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Studijní program: Imunologie

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Mechanismus, regulace a využití TRAILem indukované apoptózy nádorových buněk

Mechanism, regulation and use of TRAIL-induced apoptosis in cancer cells

Disertační práce

Vedoucí disertační práce:

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Prohlášení:

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

V Praze, 30. května 2013

Vladimíra Horová

Děkuji všem, kteří mi umožnili vypracování této studie a její dokončení.

Obsah

Seznam použitých zkratek

ACAP1	protein obsahující α-helikální Arf-GAP, ankyrinové a PH domény 1 *ArfGAP with coiled-coil, ankyrin repeat and PH domains 1*
ADAM	protein obsahující disintegrinovou a metalloproteázovou doménu *a disintegrin and metalloproteinase domain protein*
Apaf-1	faktor aktivující apoptotické proteázy 1 *apoptotic protease activating factor 1*
AP2	adaptorový proteinový komplex 2 *adaptor protein complex 2*
APRIL	ligand indukující proliferaci *a proliferation-inducing ligand*
APP	prekurzor proteinu amyloid-β *amyloid-β precursor protein*
ARAP	protein obsahující Arf-GAP, Rho-GAP, ankyrinové a PH domény *Arf-GAP, Rho-GAP, ankyrin repeat and PH domain-containing protein*
ASM	kyselá sfingomyelináza *acid sphingomyelinase*
BafA1	bafilomycin A1
BAFF	faktor aktivující B-lymfocyty *B cell-activating factor*
Bak	inhibitor proteinu Bcl-2 *Bcl-2-antagonist/killer*
Bid	protein podporující buněčnou smrt obsahující BH3 interakční doménu *BH3 interacting domain death agonist*
Bax	protein X asociovaný s Bcl-2 *Bcl-2-associated X protein*
Bcl-2	protein B-buněčných lymfomů 2 *B-cell lymphoma 2*
BCMA	faktor pro zrání B-lymfocytů *B-cell maturation factor*
BH	Bcl-2 homologní doména *Bcl-2 homology domain*

B-Raf	onkogenní homolog retrovirového proteinu v-Raf, B izoforma *v-raf murine sarcoma viral oncogene homolog B*
CARP	s kaspázami asociovaný protein obsahující doménu RING *caspase-associated ring protein*
CCA	konkanamycin A concanamycin A
CD	názvoslovná jednotka leukocytárních antigenů *cluster of differentiation*
cIAP	buněčný inhibitor apoptózy *cellular inhibitor of apoptosis*
CRD	doména bohatá na cystein *cystein rich domain*
CTSD	katepsin D *cathepsin D*
DcR	tlumivý receptor *decoy receptor*
DD	doména smrti *death domain*
DED	efektorová doména smrti *death effector domain*
DIABLO	protein s nízkým pI, který přímo váže inhibitory apoptózy *direct IAP-binding protein with low pI*
DISC	signalizační komplex indukující buněčnou smrt *death inducing signaling complex*
DN	dominantně negativní *dominant negative*
DNA	deoxyribonukleová kyselina *deoxyribonucleic acid*
cDNA	přepisovaná DNA *complementary DNA*
DR	receptor smrti *death receptor*
EEA1	antigen raných endozómů 1 *early endosome antigen 1*

EDAR	receptor proteinu ektodysplazin A *ectodysplasin A receptor*
EMCV	virus encefalomyokarditidy *encephalomyocarditis virus*
Erk	kináza regulovaná extracelulárními signály *extracellular signal-regulated kinase*
EST	krátká sekvence cDNA *expressed sequence tag*
FADD	protein asociovaný s receptorem Fas a obsahující doménu smrti *Fas-associated protein with death domain*
FLIP	protein inhibující kaspázu 8 *FLICE/CASP8 inhibitory protein*
GAP	protein aktivující GTPázy *GTPase-activating protein*
GITR	protein indukovaný glukokortikoidy a příbuzný receptoru TNFR *glucocorticoid-induced TNFR-related protein*
GPI	glykofosfatidylinositolová kotva *glycophosphatidylinositol anchor*
GTPáza	fosfohydroláza guanosintrifosfátu
HVEM	protein zprostředkovávající vniknutí herpesvirů do buňky *herpesvirus entry mediator*
ICP	intracelulární část *intracellular part*
IFN	interferon
IKK	kináza faktoru IκB *IκB kinase*
IL	interleukin
IRF1	faktor regulující interferony 1 *interferon regulatory factor 1*
JNK	kináza faktoru c-Jun *c-Jun N-terminal kinase*
KRAS	onkogen izolovaný z viru způsobujícího nádory *Kirsten rat sarcoma viral oncogene homolog*

LAMP	protein asociovaný s lyzozomální membránou *lysosomal-associated membrane protein*
LMP	proděravění lyzozomální membrány *lysosomal membrane permeabilization*
LPS	bakteriální lipopolysacharid
LT	lymfotoxin
MAPK	mitogeny aktivovaná proteinkináza *mitogen-activated protein kinase*
MMP	metaloproteáza *matrix metalloproteinase*
MMP	proděravění mitochondriální membrány *mitochondrial membrane permeabilization*
mRNA	mediátorová RNA *messenger RNA*
MVB	multivezikulární tělíska *multivesicular bodies*
MYC	oncogen izolovaný z viru ptačí myelocytomatózy *myelocytomatosis oncogene*
NECAP	protein spjatý s endocytózou *endocytosis-associated protein*
NEMO	protein nezbytný pro modulaci funkce faktoru NF-κB *NF-κB essential modulator*
NF-AT	transkripční faktor aktivovaných T-lymfocytů *nuclear factor of activated T-cells*
NF-κB	transkripční faktor zesilující expresi lehkého řetězce-κ v B-lymfocytech *nuclear factor of κ-light chain gene enhancer in B cells*
NGFR	receptor pro nervový růstový faktor *nerve growth factor-receptor*
NK	"*natural killer*" buňka
OPG	osteoprotegerin
PARP	poly(ADP-ribóza)-polymeráza *poly(ADP-ribose)-polymerase*

PH	plecstrin homologní doména *plecstrin homology domain*
pH	vodíkový exponent *potential of hydrogen*
PI3K	fosfatidylinositol-3-kináza *phosphatidylinositol-3-kinase*
PIK3CA	fosfatidylinositol-3-kináza, katalytická podjednotka α *phosphatidylinositol-3-kinase, catalytic subunit alpha*
PKB	proteinkináza B, Akt *protein kinase B, Akt*
PLAD	doména umožňující sestavení komplexu s ligandem *pre-ligand assembly domain*
PTEN	fosfatáza a homolog proteinu tensin *phosphatase and tensin homolog*
Puma	proapoptotický protein závislý na transkripčním faktoru p53 *p53 up-regulated modulator of apoptosis*
RANK	receptor aktivující faktor NF-κB *receptor activator of NF-κB*
RELT	receptor exprimovaný v lymfoidních tkáních *receptor expressed in lymfoid tissues*
RIP	protein-kináza interagující s receptory *receptor-interacting protein kinase*
RNA	ribonukleová kyselina *ribonucleic acid*
SCID	syndrom těžké kombinované imunodeficience s*evere combined immunodeficiency*
siRNA	malá interferující RNA (krátká interferující RNA, umlčující RNA) *small interfering RNA (short interfering RNA, silencing RNA)*
Smac	druhý aktivátor kaspáz odvozený od mitochondrií *second mitochondria-derived activator of caspase*
SNP	jednonukleotidový polymorfizmus *single nucleotid polymorphism*
STAT1	přenašeč signálu a transkripční aktivátor 1 *signal transducer and activator of transcription 1*

TACI	transmembránový protein aktivující a interagující s proteinem CAML *transmembrane activator and CAML interactor*
tBid	zkrácená forma proteinu Bid *truncated Bid*
TCR	antigenně specifický receptor T-lymfocytů *T-cell receptor*
THD	TNF-homologní doména *TNF-homology domain*
TL1	molekula příbuzná ligandu TNF *TNF ligand-related molecule-1*
TNF	faktor nekrotizující nádory *tumor necrosis factor*
TNFR	receptor faktoru nekrotizujícího nádory *tumor necrosis factor-receptor*
TRADD	protein asociovaný s receptorem TNFR1 a obsahující doménu smrti *TNFR1-associated death domain protein*
TRAF	faktor asociovaný s receptorem TNFR1 *TNFR1-associated factor*
TRAIL	ligand příbuzný faktoru TNF indukující apoptózu *TNF-related apoptosis-inducing ligand*
TRAIL-R	receptor ligandu TRAIL *TRAIL-receptor*
TWEAK	protein příbuzný faktoru TNF a slabě indukující apoptózu *TNF-related weak inducer of apoptosis*
V-ATPáza	vakuolární protonová pumpa hydrolyzující ATP *vaculolar ATPase*
VEGI	inhibitor růstu cévních endoteliálních buněk *vascular endothelial growth inhibitor*
XEDAR	receptor proteinu ektodysplazin A vázaný na chromozóm X *X-linked ectodysplasin A receptor*
XIAP	inhibitor apoptózy vázaný na chromozóm X *X-linked inhibitor of apoptosis*
Y2H	kvasinkový dvouhybridní systém *yeast-two-hybrid system*

I. Úvod a cíle práce:

Proteiny z rodiny TNF (*tumor necrosis factor,* faktor nekrotizující nádory) hrají roli ve velkém spektru fyziologických procesů. Podílí se na udržování homeostázy organizmu, ovlivňují buněčné dělení, buněčnou diferenciaci i programovanou buněčnou smrt. Posledně jmenovaná funkce je antagonisticky regulována zejména aktivací proapoptotických a antiapoptotických signálních drah.

Člen této početné rodiny – ligand TRAIL (*TNF-related apoptosis-inducing ligand*) – je s membránou asociovaný glykoprotein exprimovaný zejména hematopoetickými buňkami, který může u vnímavých buněk indukovat apoptózu. *In vitro* a *in vivo* studie odhalily schopnost ligandu TRAIL vyvolávat buněčnou smrt přednostně u nádorových nebo virově infikovaných buněk. Tato potenciální role ligandu TRAIL v protinádorové imunitní odpovědi vedla k důkladnému výzkumu jak regulace exprese jeho receptorů, tak jím aktivovaných signálních drah. Současně se rozbíhají klinické studie, ve kterých jsou testovány rekombinantní rozpustná forma ligandu a agonistické protilátky proti receptorům ligandu TRAIL.

Součástí signálních kaskád spouštěných ligandem TRAIL je vedle indukce programované buněčné smrti závislé na kaspázách i aktivace transkripčního faktoru NF-κB, c-Jun N-terminální kinázy (JNK), proteinové kinázy C či MAP kináz. Výslednou rezistenci či citlivost buňky na apoptotický signál ovlivňuje kinetika a kombinace těchto aktivovaných drah. Pro jiné členy TNF rodiny (FasL a TNFα) byl také popsán významný vliv internalizace trimerizovaného receptoru, signalizace z nitrobuněčných váčků či zásadní role lyzozómů a propustnost jejich membrán. Výzkum podobných regulačních procesů v případě ligandu TRAIL však zůstává nadále výrazně nepřehledný.

Cílem naší práce bylo přispět k pochopení regulačních mechanizmů vedoucích k rezistenci některých buněčných typů na apoptotickou signalizaci indukovanou ligandem TRAIL a v důsledku se tak podílet na širším poznání tohoto potenciálního protinádorového terapeutika. Jako modelový systém jsme si vybrali buňky kolorektálního karcinomu, jehož incidence je v České republice na velmi vysoké úrovni. Dílčí cíle naší studie byly následující:

1. Určit význam endocytózy a post-endocytických procesů v signalizaci spuštěné ligandem TRAIL.

2. Analyzovat složení nitrobuněčného proteinového komplexu apoptotických receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5 a jeho regulační roli.
3. Prostudovat vliv vakuolární ATPázy a lyzozómů na buněčnou smrt vyvolanou ligandem TRAIL.

II. Abstrakt

Člen proteinové rodiny faktorů příbuzných s TNF - transmembránový ligand TRAIL (*tumour necrosis factor-related apoptosis inducing ligand*) - přitahuje pozornost díky své schopnosti specificky a efektivně vyvolávat apoptotickou smrt u různých typů rakovinných buněk. Přesto se často setkáváme se sekundární rezistencí, kterou rakovinné buňky vyvíjejí k působení ligandu TRAIL, přičemž molekulární mechanizmy související s tímto jevem nejsou doposud uspokojivě objasněny.

V první publikaci jsme se zaměřili na analýzu role endozomální acidifikace v proximálních signalizačních drahách ligandu TRAIL. Pomocí specifických inhibitorů bafilomycinu A1 (Baf A1) a konkanamycinu A (CCA) jsme u několika vnímavých kolorektálních buněčných linií zablokovali vakuolární ATPázu, která zodpovídá za vnitřní okyselování endocytických váčků a lyzozómů. Zjistili jsme, že citlivost takto ošetřených buněk k cytotoxickému působení ligandu TRAIL je významně a reprodukcibilně potlačena. Buňky vykazovaly minimální štěpení prokaspázy-8 a výrazně zpomalena (i když pouze dočasně) byla i agregace receptorových komplexů a jejich pohyb po buňce v klatrinem obalených váčcích. Povrchová exprese receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5, jejich internalizace po navázání ligandu TRAIL i aktivace antiapoptotických NF-κB a MAP kinázových signalizačních drah však zůstaly nezměněny. Ověřili jsme, že na supresivní efekt acidifikačních inhibitorů nemá vliv mitochondriální amplifikační smyčka, lyzozomální permeabilizace a ani aktivace kyselé sfingomyelinázy. Naše práce tímto dokazuje, že funkce vakuolární ATPázy může představovat regulační uzel, který spolurozhoduje o výsledné buněčné odpovědi na působení ligandu TRAIL.

Ve druhé práci jsme studovali regulaci exprese receptoru TRAIL-R1/DR4 na plazmatické membráně a popisujeme charakterizaci jeho nového interagujícího proteinu ARAP1, který se účastní transportu TRAIL-R1/DR4 na buněčný povrch. V poslední studii jsme se věnovali testování tří různých cytostatik (TRAIL, 17-AAG, PLX4720) na rezistentních buňkách střevního karcinomu a na objasnění molekulárních mechanizmů jejich působení na základě genetického pozadí jednotlivých buněčných linií.

Věříme, že představované výsledky mohou přispět k objasnění vzniků rezistencí rakovinných buněk k indukované apoptóze a k racionálnějšímu využívání ligandu TRAIL v lidské medicíně.

III. Abstract

Tumour necrosis factor-Related Apoptosis Inducing Ligand (TRAIL), a membrane-bound ligand from the TNF family, has attracted significant attention due to its rather specific and effective ability to induce apoptotic death in various types of cancer cells via binding to and activating its pro-apoptotic death receptors (DRs). However, a significant number of primary cancer cells often develop resistance to TRAIL treatment, and the signalling platform behind this phenomenon is not fully understood.

In the first paper we focused on the influence of endosomal acidification. Upon blocking endosomal acidification by the vacuolar ATPase (V-ATPase) inhibitors bafilomycin A1 (BafA1) or concanamycin A (CCA) we observed a significantly reduced initial sensitivity of several, mainly colorectal, tumour cell lines to TRAIL-induced apoptosis. In cells pre-treated with these inhibitors, the TRAIL-induced processing of caspase-8 and the aggregation and trafficking of the TRAIL-receptor complexes were temporary attenuated. The cell surface expression of TRAIL receptors and their TRAIL-induced internalization were not affected by V-ATPase inhibitors. NF-κB or MAP kinase signalling from the activated TRAIL receptors remained unchanged, and neither possible lysosomal permeabilization, mitochondrial amplification loop nor acid sphingomyelinase were involved in this process. Altogether, the obtained data provide the first evidence that endosomal acidification could represent an important regulatory node in the proximal part of TRAIL-induced pro-apoptotic signalling.

In the second presented project we studied regulation of death receptors expression on the plasma membrane and we identified and characterized the novel TRAIL-R1/DR4 interacting adaptor protein ARAP1, which is involved in receptor trafficking to the cell surface. The last study was aimed on testing three different widely used cytostatics and inhibitors (TRAIL, 17-AAG and PLX4720) in resistant colon cancer model cell lines. We analyzed the combinatory effect on TRAIL induced apoptosis and molecular mechanisms in various genotype backgrounds.

Results presented in this thesis thus should contribute to the better understanding of TRAIL-triggered cell signaling and hopefully also to its rational application in the clinical practice.

IV. Literární přehled

IV.1 Proteiny rodiny TNF

IV.1.1 Historie

Lidské proteiny příbuzné s faktorem nekrotizujícím nádory (TNF) tvoří rozsáhlou skupinu čítající 19 ligandů a 29 odpovídajících receptorů. Tato superrodina je od roku 1984, kdy byl izolován její první člen TNFα, intenzivně studována a za posledních 30 let bylo publikováno více než 100 000 prací věnovaných molekulární a funkční charakterizaci těchto významných proteinů.

