner. Engagement of TRAIL agonistic receptors induces the formation of a molecular platform called the DISC (death-inducing signaling complex) within minutes, through homotypic interactions.[2] This platform includes the adapter protein FADD and caspase-8, an initiator caspase that is activated and subsequently released from the DISC to the cytosol for dismantling of the cells. The amount of caspase-8 generated within the DISC in type I cells is sufficient to trigger apoptosis through the direct activation of the effector caspase-3. Type II cells require the engagement of a mitochondrial amplification loop, which is activated by caspase-8-dependent cleavage of Bid, a BH3-only protein that targets the intrinsic pathway through Bax and Bak, allowing the formation of the apoptosome. However, enforced aggregation of TRAIL agonistic receptors in these cells enhances caspase-8 activation at the DISC level and overcomes mitochondrial checkpoints.[3] Likewise, enhanced caspase-8 recruitment and activation at the TRAIL DISC by chemotherapeutic drugs has been associated with the restoration of TRAIL sensitivity in hepatocellular and colon carcinomas.[4,5]

Cellular resistance to TRAIL-induced cell death arises from a large variety of events, ranging from defects in DISC formation, or inhibition of more distal events, including mitochondrial block.[6,7]

TRAIL-induced cell death can be specifically inhibited by two membrane-bound antagonistic receptors, TRAIL-R3 (DcR1, LIT or TRID) or TRAIL-R4 (DcR2 or TRUNDD).[2] These receptors have been shown to be expressed and to prevent TRAIL-induced cell death in various human primary tumor cells, including lymphomas, lung, breast and prostate carcinomas,[8–10] but the inhibitory potential of this receptor still remains controversial.[11] Although TRAIL-R3 is a GPI-anchored receptor that sequesters TRAIL into lipid rafts, TRAIL-R4 interacts with TRAIL-R2 within the DISC, and impairs caspase-8 processing,[12] thus, inhibiting TRAIL-induced apoptosis.[13,14]

The efficacy of recombinant hAPO2L/TRAIL in association with chemotherapy is evaluated in ongoing clinical trials.[1] It remains unknown whether TRAIL-R4 expression may compromise the efficacy of TRAIL.

We demonstrate here that TRAIL-R4 efficiently inhibits TRAIL, and that chemotherapeutic drugs can overcome this resistance. Restoration of apoptosis primarily occurs at the membrane level, irrespective of the mitochondria, through enhanced caspase-8 recruitment and activation at the TRAIL DISC. TRAIL-R4 expression also impairs TRAIL-induced tumor regression *in vivo*, but sequential treatments associating

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Keywords: TRAIL; TRAIL-R4; c-FLIP; chemotherapy; apoptosis

Abbreviations: 5FU, 5-fluorouracil; CDDP, cisplatin; DISC, death-inducing signaling complex; TRAIL-R, trail Receptor ; VP16, etoposide

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CDDP and TRAIL prevent tumor growth in nude mice. Altogether, our results demonstrate that TRAIL-R4 is a negative regulator of TRAIL, whose inhibitory function can be overcome by chemotherapy.

Results

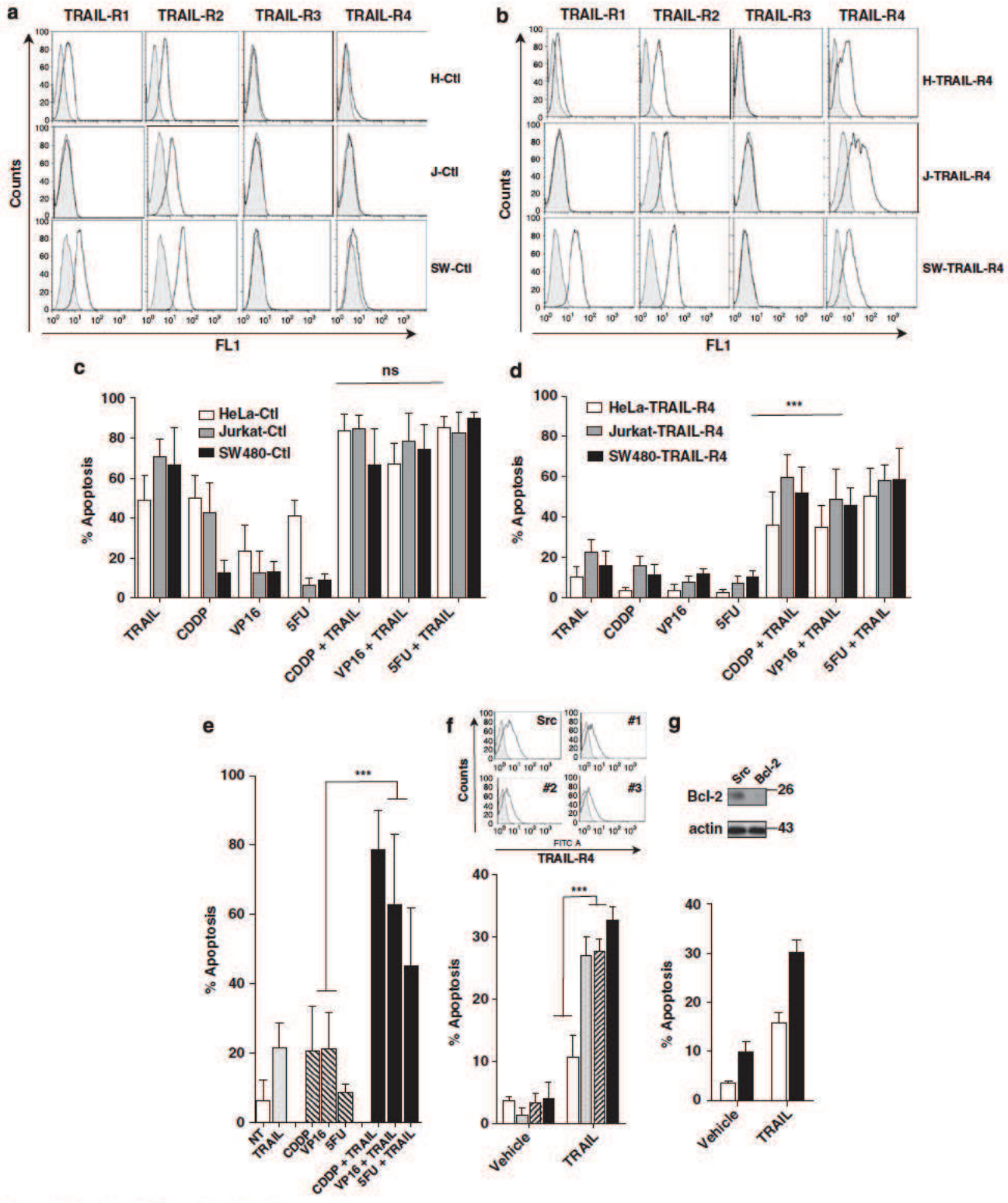
TRAIL and chemotherapeutic drugs synergistically induce apoptosis in TRAIL-R4-expressing cells. We have demonstrated previously that, ectopic expression of TRAIL-R4 impairs TRAIL-induced cell death through the formation of a heteromeric complex with TRAIL-R2, leading to the inhibition of caspase-8 activation within the TRAIL DISC.[12] Owing to the ability of TRAIL-R4 to inhibit TRAIL-induced cell death, we evaluated whether its expression may compromise combination therapies associating TRAIL with conventional chemotherapeutic drugs. To test this hypothesis, TRAIL-R4 was ectopically expressed using retroviruses in three TRAIL-sensitive tumoral cell lines, HeLa, Jurkat and SW480. Cell surface expression was assessed by flow cytometry (Figure 1a and b). TRAIL-R4 inhibited TRAIL-induced apoptosis in these cells (Figure 1c and d). Strikingly, TRAIL-R4 also inhibited death induced by chemotherapeutic drugs in some cell lines. Pre-treatment with pharmacological concentrations of CDDP, VP16 for 3 h or 5FU for 72 h, however, restored TRAIL sensitivity in these cells (Figure 1c and d). Similar results were obtained in the B-lymphoma cell line VAL, in which the cells are poorly sensitive to TRAIL-induced cell death (Figure 1e). VAL cells endogenously express TRAIL-R4 at the cell surface and high levels of Bcl-2, because of the t(14;18) chromosomal translocation (Figure 1f and g). Cells sensitivity to TRAIL-induced cell death was restored in VAL cells after pre-treatment with CDDP, VP16, or 5FU (Figure 1e). siRNA-mediated downregulation of TRAIL-R4 or Bcl-2 expression in VAL cells also restored sensitivity to TRAIL (Figure 1f and g), demonstrating that both TRAIL-R4 and Bcl-2 are functional in these cells.

Sequential chemotherapy and TRAIL treatments restore caspase activation. Chemotherapeutic drugs enhanced caspase activation upon TRAIL stimulation (Figure 2a) without changing TRAIL receptor expression (Supplementary Figure S1). In HeLa control cells, TRAIL alone triggered the activation of caspase-8, caspase-9 and caspase-3, and induced Bid and PARP cleavage, as demonstrated by the disappearance of their proform or the appearance of cleaved fragments (Figure 2a). However, in HeLa cells expressing TRAIL-R4 (H-TRAIL-R4 cells), TRAIL induced only a modest cleavage of caspase-8 and caspase-9, resulting in the poor activation of caspase-3 (Figure 2a). Pre-treatment of these cells with CDDP, VP16, or 5FU restored caspase-3 activation upon TRAIL stimulation, as demonstrated by the appearance of the caspase-3 p17 fragment and an increase in PARP cleavage (Figure 2a). Restoration of caspase-3 activation by chemotherapeutic drugs in HeLa-TRAIL-R4 cells was associated with partial activation of both caspase-8 and caspase-9 (Figure 2a).

Activation of the mitochondrial intrinsic pathway is not required to restore sensitivity to TRAIL in response to chemotherapy. As most chemotherapeutic drugs engage the mitochondrial pathway to trigger apoptosis, we next analyzed its contribution with regard to chemotherapy-mediated sensitization to TRAIL-induced cell death. TRAIL stimulation in control HeLa cells triggered the activation of the intrinsic pathway, as evidenced by the disappearance of Bid (Figure 2a), the release of cytochrome *c*, Smac/DIABLO and omi to the cytosol (Figure 2b), and by the activation of Bax (Figure 2d and e). Release of cytochrome *c*, Smac/DIABLO and omi were much weaker in H-TRAIL-R4 cells as compared with control cells (Figure 2c), however, chemotherapy combined with TRAIL nearly completely restored Bax activation in these cells (Figure 2d and e). Overexpression of Bcl-2 or Bcl-xL in H-TRAIL-R4 failed to protect cells from TRAIL-induced apoptosis after chemotherapy (Figure 3a and b). These results are consistent with the demonstration that chemotherapeutic drugs can restore TRAIL sensitivity in VAL cells, despite large amounts of Bcl-2 expression (Figure 1f). To determine the role of Bax in drug-mediated sensitization to TRAIL-induced cell death, we performed the same experiments in the Bax-deficient or parental WT HCT116 cells engineered to express TRAIL-R4 (Figure 3c). TRAIL-mediated apoptosis in HCT116 cells was shown to rely on Bax but not Bak activation.[15] According to these findings, TRAIL alone, or simultaneous combinations of TRAIL and 5FU, failed to induce apoptosis in Bax-deficient cells (Figure 3d and

Figure 1 Chemotherapeutic drugs restore TRAIL-induced cell death in TRAIL-R4-expressing cells. (**a**) and (**b**), HeLa, Jurkat or SW480 cancer cell lines were infected with empty vector (H-Ctl, J-Ctl or SW-Ctl) or with a vector encoding TRAIL-R4 (H-TRAIL-R4, J-TRAIL-R4 or SW-TRAIL-R4). Expression of TRAIL receptors was analyzed by flow cytometry (gray line) against an isotype control (filled curve). (**c**) and (**d**), control cells or TRAIL-R4-expressing cells were stimulated with His-TRAIL (500 ng/ml, 6 h), Cisplatin (CDDP, 20 μM, 3 h), etoposide (VP16, 10 μM, 3 h) or 5-fluorouracil (5FU, 1 μg/ml, 72 h). Apoptosis was evaluated after 6 h (TRAIL), 48 h (CDDP or VP16) or 72 h (5FU) by Hoechst staining in HeLa (white), Jurkat (gray) or SW480 (black). Sequential stimulation with chemotherapeutic drugs and TRAIL was performed as follows. Cells were pre-treated with CDDP or VP16 for 3 h, in serum-free medium, then washed and allowed to recover at 37 °C in complete medium for 48 h before stimulation with His-TRAIL (500 ng/ml) for an additional 6 h. Alternatively, cells were stimulated for 72 h with 5FU, then His-TRAIL for 6 h. (**e**) VAL cell sensitivity to His-TRAIL, chemotherapy or sequential treatments was analyzed as described above. (**f**) Deregulation of TRAIL-R4 expression in VAL cells using three different siRNAs (scramble siRNA, Src; TRAIL-R4 siRNA, #1, #2 and #3) as analyzed by Facs for TRAIL-R4 expression using an anti-TRAIL-R4 antibody (gray line) or a control isotype (filled curved). The effect of TRAIL-R4 downregulation was assessed by Hoechst staining 6 h after His-TRAIL treatment (500 ng/ml), scramble (white) and TRAIL-R4 siRNA (#1 gray; #2 dashed and #3 black). (**g**) Bcl-2 expression in VAL cells after transfection with the scramble siRNA (Src) or the Bcl-2 siRNA (Bcl-2) and corresponding Hoechst staining 6 h after His-TRAIL treatment (500 ng/ml), Bcl-2 siRNA (in black) or a scramble siRNA (in white). These results are representative of at least three independent experiments. Mean percentage of apoptotic cells and S.D. shown (mean ± S.D.). Differences between selected groups were compared by non-parametric analysis of variance (ANOVA) with Bonferroni *post hoc* multiple comparison test, $^{***}P < 0.001$. Molecular size markers are shown on the right in kDa. (For figure refer next page)

Supplementary Figure S2). Nevertheless, pre-treatment for 72 h with 5FU before adding TRAIL efficiently induced cell death in these cells (Figure 3d). Likewise, treating Bax-deficient cells sequentially for 3 h with CDDP or VP16, and stimulating with TRAIL, 48 h after the onset of the treatment in drug-free medium (see Materials and Methods) restored TRAIL-induced apoptosis (Figure 3d). As in HeLa cells, sequential use of chemotherapy and TRAIL afforded sensitization to TRAIL-induced cell death in Bax-proficient cells expressing TRAIL-R4 ectopically (Figure 3d). However,



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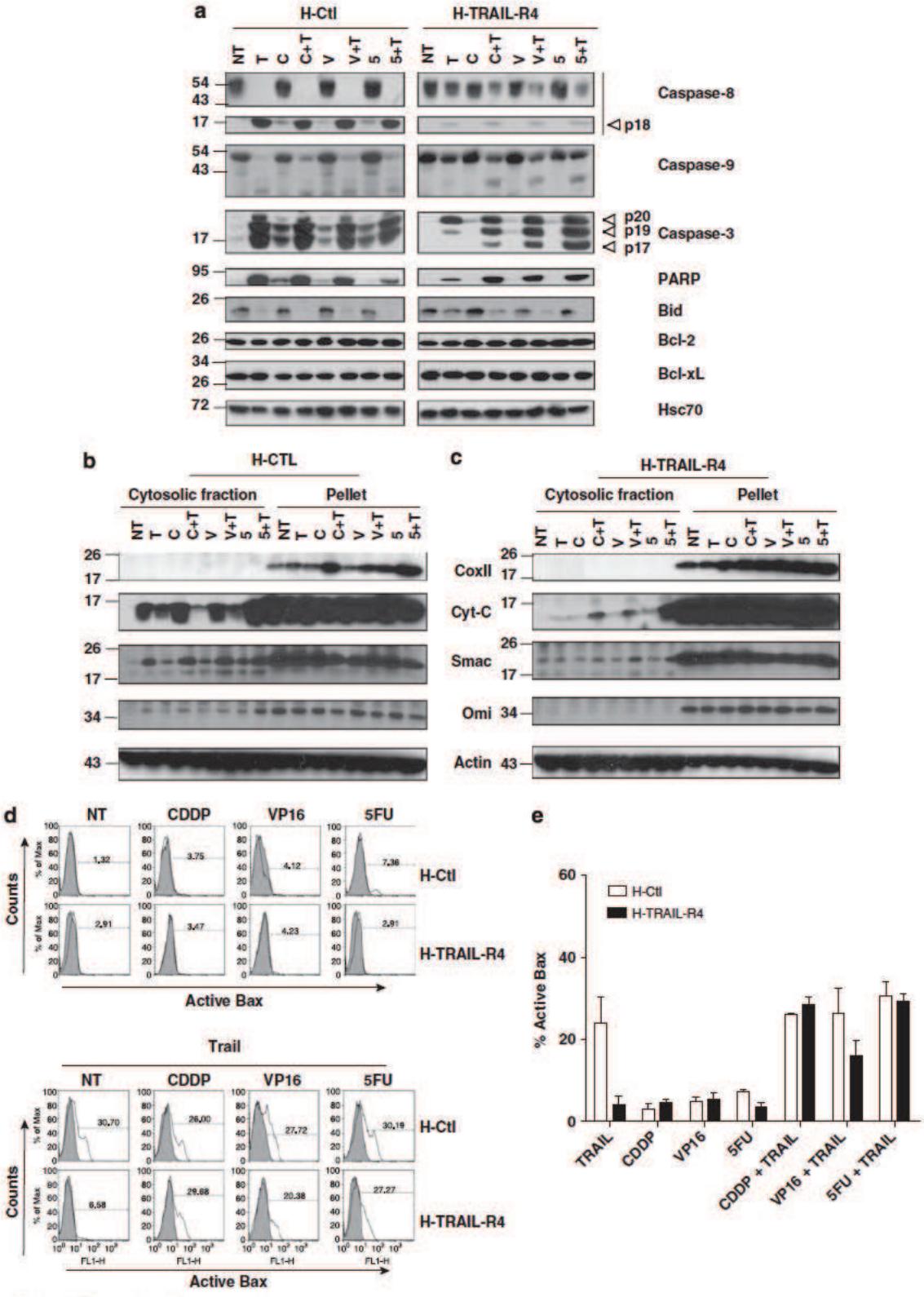
in the absence of Bax, TRAIL-R4 overexpression induced resistance to TRAIL after CDDP or VP16 pretreatment, but not upon 5FU stimulation (Figure 3d).

Sensitization to TRAIL-induced cell death by 5FU has previously been described to involve the deregulation of c-FLIP.[4,16] We, therefore, analyzed c-FLIP expression after



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chemotherapy at the time when the cells were exposed to TRAIL stimulation. Contrary to our expectations, we found that 5FU poorly induced c-FLIP deregulation in our settings (Figure 3e), but that CDDP and VP16 induced c-FLIP upregulation in these cells (Figure 3e). Consistent with these findings, expression of c-FLIP abrogated sensitization to TRAIL after 5FU treatment, irrespective of TRAIL-R4 or Bax expression in HCT116 cells (Figure 3f), indicating that the mere upregulation of c-FLIP is probably sufficient to impair the synergy irrespective of the mitochondria. In agreement with this finding, the caspase-9 inhibitor z-LEHD-fmk failed to protect TRAIL-R4-expressing cells from TRAIL-induced cell death after chemotherapy, while the pan-caspase inhibitor z-VAD-fmk completely abrogated the synergy (Supplementary Figure S3).

Chemotherapeutic drugs enhance caspase-8 recruitment and activation within the TRAIL DISC. To determine the contribution of TRAIL DISC formation and caspase-8 activation in the synergy, c-FLIP was co-expressed in H-TRAIL-R4 cells and cells were stimulated with TRAIL after chemotherapy. Like TRAIL-R4, expression of c-FLIP (Figure 3g) reduced cell sensitivity to TRAIL, but alone failed to block apoptosis induced by TRAIL upon chemotherapy (Figure 3h). However, combined expression of TRAIL-R4 and c-FLIP severely impaired TRAIL-induced apoptosis by chemotherapeutic drugs, indicating that activation of caspase-8 *per se* has a prominent role in the synergy (Figure 3h). In agreement with these findings, DISC analysis in cells subjected to chemotherapy and TRAIL treatments revealed that chemotherapeutic drugs enhanced caspase-8 recruitment and activation within the TRAIL DISC in HeLa control cells (Figure 4a) but, probably more importantly, also in HeLa expressing TRAIL-R4 (Figure 4b) and VAL cells (Figure 4c), which express TRAIL-R4 endogenously. Altogether, these results clearly demonstrate that TRAIL-R4 inhibits TRAIL-induced cell death, and that chemotherapy can restore tumor cell sensitivity to apoptosis, mainly through the restoration of caspase-8 recruitment and activation within the DISC.

TRAIL-R4 inhibits TRAIL-induced cell death but not chemotherapy-induced sensitization to TRAIL *in vivo*. Ability of TRAIL-R4 to prevent TRAIL-induced tumor regression, combined or not with chemotherapy, was next evaluated in nude mice using xenografts of HCT116 cells expressing TRAIL-R4. Mice were implanted, in both flanks, with HCT116-Ctl cells (right flank) and HCT116-TRAIL-R4 (left flank). When the tumor volume reached 20 mm^3, mice were treated with phosphate-buffered saline (PBS), CDDP, recombinant TRAIL or treated sequentially with CDDP and TRAIL as described in the Materials and Methods section. Compared with PBS-treated mice, HCT116-Ctl tumor growth was inhibited in mice receiving injections of TRAIL, CDDP and by the combined treatment (Figure 5a). However, TRAIL, and to a lesser extent CDDP, failed to induce tumor regression in TRAIL-R4-expressing cells (Figure 5b), but combined treatments induced a marked inhibition of the tumor growth of HCT116 cells expressing TRAIL-R4 (Figure 5b), with statistically significant *P*-values <0.001 as compared with PBS-treated mice. These results demonstrate that TRAIL-R4 efficiently inhibits TRAIL-induced cell death not only *in vitro*, but also *in vivo*. However, chemotherapeutic drugs, including CDDP, can overcome TRAIL-R4 mediated resistance, highlighting the potential therapeutic value of these combined therapies for cancer.

Discussion

TRAIL-based combinatorial therapies are emerging paradigms for cancer treatment as synergistic activation of TRAIL-induced apoptosis by chemotherapeutic drugs generally affords to overcome tumor cell resistance, whereas monotherapies are most of the time poorly successful. Preclinical studies and clinical trials are giving promising results, supporting the potential of these combining approaches.[17,18]

Cell surface expression of TRAIL agonistic receptors is the first requirement in order to trigger the TRAIL apoptotic machinery but, to date, the expression of TRAIL receptors in primary tumors remains poorly studied and the anti-apoptotic function of TRAIL-R4 remains controversial. It was found, however, in a few studies that primary lymphomas could express functional TRAIL antagonistic receptors at the cell surface.[10] In solid tumors, analysis of TRAIL receptor expression was often performed by immunohistochemistry, and although this method does not provide the information whether the receptors are expressed at the cell surface, these studies indicate that the extent of expression of the antagonistic receptors TRAIL-R3 and TRAIL-R4 is probably underestimated.[9,19–21]

Engagement of apoptosis upon TRAIL stimulation in a given tissue type, primary tumor or cell line, relies on the contribution of multiple players, including proapoptotic and prosurvival factors, which ultimately determine cell fate. It has

Figure 2 Chemotherapeutic drugs activate the mitochondrial apoptotic pathway. (**a**) Western blot analysis of caspase-8, caspase-9, and caspase-3, PARP, Bid, Bcl-2, Bcl-xL and hsc70 in control HeLa cells (H-Ctl) or cells expressing TRAIL-R4 (H-TRAIL-R4) after stimulation with His-TRAIL (T) and/or chemotherapeutic pretreatments with cisplatin (C), etoposide (V) or 5FU (5). White arrows indicate cleavage fragments. Molecular size markers are shown on the left in kDa. These results are representative of at least three independent experiments. (**b**) and (**c**) A digitonin-based permeabilisation experiment followed by western blot analysis of the different fractions (cytosolic or pellet) was performed to analyze the release of cytochrome *c*, Smac and Omi from the mitochondria. CoxII antibody was used as a control for efficient subcellular fractionation and the actin was probed for normalization. Control HeLa cells and H-TRAIL-R4 cells were treated as previously with cisplatin (C), etoposide (V) or 5-fluorouracil (5) plus or minus His-TRAIL (T). Molecular size markers are shown on the left in kDa. (**d**) Control HeLa cells and H-TRAIL-R4 cells were pre-treated as above with cisplatin (CDDP), etoposide (VP16) or 5-fluorouracil (5FU) then subsequently stimulated or not with His-TRAIL (TRAIL), as in Figure 1. After treatment, cells were permeabilized and stained with an antibody recognizing active Bax and analyzed by flow cytometry. (**e**) The percentage of cells containing active Bax was determined by FACS in control HeLa cells (H-Ctl, white bars) or TRAIL-R4-expressing cells (H-TRAIL-R4, black bars). These results are representative of at least three independent experiments. Mean %Active Bax values and S.D. are shown (mean ± S.D.). (For figure refer previous page)

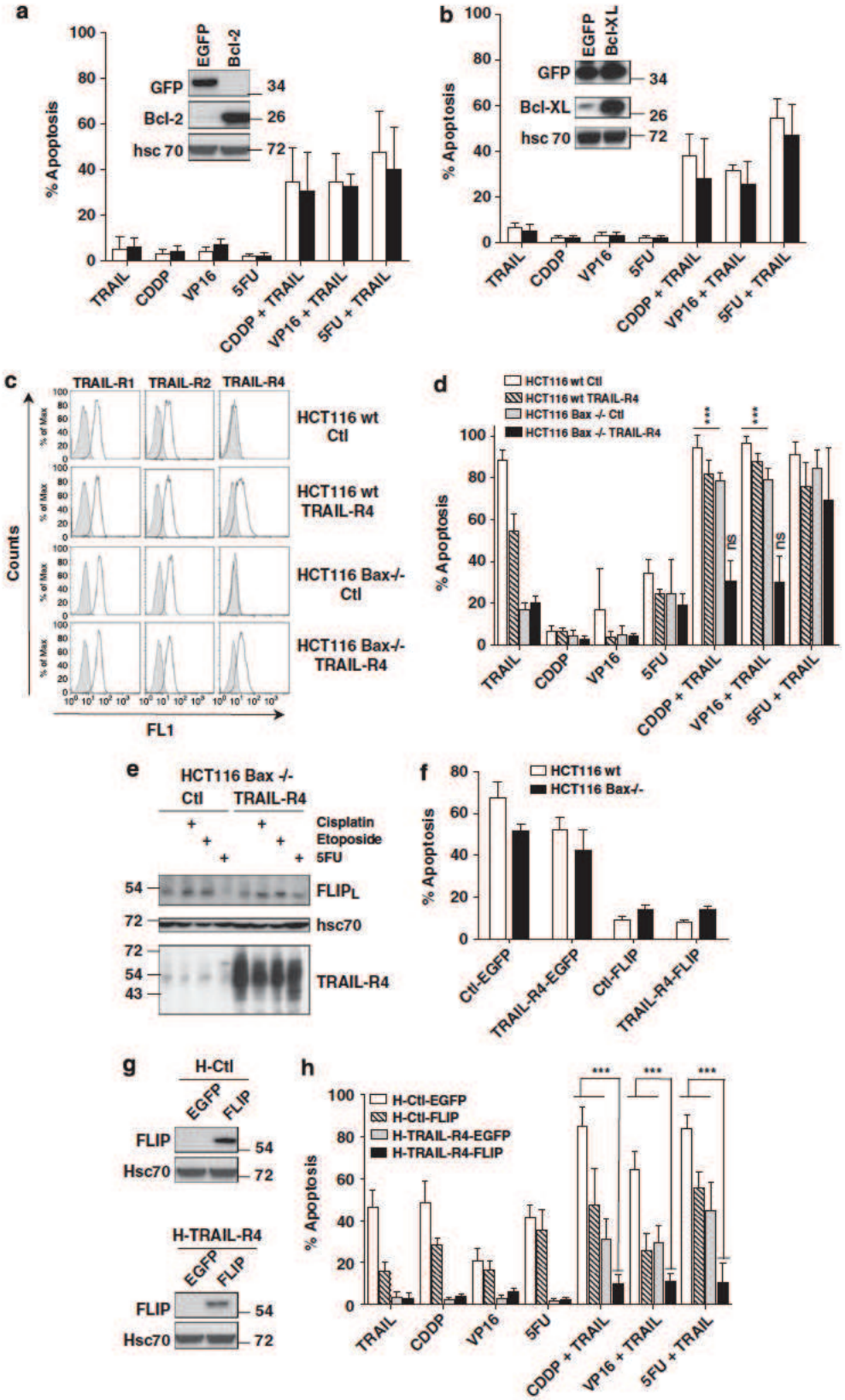

Figure 3 Continued. (For caption refer next page)

recently been demonstrated that naturally occurring differences in the levels or states of proteins regulating TRAIL signaling are the primary causes of cell-to-cell variability.[22] The large variety of cellular changes in protein levels or status induced by chemotherapeutic drugs may explain why these drugs, restore TRAIL sensitivity in resistant cells, albeit targeting different signaling pathways. Sensitization to TRAIL by chemotherapeutic drugs, has been attributed to multiple molecular mechanisms including the upregulation of TRAIL-R2,[23] activation of the mitochondrial pathway[24,25] inhibition of c-FLIP expression[26] or enhanced caspase-8 recruitment to the TRAIL DISC.[4,5]

We demonstrate here that chemotherapy overcomes TRAIL resistance induced by TRAIL-R4 at the level of the DISC, and provide strong evidence that the mitochondrial pathway is dispensable for the restoration of TRAIL sensitivity by chemotherapeutic drugs (Figure 6). Chemotherapeutic drugs afforded sensitization to TRAIL of aggressive B-cell follicular lymphomas such as VAL cells, despite endogenous expression of functional TRAIL-R4 and Bcl-2. Likewise, these compounds restored TRAIL sensitivity of epithelial-derived tumor cell lines harboring either a deficiency for Bax expression, or engineered to express Bcl-2 or Bcl-x_L, irrespective of TRAIL-R4 expression levels. Bax deficiency however, may be detrimental to some chemotherapeutic drugs in cells that express TRAIL-R4 and low but significant levels of c-FLIP. Accordingly, restoration of TRAIL sensitivity in HCT116 Bax-deficient cells expressing TRAIL-R4 was only observed with 5FU, but not CDDP or VP16 because of their ability to induce c-FLIP expression. These results could explain some discrepancies regarding the lack of correlation regarding TRAIL-R4 expression and cell sensitivity to TRAIL-induced cell death. In particular, c-FLIP expression levels have scarcely been taken into consideration in these studies.

Our results demonstrate that TRAIL-R4 can inhibit TRAIL-induced cell death both *in vitro* and *in vivo*, and cooperate with c-FLIP to inhibit chemotherapy-mediated sensitization to TRAIL-induced apoptosis (Figure 6). These findings not only have important implications for the understanding of the molecular mechanisms involved in the regulation of TRAIL signaling, but also for therapeutic approaches aiming at utilizing recombinant TRAIL to cure patients suffering from cancer.

The physiological function and relevance of TRAIL-R4 is still unclear. Our study is probably the first demonstration that TRAIL-R4 can confer TRAIL resistance *in vivo*, as we demonstrate that ectopic expression of this receptor in the colon carcinoma cell line HCT116 efficiently impairs TRAIL-induced tumor killing in nude mice. At the physiological level, TRAIL-R4 could protect cells selectively from TRAIL-induced cell death. Noteworthy, it has been demonstrated that NK and CD8+ T cells are induced to express TRAIL, TRAIL-R2, TRAIL-R4 and c-FLIP upon activation.[27] Despite high expression levels of TRAIL, these cells are resistant to TRAIL, but selective inhibition of c-FLIP expression induced TRAIL sensitivity.[27] It should be noted, however, that selective TRAIL-R4 downregulation was not assessed in this study, therefore, it is conceivable that TRAIL-R4 may also have a role in protecting these cells from TRAIL-induced cell death. Although the function of TRAIL-R4 remains to be determined in a physiological context, our results indicate that this receptor in pathological conditions, such as overexpression in primary tumor cells, could represent a problem in oncology. Our results clearly support the inhibitory potential of TRAIL-R4 and, in agreement with previous studies,[4,5] sustain the demonstration that chemotherapy sensitize tumor cells to TRAIL mainly through the regulation of caspase-8 activation at the DISC level.

The molecular mechanisms leading to the restoration of caspase-8 recruitment and enhancement of caspase-8 activation within the TRAIL DISC after chemotherapy remains an open question. Some reports indicate that chemotherapeutic drugs could enhance TRAIL receptor clustering at the cell surface, through ceramide production and receptor partitioning into lipid rafts.[28,29] Work is currently in progress to address this question in our laboratory.

Figure 3 The mitochondrial pathway is dispensable for the synergy in TRAIL-R4 expressing HeLa cells. (**a**) H-TRAIL-R4 cells were infected using the pBabe-blasticidin retroviral vector encoding EGFP or Bcl-2. The expression of the different transgenes was checked by western blot using an anti-Bcl-2 or anti-GFP antibody. Hsc70 was used as a loading control. Molecular size markers are shown on the right in kDa. TRAIL-R4 HeLa cells overexpressing EGFP (EGFP, in white) or Bcl-2 (Bcl-2, in black) were pre-treated with the chemotherapeutic drugs as described in Figure 1 and sequentially treated with His-TRAIL (500 ng/ml for 6 h). Apoptosis was quantified by Hoechst staining. (**b**) H-TRAIL-R4 cells were infected with pMIG empty vector (EGFP) or pMIG-Bcl-xL and analyzed by western blot. Molecular size markers are shown on the right in kDa. Sensitivity to apoptosis induced by His-TRAIL, chemotherapy or sequential treatments (H-TRAIL-R4-EGFP, white; H-TRAIL-R4-Bcl-xL, black) was assessed by Hoechst staining. (**c**) HCT116 parental (HCT116 wt) and HCT116 Bax / cells were infected with an empty pMSCV-vector (HCT116 wt Ctl and HCT116 Bax / Ctl) or with pMSCV-vector encoding TRAIL-R4 (HCT116 wt TRAIL-R4 and HCT116 Bax / TRAIL-R4). Expression of TRAIL receptors was analyzed by flow cytometry. (**d**) Apoptosis induced by His-TRAIL (500 ng/ml, 6 h) after chemotherapeutic treatment was measured by Hoechst staining in HCT116 parental Bax wt Ctl (white), HCT116 wt overexpressing TRAIL-R4 (HCT116 wt TRAIL-R4, dashed), HCT116 Bax / Ctl (gray) and HCT116 Bax / overexpressing TRAIL-R4 cells (HCT116 Bax / TRAIL-R4, black). These results are representative of three independent experiments performed in triplicate. Mean percentage of apoptotic cells values and S.D. are shown (mean ± S.D.). Differences between selected groups were compared by non-parametric analysis of variance (ANOVA) with Bonferroni *post hoc* multiple comparison test, $^{***}P<0.001$, compared with TRAIL stimulation alone in HCT116 Bax-deficient or HCT116 Bax-deficient expressing TRAIL-R4 cells, ns (not statistically significant). (**e**) Cells were stimulated as above for 3 h with treatments CDDP or VP16 or 72 h with 5FU, and c-FLIP or TRAIL-R4 expression was analyzed by western blotting 48 h or immediately after stimulation, respectively. Molecular size markers are shown on the left in kDa. (**f**) HCT116 Bax wt and Bax / control (Ctl) or TRAIL-R4 (TRAIL-R4) were infected with pMIG-$FLIP_L$ (FLIP) or an empty vector (EGFP), and sorted by flow cytometry based on GFP positivity. Sensitivity to TRAIL-induced apoptosis after a 72 h pre-treatment with 5FU was measured by Hoechst staining 6 h after His-TRAIL (500 ng/ml) treatment. (**g**) HeLa control (H-Ctl) and HeLa overexpressing TRAIL-R4 (H-TRAIL-R4) were infected with pBabe-EGFP or pBabe-FLIP. Expression of the different transgenes was checked by western blot. (**h**) Cells overexpressing EGFP (H-Ctl-GFP in white bars and H-TRAIL-R4-GFP in gray bars) or FLIP (H-Ctl-FLIP dashed bars and H-TRAIL-R4-FLIP in black bars) were stimulated with the chemotherapeutic agents, as described previously, and sequentially treated with His-TRAIL (500 ng/ml) for 6 h. Apoptotic cells were counted after Hoechst staining. These results are representative of at least three independent experiments. Mean percentage of apoptotic cells and S.D. are shown (mean ± S.D.). Differences between selected groups were compared by non-parametric analysis of variance (ANOVA) with Bonferroni *post hoc* multiple comparison test. $^{***}P<0.001$, H-TRAIL-R4-FLIP compared with H-Ctl-Mock, H-Ctl-FLIP or H-TRAIL-R4-Mock. (For figure refer previous page)

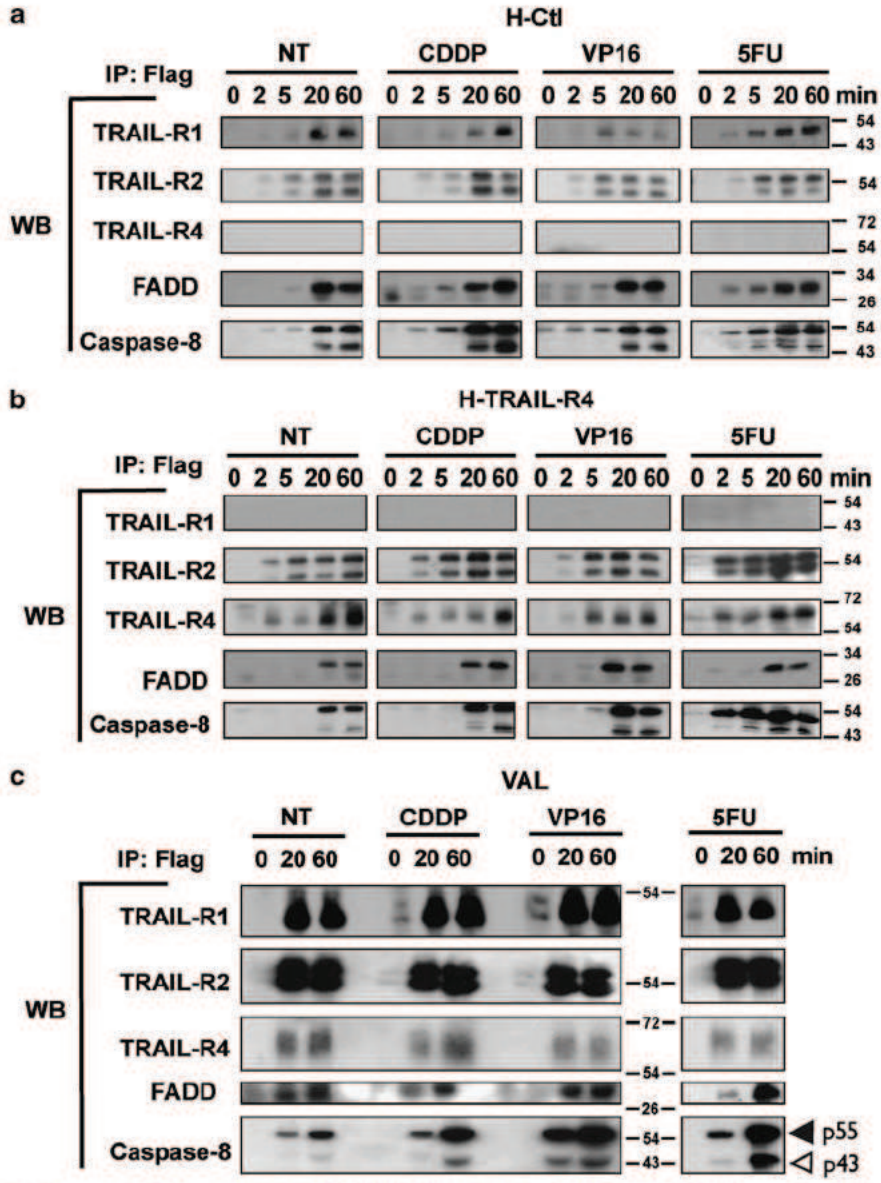

Figure 4 Chemotherapeutic drugs restore TRAIL sensitivity at the DISC level. (a) control HeLa cells (H-Ctl), (b) TRAIL-R4 expressing HeLa cells (H-TRAIL-R4) or (c) VAL cells were pre-treated with CDDP, VP16 or 5FU or left untreated as described in Figure 1, then stimulated with TRAIL for the indicated time. TRAIL DISC was immunoprecipitated (see Materials and Methods section) and analyzed by western blot. Molecular size markers are shown on the right in kDa

Remarkably, like c-FLIP,[30,31] ectopic expression of TRAIL-R4 induced cross-resistance to some chemotherapeutic drugs *in vitro* and *in vivo*. How TRAIL-R4 impairs chemotherapy-induced apoptosis remains to be determined. Some reports point to the observation that forced aggregation of some death receptors of the TNF family including Fas, or downstream effectors like Bid, may contribute to genotoxic drug-induced apoptosis in a ligand-independent manner.[31–33] Combined expression of TRAIL-R4 and c-FLIP may, therefore, not only impair TRAIL-induced cell death after chemotherapy, but may also alter chemotherapy itself. In line with this hypothesis, it has been demonstrated that c-FLIP and TRAIL-R4 are overexpressed in a growing number of primary tumors and that their expression levels has recently been defined as a poor prognostic marker in colorectal[34] and prostate cancer patients.[35]

Altogether our results clearly demonstrate that TRAIL-R4 is a negative regulator of TRAIL whose inhibitory function can be overcome using chemotherapy to restore TRAIL-induced cell death. However, we also demonstrate that TRAIL-R4 cooperates with c-FLIP to inhibit TRAIL-induced apoptosis after chemotherapy. Their ability to cooperate and to efficiently inhibit TRAIL-induced apoptosis needs to be taken into consideration both *in vitro* and in future clinical trials to assess the efficacy of combinatorial treatments associating recombinant TRAIL with chemotherapy. It is anticipated that patients expressing both TRAIL-R4 and c-FLIP may respond better to alternative therapeutic approaches, including

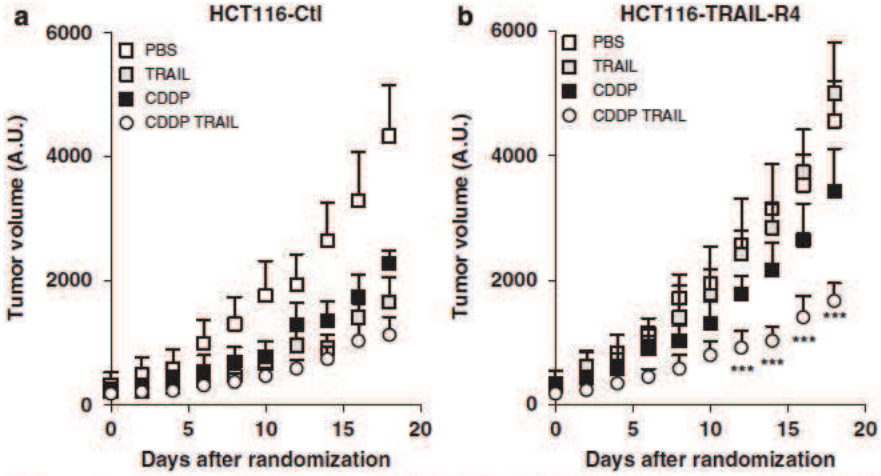

Figure 5 Chemotherapeutic drugs restore TRAIL sensitivity *in vivo*. (**a**) and (**b**), HCT116-Ctl or HCT116-TRAIL-R4 cells were implanted into NMRI nu/nu mice and allowed to reach 20 mm^3. After randomization (day 0), mice were either injected with PBS (white squares), His-TRAIL alone at 8 mg/kg (gray squares), CDDP at 4 mg/kg (black squares) or sequentially with CDDP and 2 days later with His-TRAIL 8 mg/kg (white circle). Mice were subjected to two treatments spaced within 2 days. Tumors were measured every 2 days using a caliper. The combination was found statistically different from single treatments (***$P < 0.001$) at days 14, 16, 18 and 20 as analyzed by ANOVA, two-sided. These results represent mean tumor volume in arbitrary units ± S.D. of 9 to 10 mice per group from three independent experiments

non-conventional chemotherapeutic drugs, TRAIL derivatives, targeting specifically TRAIL-R2, or to strategies aiming at inhibiting c-FLIP expression or blocking TRAIL-R4.