Výzkum proteinů z rodiny TNF se datuje až do předminulého století, kdy německý lékař P. Bruns pozoroval regresi nádorů u pacientů s prodělanou bakteriální infekcí (Bruns, 1868). Bakteriální extrakty byly posléze s úspěchem používány v protinádorové léčbě (Coley, 1891) a roku 1943 Shear izoloval bakteriální lipopolysacharid, který byl odpovědný za nádorovou regresi (Shear et al., 1943). Roku 1963 O´Maley dokázal, že tento jev není přímý, ale je zprostředkován neznámým toxinem v lidském séru, který poprvé nazval faktorem nekrotizujícím nádory (TNF) (O'Malley et al., 1963). Teprve později se jeho zdrojem ukázaly být aktivované makrofágy (Carswell et al., 1975), a až tento objev otevřel cestu k izolaci TNFα a k určení jeho aminokyselinové sekvence roku 1984 (Aggarwal et al., 1985; Aggarwal et al., 1984).

Pomocí biochemických přístupů i díky počítačovému vyhledávání homologních sekvencí v databázích pak byli velmi rychle identifikováni a charakterizováni další a další členové této rodiny (Obr. 1). Jejich vlastnosti jsou shrnuty v následujících odstavcích.

IV.1.2 Zástupci rodiny TNF/TNFR a jejich fyziologická role

IV.1.2.1 Ligandy

Ligandy rodiny TNF se řadí mezi transmembránové proteiny II. třídy s jednou transmembránovou doménou a C-koncovou extracelulární částí. Výjimku tvoří pouze lymfotoxin α (LTα) a inhibitor růstu cévních endoteliálních buněk (VEGI), které jsou sekretované (Aggarwal, 2003). Některé z transmembránových proteinů mohou být z povrchu buněk odštěpované proteázami, jako je to v případě ligandů TNF a RANKL pomocí proteázy ADAM (Black et al., 1997; Lum et al., 1999) nebo v případě FasL

pomocí matrilysinu (Powell et al., 1999). Posledních 150 aminokyselin v C-terminální oblasti těchto ligandu zastupuje vysoce konzervovanou TNF-homologní doménu (THD), vytvářející terciární strukturu beta barelu. Tato doména obsahuje 20-30% aminokyselin shodných pro všechny ligandy z TNF rodiny a je odpovědná za vazbu na příslušné receptory (Magis et al., 2012).

Ligandy bývají exprimovány ve valné většině buňkami imunitního systému, najdeme je na površích B buněk, T buněk, monocytů i dendritických buněk. Výjimku tvoří ligand VEGI, který se vyskytuje na endoteliálních buňkách (Aggarwal, 2003).

IV.1.2.2 Receptory

Ve srovnání s omezenou expresí ligandů, receptory z rodiny TNFR nalezneme na površích širokého spektra normálních i transformovaných lidských buněk.

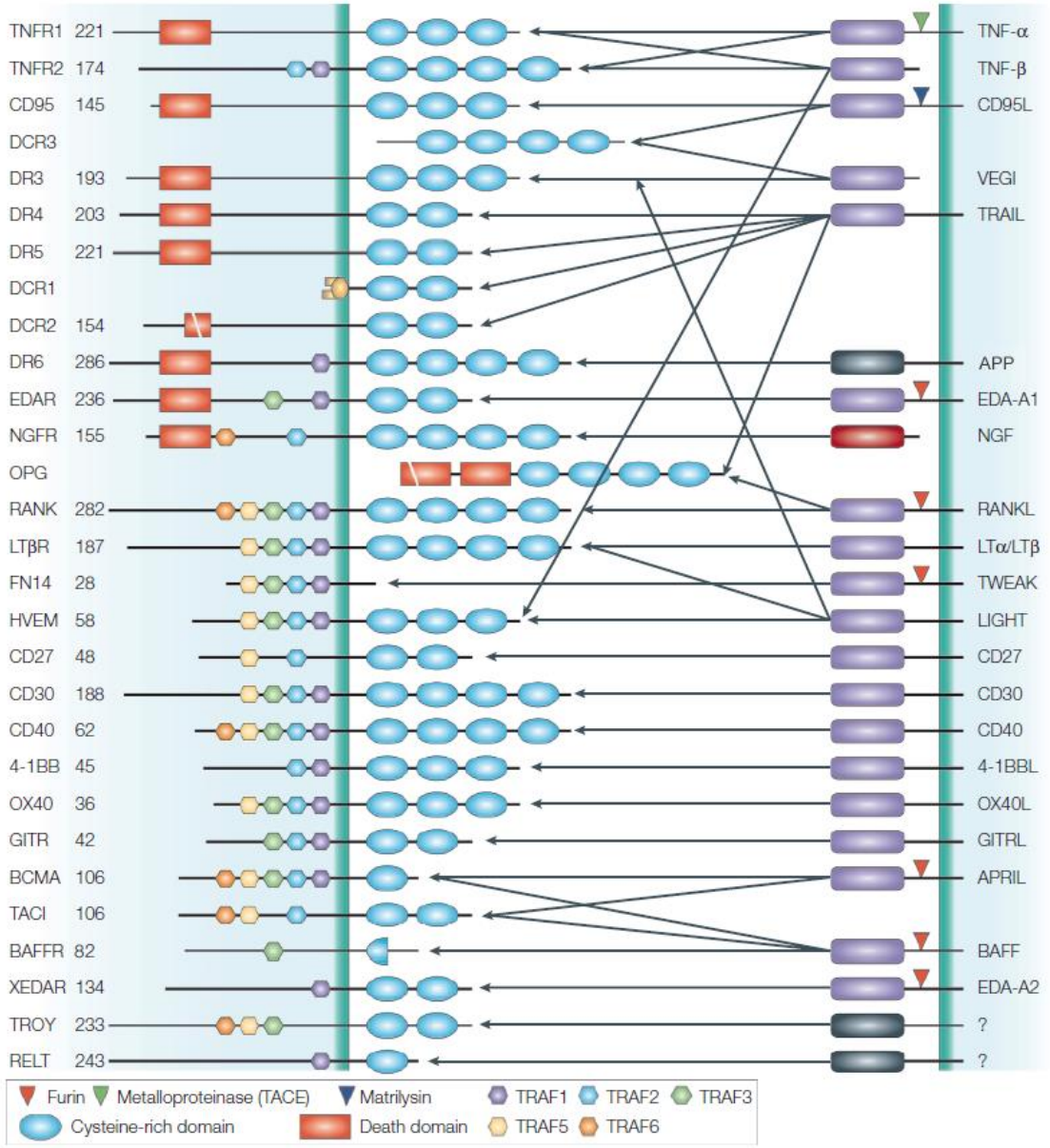
Receptory z rodiny TNFR jsou transmembránové proteiny I. třídy, pro které je typická N-koncová extracelulární část. Receptorům BCMA, TACI, BAFFR a XEDAR chybí signální peptid a řadíme je mezi speciální III. třídu transmembránových proteinů. Speciálním případem je i receptor TRAIL-R3/DcR1, který je s plazmatickou membránou spojen pouze pomocí GPI-kotvy. Sekretované jsou receptory OPTG a DcR3, které transmembránovou doménu zcela postrádají (Aggarwal, 2003). Také se vyskytují i rozpustné formy ostatních receptorů, které vznikly odštěpením pomocí metaloproteáz nebo alternativním sestřihem (Fas, 4-1BB), ale tyto formy vykazují posléze pozměněnou, inhibiční funkci (Smith et al., 1994).

Pro všechny receptory TNFR je však charakteristická přítomnost domén bohatých na cystein (*cystein rich domains*, CRD) v extracelulární části. Jde o poměrně krátké sekvence 30-40 aminokyselin, které jsou zodpovědné za vazbu na THD doménu TNF ligandů. Jejich počet se pohybuje v rozmezí 1-6, ale většina receptorů obsahuje 3 nebo 4 CRD domény. Vysoké zastoupení cysteinu v doméně je dáno přítomností většinou tří disulfidických můstků v každé CRD doméně (Bazan, 1993).

Vazba ligandu na receptor probíhá v poměru 3:3, vyžaduje tedy tři receptory a tři ligandy. Pouze tento trimerní komplex je aktivní ve vazbě dalších proteinů v cytoplazmatické části a v signální transdukci. Některé receptory můžeme nalézt na povrchu buněk již ve formě trimerů i bez navázaného ligandu. Na této oligomerizaci se podílí doména PLAD (*pre-ligand assembly domain*) v jejich extracelulární části a je známo, že kromě akumulace receptorů jsou takto vzniklé komplexy často efektivnější v pozdější signalizaci po připojení ligandu. Formování receptorových komplexů díky

doméně PLAD bylo pozorováno u receptorů TNFR1, TNFR2, CD40, Fas, TRAIL-R1/DR4, TRAIL-R2/DR5 a TRAIL-R4/DcR2 (Chan, 2007; Chan et al., 2000; Clancy et al., 2005).

Většina ligandů se za určitých podmínek může vázat na několik různých receptorů, v případě ligandu TRAIL až 5, jiné vazby ligand/receptor jsou jedinečné. Do rodiny TNFR patří i receptory, jejichž odpovídající ligand ještě nebyl objeven - jako například TROY a RELT (Bossen et al., 2006).



Obrázek 1: Ligandy a receptory rodiny TNF

Receptory z rodiny TNFR (v levé části diagramu) jsou transmembránové proteiny I. třídy, které se vyznačují přítomností několika CRD domén (*cystein-rich domains*) v extracelulární části. Intracelulární část u *death* receptorů obsahuje DD domény (*death domain*) odpovědné za zprostředkování apoptotické signalizace. Receptory až na pár výjimek (DR6, NGFR) váží ligandy z rodiny TNF. Ligandy představují transmembránové proteiny II. třídy, které jsou exprimovány většinou na površích buněk imunitního systému. Charakteristická je pro ně TNF-homologní doména v extracelulární části. (Upraveno podle *Aggarwal, Nature Reviews in Immunology, 2003.*)

IV.1.2.3 Signalizace a fyziologická funkce

Žádný z receptorů nemá enzymatickou aktivitu, ale jejich cytoplazmatická část slouží k přenosu signálu, který je zprostředkován adaptorovými proteiny typickými pro jednotlivé receptory. Tyto proteiny se váží na trimerizované cytoplazmatické domény a umožňují vazbu dalších signalizačních a efektorových proteinů. Adaptorový protein FADD váže a podporuje aktivaci iniciačních a posléze efektorových kaspáz, adaptorové proteiny z rodiny TRAF umožňují vazbu proteinu RIP a následnou aktivaci antiapoptotického faktoru NF-κB. Protein TRADD, který rozpoznává cytoplazmatickou doménu TNRFI je schopen se za určitých okolností podílet na obou těchto signalizačních kaskádách (Dempsey et al., 2003).

Výsledkem aktivovaných signalizačních drah, pak v případě TNF/TNFR rodiny bývá

a) programovaná buněčná smrt (TNF, LT, FasL, TRAIL, VEGI, TWEAK, LIGHT)
b) buněčný růst (RANKL, BAFF)
c) diferenciace (TNF, RANKL, DR6)
d) proliferace (TNF, CD27L, CD30L, CD40L, OX40L, 4-1BBL, APRIL, BAFF).

Všechny zmiňované procesy se stávají součástí komplexních fyziologických dějů jako je organogeneze, hematopoéza, regulace imunitního systému, obrana proti rakovinnému bujení nebo obrana proti infekcím. A samozřejmě jakýkoliv zásah do správného fungování vede k širokému spektru patologických projevů a onemocnění (Hehlgans & Pfeffer, 2005).

Z důležitých fyziologických procesů můžeme zmínit vytváření lymfatických uzlin a Peyerových plaků během embryogeneze, na kterém se podílí lymfotoxin α a β (Fütterer et al., 1998), tvorbu sekundárních lymfatických tkání ve slezině a rozvoj protilátkové imunitní odpovědi v případě TNFα (Fu & Chaplin, 1999; Nedospasov, 2003). Nebo také zajištění správného průběhu imunoglobulínového přesmyku, na kterém se podílejí ligandy CD40, OX40 a APRIL (Castigli et al., 2004; Grewal & Flavell, 1996; Stüber & Strober, 1996). Ligandy TNF α, LT α s FasL hrají roli v potlačování nádorového růstu, imunitní odpovědi na nitrobuněčné patogeny a TNFα je zároveň základním cytokinem zprostředkovávajícím zánětlivé procesy jako jsou horečka, nízký tlak či septický šok (Malik & Balkwill, 1992).

Naopak k patologiím a zásadnímu poškození tkání vedou různé mutace v genech, chyby v regulaci exprese proteinů a deregulované signalizační dráhy. Nadprodukce TNF ligandů a receptorů byly popsány u širokého spektra chronických zánětlivých chorob, například trávicího traktu v případě TNFα, LTα/β, CD40 a LIGHT (ulcerózní kolitida, Crohnova choroba (Baert & Rutgeerts, 2000), (Maerten et al., 2003)). Aberantní signalizace spuštěná ligandy LT a LIGHT se podílí na vzniku artritid (Fava et al., 2003) a ligandy OPTG a RANKL na vzniku osteoporózy (Saidenberg-Kermanac'h et al., 2004). Zvýšené množství TNFα nebo LTα v séru pacientů bylo popsáno u roztroušené sklerózy (Tsukada et al., 1993) a psoriázy (McDevitt et al., 2002), u systémového lupusu je většinou zachycen zvýšený ligand BAFF (Cheema et al., 2001). Polymorfismy v genech pro TNFα/LTα byly pozorovány u cukrovky obou typů (Boraska et al., 2010). Dále se špatnou funkcí TNF signalizace souvisí také například odhojování štěpů po transplantaci, Alzheimerova choroba, astma, vytváření metastáz a ischemické choroby.

IV.1.3 Receptory smrti a jejich ligandy

Proteiny obsahující ve své intracelulární části *death* doménu (DD) řadíme mezi tzv. receptory smrti (*death receptor*s, DRs), neboť po své aktivaci ligandy mohou indukovat buněčnou smrt. Patří mezi ně receptory Fas, TNFRI, TRAIL-R1/DR4, TRAIL-R2/DR5, DR3, DR6, EDAR a NGFR. Kromě DR6 a NGFR, které jsou aktivovány pravděpodobně beta-amyloidním prekurzorovým proteinem APP (Nikolaev et al., 2009) respektive NGF (Bothwell, 1996), ostatní *death* receptory váží ligandy z rodiny TNF. Po navázání ligandu se k cytoplazmatické *death* doméně připojí adaptorové proteiny FADD nebo TRADD, které aktivují kaspázovou signalizační kaskádu vedoucí k apoptóze (Chinnaiyan et al., 1996; Hsu et al., 1995; Kischkel et al., 2000). Zároveň však většina z nich umí za určitých podmínek navázat i adaptorové proteiny TRAF, které po vazbě na RIP1 zapnou antiapoptotické procesy směřující k aktivaci jaderného faktoru NF-κB a Jun N-terminální kinázy JNK. Rovnováha mezi oběma těmito ději, které se rozbíhají současně, je velmi přísně regulována a odehrává se na mnoha úrovních od exprese receptoru na membráně, posttranslačních modifikacích jednotlivých komponent až po zapojení cytoplazmatických inhibitorů. Pro svoji vysokou míru homologie a podobnost signalizačních kaskád, jsou většinou ligandy TNFα, FasL a TRAIL zmiňovány společně. Naše práce se zaměřuje na specifika buněčných dějů spouštěných ligandem TRAIL.

IV.2 TRAIL

Ligand TRAIL byl objeven v letech 1995 a 1996 dvěma nezávislými skupinami pomocí počítačového srovnávání homologních struktur s TNFα v EST databázích (Pitti et al., 1996; Wiley et al., 1995) jako třetí člen TNF rodiny, který indukuje apoptózu. Shoda v aminokyselinové sekvenci mezi ligandem TRAIL a dalšími členy této rodiny TNFα a FasL je 23%, respektive 28%. Obdobně i v signálních kaskádách aktivovaných těmito cytokiny najdeme mnoho podobností.

IV.2.1 Exprese a regulace genu

TRAIL je na mRNA úrovni široce exprimován v dospělých i embryonálních tkáních, ať už se jedná o slezinu, brzlík, prostatu, placentu nebo tenké střevo (Wiley et al., 1995), jako protein je ale exprimován zejména na povrchu hematopoetických buněk jako jsou NK buňky, B lymfocyty, monocyty, dendritické buňky a cytotoxické T lymfocyty (Allen & El-Deiry, 2012).

V jeho promotorové oblasti bylo nalezeno několik vazebných míst pro transkripční faktory, jako jsou STAT1,NF-AT, FOXO, NF-κB, p53, z nichž většina je aktivována cytokinovou signalizací (Wang et al., 2000). Například IFN-γ pomocí faktorů STAT1 a IRF1 nastartoval produkci ligandu TRAIL a následnou zvýšenou apoptózu plicních rakovinných buněk (Kim et al., 2002). Také IFN-α byl popsán jako induktor produkce TRAILu u makrofágů (Solis et al., 2006) a IFN-β u kolorektálních rakovinných buněk (Choi et al., 2003).

Gen pro ligand TRAIL obsahuje 5 exonů a 4 introny a alternativní sestřih dává vzniknout 9 izoformám, z nichž všechny obsahují N-terminální oblast a transmembránový helix, ale liší se C-koncovou doménou. (Krieg et al., 2003; Wang et al., 2011). Zkrácené formy v různé míře váží TRAIL receptory a aktivují NF-κB dráhu, ale pouze nejdelší varianta vykazuje apoptotické vlastnosti, ostatní pravděpodobně hrají roli v regulaci signalizace.

Co se týče chorob spojených s aberantní TRAILem indukovanou signalizací, tak změněná exprese ligandu TRAIL byla popsána u roztroušené sklerózy, zvýšená hladina rozpustného ligandu u systemického lupusu, naopak snížená tvorba mRNA u rakoviny prostaty a střeva (Allen & El-Deiry, 2012). TRAIL se podílí také na vzniku arterosklerózy, diabetu či astmatu (Corallini et al., 2007; Michowitz et al., 2005; Schoppet et al., 2006).

Z důvodu zmíněných patologických projevů spjatých s ligandem TRAIL a jeho signalizací byly pečlivě analyzovány jednonukleotidové polymorfizmy (*single nukleotid polymorphysms*, SNPs) v genu pro protein TRAIL. Byla zaznamenána souvislost nalezených SNPs se sníženou expresí mRNA ligandu TRAIL u rakoviny prsu (Pal et al., 2011), ale většinou nebyly negativní dopady SNPs potvrzeny. I zdravé osoby mají oblast pro gen TRAIL velmi polymorfní (Weber et al., 2004)

IV.2.2 Receptory pro TRAIL

Krystalografické studie ukázaly, že trimer ligandu TRAIL je stabilizován jedním atomem zinku a může interagovat s pěti receptory, z nichž pouze dva jsou schopny efektivně zprostředkovat apoptotickou signalizaci.

TRAIL-R1/DR4 a TRAIL-R2/DR5 obsahují ve své cytoplazmatické části *death* doménu (DD), motiv obsahující 6-7 α-helixů, který je schopen homotypicky vázat další DD jiných interagujících proteinů. TRAIL-R3/DcR1 tuto DD postrádá a TRAIL-R4/DcR2 ji má výrazně zkrácenou (Degli-Esposti et al., 1997a; Degli-Esposti et al., 1997b). Zatímco vazba *decoy* receptorů spouští NF-κB signalizaci, po navázání ligandu na TRAIL-R1/DR4 a TRAIL-R2/DR5 se vytváří signalizační komplex indukující buněčnou smrt – tzv. DISC (*death-inducing signaling complex)* (Mérino et al., 2006).

S daleko nižší afinitou se ligand TRAIL váže také na solubilní receptor osteoprotegerin, který se uplatňuje v regulaci vývoje kostí a byl popsán v literatuře jako *decoy* receptor pro RANKL. RANKL po vazbě na receptor RANK spouští NF-κB signalizaci regulující kostní metabolismus a formování osteoklastů (Vitovski et al., 2007). TRAIL by se tedy mohl účastnit této signalizace jako kompetitivní regulátor, tato hypotéza ale nebyla zatím věrohodně potvrzena.

Oproti tomu u myši byl nalezen pouze jeden homolog k proapoptotickým TRAILovým receptorům (Wu et al., 1999) a dva *decoy* receptory (Schneider et al., 2003).

IV.2.3 Fyziologický význam a myší model

Fyziologická role ligandu a jeho receptorů nebo s nimi spojené choroby byly intenzivně studovány také na zvířecích modelech.

V roce 2002 byla získána první data z myšího modelu s deletovaným genem pro ligand TRAIL. Tato zvířata nevykazovala žádné zásadní vývojové vady, byla životaschopná a fertilní (Cretney et al., 2002; Sedger et al., 2002). Stejně na tom byly i myši, kterým chyběl receptor pro ligand TRAIL (Diehl et al., 2004; Finnberg et al.,

2005). Mladé myši s vyřazeným genem pro ligand či receptory nevykazovaly ani vyšší frekvenci vzniku spontánních nádorů, pouze s přibývajícím věkem byl pozorován větší počet lymfomů (Zerafa et al., 2005). TRAIL$^{-/-}$ myši byly ale výrazně náchylnější ke vzniku nádorů po podání karcinogenů (Cretney et al., 2002). V souvislosti s tím je třeba zmínit, že u SCID myší, do kterých byly vpraveny rakovinné buňky rezistentní k apoptotickému působení ligandu TRAIL, byla po stimulaci ligandem TRAIL zachycena zvýšená tvorba metastáz (Malhi & Gores, 2006). Antiapoptotická signalizace od TRAILových receptorů vedla ke zvýšené proliferaci a migraci těchto buněk. Signalizační kaskády spuštěné ligandem TRAIL se také podílí na vzniku autoimunitních onemocnění. Blokování TRAILové signalizace u indukovaných experimentálních autoimunit výrazně zpomalilo vznik a průběh těchto onemocnění u myších modelů – např. roztroušené sklerózy (Cretney et al., 2005), autoimunitní thyreotitidy (Wang et al., 2005), diabetu nebo artritidy (Lamhamedi-Cherradi et al., 2003).

Exprese ligandu TRAIL je na hematopoetických buňkách aktivována a regulována plejádou fyziologických i patologických podnětů. V buněčných kulturách monocytů a i na myších modelech byla zjištěna zvýšená exprese ligandu TRAIL po stimulaci lipopolysacharidem a interferony (Ehrlich et al., 2003). Interferony společně s CpG oligodeoxynukleotidy také aktivovaly expresi ligandu TRAIL na dendritických buňkách (Kemp et al., 2003; Liu et al., 2001). Podobně IFN-γ indukoval expresi TRAILu na povrchu NK buněk. Společně s cytotoxickými T lymfocyty, které využívají ligand TRAIL, se všechny tyto procesy uplatňují v odpovědi buňky na virovou infekci. Myši infikované virem EMCV, kterým byly podávány inhibiční protilátky proti ligandu TRAIL umíraly výrazně rychleji než kontrolní zvířata (Sato et al., 2001).

Hlavní fyziologickou roli má tedy ligand TRAIL a jeho receptory v kontextu imunitního systému, ať už se jedná o regulaci odpovědi na infekce nebo imunitní dohled nad nádory a metastázemi (Schaefer et al., 2007).

IV.2.4 DISC a signalizační kaskády

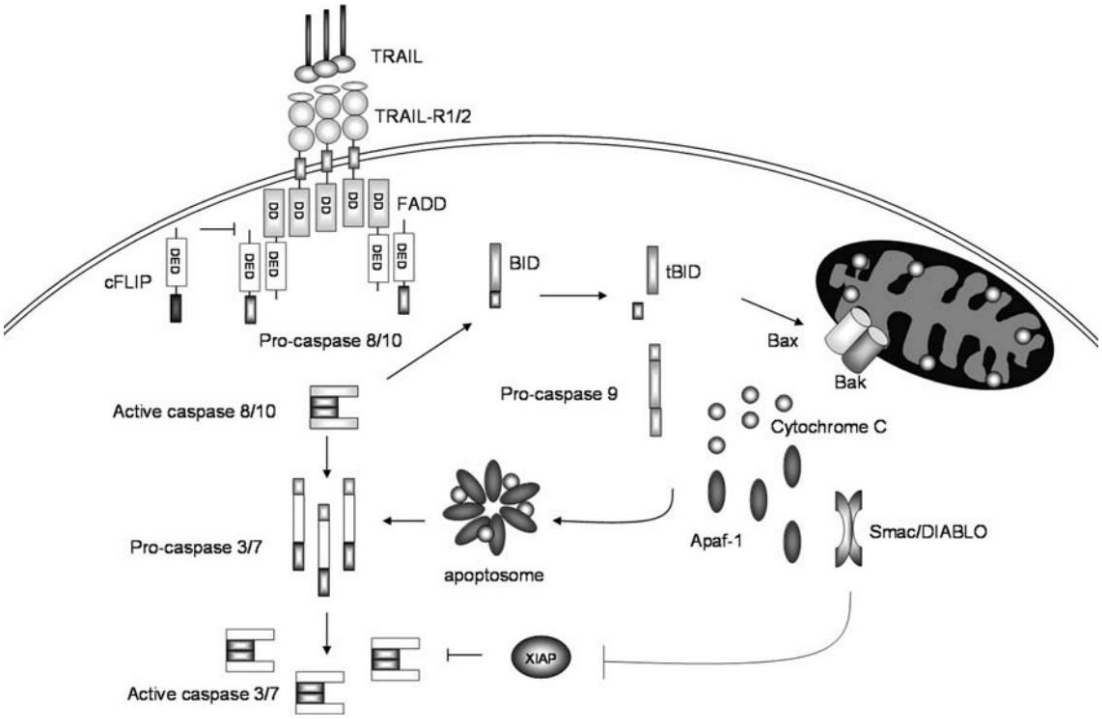
Navázání trimerního ligandu TRAIL k proapoptotickým receptorům TRAIL-R1/DR4 či TRAIL-R2/DR5 vede k DD zprostředkované vazbě adaptorového proteinu FADD, který posléze přes své efektorové domény smrti (*death effector domain*, DED) umožňuje vazbu dalších proteinů, především prokaspázy-8, prokaspázy-10 a proteinu

cFLIP. Výsledný multiproteinový komplex, který zprostředkovává apoptotickou signalizaci, se nazývá DISC ((Kischkel et al., 1995; Kischkel et al., 2000; Sprick et al., 2000).

Kaspázy jsou proteázy, které jsou produkovány jako neaktivní proenzymy a teprve aktivovaným receptorem zprostředkovaná oligomerizace iniciačních kaspáz (např. kaspázy-8 či -9) umožňuje jejich autokatalytické štěpení. Vznikají aktivní formy, které jsou schopny štěpit další většinou již efektorové prokaspázy-3, -7 a -6. Tyto kaspázy pak štěpí řadu dalších substrátů, což ve výsledku vede k celkové dezintegraci buňky projevující se fragmentací jádra a k rozpadu buňky na jednotlivé váčky, které jsou fagocytovány makrofágy. Kaspáza-10 se štěpí většinou s podobnou kinetikou jako prokaspáza-8, ale její úloha v komplexu DISC je stále nejasná. Nebylo prokázáno, že by byla schopna zcela zastoupit roli kaspázy-8 v buňkách, kterým tento protein chybí (Sprick et al., 2002).

Dalším proteinem, který se přes DED doménu váže na FADD ve stejném místě jako prokaspáza-8, je antiapoptotický protein cFLIP. cFLIP je velmi homologní s prokaspázou-8, ale postrádá jakékoliv katalytické domény. Je exprimován ve třech sestřihových variantách *l*, *r* a *s*, z nichž zejména kratší varianty *r* a *s* obsahující jen dvě DED domény velmi účinně inhibují vazbu, a tudíž i aktivaci kaspázy-8 (Thome et al., 1997) . Nadprodukce delší varianty $cFLIP_L$ také inhibuje apoptotickou signalizaci, ale naproti tomu nízká exprese $cFLIP_L$ může paradoxně umocňovat autoaktivaci kaspázy-8 pravděpodobně vytvářením nukleačních center (Oztürk et al., 2012).

Buňky, kterým stačí k indukci apoptózy aktivovat přímou kaspázovou kaskádu, jsou označovány jako typ I. Oproti tomu buňky typu II vyžadují ještě zapnutí mitochondriální amplifikační smyčky (Barnhart et al., 2003). Aktivovaná forma kaspázy-8 štěpí protein Bid na zkrácenou verzi tBid, která jednak kompetitivně inhibuje antiapoptotické proteiny z rodiny Bcl-2 (např. Bcl-2, Bcl-XL či Mcl-1), jednak interaguje s proapoptotickými proteiny Bax a Bak na mitochondriální membráně a společně umožňují vytvoření póru a uvolnění cytochromu c do cytosolu. Zde cytochrom c váže protein Apaf-1 a kaspázu-9 a společně vytváří proteinový komplex zvaný apoptozóm, ve kterém dochází k proteolytické aktivaci kaspázy-9 a ta následně štěpí efektorové kaspázy (Yin, 2006). Permeabilizace vnější mitochondriální membrány také umožňuje uvolňování proteinu Smac/DIABLO, který kompetitivně inaktivuje protein XIAP, inhibitor efektorových kaspáz, a který také přispívá ke zdárnému průběhu apoptotické signalizace (Waterhouse et al., 2002). Viz Obrázek 2.



Obrázek 2: Model apoptotické signalizace spouštěné ligandem TRAIL.

Po navázání trimerního ligandu k receptorům TRAIL-R1/R2 se k cytoplazmatickým *death* doménám (DD) receptorů naváže adaptorový protein FADD, který umožní vazbu prokaspáz-8/-10 a inhibitoru cFLIP. Dohromady tyto proteiny tvoří komplex DISC. V přímé apoptotické dráze u buněk typu I jsou následně autokatalyticky odštěpeny aktivní formy kaspáz, které štěpí další efektorové kaspázy. Buňky typu II vyžadují pro plnou efektivitu proapoptotických procesů změny na mitochondriální membráně. Aktivní formy kaspázy-8 a -10 štěpí protein Bid na tBid, které společně s proteinem Bak zprostředkují permeabilizaci mitochondriální membrány a uvolnění dalších proapoptotických faktorů jako je Smac/DIABLO nebo proteiny vytvářející apoptozóm. (Převzato z *Newsom-Davis et al., Apoptosis, 2009.*)

Od aktivovaných receptorů pro TRAIL se rozbíhají i signální kaskády, které nesměřují k apoptotické smrti, ale mohou iniciovat proliferaci, diferenciaci či migraci buňky. Tyto signální dráhy, mezi něž patří aktivace transkripčního faktoru NF-κB a mnohých kináz (jako jsou MAP kináza, *stress* kinázy JNK1/2 a p38 či protein kináza C), jsou ligandem TRAIL indukovány v závislosti na buněčném typu a podmínkách. Významnou roli při jejich aktivaci hraje protein RIP1 (*receptor interacting protein*). Ve speciálním sekundárním od membrány odtrženém komplexu, který obsahuje cytoplazmatické domény receptorů TRAIL-R1/DR4 nebo TRAIL-R2/DR5, FADD i

kaspázu-8 a který napomáhá zapnutí výše zmíněných drah, byly zachyceny kromě proteinu RIP1 také adaptorové proteiny TRAF2, TRADD a IKKγ (Varfolomeev et al., 2005). TRAILem indukovaná antiapoptotická signalizace je kromě NF-κB spojována také s aktivací Erk1/2 (Secchiero et al., 2004) či Akt (Secchiero et al., 2003) kináz.

IV.2.5 Faktory ovlivňující rezistenci buněk k apoptóze indukované ligandem TRAIL

Rovnováha mezi buněčným dělením a programovanou smrtí je nezbytná pro zachování tkáňové homeostázy, proto i apoptóza je přísně regulovaný děj. Rezistenci či citlivost buňky na apoptotický signál ovlivňuje buněčný typ i celkový biochemický kontext a v případě signalizace spuštěné ligandem TRAIL se často jedná o nadprodukci apoptotických receptorů, změnu proteinové exprese inhibitoru cFLIP a kaspázy-8 nebo podporu vytváření komplexu DISC. Dále hrají roli komponenty antiapoptotických drah, proteiny Bcl-2, BCL-XL, Mcl-1, survivin, IAP nebo dráhy Akt a NF-κB (Shirley et al., 2011). Regulace se tedy v buňce odehrává na několika různých úrovních, z nichž některé jsou rozebírány dále.

a) exprese receptoru a procesy na plazmatické membráně

Jednou z možností regulace TRAILem indukované signalizace je změna povrchové exprese jeho receptorů (Ravi et al., 2001). Například u rezistentních buněk rakoviny vaječníků byla popsána hypermetylace promotoru pro TRAIL-R1/DR4 a jeho výsledná snížená produkce (Horak et al., 2005a; van Noesel et al., 2002). Další možnost regulace představují takzvané *decoy* receptory, které vyvazují ligand, ale jejichž součástí není funkční DD, takže nedokáží buňce zprostředkovat apoptotický signál (Kimberley & Screaton, 2004; LeBlanc & Ashkenazi, 2003). Receptor TRAIL-R4/DcR2 také může vytvářet s proapoptotickým receptorem TRAIL-R2/DR5 heterotrimer, který ale následně není schopen iniciovat tvorbu aktivního komplexu DISC (Mérino et al., 2006).

O tom, zda převládne pro- či anti-apoptotický program, může rozhodovat i přesné umístění receptorů pro TRAIL v cytoplazmatické membráně Například lokalizace Fas receptoru do lipidových raftů může pozitivně ovlivnit jeho proapoptotickou signalizaci. V submembránových lokalizacích hrají důležitou roli posttranslační modifikace receptorů, jako je glykosylace a palmitylace, které mohou podporovat agregaci *death*

receptorů a směřovat je dle stavu buňky do různých částí plazmatické membrány (Chakrabandhu et al., 2007). Také pro TRAILem indukovanou apoptotickou signalizaci byl publikován pozitivní vliv lokalizace receptorů do membránových domén bohatých na cholesterol *(Song et al., 2007)*, TRAIL-R1/DR4 je na rozdíl od TRAIL-R2/DR5 za určitých okolností palmitylován (Rossin et al., 2009), ale zásadní vliv této posttranslační modifikace na aktivaci kaspázy-8 nebyl zatím náležitě prokázán.