Materials and Methods

Ligand production and antibodies. Flag-tagged recombinant soluble human TRAIL, his-tagged TRAIL and FasL were produced and used as described previously.[36] Anti-Flag (M2) and staurosporin were from Sigma-Aldrich (Lyon, France). For western blot analysis, anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3 and anti-TRAIL-R4 antibodies were purchased from Chemicon (Millipore, Molsheim, France), anti-FADD was obtained from Transduction Laboratories (BD biosciences, Le Pont de Claix, France), anti-caspase-8 and anti-caspase-10 were from Medical & Biological Laboratories (Clinisciences, Montrouge, France). Antibodies against active cleaved fragment of caspase-3, and PARP were from Cell Signaling (Millipore), anti-GFP, Bcl-2, cytochrome c, Bax (N-20) and HSC-70 were from Santa Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France) and anti-caspase-9 was from Upstate (Millipore). Anti-Bid, anti-Bcl-xL and anti-FLIP (NF6) antibodies were purchased from BD Pharmingen, Transduction Lab (BD Biosciences), Calbiochem (VWR, Fontenay-sous-Bois, France) and Alexis (Coger, Paris, France), respectively. Anti-CoxII, anti-Smac/DIABLO and anti-Omi/HtrA2 were from Molecular probes (Invitrogen, Cergy Pontoise, France), Proscience (Coger, Paris, France) and R&D systems (Lille, France), respectively. For flow cytometry experiments, the anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3 and anti-TRAIL-R4 (clones wB-K32, B-L27, wB-B44 and wB-P30 respectively), were kindly provided by Diaclone (Besançon, France). The secondary antibody was an Alexa-488 coupled-goat anti-mouse from Molecular Probes (Invitrogen). The pan-caspase inhibitor (z-VAD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were purchased from Alexis.

Cell culture. The HeLa (human cervix carcinoma) and SW480 (human colon adenocarcinoma) cell lines were cultured with high-glucose Dulbecco's modified Eagle's medium medium (Lonza, Levallois-Perret, France) supplemented with 10% fetal bovine serum (Lonza) and penicillin/streptomycin (100 mg/ml of each). The Jurkat (human T lymphoma) cells, VAL (human B lymphoma) and HCT116 human colon adenocarcinoma cell lines were cultured in RPMI 1640 medium (Lonza) containing 10% fetal bovine serum and penicillin/streptomycin. All these cell lines were grown in 5% CO_2 at 37°C. HCT116 Bax+/ or Bax / are kind gifts of Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD, USA).

Retrovirus production and cell transduction. The retroviral vector pMSCV-puro for TRAIL-R4 expression and generation of viruses has been previously described.[37] Cells were transduced for 16 h with viral supernatants containing polybrene (8 mg/ml), washed in PBS, and cultured in complete medium containing puromycin (2.5 mg/ml). EGFP, $FLIP_L$ and Bcl-2 were cloned into pBabe-Blasticidin. Transduced cells were then selected with blasticidin (2.5 μg/ml). pMIG-Bcl-xL expression vector[38] was purchased from Addgene (plasmid 8790, Cambridge, MA, USA). pMIG-$FLIP_L$ was obtained as previously described.[14] After transduction, cells were sorted using a cell sorter Coulter Epics Elite ESP (Beckman-Coulter-France, Villepinte, France).

Treatments with chemotherapy and TRAIL. For sequential treatments, cells were treated for 3 h with CDDP (20 μM) or VP16 (10 μM) in serum-free medium and then washed. Cells were cultured for 48 h in complete medium before being treated for 6 h with His-TRAIL (500 ng/ml). 5FU was added in complete medium 72 h before TRAIL treatments and the Hoechst analysis.

Hoechst analysis. Apoptosis was assessed by Hoechst staining and determination of the percentage of condensed and fragmented nuclei from at least 300 cells per conditions. Experiments were repeated at least three times.

Bcl-2 and TRAIL-R4 gene silencing by siRNA. TRAIL-R4 siRNA #1 (5′-UCCUUAAGUUCGUCGUCUU-3′), TRAIL-R4 siRNA #2 (5′-UCACUACCUUAU CAUCAUA-3′) and TRAIL-R4 siRNA #3 (5′-GGGUGUGGAUUACACCAUU-3′) were purchased from Eurogentec (Angers, France). Bcl-2 siRNA was purchased from Invitrogen. Cells were transfected with a scramble, Bcl-2 or TRAIL-R4 targeting siRNAs using Amaxa cell line nucleofector kit V (Lonza) with transfection program N016. 48 h after transfection, Bcl-2 and TRAIL-R4 expression were monitored either by western blotting or by flow cytometry, and sensitivity to TRAIL was assessed by Hoechst.

Bax activation by flow cytometry analysis. Cells, treated or untreated with His-TRAIL and/or chemotherapy were fixed with 4% PFA, permeabilized (PBS, BSA 1% and saponin 0.1%) for 10 min at room temperature and stained with an anti-Bax antibody which recognizes the active N-terminal form of Bax (clone 6A7, Tebu-bio). In all, 10 000 events were analyzed using a LSR2 flow cytometer (BD Biosciences).

Digitonin permeabilization. After treatment, cells were washed in PBS and lysed in buffer containing 75 mM KCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4 and 250 mM sucrose containing 400 μg/ml digitonin. Cells were kept on ice to obtain 90 95% of

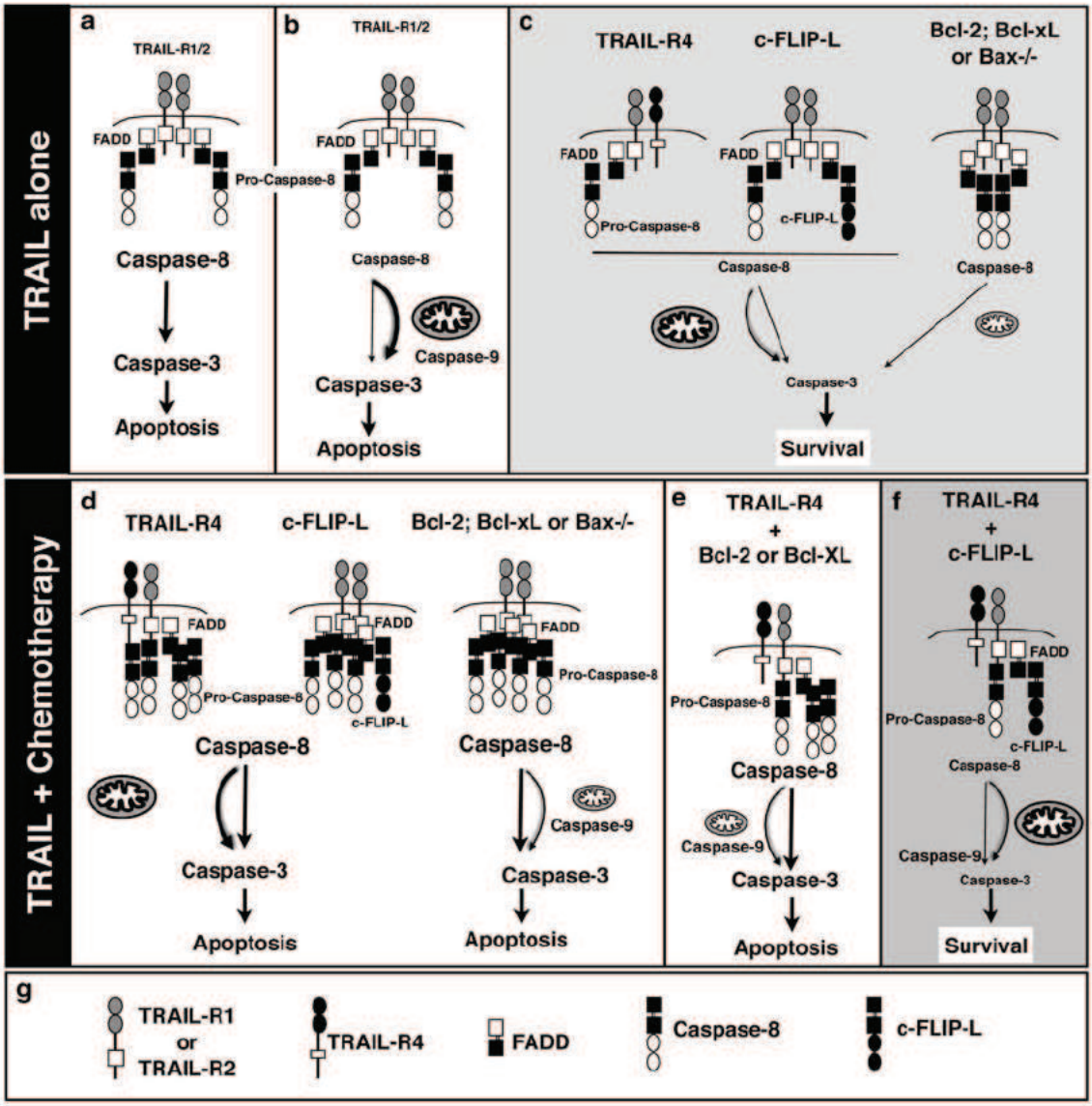

Figure 6 Proposed model of TRAIL-induced cell death regulation. (**a**) Direct activation of caspase-8 by TRAIL in type I cells. (**b**) A mitochondrial amplification loop of caspase activation in type II cells is required because of reduced caspase-8 activation upon TRAIL engagement. (**c**) Overexpression of TRAIL-R4, FLIP-L or mitochondrial block, protects type II cells from TRAIL-induced cell death. TRAIL-R4 and c-FLIP-L limit caspase-8 activation within the TRAIL DISC, which impairs mitochondrial activation, leading to low caspase-3 activation and survival. Mitochondrial block in type II cells, induced by Bcl-2 or Bcl-xL overexpression or Bax-deficiency inhibit amplification of the signal. Caspase-8 is activated but much less efficiently than in type I cells, leading to low caspase-3 activation and survival. (**d**) Chemotherapeutic drugs restore TRAIL sensitivity mainly through enhanced capase-8 recruitment to and activation at the DISC. Thus, the threshold of active caspase-8 required to induce direct caspase-3 activation can be reached and cells undergo apoptosis, overcoming TRAIL-R4- and c-FLIP-mediated inhibition of caspase-8, but also inhibition induced by Bcl-2 or Bcl-x_L overexpression or Bax-deficiency. (**e**) Inhibition of the mitochondrial pathway by Bcl-2 or Bcl-xL overexpression in TRAIL-R4-expressing cells fails to compromise chemotherapy-induced sensitization to TRAIL. (**f**) Forced inhibition of caspase-8 activation in TRAIL-R4 and c-FLIP-L-expressing cells abrogates apoptosis induced by TRAIL after chemotherapy. (**g**) Schematic representation of TRAIL receptors, FADD, c-FLIP and caspase-8

trypan blue-permeabilized cells. After 5 min at 16 000 × *g*, supernatants were collected as the cytosolic fraction. Pellets were then lysed in buffer containing 1% Triton-X100. After centrifugation for 20 min at 16 000 × *g*, supernatants were collected.

Immunoprecipitations. For DISC analysis, 10^8 cells in 1 ml of medium were stimulated with 5 μg Flag-TRAIL cross-linked with 10 μg of M2 antibody for the indicated times at 37°C. Cells were then washed with cold phosphate saline buffer, lysed in 1 ml of lysis buffer containing 1% NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 10% glycerol. Lysates were pre-cleared with Sepharose 6B (Sigma-Aldrich), and immunoprecipitated overnight at 4 °C with G-protein Sepharose beads (Amersham Biosciences, Les Ullis, France). Beads were then washed four times with the respective detergent, and immunoprecipitates were eluted in lysis buffer (Tris-HCl 63 mM, SDS 2%, phenol red 0.03%, glycerol 10% and DTT 100 mM of pH 6.8), boiled for 5 min and processed for immunobloting.

Western blotting. Immunoprecipitates or cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in PBS containing 0.05% of Tween 20 and 5% of milk powder. Immunoblots were then incubated with specific primary antibody followed by HRP-conjugated secondary antibody and were developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

***In vivo* evaluation of the combination of CDDP and TRAIL.** 5-week-old athymic female mice (NMRI nu/nu) were obtained from Janvier (Le Genest Saint-Isle, France). This protocol was approved by the local Animal Ethical committee (Université de Bourgogne, Dijon, France). Mice were subcutaneously xenografted with 4×10^6 HCT116-Ctl cells in the right flank and 4×10^6 HCT116-TRAIL-R4 in the left flank. Mice were weighed and tumor volume was evaluated every 2 days by caliper measurement using the following formula: $(l \times l \times L)/2$, with l the lower and L the higher dimension. When the tumor volume reached 20 mm^3, mice were divided randomly into four groups with four mice per group (day 0). The first group served as a control and received 0.2 ml PBS as vehicle at days 0 and 8, and 0.1 ml PBS containing 10 mM β-mercaptoethanol at days 2, 3, 4, 5 and 10, 11, 12, 13. The second group was injected as group 1, but received 4 mg/kg CDDP at day 0 and day 8. The third group received 8 mg/kg recombinant His-TRAIL at days 2, 3, 4, 5 and days 10, 11, 12, 13 and PBS at days 0 and 8. The fourth group received 4 mg/kg CDDP at days 0 and 8 and 8 mg/kg recombinant His-TRAIL at days 2, 3, 4, 5 and days 10, 11, 12, 13. All administrations were done intraperitoneally. The initial value for each group (day 0) was arbitrarily established as 100, and all subsequent changes in tumor volume for each group were expressed as a percentage change in comparison with the starting tumor volume [(Tumor volume day 1) $\times$ 100/ (Tumor volume at day 0)], and are referred as arbitrary tumor volume.

Conflict of interest

The authors declare no conflict of interest.

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TRAIL-R4 Promotes Tumor Growth and Resistance to Apoptosis in Cervical Carcinoma HeLa Cells through AKT

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Abstract

Background: TRAIL/Apo2L is a pro-apoptotic ligand of the TNF family that engages the apoptotic machinery through two pro-apoptotic receptors, TRAIL-R1 and TRAIL-R2. This cell death program is tightly controlled by two antagonistic receptors, TRAIL-R3 and TRAIL-R4, both devoid of a functional death domain, an intracellular region of the receptor, required for the recruitment and the activation of initiator caspases. Upon TRAIL-binding, TRAIL-R4 forms a heteromeric complex with the agonistic receptor TRAIL-R2 leading to reduced caspase-8 activation and apoptosis.

Methodology/Principal Findings: We provide evidence that TRAIL-R4 can also exhibit, in a ligand independent manner, signaling properties in the cervical carcinoma cell line HeLa, through Akt. Ectopic expression of TRAIL-R4 in HeLa cells induced morphological changes, with cell rounding, loss of adherence and markedly enhanced cell proliferation *in vitro* and tumor growth *in vivo*. Disruption of the PI3K/Akt pathway using the pharmacological inhibitor LY294002, siRNA targeting the p85 regulatory subunit of phosphatidylinositol-3 kinase, or by PTEN over-expression, partially restored TRAIL-mediated apoptosis in these cells. Moreover, the Akt inhibitor, LY294002, restituted normal cell proliferation index in HeLa cells expressing TRAIL-R4.

Conclusions/Significance: Altogether, these results indicate that, besides its ability to directly inhibit TRAIL-induced cell death at the membrane, TRAIL-R4 can also trigger the activation of signaling pathways leading to cell survival and proliferation in HeLa cells. Our findings raise the possibility that TRAIL-R4 may contribute to cervical carcinogenesis.

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Introduction

TRAIL/Apo2L is regarded as a promising anticancer agent for cancer therapy and is currently being evaluated in clinical trials [1]. TRAIL binds to four membrane anchored receptors: TRAIL R1 (DR4), TRAIL R2 (DR5, Killer, TRICK), TRAIL R3 (DcR1, LIT, TRID) or TRAIL R4 (DcR2, TRUNDD), and one soluble receptor, osteoprotegerin (OPG) [1]. TRAIL induces cell death through its interaction with either TRAIL R1 or TRAIL R2. These two agonistic receptors harbor, within their cytoplasmic region, a relatively small amino acid stretch called the death domain (DD), which is necessary and sufficient to transduce the death signal [2]. Activation of TRAIL R1 or TRAIL R2 by trimeric TRAIL induces the recruitment of the adaptor protein FADD (Fas associated death domain protein) *via* homotypic interactions with their respective DD, allowing in turn the recruitment of the initiator caspases, procaspases 8 and 10 [3,4], leading to the formation of the death inducing signaling complex (DISC) [5]. Within the DISC, the initiator caspases 8 and 10 undergo catalytic cleavage inducing their release to the cytosol and the triggering of the caspase cascade that ultimately leads to apoptosis. In contrast, TRAIL binding to TRAIL R3 or TRAIL R4 fails to induce the apoptotic machinery because none of these receptors harbor a functional DD [6]. TRAIL R3 is anchored to the membrane via its glycosyl phosphatidylinositol tail (GPI), whereas TRAIL R4 is addressed to the cell surface through a transmembrane domain but includes a truncated DD that is unable to recruit the adaptor protein FADD [7].

Expression of TRAIL R3 or TRAIL R4 confers resistance to TRAIL induced cell death in several tumor cell lines and primary tumors [8,9,10,11,12,13]. These antagonistic receptors, coined "decoy receptors", were initially proposed to act as competitors to

TRAIL R1 and TRAIL R2 for TRAIL binding [14]. However, we and others have provided evidence that TRAIL R4 should rather be considered as a regulatory receptor, because TRAIL R4 is able to interact with TRAIL R2 within the TRAIL DISC and to impair caspase 8 activation [10,12,15]. In this study, we provide new evidence that TRAIL R4 exhibits a TRAIL independent signaling activity that gives rise to oncogenic like properties in HeLa cells, mainly through the activation of Akt.