Také O-glykosylace extracelulárních částí receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5 pozitivně ovlivňuje jejich agregaci a je spojována s efektivním formováním komplexu DISC a spouštěním apoptózy (Wagner et al., 2007).

b) internalizace

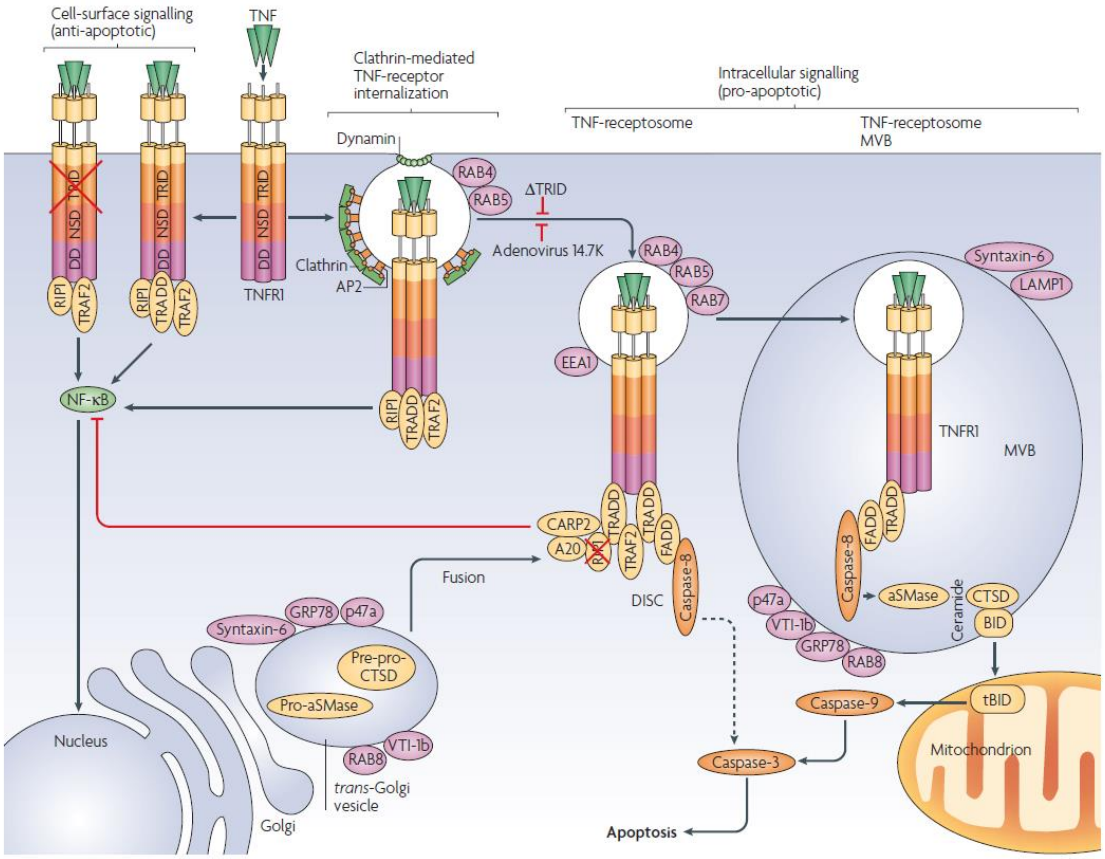
Internalizace aktivovaných receptorů může být dalším regulačním faktorem v *death* receptory indukované apoptotické signalizaci. Donedávna byla považována za pouhý nástroj k ukončení signalizace, kdy receptory jsou buď degradovány v lyzozomech, nebo zbaveny ligandu a vráceny zpět na povrch. Je však známo, že například pro plnou aktivaci receptoru pro epidermální růstový faktor či aktivaci inzulinového receptoru je nezbytná jejich internalizace, teprve na povrchu endocytických váčků dochází ke správnému kontaktu potřebných signálních molekul (Miaczynska et al., 2004).

V případě aktivovaného receptoru TNFR1 neinternalizovaný membránový komplex spouští zejména NF-κB signalizaci, zatímco internalizovaný cytoplazmatický receptorový komplex vytváří DISC a aktivuje štěpení kaspázy-8 (Micheau & Tschopp, 2003). Tyto výsledky potvrdila i práce z laboratoře Stephana Schützeho, kdy zablokování klatrinové endocytózy v epiteliálních buňkách zabránilo po navázání ligandu na TNFR1 tvorbě DISCu a inhibovalo spuštění programované buněčné smrti, zatímco aktivace NF-κB zůstala nedotčena (Schneider-Brachert et al., 2004).

Podobně i stimulace ligandem FasL vede k indukované klatrinové endocytóze Fas receptoru (Lee et al., 2006). Už 3 minuty po přidání FasL kolokalizuje s proteiny typickými pro rané endozómy jako je *endosome-associated protein 1* (EEA1). DISC je v tomto případě formován nejspíš již na cytoplazmatické membráně, ale prokaspáza-8 je štěpena až ve vnitrobuněčném prostředí. Buňky, kterým bylo zabráněno v internalizaci receptorových komplexů, vykazovaly zvýšenou aktivaci proliferačních signálních drah proteinů Erk a NF-κB.

Následně bylo zjištěno, že internalizace receptorových komplexů TNFR1 a aktivované kaspázy-8 je nezbytná pro funkci kyselé sfingomyelinázy. Kaspázou-8 aktivovaná kaspáza-7 v prostředí multivezikulárních endozómů štěpí přímo proenzym kyselé sfingomyelinázy a ten pak produkuje ceramidy. Ty se pak podílejí na efektivním průběhu apoptotické signalizace a aktivují lyzozomální proteázy katepsiny a napomáhají štěpení proteinu Bid (Edelmann et al., 2011). Pozitivní role kyselé sfingomyelinázy, která je obsažena většinou v trans-Golgi váčcích, byla popsána i pro ligand Fas a TRAIL (Dumitru & Gulbins, 2006; Lin et al., 2000).

Průběh internalizace TNFR1 po navázání ligandu a následné signalizační děje znázorňuje Obrázek 3.



Obrázek 3: Vliv internalizace a kyselé sfingomyelinázy (ASM) na TNF-R1 signalizaci.

Membránově vázaný receptor TNF-R1 s navázaným ligandem TNFα vytváří proteinový komplex, jehož součástí jsou adaptorové proteiny TRADD, TRAF-2 a RIP-1 a který vede k aktivaci jaderného faktoru NF-κB. Během několika minut je tento komplex endocytován do klatrinem obalených váčků a NF-κB signalizace je ukončena pomocí ubiquitinylace a degradace proteinu RIP-1, které zprostředkovávají E3 ligázy CARP-2/-1 a A20. K internalizovanému receptoru se naváží další proteiny vytvářející smrt indukující signalizační komplex (*death inducing signaling complex*, DISC): adaptorové proteiny TRADD a FADD a prokaspáza-8. V rámci DISCu je kaspáza-8 štěpena, aktivována a následně může štěpit prokaspázu-3. Během endocytické dráhy TNF-receptozómy fúzují s trans-Golgi váčky obsahujícími proenzymy kyselé sfingomyelinázy (pro-ASM) a katepsinu D (pre-proCTSD) a společně vytváří multivezikulární tělíska (MVB). Uvnitř MVB aktivuje kaspáza-8 efektorovou kaspázu-7, která umožňuje štěpení pro-ASM, aktivovaná ASM pak produkuje ceramidy, které zprostředkovávají aktivaci katepsinů D. Katepsiny se pak podílejí na spuštění mitochondriální amplifikační smyčky pomocí proteinů tBid a kaspázy-9 vedoucí k plnému rozvinutí programované buněčné smrti. Na obrázku jsou vyznačeny charakteristické povrchové proteiny jednotlivých váčků. (Převzato z *Schütze et al., Nature Reviews Molecular Cell Biology, 2008.*)

Vztah mezi internalizací receptorů pro TRAIL a apoptózou není doposud příliš jasný. Recentní práce k dané tematice od Susan L. Kohlhass například ukazuje, že v prostředí buněčné linie BJAB dochází k ligandem TRAIL indukované formaci komplexu DISC, aktivaci kaspáz a spuštění apoptózy i při zablokování klatrinové endocytózy. Do buněk však tyto receptory mohou pravděpodobně vstupovat několika různými endocytickými cestami, nejen klasickou klatrinovou (Kohlhaas et al., 2007). Situace je navíc komplikována faktem, že proteiny endocytické dráhy mohou být zároveň substráty aktivovaných kaspáz, takže TRAILem indukovaná apoptotická signalizace může endocytózu aktivovaných receptorů následně inhibovat (Austin et al., 2006). Ze zmiňovaných prací tedy vyplývá, že endocytóza není rozhodujícím krokem k aktivaci apoptotických drah spouštěných ligandem TRAIL.

V rozporu s těmito zjištěními je práce Akazawy a spol. z roku 2009 na buňkách odvozených karcinomu jater Huh-7, která tvrdí, že internalizace TRAIL-R2/DR5 a směřování receptorových komplexů do lyzozómu je nezbytná k uvolnění lyzozomálních proteáz do cytosolu a k úspěšnému průběhu programované buněčné smrti (Akazawa et al., 2009).

Přesný molekulární základ většiny těchto dějů však zůstává nejasný a zdá se být navíc buněčně specifický.

c) vytváření komplexu DISC a aktivace kaspázy-8

Významným prvkem v TRAILové signalizaci je vytváření komplexu DISC a aktivace kaspázy-8. Důležitým regulátorem je protein cFLIP, který se váže do stejného místa na adaptorové proteiny v rámci komplexu DISC jako prokaspáza-8, ale postrádá další katalytické schopnosti. Některé viry jsou dokonce schopny si kódovat vlastní variantu tohoto antiapoptotického proteinu (Thome et al., 1997). Zvýšená exprese proteinu cFLIP tedy představuje základní nástroj inhibice apoptotické signalizace, například až 40% buněčných vzorků odvozených z rakoviny vaječníků vykazovalo výrazně zvýšenou hladinu proteinu cFLIP (Horak et al., 2005b). Bylo potvrzeno, že snížení produkce proteinu cFLIP pomocí malých RNA zcitlivuje rezistentní buňky k působení ligandu TRAIL (Geserick et al., 2008). Jiné rakovinné buněčné typy zase vykazují sníženou proteinovou expresi kaspázy-8 (Hopkins-Donaldson et al., 2000). Poměr mezi zastoupením proteinů cFLIP a kaspázou-8 v rámci komplexu DISC hraje tedy zásadní roli.

Další mechanizmus regulace přestavuje polyubiquitinylace kaspázy-8 pomocí E3 ligázy cullin3 po navázání ligandu na receptory TRAIL-R1/DR4 a TRAIL-R2/DR5, která vede k její rychlejší agregaci a efektivnímu štěpení (Jin et al., 2009). Na druhou stranu vazba adaptorového proteinu TRAF2 na kaspázu-8 iniciuje její směřování do proteazómu a degradaci (Gonzalvez et al., 2012).

Funkci aktivačního komplexu DISC ovlivňují i další proteiny, které byly objeveny většinou pomocí protein-protein interakčních studií. Jedná se například o IG-20, RassFA1, DEDD2, PEA-15, MADD a PRMT5 (Shirley et al., 2011).

d) aktivace efektorových kaspáz a mitochondriální amplifikační smyčka

Jiný z regulačních bodů představují inhibiční proteiny z rodiny IAP (*inhibition of apoptosis protein*), které interagují s aktivovanými kaspázami a blokují jejich enzymatickou aktivitu. Velmi významnými regulátory apoptotické signalizace jsou také proteiny z rozsáhlé rodiny Bcl-2 (*B-cell lymphoma 2*), která zahrnuje jak proapoptotické tak antiapoptotické členy a ovlivňuje uvolňování kaspázových aktivátorů (např. cytochrom c a SMAC/Diablo) z mitochondrií.

Zvýšená exprese IAP proteinu XIAP byla pozorována u rakovinných buněk prostaty (Huang et al., 2004; Makhov et al., 2008) a antiapoptotických proteinů Bcl-2 a Bcl-XL u rezistentních prostatických buněk (Fulda et al., 2002; Hinz et al., 2000). Další antiapoptotický člen Bcl-2 rodiny protein Mcl-1, který váže a negativně ovlivňuje funkci proapoptotických proteinů z rodiny BH3 jako je Bim, Bid, Puma a Bak, způsobuje rezistenci k ligandu TRAIL zase u rakovinných buněk žlučových cest (Taniai et al., 2004).

IV.2.6 Klinické studie

Na rozdíl od TNFα a FasL, které v myších modelech vykazovaly značnou cyto- a hepatotoxicitu (Leist et al., 1996), rekombinantní TRAIL sám o sobě nevykazuje tyto vedlejší účinky, a to jak v myších modelech, tak i u pacientů (Ashkenazi et al., 1999; Herbst et al., 2006; Walczak et al., 1999). Obdobně dobře jsou v rámci klinických testů snášeny agonistické protilátky proti TRAILovým receptorům

V klinických testech jsou vedle rekombinantního TRAILu a agonistických protilátek testovány i genové transfery ligandu TRAIL do nádorových buněk (Griffith et al., 2008).

IV.2.6.1 Monoterapie rekombinantním ligandem či agonistickými protilátkami

Po preklinických zkouškách byly do klinických testů uvedeny rekombinantní TRAIL (aminokyseliny 114-281, Dulanermin, Genentech) a agonistické anti-TRAIL-R1/DR4 a anti-TRAIL-R2/DR5 humanizované monoklonální protilátky (Kelley & Ashkenazi, 2004)

Mezi nejpoužívanější preparáty z řady agonistických protilátek patří Mapatumumab (anti-TRAIL-R1/DR4, HGS), Lexatumumab (anti-TRAIL-R2/DR5, Amgen) a Apomad (anti-TRAIL-R2/DR5, Genentech). Oproti rekombinantnímu ligandu je jejich výhodou receptorová selektivita a také jejich stabilita v lidském séru (14-21 dní, ve srovnání s 30-60 minutami v případě rekombinantního ligandu) (Walczak et al., 1999).

Nadějné výsledky preklinických studií, kdy rekombinantní ligand či agonistické protilátky úspěšně eliminovaly rakovinné buňky odvozené z různých malignit: leukémie, mnohočetného myelomu, plic, jater, střeva, prsu, prostaty, pankreasu, ledvin i štítné žlázy (Newsom-Davis et al., 2009) ale nebyly reflektovány ve fázi I a II klinických testů u nádorových pacientů. Samotný ligand či agonistické protilátky např. Mapatumumab u pacientů s non-Hodginovým lymfomem (Younes et al., 2005) a pacientů se solidními nádory tlustého střeva, vaječníků a prostaty (Greco et al., 2008; Hotte et al., 2008; Le et al., 2004) vykazovaly jen velmi limitní pozitivní výsledky (částečná remise nebo stabilizace choroby). Léčba byla ale většinou dobře snášena s minimálními vedlejšími účinky a toxicita užívaných preparátů byla zaznamenána pouze při podání vysokých dávek.

Rezistenci některých primárních nádorů k monoterapii se poslední dobou snaží mnohé studie obejít kombinací s dalšími cytostatiky.

IV.2.6.2 Kombinovaná testovací terapie

Velmi častá necitlivost primárních rakovinných buněk k indukované apoptóze vede k výzkumu kombinované léčby a jsou hledány terapeutické přístupy, další cytostatika či imunologicky aktivní látky, které by podpořily protinádorové vlastnosti ligandu TRAIL (Ganten et al., 2006). Mezi cytostatika, která jsou používána spolu s ligandem TRAIL patří karboplatina, paclitaxel (Chow et al., 2006), cisplatina (Oldenhuis et al., 2008), 5-fluorouracil, doxorubicin (Sikic et al., 2007) či ionizující záření. Úspěch v překonávání rezistence rakovinných buněk zaznamenalo i používání následujících látek: protilátek proti CD20 rituximab (Daniel et al., 2007), inhibitorů

proteazómu bortezomib a MG321, které podporují mitochondriální amplifikační smyčku (Ganten et al., 2005; Leverkus et al., 2003), inhibitorů histondeacetylázy, inhibitorů PI3K-Akt signalizační dráhy jako je LY294002 (Secchiero et al., 2003) nebo rapamycin (Panner et al., 2005).

Další látky jsou neustále testovány (Hellwig & Rehm, 2012), v Tabulce 1 jsou uvedeny chemikálie, které úspěšně zcitlivovaly buněčné linie odvozené od rakoviny tlustého střeva, a mechanismus jejich působení na signalizační kaskádu ligandu TRAIL.

Drug-induced effects to overcome TRAIL-resistance in colorectal cancer and cell lines

Drug	Effect to overcome TRAIL-resistance	Cell line(s) studied
Cisplatin	DISC formation ↑	HT29
Camptothecin	TRAIL-receptors ↑	HCT116
CPT-11	TRAIL-receptors ↑ Type II → type I cells	colon tumors HCT116
Etoposide	TRAIL-receptors ↑ DISC formation ↑	HCT116 HT29
Doxorubicin	DISC formation ↑ Unknown	HT29 SW480
Interferon-γ	Caspase 8 ↑	HT-29 SW480
NSAIDs (sulindac)	Mitochondrial pathway ↑	HCT116 HCT15 SW480 HT29
PS-341	TRAIL-receptors ↑ Mitochondrial pathway ↑	HCT116
MG132	TRAIL-receptors ↑ Mitochondrial pathway ↑	HCT116
Resveratrol	Formation death receptors in lipid rafts	HT29 HCT116
Butyrate	TRAIL-receptors ↑ c-FLIP ↓	HT29 HCT116 KM12C KML4A KM20
Tunicamycin	TRAIL-receptors ↑	SW480
Nitroprusside	Mitochondrial pathway ↑	CX-1

Tabulka 1: Cytostatika používaná v kombinované léčbě a jejich efekt na zcitlivování kolorektálních buněk a buněčných linií rezistentních na působení ligandu TRAIL. (Převzato z *Van Geelen et al., Drug Resistance Updates, 2004.*)

V. Výsledky

V.1 Inhibice vakuolární ATPázy zpomaluje aktivaci kaspázy-8 spuštěnou ligandem TRAIL a zároveň ovlivňuje buněčný transport receptorových komplexů obsahujících tento ligand.

(komentář k publikaci **Horová V.**, Hradilová N., Jelínková I., Koc M., Švadlenka J., Bražina J., Klíma M., Slavík J., Hyršlová Vaculová A., Anděra L.: Inhibition of vacuolar ATPase attenuates the TRAIL-induced activation of caspase-8 and modulates the trafficking of TRAIL receptosomes. FEBS Journal, 2013)

Regulační vliv internalizace aktivovaného receptoru a jeho následného pohybu uvnitř buňky na výslednou signalizaci je široce diskutován, ale v případě ligandu TRAIL je situace poměrně nepřehledná (Akazawa et al., 2009; Austin et al., 2006; Kohlhaas et al., 2007). Nejednoznačnost doposud publikovaných dat nás vedla k bližší analýze této zajímavé problematiky.

Nejprve jsme na modelových kolorektálních nádorových buněčných liniích sledovali kinetiku a buněčné umístění TRAILem aktivovaných receptorových komplexů. Pro tyto účely jsme připravili rekombinantní rozpustný protein TRAIL značený fluorescenční značkou Alexa 647 a pomocí konfokální mikroskopie jsme analyzovali jeho lokalizaci v buňkách. Použili jsme protilátky proti složkám a specifickým proteinům jednotlivých endocytických drah jako jsou obalové proteiny klatrin, kaveolin, antigen raných endozómů EEA-1, antigen Golgiho aparátu GM130 či trans-Golgiho aparátu Vti1B, antigen lyzozomálních váčků LAMP2 a také fluorescenčně značený protein transferrin, který je endocytován klasickou cestou závislou na klatrinu. Dospěli jsme k závěru, že v modelových buněčných liniích HCT-116 a DLD-1 je ligand TRAIL takřka okamžitě po navázání na receptor internalizován do klatrinem obalených endozómů, které se postupně okyselují a nejdříve po dvou hodinách splývají s lyzozómy. Nepozorovali jsme žádné shodné barvení váčků zároveň ligandem TRAIL a kaveolinem nebo povrchovými antigeny pro Golgiho aparát.

V rámci studia role lyzozómu v signalizaci indukované ligandem TRAIL jsme se v dalším kroku, který je již obsahem komentované publikace, pokusili zablokovat fúzi pozdních endozómů s lyzozómy a ovlivnit výsledek apoptotické signalizace u senzitivních buněk HCT116, Colo 206F a DLD-1. Za tímto účelem jsme použili chemický inhibitor E64d, který je schopen inhibovat aktivitu katepsinů, tedy

lyzozomálních proteáz. Dále jsme pomocí malých RNA snížili v buňkách zastoupení proteinu rab7, malé GTPázy, která se vyskytuje na membránách pozdních endozómů a zajišťuje průběh fúze s lyzozómem. V neposlední řadě jsme pomocí chemických inhibitorů vakuolární ATPázy bafilomycinu A1 a konkanamycinu A blokovali acidifikaci endozómů a lyzozómů.

Sledovali jsme vliv zvolených metod na aktivaci efektorové kaspázy-3 a štěpení jejího substrátu cytokeratinu 18 (metoda barvení protilátkou M30, která rozeznává štěpenou formu cytokeratinu, a stanovování zastoupení M30 pozitivních apoptotických buněk v kultuře pomocí průtokové cytometrie).

Ovlivnění katepsinů ani omezení produkce proteinu rab7 nemělo na aktivaci apoptózy zásadní vliv, ale zastavení acidifikace vnitrobuněčných kompartmentů pomocí bafilomycinu A1 výrazně snížilo TRAILem indukovanou proapoptotickou signalizaci reflektovanou aktivací kaspázy-3.

Inhibice V-ATPázy účinně potlačovala spuštěnou apoptotickou signalizaci nejen po hodinové preinkubaci buněk s bafilomycinem A1, ale i v případě, kdy byl inhibitor dodán zároveň s ligandem TRAIL nebo když byl inhibitor přidán 20 minut po aplikaci ligandu. Účinek bafilomycinu A1 a konkanamycinu A jsme testovali i na kulturách jiných buněčných typů (například liniích odvozených z rakoviny děložního čípku HeLa, jater HUH-7 či prostaty PC3). Výsledek byl vždy srovnatelný s předchozími zjištěními, předcházející inkubace s inhibitory intravezikulární acidifikace snižovala citlivost těchto buněk k působení ligandu TRAIL. Byli jsme tedy svědky obecného a velmi rychlého inhibičního efektu.

Současně se ale jedná o jev relativně časově omezený projevující se zejména během 2-3 hodin působení ligandu TRAIL. Posléze cytotoxicita ligandu převládne a po 4 hodinách je zastoupení apoptotických buněk v populaci srovnatelné s kontrolou bez přidaného inhibitoru.

Tento fenomén nás přivedl k domněnce, že BafA1 ovlivňuje mitochondriální amplifikační smyčku, která za normálních okolností zrychluje průběh programované buněčné smrti dalším štěpením kaspázy-3. Testovali jsme tedy vliv BafA1 i na HCT-116 buňkách s deletovaným genem pro protein Bax, které nejsou schopny zapnout mitochondriální signální kaskádu. Překvapivě jsme u těchto buněk zaznamenali ještě výraznější inhibiční vliv BafA1 na TRAILem indukovanou apoptotickou signalizaci. To potvrdilo náš předpoklad, že funkční mitochondriální smyčka je tím prvkem, který časově omezuje a posléze překonává inhibiční vliv blokované endozomální acidifikace.

Na druhou stranu se ukázalo zřejmým, že BafA1 působí na jiné aktivační respektive regulační složky v signalizaci od receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5 než je ta mitochondriální.

Abychom se vrátili k myšlence ovlivňování fúze intracelulárních váčků s lyzozómy, provedli jsme soubor pokusů, které měly za cíl vizualizovat, co se děje v buňce po přidání inhibitoru BafA1 a co společně s ligandem TRAIL. Pomocí Lysosensoru, fluorescenční značky rozeznávající kyselé prostředí, jsme si potvrdili, že BafA1 výrazně snižuje pH v běžně acidických kompartmentech. Mikroskopické studie, kdy jsme použili protilátky proti lyzozomálním markerům LAMP3 a LAMP2, současně naznačily, že po použití BafA1 dochází i k morfologickým a kvantitativním změnám těchto intracelulárních kompartmentů. Inhibice vakuolární ATPázy tedy opravdu výrazně mění složení a tvorbu lyzozómů respektive kyselých váčků.

Dále jsme sledovali, zda se mění endocytóza rozpustného fluorescenčně značeného ligandu TRAIL po přidání inhibitoru a závěry z imunofluorescenčních pokusů by se daly shrnout do následujících bodů. Po hodinové inkubaci buněčné kultury s bafilomycinem A1 následované dodáním ligandu TRAIL:

1. se snižuje kyselost Lysosensorem rozeznávaných nitrobuněčných váčků a snižuje se i počet váčků barvených lyzozomálním markerem LAMP3
2. se tvoří výrazně opožděně jasné váčky obsahující značený ligand TRAIL (cca o 30 - 45minut)
3. zároveň tyto váčky obsahující ligand TRAIL nejsou barveny v prvních dvou hodinách lyzozomálními markery

Z tohoto pohledu se zdá, že jsme nezablokovali fúzi s lyzozómy, ale že také v dotčené době (v prvních 2 hodinách po přidání ligandu TRAIL) k žádné nedochází ani za normálních okolností. Ukazují na to experimenty, kdy jsme barvili zároveň štěpenou formu kaspázy-8 a antigeny na povrchu lyzozómů. Aktivovaná kaspáza-8 je pozorovatelná ve stejných váčcích jako fluorescenčně barvený ligand TRAIL již po 20 minutách jeho působení, zatímco ke shodnému barvení s lyzozomálními markery nedochází ani po 90 minutách. Také z pokusů s E64d a siRNA proti rab7 není zřejmé, že by omezené působení lyzozomálních proteáz po permeabilizaci lyzozomální membrány (LMP) respektive zablokovaná fúze s lyzozómy měly vliv na aktivaci apoptózy spuštěné ligandem TRAIL.

Inhibice intravezikulárního okyselování tedy zásadně ovlivňuje vytváření endozomálních váčků obsahujících ligand TRAIL poblíž jádra, ale rozhodně se nejedná o lyzozómy. Spíše jsou zasaženy pozdní endozómy nebo multivezikulární tělíska.

S postupným okyselováním endocytických váčků je spjata také aktivace kyselé sfingomyelinázy (ASM) a následující produkce ceramidů. Ceramidy byly popsány jako druzí poslové, kteří amplifikují a zrychlují apoptotickou signalizaci od mnohých *death* receptorů (Zeidan & Hannun, 2010). Rozhodli jsme se proto ověřit hladinu buněčných sfingolipidů za přítomnosti BafA1 a bez něj. Roli ASM v pozorovaném ději jsme ověřili použitím jejího inhibitoru desipraminu.

Data získaná z hmotnostní spektrometrie nás však nedovedla k žádnému zásadnímu zjištění. Koncentrace ceramidů 16, 18 a 24 ani sfingosinu C-18 a sfingosin-1-fosfátu se v buňce během inkubace s BafA1 nemění a ani přidání inhibitoru ASM desipraminu nemá výrazný vliv na snížení citlivosti buněk k ligandu TRAIL způsobené inhibitorem BafA1. Přidání externího nejběžnějšího ceramidu C-18 nebo sfingosinu C-18 také nezajistilo zvrácení inhibičního efektu BafA1.

Po vyloučení vlivu mitochodriální amplifikační smyčky, LMP i aktivity ASM jsme se zaměřili na nejranější fáze signální kaskády po navázání ligandu TRAIL k odpovídajícímu receptoru. Studovali jsme možné změny v expresi receptorů, jejich internalizaci a formování signalizačního komplexu DISC, a postupující aktivaci pro- i anti-apoptotických drah.

A zjistili jsme následující - po hodinové inkubaci buněčné kultury s bafilomycinem A1 následované přidáním ligandu TRAIL:

1. exprese *death* receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5 i jejich internalizace zůstávají nezměněné
2. v buňce je v první hodině po přidání cytotoxického činidla méně štěpena kaspáza-8 a -3 i jejich substráty PARP-1 a cFLIP
3. NF-κB dráha zůstává beze změn, stejně tak fosforylace Erk1/2 či JNK
4. k inhibici a pomalejšímu štěpení prokaspázy-8 ve srovnání s kontrolou dochází již v rámci proteinového komplexu DISC (spolu s prokaspázou-10), jinak je tento komplex nezměněn

Aktivaci kaspázy-8 vázanou na komplex DISC velkou měrou ovlivňuje i působení inhibitoru cFLIP, který se běžně váže k intracelulárním doménám receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5. Jeho roli jsme otestovali pomocí klonů buněk DLD-1,

ve kterých jsme využitím krátkých RNA vnesených do genomu virovou transdukcí stabilně snížili expresi proteinu cFLIP. Zaznamenali jsme, že klony s blokovanou produkcí cFLIP vykazují jednak výrazně zvýšenou citlivost na cytotoxické působení ligandu TRAIL, ale zároveň přestávají být citlivé k inhibičním účinkům BafA1. To by mohlo podporovat teorii, že jen velmi malé vychýlení stochiometrických poměrů v zastoupení jednotlivých proteinů v komplexu DISC dokáže změnit celkové vyznění apoptotické signalizace a to nehledě na změny v acidifikaci endozómů.

Přínosem prezentované publikace je tedy zjištění, že v případě signalizace vyvolané ligandem TRAIL hraje zásadní roli správná funkce vakuolární ATPázy a postupná intravezikulární acidifikace. Inhibice těchto regulačních komponent má za následek výrazně nižší citlivost rakovinných buněk k působení ligandu TRAIL, která se projevuje neefektivním štěpením prokaspázy-8 a zpomaleným formováním nitrobuněčných váčků obsahujících proteinový komplex s ligandem TRAIL. Zjistili jsme, že tento jev je nezávislý na NF-κB dráze, mitochodriální amplifikační smyčce i lyzozomálních proteázách, stejně tak jako na aktivaci kyselé sfingomyelinázy a produkci ceramidů.

V.2 Adaptorový protein ARAP1 se účastní mobilizace receptoru TRAIL-R1/DR4 k plazmatické membráně

(komentář k publikaci Šímová Š., Klíma M., Čermák L., **Šourková V.**, Anděra L.: Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane. Apoptosis, 2008)

Konečná odpověď buňky po navázání ligandu TRAIL k jeho odpovídajícím receptorům na povrchu je souhrou působení mnoha proapoptotických a antiapoptotických signalizačních drah. Regulace těchto drah probíhá na několika úrovních a jeden z významných mechanizmů představuje už ovlivnění membránové exprese apoptických receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5 a jejich efektivní trimerizace.

Základní funkční složení proteinového komplexu DISC, který se vytváří okamžitě po oligomerizaci receptorů, je již důkladně prostudováno. Skládá se z cytoplazmatických *death* domén (DD) transmembránových receptorů, adaptorového proteinu FADD a iniciační kaspázy-8 společně s jejím inhibitorem cFLIP. Současně se ale objevují další a další proteiny, které se váží na jednotlivé komponenty komplexu DISC a které se různou měrou podílejí na jeho aktivaci či inhibici. Pokusili jsme se proto najít nové partnery *death* receptorů pomocí kvasinkového dvouhybridního systému. Provedli jsme rozsáhlé prohledávání savčí cDNA knihovny a zachytili jsme nový protein interagující s receptorem TRAIL-R1/DR4, který jsme posléze identifikovali jako ARAP1 (centaurin-δ2).

ARAP1 je adaptorový protein vykazující aktivity Arf GAP a Rho GAP z rodiny AZAP. Tyto proteiny fungují jako GTPázy a většinou pomáhají disociaci a recyklaci malých G-proteinů a účastní se cytoskeletárních změn uvnitř buňky. Patří mezi ně například ArfGAP1, který se podílí na tvorbě obalených transportních váčků (Randazzo & Hirsch, 2004). Další člen této rodiny ACAP1 se váže na cytoplazmatickou část transferrinového receptoru a umožňuje jeho recyklaci (Dai et al., 2004). ARAP1 má tři známé sestřihové varianty, přičemž námi objevená varianta interagující s receptorem TRAIL-R1/DR4 neobsahuje produkt exonu 30, kterým je jedna z C-terminálních PH (*pleckstrin homology*) domén. Další námi testované sestřihové varianty proteinu ARAP1 s receptorem TRAIL-R1/DR4 neinteragovaly, stejně jako příbuzné proteiny ARAP2 a ARAP3.

Vzájemná interakce TRAIL-R1/DR4 a ARAP1 byla potvrzena také v lidských buňkách HEK293T. Společné imunoprecipitace nadprodukovaných proteinů TRAIL-R1/DR4 a delečních mutantů ARAP1 ukázaly, že se tento adaptorový protein váže svojí C-koncovou částí do oblasti N-konce *death* domény receptoru TRAIL-R1/DR4 přilehlé k plazmatické membráně. Endogenní protein ARAP1 byl precipitován pomocí protilátek proti TRAIL-R1/DR4 pouze ve štěpené formě po přidání ligandu TRAIL k buňkám, což nasvědčuje společné interakci závislé na aktivaci kaspáz. Dalším důkazem podporujícím existenci vazby mezi těmito proteiny byla mikroskopická pozorování nadprodukovaných proteinů v NCTC buňkách. Imunofluorescenční barvení ukázala, že se ARAP1 a TRAIL-R1/DR4 společně nacházejí především na plazmatické membráně a v endoplazmatickém retikulu a po přidání ligandu TRAIL jsou společně internalizovány do raných endozómů rozeznávaných protilátkou proti proteinu rab5.