Results

TRAIL-R4 ectopic expression in HeLa cells markedly changes cell morphology, cell proliferation and tumor growth

Ectopic TRAIL R4 expression to physiological levels in HeLa cells (Figure 1A), as well as in other tumors [15], by use of retroviral vectors, affords good selective protection against TRAIL induced cell death, but not Fas ligand (Figure 1B and C). Strikingly, HeLa cells expressing TRAIL R4 (H TRAIL R4) undergo drastic morphological changes including cell rounding and loss of adherence (Figure 1D). As compared to control cells (H Ctl) infected with an empty vector, H TRAIL R4 cells exhibited a higher proliferative index (Figure 1E). This increase in cell proliferation is however most likely independent of TRAIL itself, since the recombinant fusion protein Fc TRAIL R2 failed to affect proliferation in H TRAIL R4 cells (Figure S1A). In agreement with these findings, TRAIL levels were undetectable in the supernatant or at the surface of H TRAIL R4 cells (not shown). The drastic changes in cell morphology and proliferative status prompted us to check whether TRAIL R4 overexpression confers tumor growth advantage *in vivo*. Parental (H Ctl) and TRAIL R4 expressing cells (H TRAIL R4) were implanted into nude mice in the left and the right flank, respectively of the same

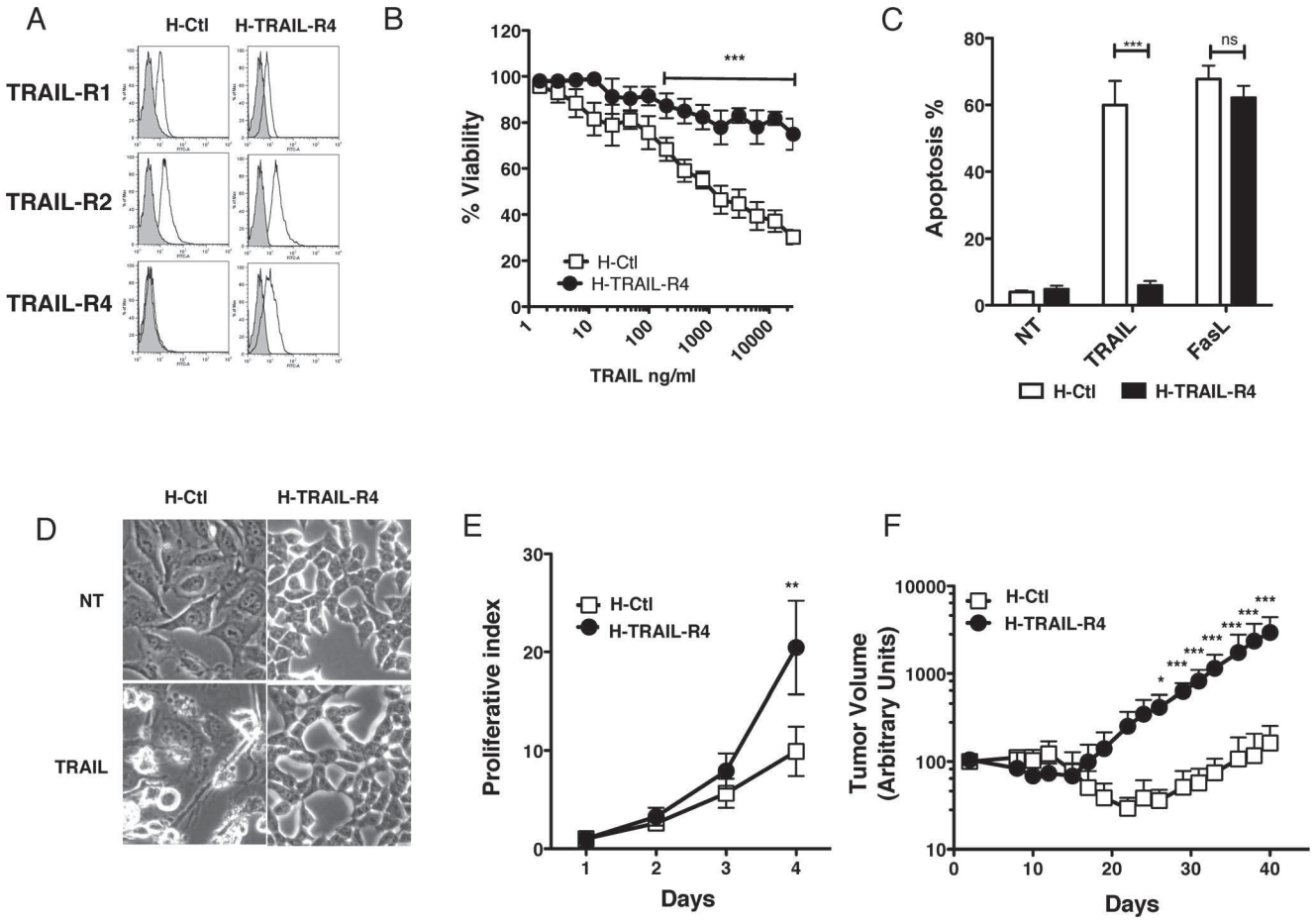

Figure 1. TRAIL R4 protects HeLa cells from TRAIL induced apoptosis and enhances tumor cell growth *in vitro and in vivo*. (A) HeLa stably transduced with retroviruses encoding TRAIL R4 (H TRAIL R4), or the empty mock retroviral vector (H Ctl), were analyzed by flow cytometry for TRAIL receptor staining as indicated. (B) Cellular viability of the populations was evaluated by PMS/MTS 24 hours after treatment with increasing concentrations of recombinant His TRAIL. HeLa control (H Ctl in open squares) and HeLa expressing TRAIL R4 (H TRAIL R4 in filled circles). Mean viability % and SD from three independent experiments are shown (mean ± SD). ***P<0.001, two way ANOVA with Bonferroni post tests, H TRAIL R4 compared with H Ctl Mock. (C) Apoptosis induced by TRAIL (500 ng/ml) in H Ctl and H TRAIL R4 cells 24 hours after stimulation. Apoptosis was determined by Hoechst staining. Data are representative of at least three independent experiments. ***P<0.001, student t test. (D) Representative light microscopic picture of H Ctl versus H TRAIL R4 treated or not (NT) with 500 ng/ml TRAIL for 16 hours. (E) Cell proliferative index was followed for 4 days and measured using CFSE by flow cytometry. Mean analysis from three independent experiments is shown. HeLa control (H Ctl in open squares) and HeLa expressing TRAIL R4 (H TRAIL R4 in filled circles). (F) Time dependent growth of HeLa control (H Ctl in open squares) and HeLa expressing TRAIL R4 (H TRAIL R4 in filled circles) in nude mice after xenograft (n = 10). These results represent the mean tumor volume in arbitrary units ± SD of a representative experiment performed with six to seven mice per group. (E) and (F) *P<0.05 and ***P<0.001, two way ANOVA with Bonferroni post tests, H TRAIL R4 compared with H Ctl Mock.
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animal (Fig. S1B) and tumor growth was followed for 32 days (Figure 1F). Remarkably, TRAIL R4 expressing HeLa cells exhibited a clear tumor growth advantage as compared to control cells in nude mice.

We next checked whether TRAIL R4 ectopic expression affected cell proliferation in two other TRAIL sensitive tumor cell lines, the Jurkat T cell lymphoma and the colon carcinoma SW480. To address this question, Jurkat and SW480 cell lines were infected with an empty vector (J Ctl and SW Ctl) or a retroviral vector encoding TRAIL R4 (J TRAIL R4 and SW TRAIL R4). Expression levels of TRAIL R4 and TRAIL R2 were analyzed by flow cytometry (Figure 2A and B) and cell sensitivity to TRAIL induced cell death was determined by PMS MTS assay. Similar to HeLa cells, ectopic expression of TRAIL R4 in Jurkat or SW480 cells inhibited TRAIL induced cell death in a dose dependent manner (Figure 2C and D). TRAIL R4 expression, however, induced no particular modification of cell proliferation as compared to control cells (Figure 2E and F), and no change in cell morphology could be observed (not shown).

TRAIL-R4-mediated constitutive Akt activation in HeLa cells contributes to cell resistance to TRAIL-induced apoptosis and to increased cell proliferation

To explore the molecular basis of the deregulated death commitment and proliferation potential in HeLa cells expressing TRAIL R4 ectopically, we next analyzed the activation status of Akt, a survival pathway that plays a central role in diverse cellular functions, including survival, growth, and proliferation [16].

Strikingly, while Akt appeared to be constitutively activated in Jurkat and SW480 cells irrespective of TRAIL R4 ectopic expression, a differential Akt phosphorylation profile was detected in H TRAIL R4 cells as compared to control parental HeLa cells (Figure 3A). Constitutive activation of Akt in HeLa cells was TRAIL independent as TRAIL stimulation only marginally induced Akt phosphorylation in H Ctl cells (Figure 3B). Activation of Akt in these cells appeared to be restricted to TRAIL R4. Accordingly, ectopic expression of TRAIL R3 (Figure 3B), TRAIL R2 (not shown) or a chimeric TRAIL receptor encoding the extracellular domain of

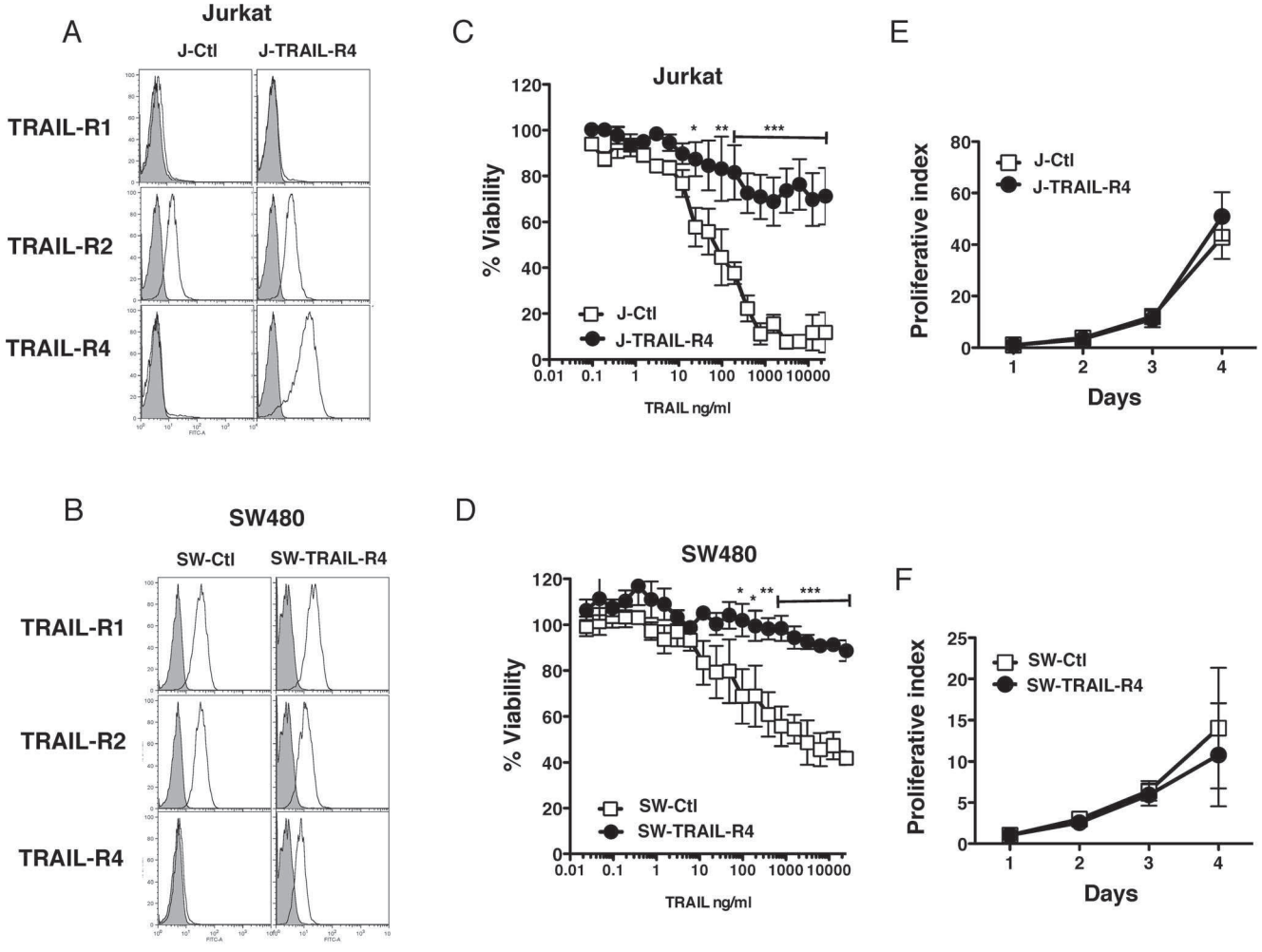

Figure 2. TRAIL R4 ectopic expression fails to promote cell proliferation in Jurkat and SW480 cells. (A and B) Jurkat and SW480 cells were stably transduced with retroviruses encoding TRAIL R4 (J TRAIL R4 and SW TRAIL R4), or the empty mock retroviral vector (J Ctl and SW Ctl), respectively. Cells were analyzed by flow cytometry for TRAIL receptor staining as indicated. (C and D) Cellular viability was evaluated by PMS/MTS 24 hours after treatment with increasing concentrations of recombinant His TRAIL. Jurkat or SW480 control cells (J Ctl or SW Ctl in open squares) and Jurkat or SW480 expressing ectopically TRAIL R4 (J TRAIL R4 or SW TRAIL R4 in filled circles). (E and F) Cell proliferative index was measured as in Figure 1. Mean proliferative index, viable cells and SD from three independent experiments are shown (mean ± SD). *P<0.05, **P<0.01 and ***P<0.001, two way ANOVA with Bonferroni post tests, J or SW TRAIL R4 compared with J and SW Ctl Mock respectively.
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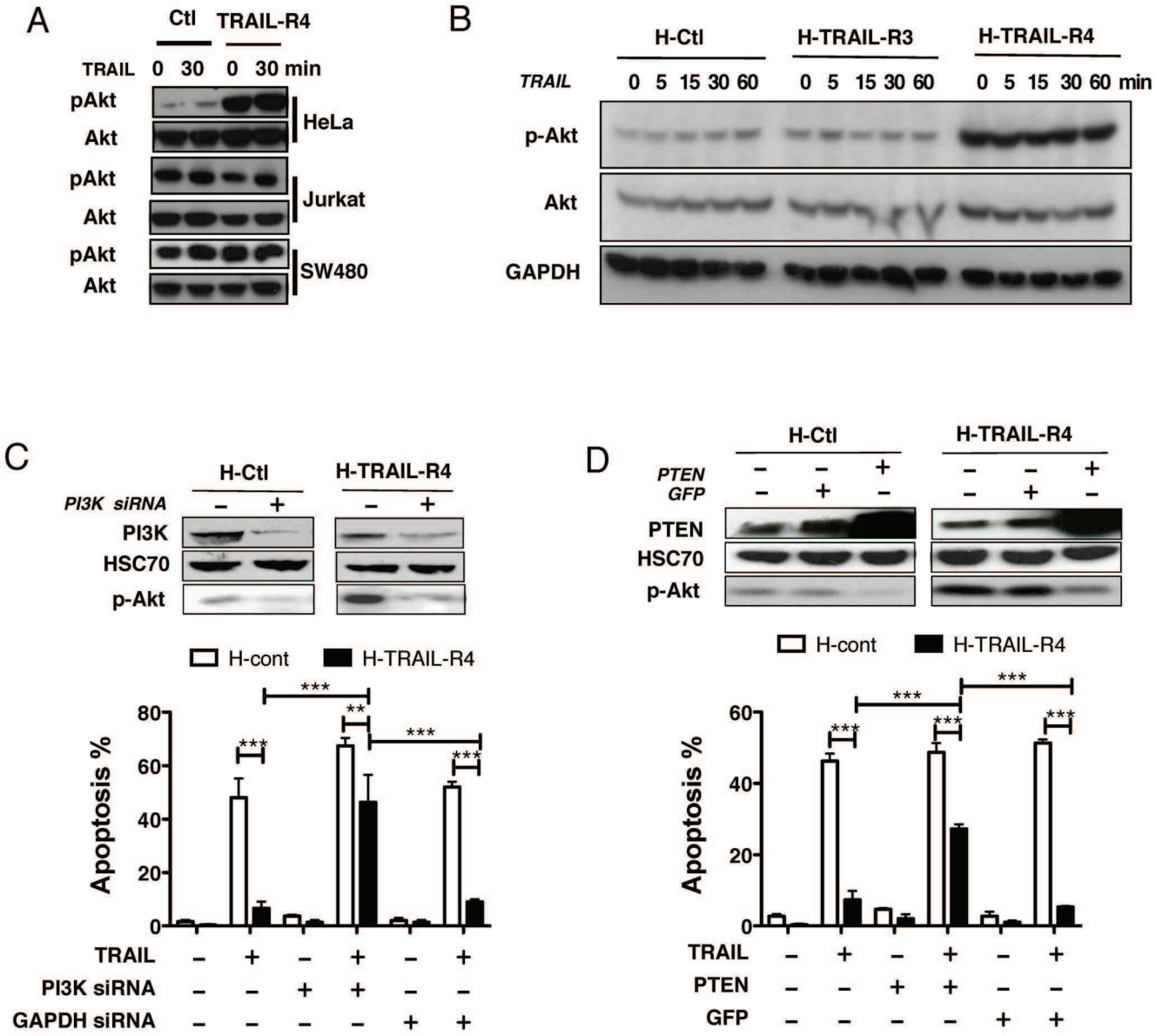

Figure 3. TRAIL R4 mediated constitutive Akt activation contributes to cell resistance to TRAIL induced apoptosis in HeLa cells. (A) Total Akt and phospho Akt were monitored by western blot from HeLa, Jurkat and SW480 cells expressing TRAIL R4 and compared to control cells (Ctl). Unstimulated or TRAIL stimulated (500 ng/ml for 30 minutes) cell samples are shown. (B) H Ctl, H TRAIL R4 or HeLa cells expressing TRAIL R3 (H TRAIL R3) were stimulated with TRAIL (500 ng/ml) for the indicated time and total Akt or phospho Akt expression was assessed as above. Sample loading was assessed using GAPDH for normalization. (C) H Ctl and H TRAIL R4 cells were transfected with a siRNA targeting PI3K or a scramble siRNA for 48 hours and stimulated or not with TRAIL (500 ng/ml) for 6 hours. Phosphorylation of Akt and PI3K expression levels were analyzed by western blot, and apoptosis was analyzed by Hoechst staining. (D) H Ctl and H TRAIL R4 cells were transfected with a GFP mock vector or with a vector encoding PTEN for 24 hours. Expression levels of phospho Akt and PTEN were analyzed by western blot and apoptosis induced by TRAIL was monitored as above. **P<0.01 and ***P<0.001, one way ANOVA with Bonferroni's multiple comparison test.
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TRAIL R1 fused to TRAIL R2 (Figure S2) induced no change in Akt activation, contrary to a chimeric construct encoding TRAIL R4 intracellular domain (Figure S2). Inhibition of Akt phosphorylation either using siRNA targeting the regulatory subunit of PI3K (Figure 3C), or by over expressing PTEN (Figure 3D) sensitized H TRAIL R4 cells to TRAIL induced cell death, indicating that cell resistance to TRAIL in these cells is, at least partly, due to the sustained activation of the Akt pathway. Accordingly, the pharmacological inhibitor of Akt phosphorylation, LY294002, significantly restored TRAIL induced apoptosis in H TRAIL R4 cells (Figure 4A). Notably, sensitization to TRAIL induced cell death was not associated with an increase in caspase 8 cleavage, but rather with an increase in caspase 3 processing (Figure 4B and C), suggesting that Akt mediated inhibition most likely occurs downstream of the TRAIL DISC probably at the mitochondrial or post mitochondrial level. Remarkably, LY294002 reduced H TRAIL R4 mediated cell proliferation to levels comparable to those of control parental cells (Figure 4D).

Altogether our findings suggest that TRAIL R4 in HeLa cells is a regulatory receptor whose anti apoptotic functions involve both TRAIL DISC targeting and activation of the Akt survival pathway.

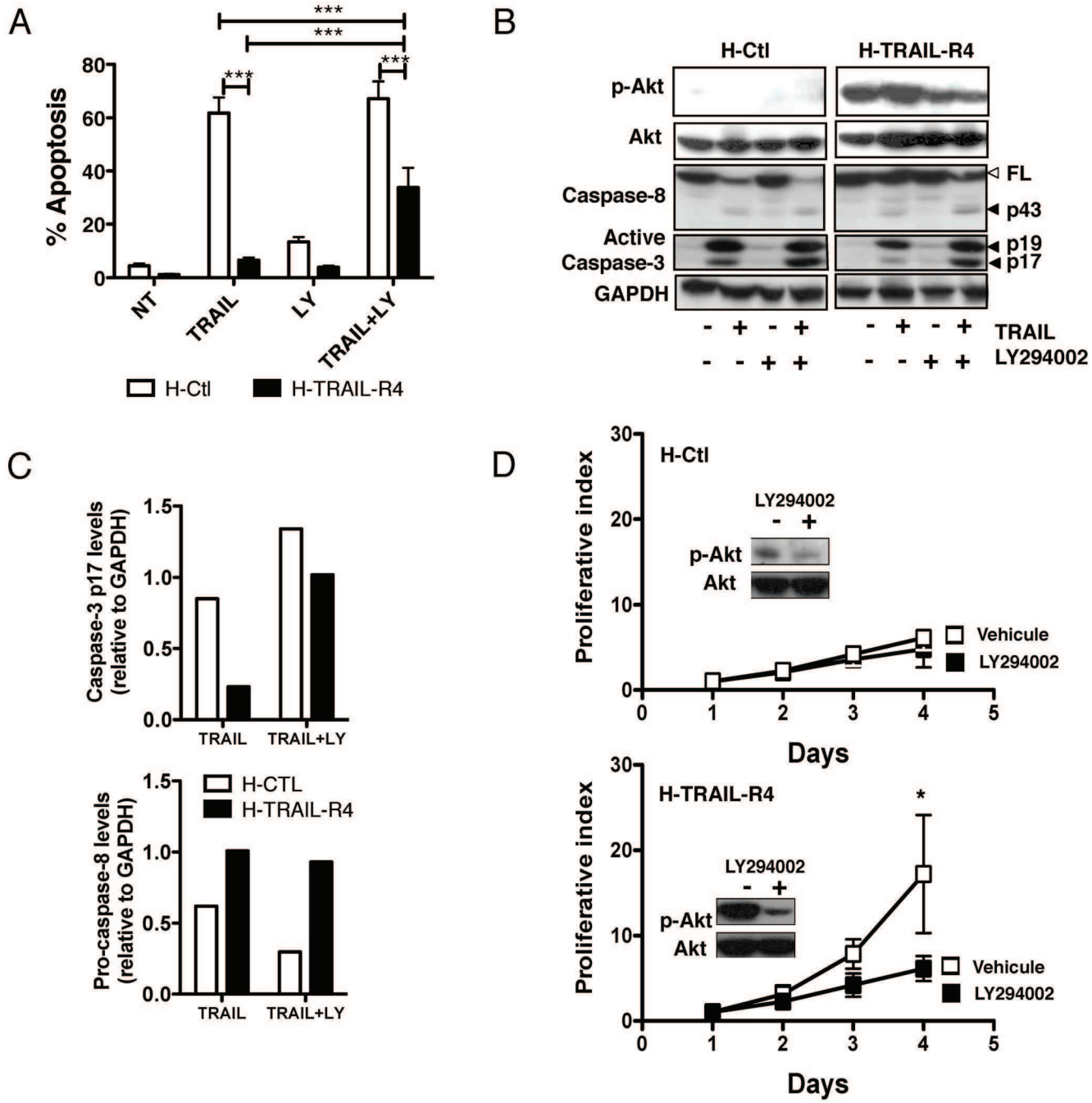

Figure 4. Inactivation of Akt restores partial sensitivity to TRAIL induced cell death in TRAIL R4 expressing cells and restores normal cell proliferation index. (A) HeLa H Ctl and H TRAIL R4 cells were left untreated or pretreated for 1 hour with the Akt inhibitor LY294002 (100 μM) then stimulated or not with TRAIL (500 ng/ml) for 24 hours and apoptosis was analyzed after Hoechst staining. These results are representative of at least 3 independent experiments. Mean percentage of apoptotic cells and SD are shown (mean ± SD). ***P<0.001, one way ANOVA with Bonferroni's multiple comparison test. (B) Cells were stimulated either with LY294002 (100 μM) then treated or not with TRAIL as above and cell lysates were processed by western blot for the analysis of Akt phosphorylation, caspase 8 and active caspase 3. Filled arrows show caspase cleavage products. The empty arrow shows full length caspase 8. Densitometry analysis of caspase 3 p17 and caspase 8 full length immunoreactive bands were obtained using ImageJ software, normalized with respect to GAPDH, and plotted in (C). (D) Cellular growth in the presence or the absence of Akt inhibitor LY294002 (10 μM) was monitored during 4 days by flow cytometry analysis using CFSE. LY294002 was applied every day to the cell culture supernatant to afford sustained inhibition of Akt.
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Discussion

TRAIL is an attractive anti tumoral agent owing to its ability to selectively induce apoptosis in tumor cells [17,18] and thus TRAIL derivatives or recombinant TRAIL preparations have entered clinical trials [1]. The molecular mechanisms governing TRAIL induced cell death or signal transduction remain, however, only partially understood. Cell resistance to TRAIL induced cell death can arise

both from the inhibition of the apoptotic machinery, or more specifically from the deregulation of the expression and/or the functionality of TRAIL receptors. Likewise, loss of TRAIL R1 or TRAIL R2 expression [19,20,21,22] or expression of TRAIL R3 or TRAIL R4, two main antagonistic receptors [9,10,11,12,13], abrogate TRAIL induced cell death selectively, without affecting the apoptotic machinery triggered by other members of the TNF family, nor the intrinsic pathway. Likewise, overexpression of TRAIL R4 protects tumor cells against TRAIL induced cell death by regulating caspase 8 activation at the DISC level [10,15].