Funkční studie naznačily, že nadprodukce proteinu ARAP1 nevede k ovlivnění povrchové exprese TRAIL-R1/DR4 ani k ovlivnění apoptotické signalizace spuštěné ligandem TRAIL, pouze ke zvýšené expozici fosfatidylserinů na plazmatické membráně. Oproti tomu snížená hladina endogenního proteinu ARAP1 pomocí siRNA snižovala u většiny testovaných buněk expresi TRAIL-R1/DR4 na povrchu buňky bez ovlivnění jeho celkového proteinové zastoupení v buňce. Současně takto potlačená exprese proteinu ARAP1 zpomalovala apoptózu vyvolanou ligandem TRAIL. Pozorovaný jev se dal kvantifikovat pomalejším štěpením cytokeratinu-18 (substrátu aktivované kaspázy-3), inhibovaným štěpením proteinů kaspáza-8, PARP1 a Bid nebo pomalejší fosforylací kinázy JNK.

Všechna tato zjištění napovídají tomu, že adaptorový protein ARAP1 se podílí na regulaci buněčného transportu TRAIL-R1/DR4 na plazmatickou membránu a ovlivňuje kinetiku programované buněčné smrti spuštěné ligandem TRAIL.

Role adaptorového proteinu ARAP1 v tvorbě endozomálních váčků není překvapivá vzhledem k funkcím jeho příbuzných proteinů. I v jeho případě už bylo publikováno, že se podílí na transportu proteinů mezi Golgiho aparátem a plazmatickou membránou (Miura et al., 2002). Také místo interakce s proteinem ARAP1 v cytoplazmatické doméně *death* receptoru TRAIL-R1/DR4 se částečně překrývá s místem pro vazbu proteinů AP2 a NECAP, adaptorových proteinů nezbytných pro tvorbu klatrinem obalených váčků (Brett et al., 2002; Ritter et al., 2007). A již několikrát v minulosti bylo popsáno, že potlačení povrchové exprese *death* receptorů

vede ke zvýšené rezistenci rakovinných buněk ke stimulaci ligandem TRAIL (Jin et al., 2004).

I tato naše práce tedy podporuje hypotézu důležitosti regulovaného vezikulárního transportu receptorů ligandu TRAIL po buňce, tentokrát směrem k plazmatické membráně.

V.3 Rezistenci rakovinných buněk způsobenou mutací genu PIK3CA k inhibitoru PLX4720 napomáhá překonat společné působení s ligandem TRAIL.

(komentář k publikaci Oikonomou E., Koc M., **Šourková V.**, Anděra L., Pintzas A.: Selective BRAFV600E inhibitor PLX4720, requires TRAIL assistance to overcome oncogenic PIK3CA resistance. Plos ONE, 2011)

Další úrovní, na které může být regulována reakce rakovinné buňky na stimulaci ligandem TRAIL, je i její genotypové pozadí, tedy souhrn konkrétních mutací, které vedly k její transformaci. Předmětem práce našich řeckých kolegů bylo popsat vliv jednotlivých mutací na citlivost různých typů buněk kolorektálního karcinomu ke kombinované léčbě ligandem TRAIL.

Rakovina tlustého střeva, tzv. kolorektální karcinom je 4. nejčastější příčinou smrti způsobenou nádorovým onemocněním na světě. Přičemž Česká republika vede světový žebříček, co se týče incidence tohoto typu rakoviny. Karcinogeneze rakoviny tlustého střeva je velmi postupný a komplexní proces, v maligních buňkách se velmi často vyskytuje mutace v genu KRAS, která s sebou nese horší průběh choroby a nižší šanci na přežití. Ještě větší transformační potenciál má mutace genu BRAF, která podporuje vznik metastází (Li et al., 2006; Oikonomou et al., 2009). Během karcinogeneze se většinou díky genomovým přestavbám stávají buňky citlivějšími ke spuštění apoptózy, ale na druhé straně i ke konstitutivnímu zapnutí MAPK, PI3K a ERK1/2 drah, které může vést k postupné rezistenci rakovinných buněk na chemoterapii (Fang & Richardson, 2005; Michl & Downward, 2005; Tran et al., 2001). Příkladem jsou kolorektální buněčné linie HT29 a RKO, které neodpovídají na léčbu apoptotickým ligandem TRAIL (Hague et al., 2005).

PLX4720 je inhibitor, který byl popsán, že cíleně brání proliferaci rakovinných buněk s mutací BRAF V600E. Například v léčbě melanomu byl velmi úspěšný (Tsai et al., 2008). Situace u karcinomu tlustého střeva však není tak jednoznačná a odlišný výsledek působení tohoto inhibitoru je přičítán rozmanitému genetickému pozadí jednotlivých typů rakovinných buněk ve střevě. Například u buněk současně nesoucích mutaci v genu KRAS může být situace dokonce opačná, dochází k aktivaci MAP kináz a podpoře buněčného růstu (Heidorn et al., 2010).

Další slibné chemoterapeutikum představuje inhibitor 17-AAG, který brání chaperonu Hsp90 v dokončování posttranslačních úprav proteinů a jejich správnému

sbalení (Grbovic et al., 2006). Mezi partnery Hsp90 patří proteiny podporující buněčný růst, jako jsou PI3K, pAkt, NF-κB, C-Raf, ale i mutovaný B-Raf V600E (Kamal et al., 2003).

V prezentované studii byly postupně zvlášť i dohromady testovány účinky tří cytostatik na proliferaci buněčných linií odvozených z různých stádií karcinogeneze tlustého střeva, lišících se i genetickým pozadím. Sledovány byly mutace v genech KRAS, BRAF a PIK3CA a testované látky zahrnovaly inhibitory PLX4720, 17-AAG a *death* ligand TRAIL. Cílem bylo překonat kombinovaným podáváním případné buněčné rezistence na jednotlivé chemikálie a dále studium molekulárních vazeb souvisejících s těmito ději.

Konkrétně bylo použito 6 buněčných linií odvozených z rakovinných buněk tlustého střeva: Caco-2 (adenom), Colo205 (adenom: mut BRAF), HT29 (adenom: mut BRAF, PIK3CA), RKO (karcinom: mut BRAF, PIK3CA), DLD-1 (adenom: mut KRAS, PIK3CA), SW620 (adenom, mut KRAS), které se liší v citlivosti k působení zkoumaných inhibitorů. Kromě toho byly také vytvořeny klony odvozené od buněčné linie Caco-2, do kterých byla pomocí virové transdukce stabilně vnesena mutace BRAF V600E. Získaná data lze shrnout do následujících bodů:

a) k působení inhibitoru PLX4720 byly citlivé pouze linie HT29 a RKO, které obsahují mutace v genech pro BRAF a PIK3CA
b) samotné působení inhibitoru 17-AAG spíše zvyšuje proliferaci buněk a míra účinku koreluje s proteinovým zastoupením kinázy pAkt v buňkách
c) rezistence k působení ligandu TRAIL souvisí s přítomností mutace v genu pro kinázu PIK3CA
d) rezistence buněk HT29 ke stimulaci ligandem TRAIL může být překonána přidáním inhibitoru 17-AAG

Tento jev provází zvýšené proteinové zastoupení kinázy pAkt v buňce a ubiquitinylace receptoru TRAIL-R1/DR4. Apoptotický signál je v tomto případě veden výhradně přes receptor TRAIL-R2/DR5.

e) zatímco Caco-2 klony jsou vnesením mutace BRAF V600E úspěšně zcitlivovány k TRAILu ve srovnání s kontrolní Caco-2 buněčnou linií

Tato aktivace proapoptotických drah se projevovala rychlejší tvorbou proteinového komplexu DISC a nadprodukcí proteinů Bad, Bid a Bax a také zvýšenou přítomností *death* receptorů TRAIL-R1/DR4 a TRAIL-R2/DR5 na plazmatické membráně.

f) samotný inhibitor BRAF mutace PLX4720 na zastavení proliferace těchto klonů nestačí a vyžaduje k vyvolání programované buněčné smrti spolupůsobení ligandu TRAIL

Potvrdilo se tedy, že buňky kolorektálního karcinomu jsou i na molekulární úrovni velmi heterogenní buněčnou strukturou, ale i přesto doufáme, že představovaná studie může přispět k objasnění vzniků rezistencí a k racionálnějšímu využívání tří zmíněných cytostatik v lidské medicíně.

VI. Diskuze a závěr

Smyslem dizertační práce bylo prostudovat různé mechanizmy regulace apoptotické signalizace od aktivovaných receptorů pro ligand TRAIL, ať už se jedná o povrchovou expresi receptorů na membráně, jejich internalizaci a pohyb uvnitř buňky pomocí obalených váčků nebo vliv transformovaného genetického pozadí jednotlivých buněčných linií odvozených z rakovinných buněk tlustého střeva.

Prvním úkolem bylo určit význam endocytózy a internalizace *death* receptorů s navázaným ligandem TRAIL na jeho následnou signalizační aktivitu. Především tedy, zda je nezbytná signalizace z nitrobuněčných váčků nebo vše probíhá již na plazmatické membráně a vytváření veziklů obsahujících receptory a jejich postupná acidifikace vede pouze k umlčení apoptotického signálu a degradaci receptorových komplexů v lyzozómech.

Tento cíl se nám nepodařilo zcela naplnit, a proto ani není součástí žádné z publikovaných prací. Nenašli jsme metodu, pomocí níž bychom zcela uspokojivě zvládli zablokovat receptory na povrchu buněk bez zásadních vedlejších účinků na jejich přežívání. Ať už to bylo použití malých siRNA proti komponentám klatrinových váčků – klatrinu a adaptorovému proteinu AP2, použití chemického inhibitoru dynaminu dynasoru či v neposlední řadě vytvoření klonů s inducibilní produkcí dominantně negativního mutantu proteinu dynamin-1 (DN K44A). Zvýšená cytotoxicita všech těchto přístupů zásadní měrou omezovala hodnocení apoptotických pokusů a získané výsledky byly velmi rozporuplné. K ujasnění podobně kontroverzní situace ohledně role endocytózy v literatuře jsme se tedy bohužel nedobrali.

Dále jsme analyzovali roli lyzozómu, permeabilizace jeho membrán po stimulaci ligandem TRAIL a vliv acidifikace intravezikulárních váčků na apoptotickou signalizaci. Data z tohoto projektu byla publikována roku 2013 v časopise FEBS Journal a zásadní zjištění shrnuje následující přehled:

1. inhibice lyzozómálních proteáz neovlivnila průběh apoptotické signalizace spuštěné ligandem TRAIL
2. váčky obsahující receptorové komplexy s ligandem TRAIL nefúzují s lyzozómy během prvních dvou hodin po přidání ligandu TRAIL
3. kyselé prostředí těchto váčků je zásadní pro správné štěpení a aktivitu kaspázy-8

4. s inhibicí vnitrobuněčné acidifikace blokováním protonové pumpy V-ATPázy souvisí také zpomalený transport ligandu TRAIL do váčků po jeho internalizaci

Naše výsledky podporují práce z nedávné doby, které považují účast lyzozómů na *death* receptory zprostředkované apoptóze za pouze podpůrnou (Oberle et al., 2010; Wattiaux et al., 2007). Podobná studie zkoumající katepsinové inhibitory u několika buněčných linií citlivých na působení ligandů FasL či TRAIL také nezaznamenala výrazné zpomalení či zablokování apoptózy (Spes et al., 2012).

Oproti tomu zásadní vliv kyselého prostředí na plnou aktivaci ligandem TRAIL indukovaných apoptotických drah je novinkou a žádá si další funkční studie. Pozorovali jsme, že tato rezistence buněk vůči cytotoxickému působení ligandu TRAIL je nezávislá nejen na lyzozomální membránové permeabilizaci, ale také na mitochondriální amplifikační smyčce a aktivitě kyselé sfingomyelinázy a její produkci ceramidů. Snížená funkce V-ATPázy má výrazný dopad na konvenční pohyb receptorových komplexů po buňce, který nejspíš souvisí se zasaženým fúzováním pozdních endozómů, respektive multivezikulárních tělísek. Za této situace je pravděpodobně zablokováno uspořádávání apoptotických faktorů do vyšších multiproteinových komplexů, které za normálních okolností urychlují průběh cytotoxických procesů. Zpomalená oligomerizace prokaspáz-8 a -10 způsobuje redukovanou apoptotickou signalizaci, neboť vzájemná blízkost katalytických domén je vyžadována pro jejich efektivní štěpení. Dalo by se spekulovat také o roli ubiquitinylace v těchto dějích.

Nicméně to, že při inhibici vakuolární ATPázy není zasažena antiapoptotická signalizační kaskáda vedoucí k aktivaci proteinu NF-κB, nás může vést k představě, že se jedná o jeden z mechanizmů, které mohou vychýlit rovnováhu mezi proapoptotickými a antiapoptotickými signály směrem k programované buněčné smrti.

Prezentované výsledky před námi otevírají ještě další téma, kterým je vliv jednotlivých apoptotických receptorů na acidifikační proces, to znamená otázku, zda se výsledek působení inhibitorů jako je bafilomycin A1 liší v případě signalizace vedoucí od TRAIL-R1/DR4 a TRAIL-R2/DR5. Pro tyto studie jsou již v naší laboratoři připraveny nástroje v podobě rekombinantních ligandů TRAIL specifických vždy pouze pro jeden typ *death* receptoru.

Teorii, že transport ligandu nebo receptoru uvnitř buňky je důležitý pro jeho správné fungování potvrdily i výsledky publikované v další práci, která je zde

zmiňována. V rámci hledání nových proteinů interagujících s receptorem TRAIL-R1/DR4 jsme nalezli adaptorový protein ARAP1. Ten se váže na cytoplazmatickou doménu receptoru TRAIL-R1/DR4 jak v dvouhybridním kvasinkovém systému tak v lidských nádorových buňkách. V rámci funkčních studií bylo pozorováno, že po stimulaci ligandem TRAIL se společně nacházejí v raných endozómech. Nově objevený interakční partner adaptorový protein ARAP1 se podílí na uvolňování receptoru TRAIL-R1/DR4 z Golgiho aporátu na plazmatickou membránu a při snížení jeho proteinové produkce pomocí malých siRNA dochází k inhibici TRAILem indukované apoptózy.

Komplexnost problému rezistence či citlivosti rakovinné buňky na působení ligandu TRAIL je studována v poslední uváděné publikaci. Ta se věnuje vlivu jednotlivých mutací nahromaděných během karcinogeneze tlustého střeva v genech pro proteiny RAS, RAF a PIK3CA. Zmiňovaná práce přináší popis účinku tří cytostatik (PLX4720 - inhibitoru BRAF mutace V600E, 17-AAG - inhibitoru chaperonu Hsp90 a ligandu TRAIL) na různé typy buněčných linií odvozených z několika stádií kolorektálního karcinomu.

Všechny tři uváděné publikace výrazným způsobem rozšiřují současné znalosti o molekulárních mechanizmech působení ligandu TRAIL v lidských nádorových buňkách a mohly by ve svém důsledku přispět k racionálnějšímu přístupu ke kombinované cytostatické terapii v humánní lékařské praxi.

VII. Použitá literatura

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VIII. Přílohy (prezentované publikace)

Horová V., Hradilová N., Jelínková I., Koc M., Švadlenka J., Bražina J., Klíma M., Slavík J., Hyršlová Vaculová A., Anděra L.: Inhibition of vacuolar ATPase attenuates the TRAIL-induced activation of caspase-8 and modulates the trafficking of TRAIL receptosomes. FEBS J. 2013 May 17.

Tumour necrosis factor-Related Apoptosis Inducing Ligand (TRAIL), a membrane-bound ligand from the TNF family, has attracted significant attention due to its rather specific and effective ability to induce apoptotic death in various types of cancer cells via binding to and activating its pro-apoptotic death receptors (DRs). However, a significant number of primary cancer cells often develop resistance to TRAIL treatment, and the signalling platform behind this phenomenon is not fully understood. Upon blocking endosomal acidification by the vacuolar ATPase (V-ATPase) inhibitors bafilomycin A1 (BafA1) or concanamycin A (CCA), we observed a significantly reduced initial sensitivity of several, mainly colorectal, tumour cell lines to TRAIL-induced apoptosis. In cells pre-treated with these inhibitors, the TRAIL-induced processing of caspase-8 and the aggregation and trafficking of the TRAIL–receptor complexes were temporary attenuated. NF-κB or MAP/stress kinase signalling from the activated TRAIL receptors remained unchanged, and neither possible lysosomal permabilization nor acid sphingomyelinase were involved in this process. The cell surface expression of TRAIL receptors and their TRAIL-induced internalization were not affected by V-ATPase inhibitors. The inhibitory effect of BafA1 was, however, blunted by the knockdown of the caspase-8 inhibitor cFLIP. Altogether, the obtained data provide the first evidence that endosomal acidification could represent an important regulatory node in the proximal part of TRAIL-induced pro-apoptotic signalling.

Šímová Š., Klíma M., Čermák L., **Šourková V.**, Anděra L.: Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane. Apoptosis, 13(3):423-36, 2008.

TRAIL, a ligand of the TNFα family, induces upon binding to its pro-death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 the apoptosis of cancer cells. Activated receptors incite the formation of the Death-Inducing Signaling Complex followed by the activation of the downstream apoptotic signaling. TRAIL-induced apoptosis is regulated at multiple levels, one of them being the presence and relative number of TRAIL pro- and antiapoptotic receptors on the cytoplasmic membrane. In a yeast two-hybrid search for proteins that interact with the intracellular part (ICP) of DR4, we picked ARAP1, an adapter protein with ArfGAP and RhoGAP activities. In yeast, DR4 (ICP) interacts with the alternatively spliced ARAP1 lacking 11 amino acids from the PH5 domain. Transfected ARAP1 co-precipitates with DR4 and co-localizes with it in the endoplasmic reticulum/Golgi, at the cytoplasmic membrane and in early endosomes of TRAIL-treated cells. ARAP1 knockdown significantly compromises the localization of DR4 at the cell surface of several tumor cell lines and slows down their TRAIL-induced death. ARAP1 overexpressed in HEL cells does not affect their TRAIL-induced apoptosis or the membrane localization of DR4, but it enhances the cell-surface presentation of phosphatidyl serine. Our data indicate that ARAP1 is likely involved in the regulation of the cell-specific trafficking of DR4 and might thus affect the efficacy of TRAIL-induced apoptosis.

Oikonomou E., Koc M., **Šourková V.**, Anděra L., Pintzas A.: Selective BRAFV600E inhibitor PLX4720, requires TRAIL assistance to overcome oncogenic PIK3CA resistance. Plos ONE, 6(6):e21632, 2011.

Documented sensitivity of melanoma cells to PLX4720, a selective BRAFV600E inhibitor, is based on the presence of mutant BRAF (V600E) alone, while wt-BRAF or mutated KRAS result in cell proliferation. In colon cancer appearance of oncogenic alterations is complex , since BRAF, like KRAS mutations, tend to co-exist with those in PIK3CA and mutated PI3K has been shown to interfere with the successful application of MEK inhibitors. When PLX4720 was used to treat colon tumours, results were not encouraging and herein we attempt to understand the cause of this recorded resistance and discover rational therapeutic combinations to resensitize oncogene driven tumours to apoptosis. Treatment of two genetically different BRAF (V600E) mutant colon cancer cell lines with PLX4720 conferred complete resistance to cell death. Even though p-MAPK/ ERK kinase (MEK) suppression was achieved, TRAIL, an apoptosis inducing agent, was used synergistically in order to achieve cell death by apoptosis in RKO (BRAFV600E/PIK3CAH1047) cells. In contrast, for the same level of apoptosis in HT29 (BRAFV600E/PIK3CAP449T) cells, TRAIL was combined with 17-AAG, an Hsp90 inhibitor. For cells where PLX4720 was completely ineffective, 17-AAG was alternatively used to target mutant BRAF (V600E). TRAIL dependence on the constitutive activation of BRAF (V600E) is emphasised through the overexpression of BRAF (V600E) in the permissive genetic background of colon adenocarcinoma Caco-2 cells. Pharmacological suppression of the PI3K pathway further enhances the synergistic effect between TRAIL and PLX4720 in RKO cells, indicating the presence of PIK3CA (MT) as the inhibitory factor. Another rational combination includes 17-AAG synergism with TRAIL in a BRAF (V600E) mutant dependent manner to commit cells to apoptosis, through DR5 and the amplification of the apoptotic pathway. We have successfully utilised combinations of two chemically unrelated BRAF (V600E) inhibitors in combination with TRAIL in a BRAF (V600E) mutated background and provided insight for new anti-cancer strategies where the activated PI3KCA mutation oncogene should be suppressed.

Inhibition of vacuolar ATPase attenuates the TRAIL-induced activation of caspase-8 and modulates the trafficking of TRAIL receptosomes

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Running title: Endosomal acidification in TRAIL-induced apoptosis

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Keywords: V-ATPase, TRAIL, caspase-8, acidification, apoptosis

Abbreviations: ASM – acid sphingomyelinase, BafA1 – bafilomycin A1, BH – Bcl-2 homology, CCA - concanamycin A, CHQ - chloroquine, DISC – death inducing signalling complex, DRs – death receptors, LMP – lysosomal membrane permeabilization, MAPK –

mitogen activated protein kinase, PARP – poly ADP ribose polymerase, TRAIL - TNFa related apoptosis inducing ligand, V-ATPase - vacuolar ATPase

Abstract

Tumour necrosis factor-Related Apoptosis Inducing Ligand (TRAIL), a membrane-bound ligand from the TNF family, has attracted significant attention due to its rather specific and effective ability to induce apoptotic death in various types of cancer cells via binding to and activating its pro-apoptotic death receptors (DRs). However, a significant number of primary cancer cells often develop resistance to TRAIL treatment, and the signalling platform behind this phenomenon is not fully understood. Upon blocking endosomal acidification by the vacuolar ATPase (V-ATPase) inhibitors bafilomycin A1 (BafA1) or concanamycin A (CCA), we observed a significantly reduced initial sensitivity of several, mainly colorectal, tumour cell lines to TRAIL-induced apoptosis. In cells pre-treated with these inhibitors, the TRAIL-induced processing of caspase-8 and the aggregation and trafficking of the TRAIL–receptor complexes were temporary attenuated. NF-κB or MAP/stress kinase signalling from the activated TRAIL receptors remained unchanged, and neither possible lysosomal permeabilization nor acid sphingomyelinase were involved in this process. The cell surface expression of TRAIL receptors and their TRAIL-induced internalization were not affected by V-ATPase inhibitors. The inhibitory effect of BafA1 was, however, blunted by the knockdown of the caspase-8 inhibitor cFLIP. Altogether, the obtained data provide the first evidence that endosomal acidification could represent an important regulatory node in the proximal part of TRAIL-induced pro-apoptotic signalling.

Introduction

Ligands from the TNFα family play prominent roles in a large spectrum of physiological processes. They participate in the maintenance of cellular homeostasis, the regulation of cell differentiation, cell division and programmed cell death [1]. TRAIL (TNF-related apoptosis inducing ligand), as a member of this family, is mainly involved in the induction of apoptosis in permissive cells. Upon binding to the appropriate receptor on the cell surface, TRAIL triggers a cascade of events eventually leading to programmed cell death [2]. Human TRAIL is expressed by hematopoietic cells (T cells, NK and dendritic cells) and preferentially induces apoptotic signalling in transformed or virally infected cells [3, 4]. This selectivity ranks TRAIL among potential biological anticancer compounds. Recombinant TRAIL protein as well as agonistic humanized anti-TRAIL receptor antibodies are currently being tested in clinical Phase I and II studies in mono- or combined-therapy against various tumours [5, 6].

The canonical signalling pathway is initiated by the ligand-dependent engagement of the death receptor TRAIL-R1 (DR4) or TRAIL-R2 (DR5) and the formation of the multimeric death-inducing signalling complex (DISC). Apart from the ligand and receptors, the core DISC contains primarily the adapter protein Fas-associated death domain (FADD), initiator pro-caspases-8 and -10, and various amounts of the caspase-8 inhibitor cFLIP [7]. Aggregation of the pro-caspases at the DISC complex triggers their self-processing [8, 9], and the active caspases can then cleave pro-apoptotic substrates, such as effector caspases-3 and -7 and the BH3-only protein Bid[10]. In addition to triggering caspase-dependent apoptosis, the activated TRAIL receptors can, similarly to other DRs, induce generally pro-survival NF-κB, MAP/stress kinase or PI3K signalling. The final outcome of TRAIL signalling leading either to cell death or survival must therefore be tightly regulated [11].

At the proximal level, TRAIL signalling is regulated by decoy receptors and, most importantly, by cFLIP inhibitors, which are well known suppressors of TRAIL-induced apoptosis [12]. TRAIL-induced apoptotic signalling can also be modulated by posttranslational modifications of the TRAIL receptors such as exo-glycosylation, membrane-proximal palmitoylation or cullin 3-dependent ubiquitinylation of caspase-8 [13-15]. A number of other proteins have been reported to affect in a cell-specific manner the effectiveness of DISC formation and the processing of the initiator caspases. These include activating proteins such as IG-20, RassFA1 and DEDD2 as well as inhibitors such as PEA-15, MADD, TRADD and PRMT5 [16]. Besides the DISC composition, the efficacy of caspase-8 self-processing can also be affected by the lipid microenvironment or by endocytosis-related events. In SCLC cells for example, the localization of activated TRAIL receptors into lipid rafts enhanced their pro-apoptotic signalling [17]. As previously shown by us and others, the chemotherapeutic drug-mediated modulation of lipid rafts is associated with colon cancer cell sensitization to TRAIL-induced apoptosis [18, 19]. In the case of Fas/CD95 or TNFR1, receptosome endocytosis has been reported to be a positive factor in enhancing pro-apoptotic signalling [20-22]. Apoptotic signalling from these DRs is also strongly amplified when the gradual acidification of the intra-vesicular space leads to the activation of acid sphingomylinase (ASM), which produces ceramides, potent pro-apoptotic signalling transducers [23, 24]. Concerning TRAIL, the role of receptor endocytosis is not so clear. In some cells such as BJAB or HeLa, the endocytosis of TRAIL receptosomes is not required for the efficient induction of TRAIL-induced apoptosis [25]. However, it has also been recently reported by Akazawa *et al.* that for hepatocellular carcinoma cells, DR5 internalization plays a role in lysosomal permeabilization and the amelioration of TRAIL-induced apoptosis [26]. Thus, the role of endocytosis and post-endocytic processing in TRAIL-induced apoptotic signalling remains obscure.

Here we analysed the role of the vacuolar proton pump V-ATPase in the regulation of the proximal events in TRAIL-triggered signalling in sensitive colon cancer cell lines. V-ATPase is a multi-protein trans-membrane complex that pumps protons to the lumen of organelles and regulates vesicular pH [27]. V-ATPase function facilitates the fusion of late endosomes with lysosomes, vesicular acidification and the activity of various acid hydrolases. We used the macrolide antibiotics bafilomycin A1 (BafA1) and concanamycin A (CCA), known as specific inhibitors of V-ATPase, which bind to the V_0 trans-membrane domain of the proton pump and mechanically block its rotation [27, 28]. We followed the trafficking of TRAIL receptors, cleaved caspase-8 and DISC formation in the early stages of TRAIL-induced apoptosis in BafA1- or CCA-treated cells. We show that in addition to the already established proximal regulatory mechanisms of TRAIL-induced pro-apoptotic signalling, endosomal acidification is another factor that contributes to the efficacy and kinetics of TRAIL DISC-assisted processing and the activation of caspase-8.

Results

Inhibitors of vacuolar ATPase attenuate TRAIL-induced apoptotic signalling, independently of the mitochondrial pathway

To test the possible role of lysosomes and signalling from acidic compartments in TRAIL-induced apoptosis, we suppressed the fusion of late endosomes with lysosomes and the acidification of these organelles. For this purpose we used the V-ATPase inhibitors BafA1 and CCA, as well as the lysosomotropic drug chloroquine (CHQ), and upon blocking vesicular acidification we evaluated the effect of these inhibitors on TRAIL-induced signalling in several TRAIL-sensitive cancer cell lines.

Surprisingly, we found that BafA1 as well as CCA effectively attenuated the TRAIL-induced activation of caspases in TRAIL-sensitive colorectal cancer cell lines (Fig. 1a, c; Fig.

S1a). Similarly, though less efficiently, the activation of pro-apoptotic signalling was suppressed by CHQ (Fig. S1 and not shown). In Colo206F and DLD-1 cells, pre-treatment with BafA1 resulted in more than a 50% reduction in caspase-3 activity at 30 and 60 minutes after the addition of TRAIL (Fig. 1a). A similar V-ATPase inhibitors-mediated attenuation of TRAIL-triggered apoptotic signalling was also observed in other TRAIL-sensitive cell lines of non-colorectal origin such as human cervical carcinoma HeLa cells, hepatocellular carcinoma HUH-7 cells and prostate cancer PC3 cells (Fig. S1b and not shown). The suppressive effect of blocked endosomal acidification on the activation of TRAIL-induced apoptosis was evident not only in cells pre-incubated with BafA1, but also in those where BafA1 was added up to 20 min after their treatment with TRAIL (although with gradually reduced efficacy) (Fig. S2a).

However, these adverse effects of V-ATPase inhibitors were only transient and, for example in BafA1- or CCA-treated Colo206F cells, the number of M30-positive apoptotic cells gradually increased and almost reached the proportion seen in untreated cells within 3-4 hrs after the addition of TRAIL (Fig. 1b). This led us to suspect that mitochondrial amplification can likely blunt and gradually overcome the adverse effect of V-ATPase inhibitors. To test this hypothesis, we used Bax-deficient HCT-116 cells with uncoupled mitochondrial amplification of the pro-apoptotic signalling and found that blocking endosomal acidification led to an even stronger suppression of the TRAIL-induced activation of effector caspases than in the parental HCT-116 cells (Fig. 1c). Thus, these data suggest that endosomal acidification affects the TRAIL-induced activation of caspases upstream of the mitochondria.

BafA1 pre-treatment changes the trafficking of TRAIL-receptor complexes and slows down their accumulation in the intracellular vesicles

BafA1 changes endosomal pH and disorganizes the acidic compartment, which we confirmed in our initial experiments in BafA1-treated HCT-116 cells stained by LAMP3 antibody or LysoSensor, an acidotropic fluorescent probe that accumulates in organelles with low pH (Fig. 2). After 15 minutes of TRAIL treatment, some LysoSensor-labeled vesicles were clearly visible close to the nucleus. In contrast, in the cells pre-treated with BafA1, the acidic compartment recognized by LysoSensor disappeared or showed a featureless diffuse pattern upon TRAIL treatment (Fig. 2a). These data were also confirmed in cells double-stained with the lysosomal marker LAMP3 and fluorescent ligand TRAIL (Fig. 2b). BafA1 thus convincingly inhibited intra-vesicular acidification and also altered TRAIL intra-vesicular trafficking to the lysosomes.

To assess the effect of V-ATPase inhibition on the endosomal fusion and trafficking of TRAIL-receptor complexes, we performed a set of fluorescent microscopy assays. Initially, to obtain a precise idea about the timing of TRAIL trafficking to the lysosomes, we analyzed the association of TRAIL-receptor complexes with acidic organelles using lysosomal markers such as LysoTracker or LAMP-3 and fluorescently-labelled TRAIL. While in TRAIL-treated HCT-116 cells we could clearly discern the co-localization of TRAIL-containing endosomes with semi-cleaved caspase-8 (the antibody recognizes a site in the large subunit unmasked upon caspase-8 self-processing in the DISC) both at 20 and 60 minutes after adding TRAIL (Fig. 2c, upper panels labelled casp-8), we did not detect any co-localization of the lysosomal marker LAMP3 with TRAIL-containing endosomes even 60 minutes after treatment (Fig. 2c, lower panels labelled LAMP3). We also could not detect any co-localization of TRAIL receptosomes with the specific Golgi/trans-Golgi markers GM130 and Vti1b at any time point (not shown). TRAIL receptosomes then co-localized with lysosomes at a relatively late time point, 2 hours after recombinant TRAIL addition (not shown).

Thus, we continued with a more detailed kinetic analysis of TRAIL receptosome trafficking in the presence or absence of V-ATPase inhibitors together with nuclear staining to assess the progression of apoptosis. TRAIL-receptor complexes in control HCT-116 or DLD-1 cells treated with fluorescently-labelled TRAIL showed a relatively dispersed pattern within the first 15 minutes of treatment. However, within 30 min of stimulation, well before reaching the lysosomal compartment (see above), TRAIL receptosomes started to fuse and accumulate in vesicular structures close to the nucleus, likely representing late endosomes or multi-vesicular bodies (Fig. 3 and Suppl. Fig. S3 left panels). In BafA1-treated HCT-116 or CCA-treated DLD1 cells, this process was rather inefficient and was delayed by at least 30-45 min when compared to the non-treated cells (Fig. 3 and Fig. S3, right panels). In agreement with our data on delayed caspase-3 activity in cells with blocked endosomal acidification, we also noticed a lack of apoptosis-related chromatin condensation in CCA-treated DLD-1 cells incubated with TRAIL for 90 minutes (Fig. S3, lower panel). Furthermore, we stained HCT-116 cells with an antibody against another lysosomal marker, LAMP2, and confirmed that similarly as shown in Fig. 2c, lysosomes do not co-localize with TRAIL-containing endosomes within the first hour of TRAIL treatment. All of these results thus show that blocking endosomal acidification not only affects the activation of effector caspases and the processing of their target proteins, but also influences the localization and trafficking of the TRAIL-receptor complexes most markedly within the initial 60 minutes of TRAIL treatment.

To definitely exclude any role of lysosomes, we also used the siRNA down-regulated expression of small GTPase Rab7, which is required for the fusion of late endosomes with lysosomes (Fig. S4). The downregulation of Rab7 was previously shown to attenuate the TRAIL-induced apoptosis of hepatocarcinoma cells [26]. However, in contrast to suppressed endosomal acidification, the downregulation of Rab7 in DLD1 cells did not have any

inhibitory effect on TRAIL-induced apoptotic signalling as documented by the similar activation of caspases in the control cells and in cells with the down-regulated expression of Rab7 (Fig. S4a, b - PARP cleavage and M30 assay).