In the present study, we provide evidence that TRAIL R4 in HeLa cells a) partially protects from TRAIL induced apoptosis and b) enhances cell proliferation index through Akt activation in a TRAIL independent manner. Remarkably, TRAIL R4 mediat ed Akt activation in HeLa cells leads to drastic morphological changes, such as reduced cell size and loss of adhesion, suggesting that TRAIL R4 may exhibit pro metastatic properties (Figure 5). These findings, however, may be restricted to tumor cell lines expressing low levels of active Akt, since contrary to HeLa cells, no morphological or proliferative changes were detected in Jurkat and SW480 cells, which express high levels of active Akt. Our findings nonetheless clearly suggest, for the first time, that TRAIL R4 exhibits unexpected signaling properties conferring cellular growth advantage both *in vitro* and *in vivo*.

Tentative model for Akt activation from TRAIL-R4 in HeLa cells

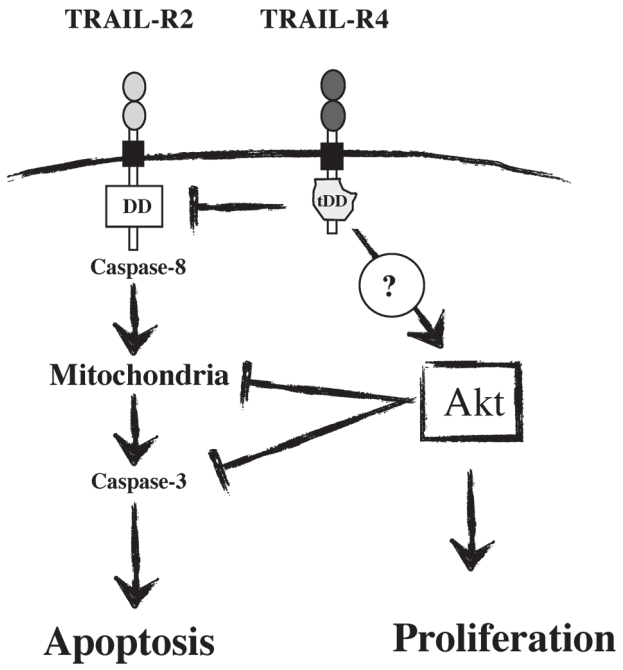

Figure 5. Model for Akt activation from TRAIL R4 in HeLa cells. TRAIL R4 is known to inhibit caspase 8 activation induced by TRAIL R2 upon TRAIL stimulation. We propose here that TRAIL R4 may, in addition, trigger Akt activation in a ligand independent manner. Constitutive activation of Akt in HeLa cells would therefore inhibit caspase 3 activation at the mitochondrial or post mitochondrial level to inhibit apoptosis induced by TRAIL, and could possibly foster cell proliferation.
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Besides apoptosis triggering, and depending on the cell line or type, TRAIL stimulation can induce non apoptotic signaling pathways including Akt [23,24,25,26], NF kB [27] or MAPK [28,29], cell proliferation [26,30,31] or differentiation [32,33,34]. It has recently been proposed that the activation of non apoptotic signaling pathways by TRAIL involve the formation of a cytosolic secondary complex which, contrary to TNF [35], allows the recruitment of adaptor proteins and kinases including TRAF2, RIP or NEMO [36]. Activation of Akt by TRAIL R4 in HeLa cells by such a secondary complex is unlikely, as this pathway appears to be activated in a TRAIL ligand independent manner. Moreover, we have not yet been able to identify this secondary complex (not shown).

How TRAIL R4 mediates Akt activation in HeLa cells remains unknown for the moment. TRAIL R4 shares relatively high homology with TRAIL R1 and TRAIL R2 [7] and is the only TRAIL antagonistic receptor that harbors an intracellular domain. Interestingly, ectopic expression of the TRAIL R4 intracellular domain in HeLa cells induces constitutive AKT activation. This domain would therefore provide an ideal docking site for a putative binding partner. It is therefore tempting to speculate that the intracellular domain of TRAIL R4 may interact with membrane associated or cytosolic proteins already known to bind to TRAIL R1 or TRAIL R2 such as GSK3 [37], ARAP1 [38], DAP3 [39], Burton's tyrosine kinase [40], or PRTM5 [41]. The exact molecular mechanism involved in this regulation therefore needs to be further investigated.

Altogether, our findings indicate for the first time that, besides inhibiting TRAIL induced cell death, TRAIL R4 is able to regulate the PI3K/Akt signaling pathway in cell type dependent manner leading to cell resistance to apoptosis and to enhanced cellular growth.

Materials and Methods

Reagents and antibodies

His tagged recombinant soluble human TRAIL and Fc TRAIL R2 were produced and used as described previously [42]. For Western blotting experiments, anti GAPDH, anti PI3K, anti HSC70 were obtained from Santa Cruz Biotechnology (CA, USA), anti phospho Akt from Upstate (Milipore, Molsheim, France), anti Akt, and anti PTEN from Cell Signaling (Ozyme, Saint Quentin Yvelines, France). Antibodies used for flow cytometry, anti TRAIL R1 (wB K32), anti TRAIL R2 (B L27), anti TRAIL R3 (wB B44), anti TRAIL R4 (wB P30), and anti TRAIL were from Diaclone (Besançon, France). The PI3K/AKt inhibitor LY294002 was purchased from Cell Signaling (Ozyme, Saint Quentin Yvelines, France).

Cell culture and transfection

HeLa (human cervix carcinoma), SW480 (Colon carcinoma) and Jurkat cells (T Lymphoma) were obtained from the ATCC. HeLa and SW480 cells were cultured in high glucose Dulbecco's modified Eagle's medium (Sigma Aldrich, Lyon, France) supplemented with 10% fetal calf serum (Gibco BRL, Erigny, France), and Jurkat cells were cultured in RMPI as above. Transfection of HeLa cells was carried out using JetsiENDO^{TM} reagent (Eurogentec, Angers, France), according to the manufacturer's instructions using the following PI3K siRNA (5′ GGGUGUGGAUUACACCAUU 3′) and siRNA control (Invitrogen, Cergy Pontoise, France). PTEN transfections were performed with the pSG5L HA PTEN construct (kindly provided by Dr William Sellers, Dana Farber Cancer Institute, Boston) using TransPEI^{TM} (Eurogentec).

Retroviral production and cell transduction

The retroviral vector pMSCVpuro and the generation of viruses have previously been described (30). TRAIL R4 full length construct

was subcloned from a pCR 3 vector (Invitrogen) to the retroviral vector pMSCV puro as a HindII XhoI fragment. HeLa cells were transduced for 16 hours with viral supernatants containing polybrene (8 μg/ml), washed in phosphate buffered saline (PBS), and selected in complete medium containing puromycin (2.5 μg/ml).

Measurement of cell viability and apoptosis

Cell viability assays were performed in 96 well plates. 10^4 cells per well were incubated at 37°C for 24 hours with increasing concentrations of His TRAIL (from 0 to 10,000 ng/ml). Cell viability was determined by the PMS/MTS method, according to the manufacturer's specifications (Promega, Madison, WI, USA.). Apoptosis was assessed by Hoechst staining by determining the percentage of condensed nuclei from at least 300 cells per condition. For Akt inhibition, cells were pretreated with LY294002 (100 μM) one hour before TRAIL treatment (500 ng/ml, 5 hours). PI3K inhibition and PTEN expression were measured 48 and 24 hours hours after transfection respectively and cells were simultaneously treated 5 hours with 500 ng/ml of His TRAIL.

Cell proliferation

To measure cell proliferation the different cellular populations were plated at the same density and counted each day. CellTrace^{TM} CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen) was used to measure cell proliferation using a LSRII flow cytometer (DB Biosciences) and the ModFIT Software (Verity Software House Topsham, ME) was applied to determine the proliferation index.

Western blotting

Lysates were resolved by sodium dodecyl sulfate polyacryl amide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked in PBS containing 0.05% Tween 20 and 5% powdered milk. Immuno blots were then incubated with specific primary antibody, followed by horseradish peroxidase conjugated secondary antibody, and developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce, Rockford, IL).

In vivo studies

Six weeks old female athymic nude mice (Harlan, Le Malcourle, Gannat) were subcutaneously xenografted with 1×10^6 H Ctl in the right flank and 1×10^6 H TRAIL R4 in the left flank (n 10). Tumor volume was obtained after caliper measurement of the tumor and the formula (l×l×L)/2 with l the smaller and L the higher dimension.

Supporting Information

Figure S1 (**A**) The proliferative index of H Ctl and H TRAIL R4 cells was measured in the presence or in the absence of 10 μg recombinant Fc TRAIL R2, as described in the manuscript Figure 1E. Fc TRAIL R2 was added to the culture daily for 4 days. (B) Representative picture of nude mice xenografted with HeLa control (H Ctl on the left flank) and HeLa expressing TRAIL R4 (H TRAIL R4 on the right flank) and the corre sponding tumors harvested from mice pictured.
(TIFF)

Figure S2 (**A**) Schematic representation of TRAIL receptor chimeric constructs (OM043, OM050 and OM051). Vectors were constructed using standard cloning procedures. TRAIL R2 and TRAIL R4 intracellular domains (icd) were obtained by polymerase chain reaction from pCRIII vectors encoding full length TRAIL R2 and TRAIL R4 as described earlier [10], with the following primer pairs: TRAIL R2 forward primer (5′ GTC GAC TGT TCT CTC TCA GGC ATC 3′); reverse primer (5′ CTC GAG CGG CCG CCA GTG TGA TGG 3′) and TRAIL R4 forward primer (5′ GTC GAC TAT CAC TAC CTT ATC ATC 3′); reverse primer (5′ CTC GAG TCA CAG GCA GGA CGT AGC 3′) containing a SalI and a XhoI site. Oligonucle otide primers and Pfu polymerase were purchased from Euro gentech (Angers, France) and Sigma Aldrich (Lyon, France) respectively. The resulting amplified fragments were subcloned into pCR Blunt (Invitrogen, Cergy Pontoise, France) and checked by sequencing. TRAIL R2 icd and TRAIL R4 icd were sub cloned between the SalI and XhoI sites of pCRIII vectors encoding the extracellular domains (ecd) of TRAIL R1 (aa 1 239, PS688), TRAIL R2 (aa 1 212, PS664) or TRAIL R4 (aa1 211, PS690), kindly provided by Dr Pascal Schneider (Lausanne, Switzerland). Resulting TRAIL R1ecd TRAIL R2icd, TRAIL R4ecd TRAIL R2icd and TRAIL R2ecd TRAIL R4icd DNA fragments were subcloned into a pMSCV Puro retroviral vector between HindIII and XhoI, generating pMSCV Puro TRAIL R1ecd TRAIL R2icd (OM043), pMSCV Puro TRAIL R4ecd TRAIL R2icd (OM051) and pMSCV Puro TRAIL R2ecd TRAIL R4icd (OM050). (**B**) Receptor expression in HeLa cells was analyzed by flow cytometry after infection of TRAIL receptor fusion constructs (OM043, OM050 and OM051) or the corresponding empty vector (OM181). (**C**) Biochemical analysis of TRAIL chimeric receptors. Immunoprecipitations were performed using a control antibody (lanes 1, 4, 7, 10, 13 and 16) or antibodies targeting TRAIL R1 (lanes 2, 3, 5 and 6), TRAIL R2 (lanes 8, 9, 11 and 12) and TRAIL R4 (lanes 14, 15, 17 and 18) from DIACLONE (Besançon, France). Antibodies were applied either after lysis (1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16 and 18), or on intact cells (2, 5, 8, 11, 14 and 17) for 1 h on ice. The latter samples were subsequently subjected to lysis in NP40 lysis buffer and samples were precleared on sepharose 6B beads before immunoprecipitation using agarose protein G beads. Samples were then washed extensively and subjected to western blot using anti TRAIL R1, R2 and R4 antibodies from Milipore (Molsheim, France). Note that TRAIL R4's extracellu lar domain is not recognized by the anti TRAIL R4 antibody from Chemicon. *ns stands for non specific. (**D**) AKT activation was analyzed by western blot as described in the text.
(TIFF)

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Author Contributions

Conceived and designed the experiments: OM NL Aymeric Morlé DM. Performed the experiments: NL Alexandre Morizot DM GJ EI Aymeric Morlé SS BR. Analyzed the data: OM NL Alexandre Morizot DM GJ EI Aymeric Morlé SS BR. Wrote the paper: OM NL DM SS ES CG.

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Early Release Paper

Quercetin-mediated Mcl-1 and survivin downregulation restores TRAIL-induced apoptosis in non-hodgkin lymphoma B-cells

by Guillaume Jacquemin, Virginie Granci, Anne Sophie Gallouet, Najoua Lalaoui, Aymeric Morle', Elisabetta Iessi, Alexandre Morizot, Carmen Garrido, Thierry Guillaudeux, and Olivier Micheau

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Quercetin-mediated Mcl-1 and survivin downregulation restores TRAIL-induced apoptosis in non-hodgkin lymphoma B-cells

Running Title: *Quercetin restores TRAIL apoptosis in B-NHL*

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Key words: follicular lymphoma, diffused large B cell lymphoma, quercetin, TRAIL, Mcl-1, surviving, apoptosis; proteasome, p53, bax, caspases.

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Abstract

Background. Non-Hodgkin's B cell lymphomas account for approximately 70% of B cell lymphomas. While its incidence is dramatically increasing worldwide, the disease is still associated with high morbidity due to ineffectiveness of conventional therapies, urging for novel therapeutic approaches. Unconventional compounds, including polyphenols and the cytokine TRAIL, are being extensively studied for their capacity to restore apoptosis in a large number of tumors including lymphomas.

Design and Methods. Molecular mechanisms of TRAIL-resistance and reactivation of the apoptotic machinery by quercetin in Non-Hodgkin lymphoma cell lines were determined by Hoescht, flow cytometry, western blot, qPCR, by use of siRNA or pharmacological inhibitors of the mitochondrial pathway and by immunoprecipitation followed by post-translational modification analysis.

Results . We demonstrate here that quercetin, a natural flavonoid, restores TRAIL-induced cell death in resistant transformed follicular lymphoma B cell lines, despite high Bcl-2 expression levels owing to the chromosomal translocation t(14;18). Quercetin rescues mitochondrial activation by inducing the proteasomal degradation of Mcl-1 and by inhibiting survivin expression at the mRNA level, irrespective of p53. Restoration of the TRAIL pathway requires Bax and Bak but is independent of enhanced TRAIL DISC formation.

Conclusions. Altogether, we demonstrate that inactivation of survivin and Mcl-1 expression by quercetin is sufficient to restore TRAIL sensitivity in resistant non–Hodgkin's lymphoma B cells. Our results suggest therefore that combining quercetin with TRAIL treatments may be useful for therapy of non–Hodgkin's lymphoma.

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Introduction:

Follicular lymphomas (FL) are indolent non-Hodgkin lymphomas that in many cases respond to first line therapy. However, the majority of patients experience recurrent relapse, leading to death (1, 2). FL are associated with Bcl-2 overexpression and chromosomal translocation t(14;18) (3), leading to dysregulated apoptosis. As Bcl-2 is an important negative regulator of the mitochondrial pathway, novel therapeutic approaches circumventing the mitochondrial block may prove useful to treat these patients (4). Unconventional anti-tumor compounds including polyphenols and the cytokine TRAIL could meet these objectives (5).

Apo2L/TRAIL is a promising anti-tumor drug owing to its ability to trigger apoptosis selectively in cancer cells. Binding of TRAIL to its cognate receptors, TRAIL-R1 or TRAIL-R2, induces the formation of a molecular platform called the DISC (Death-Inducing Signaling Complex) through homotypic interactions, enabling the recruitment of the adaptor protein FADD, which in turn allows the recruitment of caspase-8 and -10 (6). Formation of the TRAIL DISC brings together caspase monomers in close proximity, enabling their activation and subsequent release to the cytosol, inducing caspase-3 activation through proteolytic cleavage, and execution of the apoptotic program (7).

Cell dismantling heavily relies on the amount of caspase-8 that is activated within the DISC (8). Two main apoptotic signaling pathways have been described so far, based on caspase-8 and mitochondrial activation. In type I cells, caspase-3 is directly processed by the active

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caspase-8 that originates from the TRAIL DISC, while caspase-3 activation in type II cells, requires the mitochondrial amplification loop leading to the activation of caspase-9 (9). In the latter situation, caspase-8 cleaves Bid, a BH3-only protein that targets the intrinsic pathway through Bax and Bak, allowing the formation of the apoptosome, another molecular platform, in which caspase-9 is activated. Mitochondrial block in type II cells induced by Bcl-2 or Bcl-xL overexpression, or by a deficiency in Bax and/or Bak expression, impedes caspase-3 activation and thus protects tumor cells from TRAIL-induced apoptosis (10-12).

At the membrane level, TRAIL-induced cell death can also be tightly controlled by two antagonistic receptors, namely TRAIL-R3 and TRAIL-R4. These receptors can selectively compromise TRAIL-induced apoptosis (13). We have demonstrated that TRAIL-R4 can interact with TRAIL-R2 within the TRAIL DISC, where it impairs caspase-8 activation (14). Restoration of cell sensitivity to TRAIL can however be obtained in a large panel of tumor types by conventional or non-conventional anti-tumor drugs, including polyphenols (5).

We demonstrate here that two lymphoma cell lines exhibit resistance to TRAIL-induced cell death due to endogenous elevated expression of several anti-apoptotic proteins, including Mcl-1, survivin, Bcl-2 or TRAIL-R4. Interestingly, the tested cell lines, which are characterized by a robust inhibition of the mitochondrial pathway, become sensitive to apoptosis after sequential stimulation with non-cytotoxic concentrations of quercetin and TRAIL. Quercetin rescues TRAIL-induced cell death through Mcl-1-mediated proteasomal degradation and inhibition of survivin expression at the mRNA level. Our results uncover a novel molecular mechanism by which quercetin exerts synergistic activity with TRAIL.

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Design and Methods

TRAIL production and antibodies

His-tagged recombinant soluble human TRAIL was produced and used as previously described *(15)*. For western blot analysis, antibodies against TRAIL-R1, TRAIL-R2 and TRAIL-R4 were purchased from Chemicon (Millipore, Molsheim, France). Anti-FADD and anti-Bid were obtained from Transduction Laboratories (BD biosciences, Le Pont de Claix, France). Anti-caspase-8 and -10 were from Medical & Biological Laboratories (Clinisciences, Montrouge, France). Antibodies against survivin, phospho-MDM2 and cleaved fragments of caspase-3 were from Cell Signaling (Millipore). Anti-caspase-2 (C-20), Bcl-2, cytochrome c, Bax (2D2), Mcl-1 (S-19) and MDM2 antibodies were purchased from Santa Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France). Anti-Bak (ab-1), anti-caspase-9 and anti-FLIP (NF6) antibodies were purchased from EMD Biosciences (Darmstadt, Germany), Upstate (Millipore, Molsheim, France) and Alexis (Coger, Paris, France), respectively. Anti-Bcl-xL antibody was from Calbiochem (VWR, Fontenay-sous-Bois, France), anti-COXII from Molecular probes (Invitrogen, Cergy Pontoise, France), anti-p53 from Ancell (Coger, Paris, France) and anti-actin from Sigma-Aldrich (Lyon, France). For flow cytometry experiments, the antibodies directed against TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 (wB-K32, B-L27, wB-B44 and wB-P30 clones, respectively) were kindly provided by Diaclone (Besançon, France). The secondary antibody was an Alexa-488 coupled-goat anti-mouse from Molecular Probes (Invitrogen). 3,3'-dihexyloxacarbocyanine ($DiOC_6$) was purchased from Sigma-Aldrich.

Cell culture and treatments

VAL, RL and SUDHL4 cell lines (human B-cell lymphomas) were cultured in RPMI 1640 medium (Lonza, Levallois-Perret, France) containing ultraglutamine, 10 % fetal bovine serum and penicillin/streptomycin/Amphotericin B. These cell lines were grown in 5 % CO_2 at 37°C.

Quercetin (>98 % pure) was obtained from Sigma-Aldrich. A 24 mg/ml stock solution was prepared in DMSO. For sequential treatments, cells were treated for 24 hours with 20 μM quercetin in complete medium before being treated with His-TRAIL (500 ng/ml) for the indicated times. Control cells were treated with DMSO alone. Caspases inhibitors (20 μM) were added 30 min prior to TRAIL. The pan caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk)

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and caspase-9 inhibitor (z-LEHD-fmk) were purchased from Alexis. The Bax channel blocker (Santa Cruz Biotechnology) was used at 5 μM, 1 hour prior to TRAIL stimulation.

Measurement of cell viability

In 96-well plates, 5.10^4 cells were incubated for 24 hours with increasing concentrations of his-TRAIL (from 0 to 25 000 ng/ml) or staurosporin (from 0 to 1000 nM) (Sigma-Aldrich). Cell viability was assessed by the AlamarBlue® method, according to the manufacturer specifications (Invitrogen).

Quantification of apoptosis

After treatments, cells were washed twice with PBS and stained with annexin V-FITC, according to the manufacturer's protocol (BD Pharmingen). After staining with annexin V for 15 min at room temperature, the percentage of annexin V-positive cells was analyzed by flow cytometry.

Immunoprecipitation of the TRAIL DISC:

For DISC analysis, 30.10^6 cells were stimulated with 5 µg of his-TRAIL in 1 ml of complete medium, for the indicated times at 37°C. Cells were then washed with cold PBS and lysed in 1 ml of lysis buffer containing 1 % NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 10 % glycerol. Lysates were precleared with Sepharose 6B (Sigma-Aldrich) for 1 hour at 4 °C with gentle shaking, and immunoprecipitated at 4°C overnight with G protein Sepharose beads (Amersham Biosciences, Les Ullis, France), in the presence of 4 µg of anti-TRAIL-R2 antibody. Beads were then washed four times, and immunoprecipitates were eluted in lysis buffer (Tris-HCl 63 mM, SDS 2 %, phenol red 0.03 %, glycerol 10 %, DTT 100 mM, pH 6.8), boiled for 5 minutes and processed for immunoblotting.

Activation of Bax and Bak by immunoprecipitation

After treatments, cells were lysed in CHAPS buffer (10 mM HEPES ph7,4; 150 mM NaCl; 1% CHAPS) for 30 min on ice, and lysates were precleared with G-coupled sepharose beads for 1h at 4°C. Then, the conformationally active form of Bax or Bak was immunoprecipitated with 4 µg of anti-Bax (clone 6A7, BD Biosciences) or anti-Bak (clone NT, Millipore) antibodies, overnight at

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4°C on a rotating wheel. The immunoprecipitated proteins, as well as whole cell lysates, were then analyzed by western blot.

Western blot analysis

Immunoprecipitates or cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in PBS containing Tween 20 (0.05 %) and fat-free dry milk (5 %). Membranes were incubated with specific primary antibody, overnight at 4°C, followed by HRP-conjugated secondary antibody, at room temperature for 1h. Immunoblots were then developed by the enhanced chemiluminescence (ECL) reagent kit from Santa Cruz Biotechnology, according to the manufacturer's protocol.

Measurement of cytochrome c release

After treatment, cells were washed in PBS and resuspended in a permeabilization buffer containing 400 ug/ml digitonin, 75 mM KCl, 1mM NaH2PO4, 8 mM Na2HPO4 and 250 mM Sucrose, and were kept on ice for 10 min. After centrifugation (5 min at 16 000 g), supernatants were collected as the cytosolic fraction. Pellets were then lysed in buffer containing 1% Triton-X100 for 30 min on ice. After centrifugation (15 min at 16 000 g), supernatants were collected as the total extracts that contain mitochondria.

Measurement of mitochondrial membrane potential (MMP)

Cells were stimulated or not with His-TRAIL (500 ng/ml) or staurosporine (1 μM) for 16 or 6 hours. After treatment, cells were collected, resuspended in PBS and then stained for 20 min at 37°C with 50 nM $DiOC_6$, a MMP-sensitive fluorescent dye. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (CCP, Sigma) was used as positive control to quickly collapse MMP. Fluorescence related to MMP was measured by flow cytometry at 525 nm. Each measurement was conducted on 8 000 events and analyzed on Cell Quest software.

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Gene silencing using small interfering RNA

For siRNA-mediated gene knockdown, 4.10^6 cells were transfected by nucleoporation with the Amaxa nucleofector (Köln, Germany). VAL and RL cells were resuspended in 100 µL Nucleofector solution V containing 200 pm siRNA, and electroporated with the program N-016 (VAL) or X-001 (RL). Then, cells were cultured in complete medium for 48 hours before treatments with TRAIL and/or quercetin. Akt and TRAIL-R4 siRNAs were from Eurogentec (Angers, France), and have been previously described (16, 17). Mcl-1, c-FLIP, Bid, Bax and Bak SiGenome SMARTpool technology siRNAs (set of 4) were purchased from Thermo Scientific (Dharmacon Division).

Real-time PCR assay

RNA was extracted from treated cells with the RNeasy Mini Kit from Qiagen (Valencia, CA). cDNAs were synthesized from total RNA using M-MLV Reverse Transcriptase (Promega). Real time PCR was performed in triplicate using syber green PCR master Mix from Applied Biosystems (Foster City, CA) and analyzed in a 7500 Fast detection System (Applied Biosystems). The oligonucleotides used in this study were designed and synthesized (Eurogentec) as follow: Caspase-10 sense GAAGAGAACAGTGTGGGGTG, antisense GAGGTTTCCGTCTTGCTGTA; Mcl-1 sense CGTTGTCTCGAGTGATGATCCA, antisense TCACAATCCTGCCCCAGTTT; Survivin sense GCCGAGGCTGGCTTCA, antisense GAAGAAACACTGGGCCAAGTCT.

Statistics

With the exception of the experiment using AlamarBlue, Figure 1A, which was analyzed by ANOVA with Bonferroni posttesting, all other quantitative experiments were analyzed using Student *t* test. All statistical analyses were performed using Prism version 5.0a software (GraphPad Software, San Diego, CA.). Group comparisons were deemed significant for 2-tailed P values *<0.05; **<0.01 and ***<0.001.

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Results

VAL and RL B-cell lines display strong resistance to TRAIL-induced apoptosis

The non Hodgkin's B lymphoma cell lines VAL, RL and SUDHL4 exhibit differential sensitivity to TRAIL-induced cell death (Figure 1A). Follicular lymphoma VAL and RL cells were nearly completely insensitive to TRAIL-induced killing, while the viability of SUDHL4 cells, defined as a diffused large B-cell lymphoma, decreased after TRAIL stimulation in a dose dependent manner (Figure 1A). Analysis of caspases activation by western blotting after TRAIL stimulation indicated that caspase-3 was fully cleaved in the sensitive SUDHL4 cell line, but only partly processed in the resistant VAL and RL cells (Figure 1B). Strikingly, although the sensitive cell line SUDHL4, contrary to VAL and RL cells, was nearly devoid of caspase-10 (Figure 1B), activation of caspase-8, caspase-9, caspase-2 and cleavage of Bid appeared to occur to a similar extent in the three lymphoma cell lines (Figure 1B). Importantly, Bax and Bak were not significantly activated upon TRAIL stimulation in VAL and RL cells (Figure 1C). Likewise, cytochrome c was not released from mitochondria (Figure 1D), contrary to SUDHL4 cells. Therefore, since caspase-9 has been demonstrated to be a direct target of caspase-8 (18), these data suggest that activation of caspase-9 and caspase-2 in VAL and RL cells may directly result from caspase-8 activation, but not from mitochondria. In line with this hypothesis, TRAIL stimulation in these resistant cells induced no loss of mitochondrial potential (MMP) (Figure 1 E) and caspase-9 cleavage was inhibited by caspase-8 inhibitors (not shown). Moreover, VAL and RL cells were refractory to CCP- or staurosporin-induced MMP loss (Figure 1E) and were consequently resistant to apoptosis-induced by staurosporin, while MMP dropped substantially in SUDHL4 cells under similar conditions (Figure 1E), leading to apoptosis (Supplementary Figure 1).

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Resistance to TRAIL-induced apoptosis in VAL and RL cells is multifactorial

Owing to the chromosomal translocation t(14;18), follicular B-cell lymphomas express high levels of Bcl-2 (Figure 2A). We have recently shown, in addition, that besides Bcl-2, these lymphoma cell lines express different levels of TRAIL receptors (19), TRAIL-R4 in particular (Figure 2B). Inactivation of Bcl-2 by use of a specific siRNA targeting Bcl-2 (Supplementary Figure 2A), significantly restored apoptosis induced by TRAIL in VAL and RL cells (Figure 2C). Likewise, siRNA-mediated targeted inhibition of TRAIL-R4 expression in VAL cells (Supplementary Figure 2B) significantly restored sensitivity to TRAIL (Figure 2C). Conversely, inhibition of TRAIL-R4 expression in RL cells, which express low levels of TRAIL-R4 (Supplementary Figure 2B), failed to restore TRAIL-induced cell death (Figure 2C). Strikingly, SUDHL4 and VAL cells exhibited differential sensitivity to TRAIL-induced cell death, despite comparable expression levels of TRAIL-R4 (Figure 2B). The differential behavior did not result from mutations in TRAIL-R4 in SUDHL4 cells as demonstrated by DNA sequence analysis (not shown). Therefore, in order to understand why the follicular B-cell lines VAL and RL fail to engage the apoptotic machinery upon TRAIL stimulation, we have focused our attention on several additional key anti-apoptotic proteins including c-FLIP, Mcl-1 or Survivin (Figure 2A). We have recently proposed that TRAIL-R4 and c-FLIP may cooperate to inhibit TRAIL-induced apoptosis (17). In line with this hypothesis, c-FLIP long and short were both expressed to a much higher extent in the resistant cells as compared to the sensitive cell line SUDHL4 (Figure 2B). Consistently, inhibition of c-FLIP expression by use of specific siRNA (Supplementary Figure 2C) partially but significantly restored TRAIL-induced cell death in both resistant cell lines (Figure 2C), while ectopic expression of c-FLIP long in SUDHL4 inhibited TRAIL-induced cell

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death (Supplementary Figure 3). Besides TRAIL-R4 and c-FLIP, mitochondrial- or post-mitochondrial apoptotic inhibitors may play a role in controlling caspase-9 and caspase-3 activation in these resistant cells. For instance, we have found that Mcl-1 and survivin were expressed at higher levels in VAL and RL cells as compared to the sensitive cell line SUDHL4 (Figure 2B), while other inhibitors such as Bcl-xL (not shown) or XIAP (Figure 2B) were expressed at similar levels. Moreover, Mcl-1 expression appeared to increase in a time-dependent manner upon TRAIL stimulation in both resistant cells, but not in SUDHL4 cells (Figure 2B). These results prompted us to check whether inhibition of Mcl-1 or survivin expression (Supplementary Figure 2D) could restore TRAIL-induced apoptosis in VAL and RL cells. Indeed siRNA targeting of either survivin or Mcl-1 restored significantly TRAIL-induced cell death in these cells (Figure 2C). Altogether, these results highlight that VAL and RL cell resistance to TRAIL-induced cell death is a multimodal process, which takes place at the membrane-, the mitochondrial- and at the post-mitochondrial level.

Quercetin overcomes cell resistance to TRAIL-induced cell death

We next assessed the ability of quercetin to restore TRAIL-induced cell death in these resistant cells, as this flavonoid has previously been demonstrated to target survivin (20), and to synergize with TRAIL in various tumor cell types (5). Remarkably, pretreatment with 20 μM quercetin for 24 hours significantly overcame TRAIL resistance in these B lymphoma cell lines, in a caspase-dependent manner, as demonstrated by the use of the pan-caspase inhibitor zVAD (Figure 3A). This flavonoid restored full caspase-3 activation (Figure 3B). Engagement of the apoptotic machinery required both caspase-8 and caspase-9, as specific inhibitors of these initiator caspases similarly abrogated TRAIL-induced apoptosis (Figure 3C).

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Quercetin restores TRAIL sensitivity regardless of caspase-10 upregulation and recruitment to the DISC

To understand the molecular mechanisms involved in the restoration of apoptosis induced by TRAIL, after quercetin stimulation, we first evaluated whether this flavonoid might regulate TRAIL receptor expression or enhance TRAIL-DISC formation. Flow cytometry analysis demonstrated that quercetin pretreatment induced no change in the expression of any of the TRAIL receptors in VAL or RL cells (Supplementary Figure 4). TRAIL-DISC formation was also not significantly affected by quercetin stimulation, with the exception of TRAIL-R1, FADD and caspase-8 and -10 whose recruitment and activation within the DISC appeared to be slightly enhanced in VAL cells but less so in RL cells, (Figure 4A). Interestingly, quercetin induced a strong increase in caspase-10 expression in these cells, associated with an enhanced caspase-10 processing upon quercetin/TRAIL stimulation as compared to TRAIL alone (Figure 4B). As measured by qPCR analysis, quercetin-mediated caspase-10 up-regulation was controlled at the mRNA level (Figure 4C). However, caspase-10 itself appeared to be dispensable for the restoration of TRAIL-induced apoptosis by the quercetin, as inactivation of this initiator caspase, using a specific caspase-10 targeting siRNA, failed to compromise the efficacy of the combined treatment (Figure 4D and E). Altogether these results indicate that quercetin-mediated TRAIL sensitization is independent of caspase-10 and most likely independent of TRAIL DISC formation regulation.

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Sensitization mainly requires mitochondrial activation

Since enhanced TRAIL DISC formation appears to be dispensable for quercetin-mediated TRAIL-induced cell death restoration, we focused our attention on the mitochondrial pathway. Fractionation experiments to analyze cytochrome c release were performed from cells pretreated or not with quercetin and stimulated with TRAIL, for the indicated periods of time. Stimulation with quercetin enhanced cytochrome c release after TRAIL stimulation in VAL cells and induced cytochrome c release in RL cells (Figure 5A). To determine whether reactivation of the mitochondrial pathway required Bid, its expression was knocked-down using a Bid targeting siRNA (Supplementary Figure 2E). Inactivation of Bid significantly inhibited TRAIL-induced cell death after quercetin stimulation (Figure 5B), suggesting that the mitochondrial amplification loop, through Bax and/or Bak activation was required. In agreement with this hypothesis, Bax channel blockers were found to inhibit TRAIL-induced cell death after quercetin pretreatment (Figure 5C). Moreover, while inactivation of Bax or Bak alone was insufficient to fully inhibit TRAIL-induced apoptosis after quercetin pretreatment, combined Bax and Bak knockdown (Supplementary Figure 2F) completely abrogated the synergy (Figure 5D).

Quercetin reactivates the mitochondrial pathway through Mcl-1 and survivin downregulation, irrespective of p53

To elucidate the molecular events required to bypass the mitochondrial block in VAL and RL cells upon quercetin stimulation, we next assessed the expression levels of some anti-apoptotic proteins including Bcl-2 family members, by western blot analysis. While no change in Bcl-2, Bcl-xL or XIAP protein expression was found after quercetin treatment, the flavonoid induced

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the depletion of both Mcl-1 and survivin in RL and VAL (Figure 6A). In agreement with previous findings (21), we have found that Mcl-1 expression was induced upon TRAIL stimulation in both resistant cell lines, but remarkably, TRAIL-mediated Mcl-1 up-regulation was completely abrogated by quercetin (Figure 6A). Quercetin-mediated survivin and Mcl-1 down-regulation occurred in a caspase-independent manner (not shown). Since p53 is known to be a negative regulator of Mcl-1 and survivin (22, 23), we first checked whether this transcription factor might be involved in the regulation of the expression levels of these proteins upon quercetin stimulation. As shown by western blot analysis, quercetin induced p53 upregulation and a decrease in the expression of the p53 inhibitor MDM2 (Figure 6B). Interestingly, as evidenced by qPCR, survivin mRNA expression levels were reduced by more than 40% in quercetin stimulated cells as compared to non-stimulated cells, whereas Mcl-1 mRNA levels increased upon stimulation (Figure 6C). These results prompt us to assess whether p53 may promote restoration of TRAIL sensitivity, through inhibition of survivin expression. However, inactivation of p53, using specific siRNAs, had no impact on quercetin-mediated survivin or Mcl-1 expression inhibition (Figure 6D) and failed to inhibit the synergistic apoptotic activity of the combination TRAIL and quercetin (Figure 6E). Moreover, using the proteasome inhibitor MG132, we could demonstrate that inhibition of Mcl-1 expression levels, but not survivin, following quercetin treatment occurred through proteasomal degradation (Figure 6F). Accordingly, Mcl-1 was strongly ubiquitinated upon quercetin treatment (Figure 6 G).

Altogether our results demonstrate that quercetin restores TRAIL-induced apoptosis in resistant NHL-B cell lines, at least in part through inhibition of Mcl-1 and survivin expression.

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Discussion

In this study we demonstrate that quercetin synergizes with TRAIL to trigger apoptosis in FL transformed resistant B cell lines, despite strong mitochondrial inhibition due to high Bcl-2, Mcl-1 and survivin expression. Quercetin has been reported to synergize with TRAIL (24-29), but molecular mechanisms underlying this sensitization remain largely unknown. At the proximal level, quercetin-mediated sensitization to TRAIL has been correlated with TRAIL-R2 stabilization (24), increased TRAIL-R2 expression at the cell surface (25, 29), enhanced TRAIL DISC formation (27) and even c-FLIP downregulation (25). In our cellular models, regulation of proximal events is unlikely to explain the synergy since quercetin induced no change in TRAIL receptor or c-FLIP expression and only modest differences in TRAIL-R1, FADD, caspase-8 and caspase-10 recruitment within the DISC. As compared to conventional chemotherapeutic drugs, such as cisplatin or 5FU, which induce a robust increase in caspase-8 recruitment and activation within the TRAIL DISC in VAL cells (17), quercetin only weakly enhanced initiator caspase-8/10 or TRAIL-R1 recruitment. Moreover, caspase-10 up-regulation was dispensable to restore quercetin-mediated TRAIL sensitivity in both resistant cell lines. Yet, we cannot definitely exclude that the slight increase in caspase-8 or TRAIL-R1 recruitment within the TRAIL DISC might, to some extent, contribute to the restoration of the TRAIL signaling pathway. Discrepancies regarding the implication of TRAIL proximal events in restoring TRAIL-induced cell death by quercetin may merely reflect differences in drug concentrations. At this point it should be emphasized that the concentrations of quercetin used in our study, 20 μM, are lower than those used in most studies (50 to 200 μM) (24-26). Besides, cell specificities may also give rise to discrepant results. Likewise, the mitochondrial pathway is strongly inhibited in resistant B lymphoma cell lines, yet quercetin achieves restoration of the TRAIL apoptotic machinery.

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Rather, our findings suggest that the main target is the mitochondria, since quercetin treatment enhanced cytochrome c release upon TRAIL stimulation, whereas inactivation of Bid or Bax/Bak using siRNA, or inhibition of the mitochondrial pathway using a Bax channel blocker, efficiently abrogated the synergy. Quercetin-induced restoration of the mitochondrial apoptotic potential was associated with a dysregulation of Mcl-1 and survivin expression. Survivin has been proposed to act mainly at the post-mitochondrial level, through its ability to inhibit Smac release from the mitochondria, stabilizing XIAP and leading to inhibition of caspase-9 and -3 activation (30). Survivin expression has been demonstrated to be negatively regulated by a large number of transcription factors or signaling pathways, including p53, Akt or proteosomal degradation (20, 22, 29). In VAL and RL cells, neither p53 nor the proteasome or Akt (Supplementary Figure 5) appear to be required to repress survivin expression upon quercetin stimulation. Further studies will be required to elucidate how survivin expression is repressed upon quercetin stimulation.

Importantly, our results highlight a novel regulatory event controlling the restoration of TRAIL apoptotic signaling activity by quercetin. To our knowledge, we are the first to report that quercetin affords restoration of TRAIL-induced cell death in aggressive B lymphoma cell lines through Mcl-1-mediated proteasomal degradation. This Bcl-2 family member is known to sequester BH3-only proteins including Bid and Bim (31-33) but also Bak (34), affording high levels of protection against mitochondrial depolarization, cytochrome c release and activation of caspase-9. Mcl-1 has thus been proposed to protect cells from TRAIL-induced cell death by inhibiting Bak and Bid, the inhibition of which impacts on Bax activation (32, 35). This assumption is in agreement with our findings as inactivation of Bak or Bax alone by siRNA was not sufficient to inhibit apoptosis induced by the combination of quercetin and TRAIL, while

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simultaneous inhibition of Bax and Bak was required to impair the synergy. Interestingly, quercetin-mediated Mcl-1 proteasomal degradation was associated with an increase in Mcl-1 ubiquitination. Keeping in mind that quercetin has been extensively used in the past as a heat shock protein inhibitor (36), it is interesting to note that HSP70 has recently been demonstrated to impair the association of the ubiquitin ligase Mule with Mcl-1, leading to Mcl-1 stabilization and to inhibition of Bax activation (37). Our findings are particularly important since it has recently been found that Mcl-1 expression in mantle cell lymphoma was associated with high-grade morphology and proliferative state (38). Quercetin's ability to induce Mcl-1 degradation possibly represents a very important mechanism enabling restoration of the mitochondrial apoptotic pathway induced by TRAIL in human lymphomas. These findings could also apply to some leukemias, since it has been demonstrated recently that quercetin alone, at higher concentrations, could induce tumor-selective apoptosis through Mcl-1 downregulation and Bax activation (39). Therefore, therapeutic strategies associating TRAIL and quercetin to eradicate tumors and to overcome cell resistance may be close at hand (5), as quercetin and TRAIL, when applied either alone or in combination, exhibited no toxicity towards normal lymph nodes or tonsils cells (*Online Supplementary Figure 6*). Considering that these compounds alone exhibit limited side effects, and are extremely well tolerated in humans as demonstrated in clinical trials (40, 41), our results suggest that combining TRAIL with the naturally occurring flavonoid quercetin could represent an attractive therapeutic approach for NHL.

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Authorship and Disclosures

OM was the principal investigator and takes primary responsibility for the paper. GJ, VG, ASG, NL, AyM, EI, AM performed the laboratory work of this study. GJ TG and OM co-ordinated the research. GJ, CG, TG and OM wrote the paper. The authors reported no potential conflicts of interests.

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Figure legends

Figure 1. VAL and RL non-Hodgkin's B cell lymphomas are resistant to TRAIL-induced cell death because of a defect in the mitochondrial pathway of apoptosis. (A) Sensitivity to TRAIL-induced cell death of the non-Hodgkin's B lymphoma cell lines VAL, RL and SUDHL4. Cells were treated with different concentrations of His-TRAIL for 24 hours. Cell viability was measured by the AlamarBlue method. Data presented are means plus or minus SD (n =3; $P < 0.05$ for SUDHL4 as compared to VAL or RL cell lines). (B) Analysis of caspase activation and Bid by western blot after treatment with His-TRAIL (500 ng/mL) for the indicated times. (C) TRAIL-induced Bax and Bak activation. After treatment with TRAIL at 500 ng/mL for the indicated times, the active forms of Bax or Bak were immunoprecipitated and analysed by western blot. (D) TRAIL-induced cytochrome c release from the mitochondria to the cytosol. VAL, RL and SUDHL4 cells were treated with His-TRAIL (500 ng/mL) for the indicated times. Cytosolic and mitochondrial fractions were analysed by western blot to detect the presence of cytochrome c. COXII was used as a mitochondrial marker. (E) Cells were left untreated (NT), treated with His-TRAIL (500 ng/ml) or staurosporine (1 μM) for 16 or 6 hours, respectively, then incubated with the MMP-sensitive fluorescent dye $DiOC_6$ for 20 min, and fluorescence related to MMP was measured by flow cytometry. CCP was used to elicit rapid disruption of MMP (mitochondrial membrane potential), as revealed by the decrease in $DiOC_6$ fluorescence in SUDHL4 cells. Data presented are means plus or minus SD (n =3; *$P < 0.05$ or **$P<0.01$ or ***$P<0.001$ respective to NT ; ns stands for not statistically relevant).

Figure 2. Bcl-2, Mcl-1 and survivin account for the resistance to TRAIL of VAL and RL lymphoma B cel lines. (A) Western blot analysis of antiapoptotic proteins upon stimulation with His-TRAIL (500 ng/mL) for the indicated times. (B) Expression of TRAIL receptors on VAL, RL and SUDHL4 cells, at the membrane level, was measured by flow cytometry (unfilled peaks). Shaded peaks correspond to the isotype control antibody staining. (C) Effect of siRNA-mediated knockdown of Mcl-1, survivin, Bcl-2, c-FLIP or TRAIL-R4 on the sensitivity to TRAIL-induced apoptosis. 48 hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated with TRAIL at 500 ng/mL for 3 hours. Apoptosis was measured by flow cytometry after annexin V staining. Data presented are means plus or minus SD (n =3; *$P < 0.05$; **$P<0.01$ or ***$P<0.001$ respective to scramble siRNA).

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Figure 3. Quercetin sensitizes VAL and RL resistant non-Hodgkin's lymphoma B cell lines to TRAIL-induced apoptosis. (A) VAL and RL cells were treated with 20 μM quercetin (Quer) for 24 hours prior to TRAIL (500 ng/mL for 3 hours). The pan caspases inhibitor zVAD-fmk (20 μM) was added 30 min before treatment with TRAIL. Apoptosis was measured by annexin V staining. (B) Western blot analysis of caspase activation upon treatment with quercetin (20 μM, 24 hours), followed by TRAIL (500 ng/mL, 6 hours) and/or zVAD-fmk (20 μM, 30 min before TRAIL). (C) Quantification of apoptosis by annexin V staining after treatment with quercetin and TRAIL as described in (A). Caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were used at 20 μM, 30 min before TRAIL stimulation. Data presented panels (A) and (C) are means plus or minus SD (n =3; **P<0.01 or ***P<0.001 respective to quercetin alone or to quercetin+TRAIL in the presence of caspase-inhibitors).

Figure 4. Quercetin induces caspase-10 upregulation and recruitment to the DISC, but sensitization to TRAIL occurs independently of caspase-10. (A) Analysis of TRAIL-induced DISC formation. VAL and RL cells were treated with quercetin (20 μM, 24 hours) and stimulated with TRAIL (5 μ g/mL) for the indicated times. After cell lysis, the DISC was immunoprecipitated using an antibody against TRAIL-R2 and the DISC-associated proteins were analysed by western blotting. Data are representative of three independent experiments. (B) Western blot analysis of caspase-10 expression after treatment with quercetin (20 μ M, 24h), followed by TRAIL (500 ng/mL, 6 hours) and/or zVAD-fmk (20 μM, 30 min before TRAIL). (C) Relative expression of caspase-10 mRNA by qPCR after treatment with quercetin (20 μM, 24 hours). Results correspond to % fold change mRNA expression compared with cells treated with DMSO, and were normalized to L32 levels. (D-E) Effect of siRNA-mediated caspase-10 knockdown on the efficiency of the combined treatment with quercetin and TRAIL. 24 hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated with quercetin (20 μM) for 24 hours, followed by TRAIL (500 ng/mL) for 3 hours. Apoptosis was measured by flow cytometry after annexin V staining. Efficiency of the caspase-10 siRNA was evaluated by western blotting. Data presented panels (D) and (E) are means plus or minus SD (n =3; ***P<0.001 respective to TRAIL alone or to quercetin+TRAIL in the presence or the absence of Caspase-10 siRNA; ns stands for not statistically relevant).

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Figure 5. Sensitization to TRAIL by quercetin requires the mitochondrial pathway of apoptosis. (A) TRAIL-induced cytochrome c release from the mitochondria to the cytosol after quercetin pre-treatment. VAL and RL cells were treated with quercetin (20 μM) for 24 hours, followed by TRAIL (500 ng/mL) for the indicated times. Cytosolic and mitochondrial fractions were analysed by western blot for the detection of cytochrome c. COXII was used as a mitochondrial marker. (B) Effect of siRNA-mediated knockdown of Bid on the efficiency of quercetin and TRAIL combined treatment. 24 hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated with quercetin (20 μM) for 24 hours, followed by TRAIL (500 ng/mL) for 3 hours. (C) Effect of Bax channel formation on the efficacy of the combined quercetin and TRAIL treatment . VAL and RL cells were treated with quercetin (20 μM) for 24 hours. Bax Channel Blocker was added at 5 μM, 1 hour before stimulation with TRAIL (500 ng/mL for 3 hours). (D) Effect of siRNA-mediated knockdown of Bax and/or Bak on the efficiency of the combined treatment with quercetin and TRAIL. Cells were treated as in (B). (B-C-D) Apoptosis was measured by flow cytometry after annexin V staining. Data presented panels (B-D) are means plus or minus SD (n =3; *P<0.05; **P<0.01 or ***P<0.001 respective to TRAIL alone or to target siRNA as compared to scramble; ns stands for not statistically relevant).

Figure 6. Quercetin inhibits Mcl-1 through ubiquitin-dependant proteasomal degradation and downregulates survivin at the mRNA level, independently of p53. (A-B) Western blot analysis of Mcl-1, survivin, p53, phospho-MDM2 and total MDM2 expression after treatment with quercetin (20 μM, 24 hours), followed by TRAIL (500 ng/mL, 6 hours) and/or zVAD-fmk (20 μM, 30 min before TRAIL). (C) Relative expression of Mcl-1 or survivin mRNA by qPCR after treatment with quercetin (20 μM, 24 hours). Results correspond to the fold change mRNA expression (%) compared with cells treated with DMSO, and were normalized to L32 levels. (D) Effect of siRNA-mediated knockdown of p53 on Mcl-1 and survivin after TRAIL and/or quercetin stimulation. 24 hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated or not with quercetin (20 μM) for 24 hours, followed by TRAIL (500 ng/mL) for 3 hours or left untreated and p53, survivin and Mcl-1 expression was evaluated by western blotting. (E) Effect of quercetin- and TRAIL-induced apoptosis in the absence of p53 was measured by flow cytometry after annexin V staining. (F) Impact of proteasome inhibition on Mcl-1 and survivin protein levels. Cells were treated with quercetin (20 μM) or DMSO (vehicle), in the presence of the proteasome inhibitor MG132 (1 μM) for 24 hours. (G) Quercetin-mediated ubiquitination of Mcl-1. Cells were treated with quercetin (Q) or vehicle (NT) in the presence of MG132 for 24 hours as previously described. Mcl-1 was immunoprecipitated and ubiquitin residues were detected by western blot analysis. Immunoglobulin (Ig) was used as a negative control for immunoprecipitation. (F) Data presented panel (E) are means plus or minus SD (n =3; ***P<0.001 respective to TRAIL alone or to quercetin+TRAIL in the presence or the absence of p53 siRNA; ns stands for not statistically relevant).

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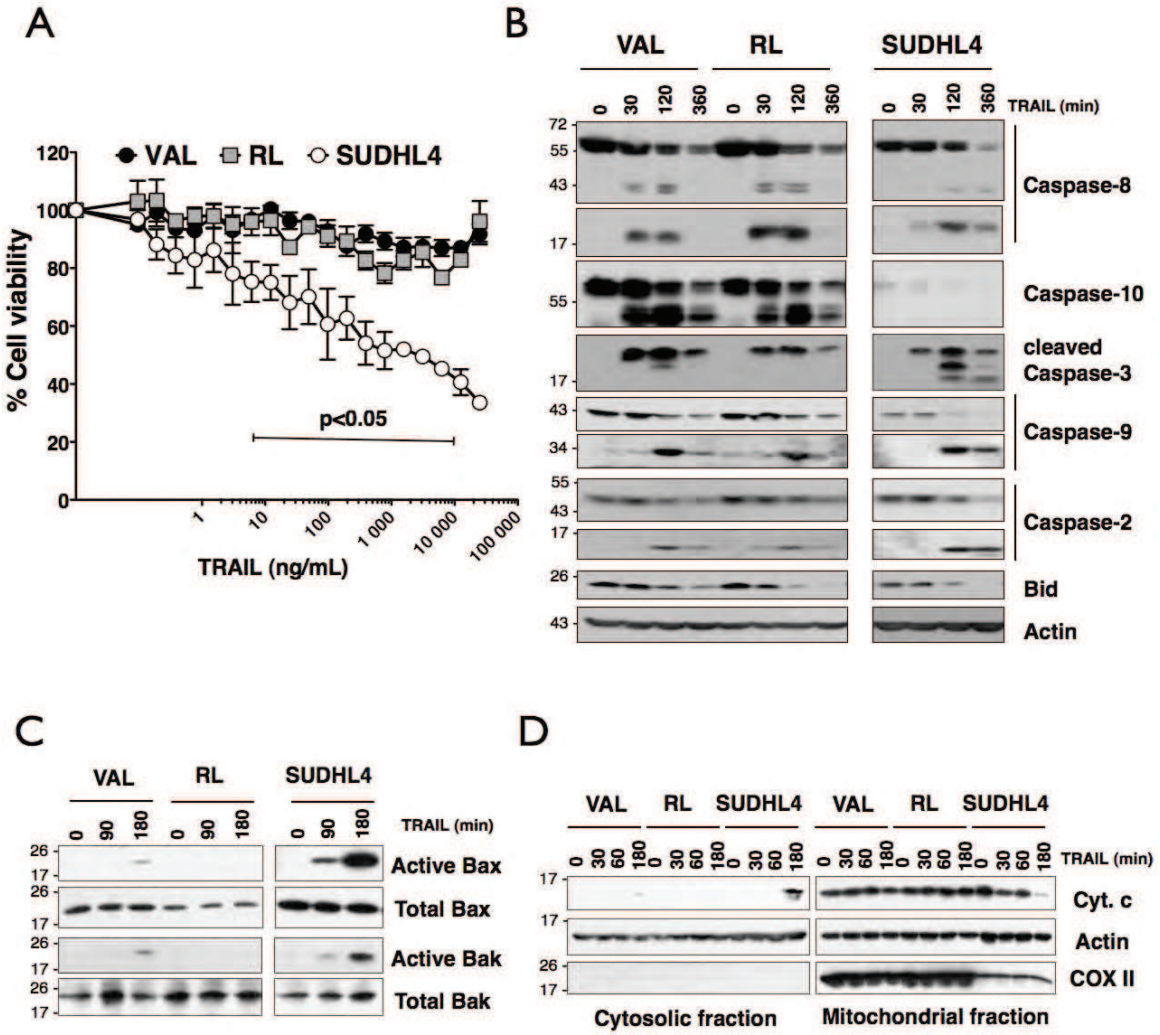

Figure 1

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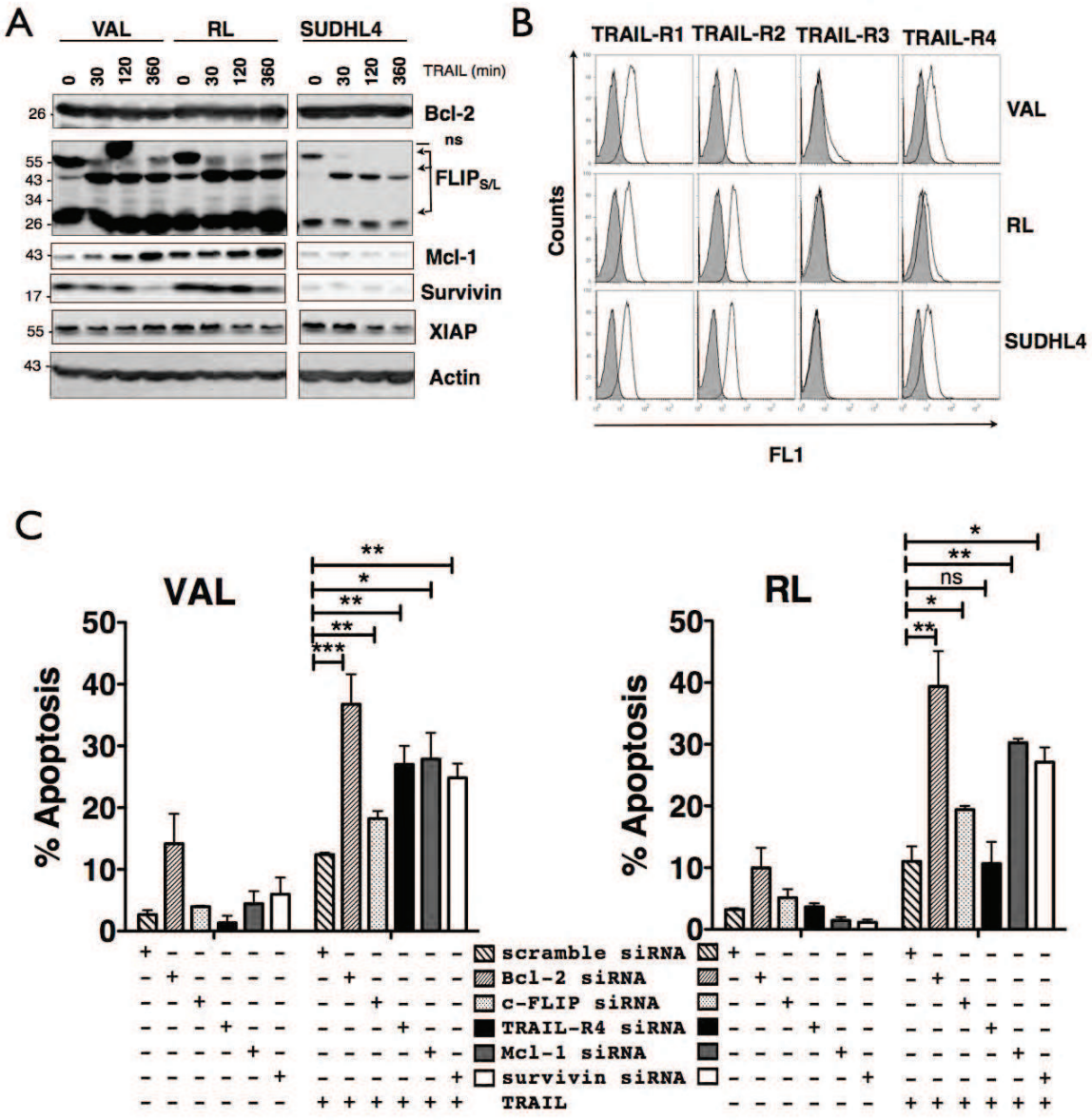

Figure 2

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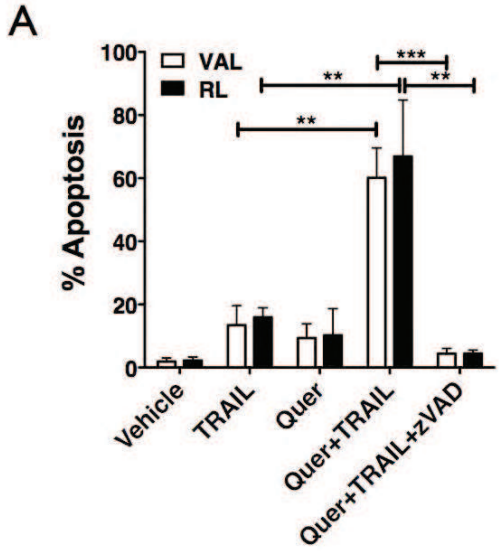

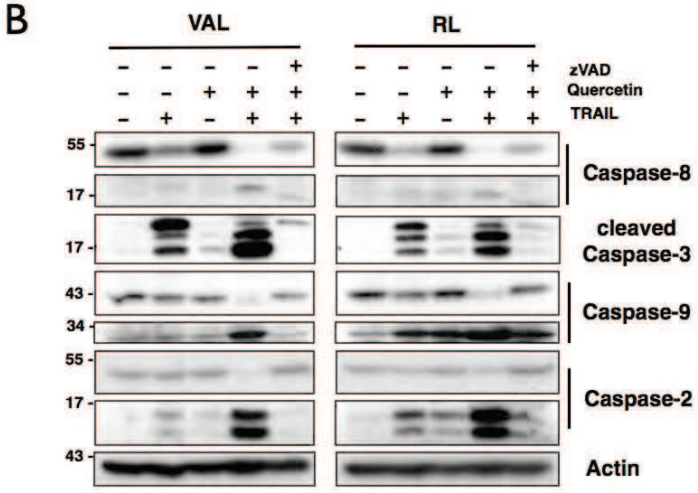

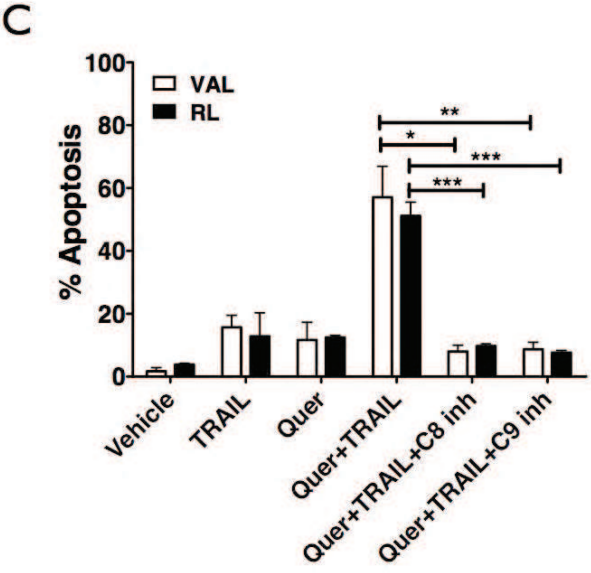

Figure #3

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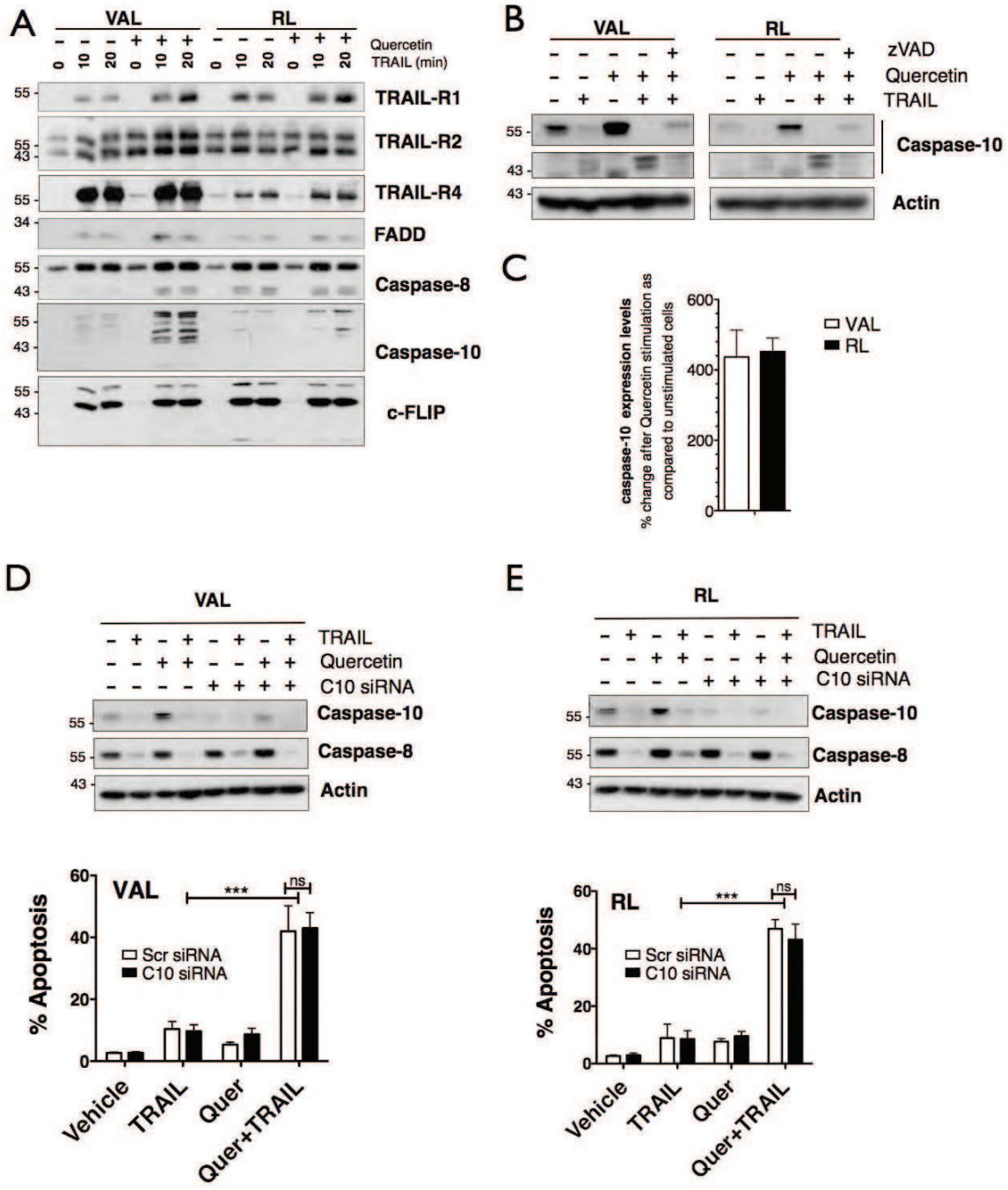

Figure #4

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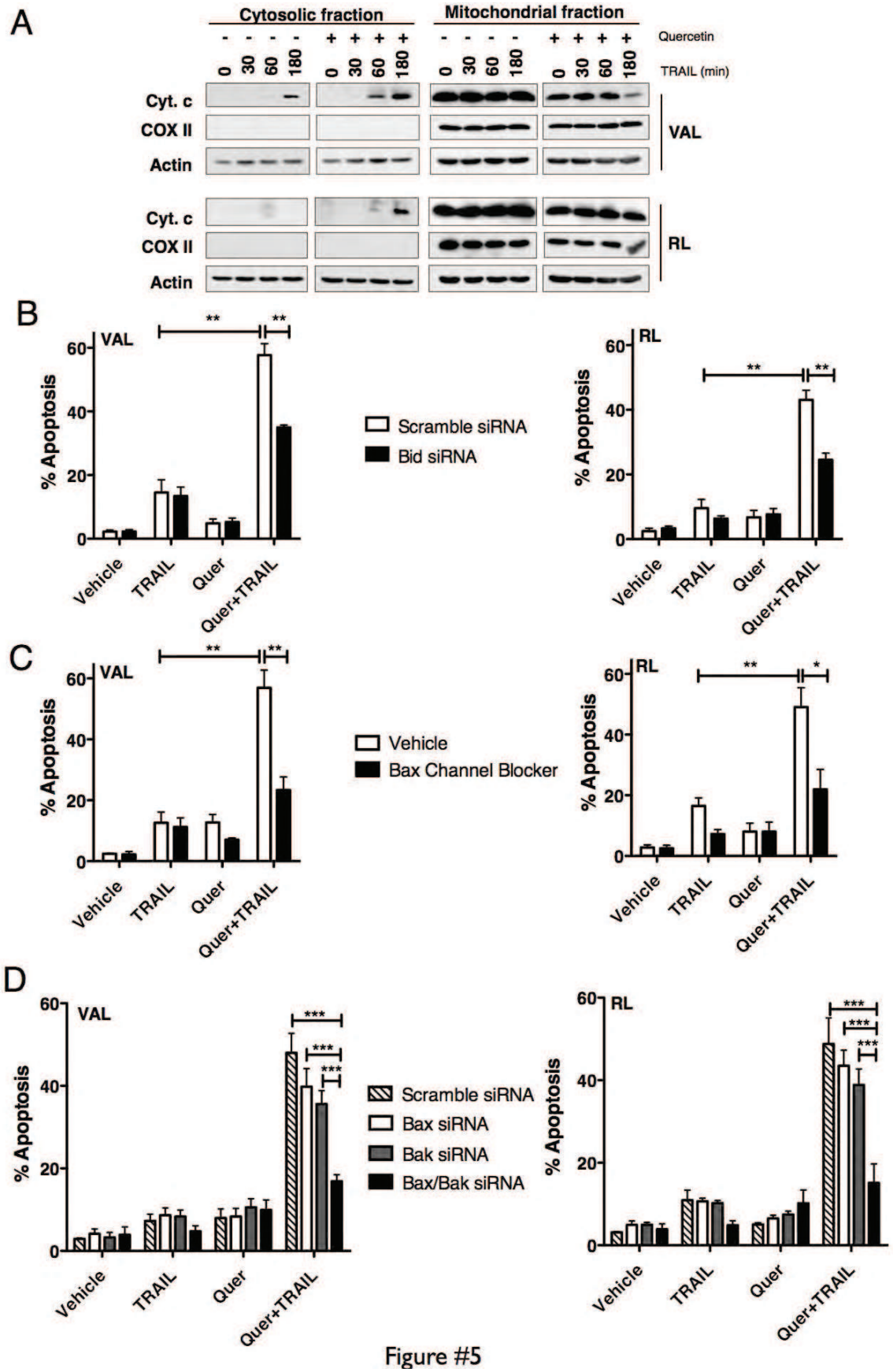

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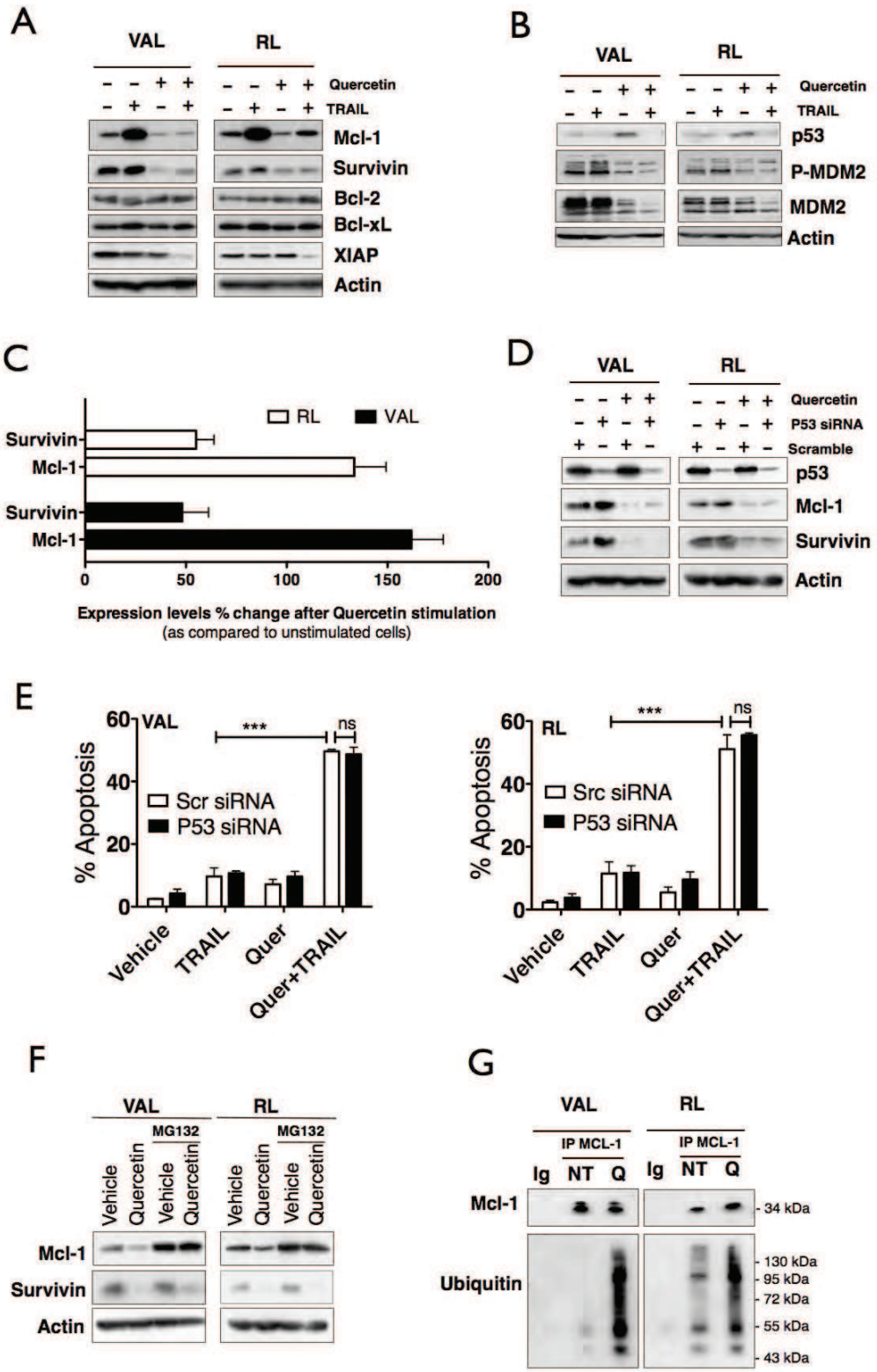

Figure #6

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SUPPLEMENTARY FIGURES

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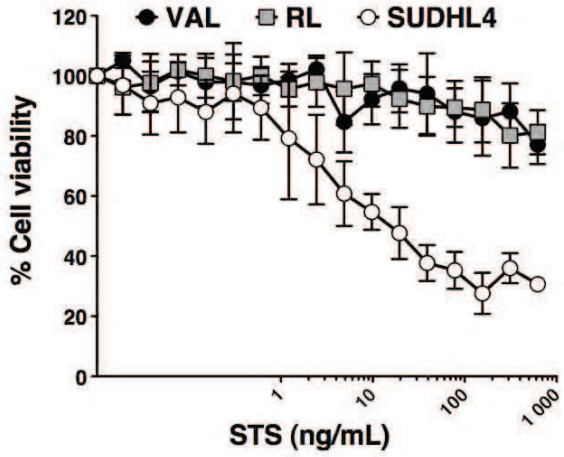
Supplemental #1



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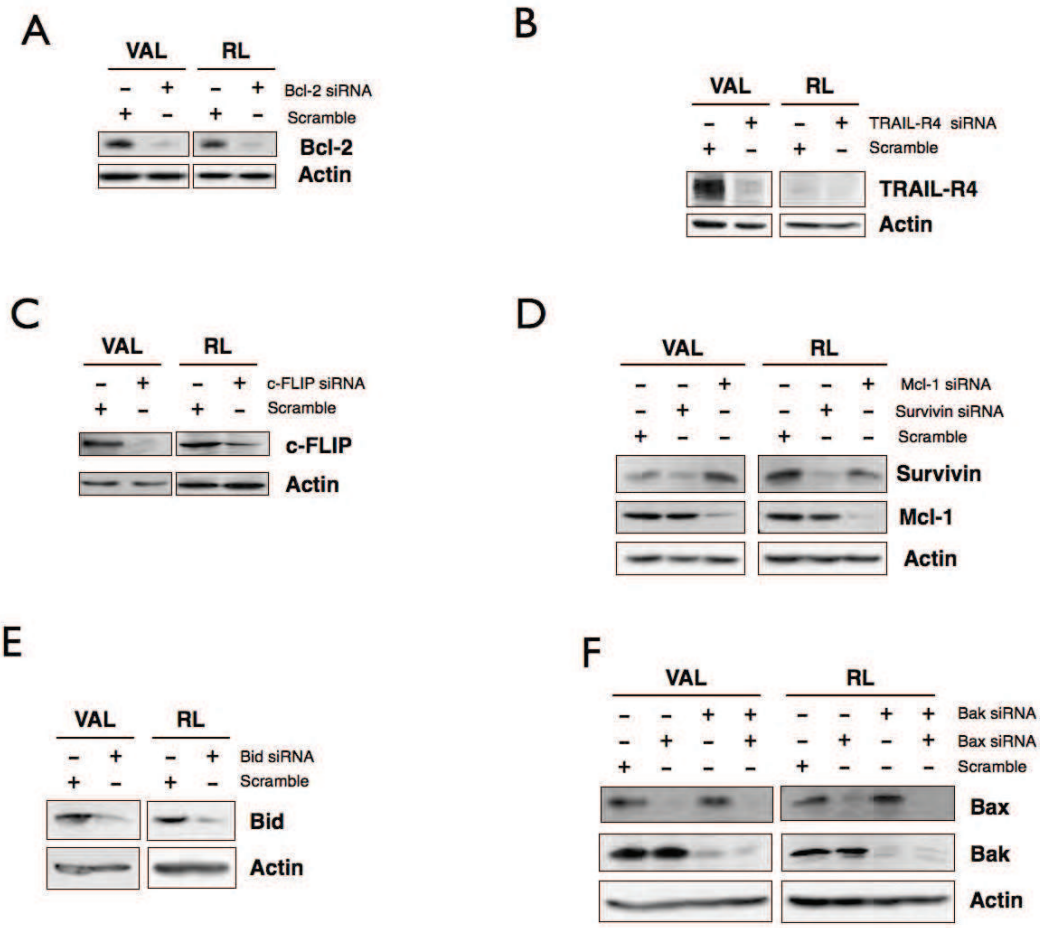

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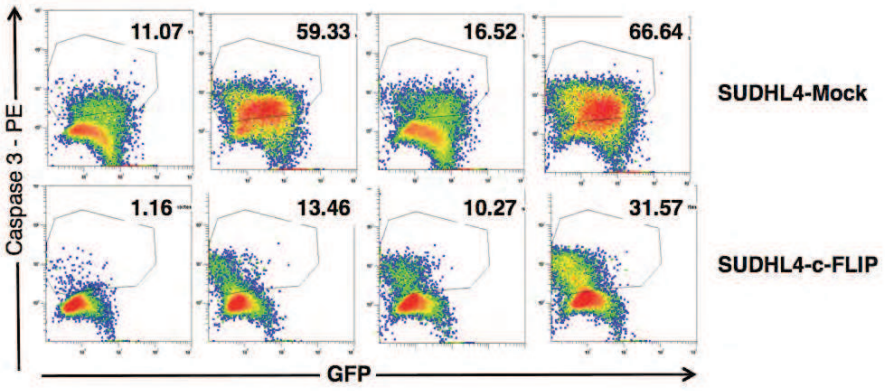

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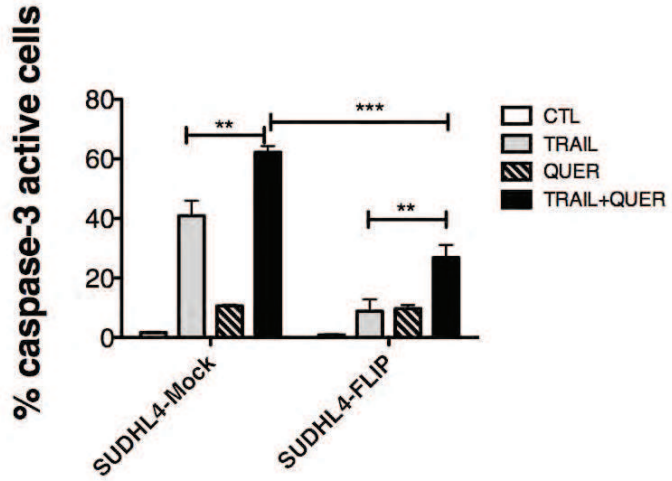

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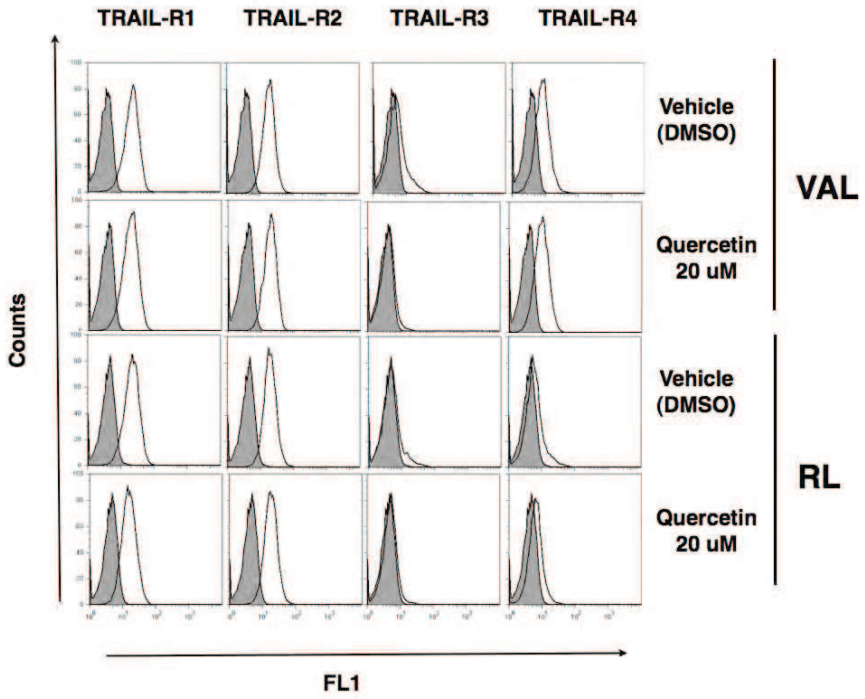

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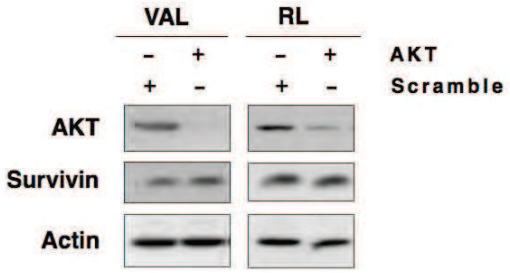

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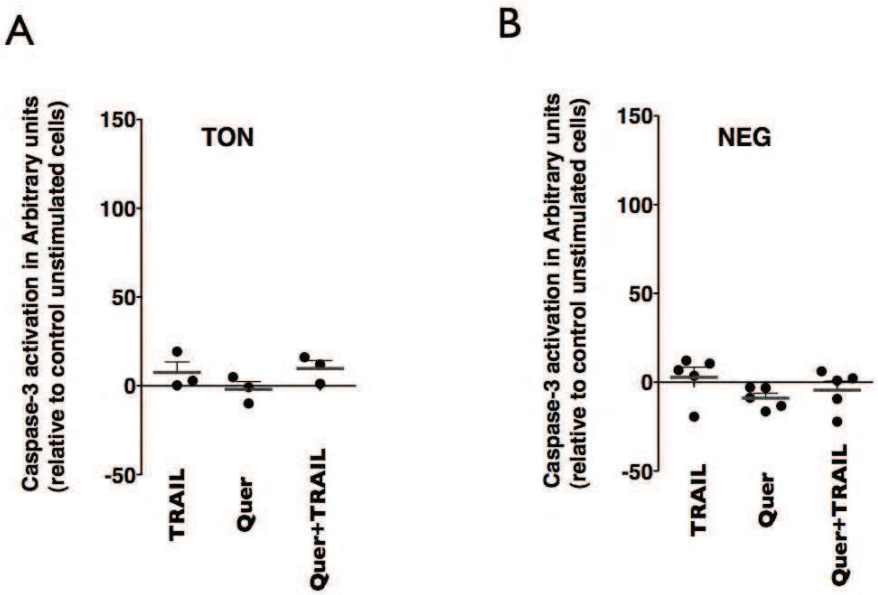

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