Acid sphingomyelinase and sphingolipids are not involved in the endosomal acidification-mediated enhancement of TRAIL-induced apoptotic signalling

Endosomal acidification is tightly connected with the activation of acid sphingomyelinase (ASM) and the subsequent increased production of ceramides (recently reviewed in [29]). Ceramides were reported to enhance the internalization and intracellular trafficking of the death receptors TNFα and CD95 as well as early events in TRAIL-induced apoptotic signalling. They were found to influence DISC formation and caspase-8 activation via the clustering of TRAIL's DISCs into ceramide-rich domains [30, 31]. Therefore, we assumed that blocking endosomal acidification and thus inhibiting the ASM-mediated production of ceramides could be at least in part responsible for the attenuation of TRAIL-induced apoptotic signalling. We evaluated whether and how could a reduction of V-ATPase activity affect cellular sphingolipid levels and compared these data with the effect of the ASM inhibitor desipramine.

Mass spectrometry analysis of the composition and levels of sphingolipids in BafA1- or desipramine-treated Colo206F cells revealed that both agents indeed caused about a 20% drop in ceramide levels (C-16 and C-24) accompanied by a very pronounced decrease in sphingosine and sphingosine phosphate levels (Fig. 4a). However, in contrast to the V-ATPase inhibitors, blocking ASM activity with desipramine caused only a subtle reduction of TRAIL-induced apoptotic signalling in Colo206F cells (Fig. 4b). Moreover, desipramine even suppressed the inhibitory effect of BafA1 on TRAIL-induced apoptotic signalling in these cells (Fig. 4b).

Supplementing some TRAIL-resistant cells with external ceramides caused their sensitization to TRAIL-induced apoptosis [32]. Therefore, we tested whether the addition of external C18-ceramide or C18-sphingosine could reverse or in any way affect the suppression of TRAIL-induced apoptotic signalling caused by blocked endosomal acidification. However, neither of these agents had any effect on this V-ATPase inhibitor-mediated attenuation of TRAIL-induced apoptotic signalling (Fig. S5 and not shown).

An altered acidic environment affects the DISC-mediated processing of the initiator caspase-8

The fast suppression of TRAIL-induced effector caspase activation and impaired vesicular formation after the inhibition of endosomal acidification prompted us to analyse the very early events in TRAIL-induced signalling. First, we measured the expression levels of the death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 on the plasma membrane and found that they were almost unaffected by BafA1 pre-treatment in any of the tested colorectal cancer cells within the first 2 hrs of BafA1 treatment (Fig. 5a and Fig. S6a). Internalization of these pro-apoptotic receptors after ligand binding also showed unchanged kinetics (Fig. 5b and Fig. S6b).

Events immediately following TRAIL receptor engagement include the formation of the DISC complex, the activation of caspases and cleavage of their substrates. At the same time, signalling pathways leading to the activation of MAP kinases and NF-κB are also triggered. Biochemical analysis of cell lysates from TRAIL-treated Colo206F or DLD-1 cells with suppressed endosomal acidification documented a reduced processing of initiator and effector caspases (-8, -9, -3) as well as the less effective cleavage of their substrates cFLIP and PARP compared to control cells in which V-ATPase was not inhibited (Fig. 6a and Fig. S7a). Using an antibody against cleaved caspase-8 in flow cytometry experiments, we also confirmed that

the inhibition of endosomal acidification led to a reproducible and significant decrease of the cellular fraction with the self-cleaved proximal initiator caspase-8 in the culture (Fig. S2b).

In contrast to the less efficient activation of caspase-8, we did not observe any major changes in the activation of NF-κB signalling (phoshorylation of p65 subunit) or the activation of ERK or JNK kinases in TRAIL-treated Colo206F or HCT-116 cells (Fig. 6b and Fig. S7b), suggesting that V-ATPases are selectively more involved in the caspase activation branch of the TRAIL receptor signalling. The only reproducibly observable difference was related to the kinetics of activation of p38 MAPK in cells pre-treated with V-ATPase inhibitors, where the TRAIL-induced phosphorylation (activation) of p38 was slightly delayed (Fig. 6b and Fig. S7b). The TRAIL-mediated induction of p38 phosphorylation is caspase-dependent [33], and thus its delayed activation correlates with the attenuated activation of caspases.

Next, we analysed the composition of DISC and the kinetics of DISC-associated initiator caspase processing and activation. In TRAIL-treated Colo206F cells, the proportions of major DISC proteins co-isolated with receptor-bound TRAIL, i.e., TRAIL-R2/DR5, FADD, cFLIP and pro-caspases-8/10, were not markedly different between untreated and CCA-treated cells (Fig. 7). However, the processing of both initiator caspases (-8, -10) in the TRAIL receptor DISC was attenuated after CCA-mediated V-ATPase inhibition.

Downregulation of cFLIP largely negates the inhibitory effects of V-ATPase inhibitors on TRAIL-induced apoptotic signalling

The caspase-8 inhibitor cFLIP plays an essential role in the regulation of DISC-mediated caspase-8 activation. Thus, we decided to examine whether lowering cFLIP levels in our model colorectal cancer cell lines would alleviate the effects of the acidification inhibitors. Using two different shRNAs, we down-regulated the expression of cFLIP in both the DLD-1 and Colo206F cell lines (Fig. 8a and not shown) and analysed the sensitivity of TRAIL

signalling in these cells to BafA1. As expected, downregulation of cFLIP enhanced the kinetics and efficacy of TRAIL-induced prime caspase activation in the mock-transfected cells. However, it also significantly reduced the inhibitory effect of V-ATPase inhibitors in these cells. Under these conditions, BafA1 pre-treatment was unable to slow down the induction of apoptosis in cells with reduced cFLIP protein expression (Fig. 8b).

Discussion

The basic mechanism of the initial phases of activation of TRAIL-induced apoptosis is known, but the role of some processes, such as endocytosis or endosomal trafficking, in fine tuning TRAIL-induced apoptosis is still nor fully understood. This part of TRAIL-induced signalling is also the subject of some controversy as Kohlhaas et al. showed that the endocytosis of activated TRAIL receptors in BJAB or HeLa cells is apparently not required for the efficient processing and activation of caspase-8 [25]. In contrast, recent reports from Gores's group point to endocytosis-requiring lysosomal membrane permeabilization (LMP) as a potent enhancer of the TRAIL-induced apoptosis of hepatocellular carcinoma cells [26, 34]. However, the participation of lysosomes in apoptosis in general and in DR-mediated apoptosis in particular is quite controversial and might play just a supportive role, not in the initial but in the late stage of apoptosis [35, 36]. Moreover, a recent study from B. Turk's group using various FasL- or TRAIL-sensitive cancer cells documented that cathepsin inhibitors neither block nor delay FasL- or TRAIL-induced apoptosis [37]. In our model colorectal cancer cells, blocking the fusion of late endosomes by siRNA-mediated downregulation of Rab7 or by using the pan-cathepsin inhibitor E64 (not shown) did not, in contrast to V-ATPase inhibitors, have any significant effect on the kinetics of TRAIL-induced apoptotic signaling. Thus, we believe that at least in the initial stages of TRAIL-induced apoptosis of colorectal cancer cells, lysosomes or lysosomal proteases do not play any essential role. Confocal microscopy studies also failed to detect any co-localization of

TRAIL or the components of DISC with lysosomal markers within a similar time frame, suggesting that vesicles containing TRAIL-complexes do not fuse with lysosomes during the first hour of TRAIL treatment. However, in contrast to blocking lysosomal proteases, we found that we could significantly and reproducibly attenuate TRAIL-induced activation using well-known vacuolar ATPase inhibitors and following suppressed endosomal acidification. The most pronounced suppression of the pro-apoptotic signaling in all tested cancer cells was observed within the first hour of TRAIL treatment. This led us to the assumption that V-ATPase/endosomal acidification enhances early TRAIL-mediated signalling by a mechanism independent of LMP. However, LMP can still play a role at later stages. We cannot, however, exclude that it has a more significant effect in other cell types.

In order to dissect the potential mechanism behind the V-ATPase inhibitor-mediated attenuation of TRAIL-induced apoptotic signalling, we used Bax-knockout HCT-116 colon cancer cells that are deficient in the mitochondrial amplification of TRAIL-induced apoptosis. Interestingly, in these cells the V-ATPase inhibitor-mediated attenuation of TRAIL-induced apoptotic signalling was not only more pronounced, but also more extended, confirming our suspicion that in cells with intact mitochondria-mediated apoptotic signalling, the mitochondrial amplification loop overcomes the attenuation of caspase activation imposed by V-ATPase inhibitors in a time-dependent manner.

Endosomal acidification is also required for the activation of ceramide-producing acid sphingomyelinase (ASM), which has been previously shown to participate in DR-mediated apoptotic signalling. For TNFR1-mediated apoptotic signalling, activated caspase-8 and then caspase-7 were responsible for the cleavage-mediated activation of ASM, followed by an increased production of apoptosis-enhancing ceramides [23]. In Fas-induced apoptosis of rat hepatocytes, caspase-8-assisted endosomal acidification and the subsequent ASM/ceramide-mediated activation of ROS production were also required for the amplification of the

apoptotic signalling [24]. TRAIL can similarly trigger the ASM-mediated production of ceramides [30], and it has been published that TRAIL-induced apoptosis was enhanced by ceramides in TRAIL-resistant colorectal carcinoma cells, doxorubicine-treated leukaemia cells and arterial endothelial cells [32, 38-40]. However, our examination of the potential connections between blocking endosomal acidification and the activation/activity of ASM in TRAIL-induced apoptotic signalling did not point to any role of ASM in the suppressive effect of V-ATPase inhibitors. The ASM inhibitor desipramine, in contrast to V-ATPase inhibitors, had only a negligible effect on early TRAIL-induced apoptotic signalling. Also, the V-ATPase inhibitor-mediated attenuation of TRAIL-induced apoptosis was not enhanced by the concomitant inhibition of ASM. Moreover, supplementing BafA1- or CCA-treated cells with external C6 ceramide or C18 sphingosine could not reverse the inhibitory effect of blocked endosomal acidification on TRAIL-induced apoptotic signalling. These results suggest that ASM, at least in our model cell lines, is not involved in the V-ATPase-mediated enhancement of the early stages of TRAIL receptor signalling.

As the V-ATPase inhibitor-mediated early attenuation of TRAIL-induced apoptotic signalling is not related to the possible suppression of lysosomal membrane permeabilization or the activity of ASM and the concentration/ratio of spingolipids, and is blunted by concurrent mitochondrial amplification of apoptotic signalling, we focused on the mechanisms and processes related to the activation of the main initiator caspase-8 – the assembly and activation of the DISC. Surprisingly, V-ATPase inhibitors did not affect the basic composition of the DISC, but we reproducibly observed the attenuation of caspase-8 processing in TRAIL-treated cells with blocked endosomal acidification. Recent reports from the MacFarlane and Lavrik laboratories claim that the efficient activation of caspase-8 at the TRAIL of FasL DISCs is accompanied by their higher order clustering and point to the delicateness of fine tuning the cFLIP:caspase-8 ratio for the regulation of caspase-8 activation

[41, 42]. In cells treated with V-ATPase inhibitors, we observed a significant delay in the formation of aggregated, TRAIL-containing structures resembling multi-vesicular bodies. The rapid formation of these structures could thus enhance the clustering of the TRAIL receptors and subsequently the DISC-mediated activation of caspase-8. These processes obviously require fine regulation, and as we also observed, the simple changing of levels or the downregulation of any of these regulators, such as cFLIP, can dramatically shift the balance towards apoptosis even in cells with suppressed endosomal acidification. Additional factors such as the caspase-8 stability-regulating ubiquitin E3 ligases cullin3 and TRAF2 or some other as-yet unidentified proteins could play a role (and be affected by the endosomal acidification-enhanced clustering) in this process, ultimately related to the kinetics and robustness of caspase-8 activation [13, 43]. Endosomal acidification could also affect posttranslational modifications of caspase-8 such as its inhibitory phosphorylation by src, p38 MAPK or RSK2 [44-46]. Obviously, further studies are required to fully address this issue. Interestingly, V-ATPase inhibition has no effect on the TRAIL-induced activation of NF-κB, suggesting that its function is restricted only to the pro-apoptotic part of TRAIL signalling. The vacuolar proton pump can therefore play a role in shifting the balance in favour of cell death instead of cell survival under normal conditions.

Numerous proximal regulatory mechanisms of TRAIL-induced pro-apoptotic signalling have already been established. They include the level of cell surface expression of pro- and anti-apoptotic TRAIL receptors, their posttranslational modification, the clustering of activated TRAIL receptors in ceramide-rich membrane domains, and the presence and posttranslational modification of the intracellular DISC components FADD, caspase-8 or cFLIP. Here we show that V-ATPase activity and endosomal acidification are other factors that contribute to the efficacy of TRAIL pro-apoptotic signalling, and for the first time we

demonstrate the impact of V-ATPase activity on DISC formation and the processing and activation of initiator caspases.

Materials and methods

Cell lines, chemicals and antibodies

HCT-116, DLD-1, Colo206F and HeLa cells were purchased from ATCC-LGC Promochem, HUH-7 (JCRB0403) from JCRB Cell Bank and human immortalized keratinocytes NCTC were provided by Dr. L. LoMuzio. Cells were cultivated in DMEM (HCT-116, HeLa), IMDM (HUH-7, NCTC) or RPMI (DLD-1, Colo206F) medium supplemented with 10% FCS and antibiotics.

Human recombinant TRAIL was provided by Apronex (Vestec,, Czech Republic), desipramine by Enzo LS (Farmingdale, New York, USA) and bafilomycin A1, concanamycin and chloroquine by Sigma-Aldrich (St. Louis, Missouri, USA). C18 sphingosine was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and C6 and C18 ceramides from Enzo LS. The following antibodies were used for Western blotting: caspase-3 (mouse, 31A1067, Enzo LS), cleaved caspase-3 (rabbit, 9661, Cell Signaling, Danvers, Massachusetts, USA), caspase-8 (rabbit, 559932, BD Pharmigen, Franklin Lakes, New Jersey, USA; mouse, 12F5, ENZO LS; mouse 9746, Cell Signaling), caspase-9 (rabbit, 9602, Cell Signalling), cleaved caspase-9 (rabbit, 9505, Cell Signaling), caspase-10 (mouse, 4C1, MBL, Woburn, Massachusetts), DR5 (rabbit, D3938, Sigma-Aldrich), FADD (rabbit, 610399, BD Transduction Laboratories), PARP (rabbit, 7150, Santa Cruz Biotechnology, Dallas, Texas, USA), cFLIP (mouse, NF-6, Enzo LS), XIAP (mouse, 610762, BD Transduction Laboratories), P-NF-κB p65 (rabbit, 3033, Cell Signalling), NF-κB p65 (rabbit, 7151, Santa Cruz Biotechnology), P-SAPK/JNK (rabbit, 9251S, Cell Signalling), P-p44/42 MAPK (Erk1/2) (rabbit, 4370, Cell Signalling), p44/42 MAPK (Erk1/2) (rabbit, 4695, Cell

Signalling), P-p38 MAP Kinase (rabbit, 9211, Cell Signalling), p38 MAP Kinase (rabbit, 9212, Cell Signalling) and actin (goat, C-11, Santa Cruz Biotechnology); for immunocytochemical analysis: LAMP3 (mouse, prepared in Dr. Horejsi's lab), LAMP2 (mouse, H4B4, Exbio, Vestec, Czech Republic), cleaved caspase-8 (rabbit, 18C8, Cell Signalling) and fluorochrome conjugated goat anti-mouse IgG and anti-rabbit IgG secondary antibodies (Molecular Probes, Life Technologies, Carlsbad, California, USA); and for flow cytometry: M30-Cytodeath FITC (mouse, ALX-804-590F, Enzo LS), DR4 (mouse, DR-4-02, Exbio), DR5 (mouse, DR5-01-1, Exbio), Fas (mouse, DX2, BD Transduction Laboratories) and cleaved caspase-8 (rabbit, 18C8, Cell Signalling).

Cell lysis, Western blotting and DISC immunoprecipitation

Cells (either in the cultivation vessels or pellets of trypsinized cells) were directly lysed in SDS sample buffer; the lysates were cleared by filtering through filter tips to remove DNA and then boiled for 2 minutes. Clarified lysates corresponding to an equal number of cells (usually 40-60 μg of total protein) were run on SDS-polyacrylamide gels, blotted to nitrocellulose membranes, and then analysed by immunodetection using the appropriate antibodies.

For DISC precipitation, cells in ice-cold cultivation medium were incubated with biotinylated TRAIL (1 μg/ml) on ice for 15 minutes, then the medium was exchanged for warm medium (37°C) and the cells were incubated for defined time periods. At the relevant time points the cells were scraped into ice-cold PBS, washed with PBS and lysed in ice-cold 1% NP40 lysis buffer (1% NP40; 20 mM Tris; 150 mM NaCl; 10% glycerol; 10 mM EDTA; 1x Complete protease inhibitor cocktail, Roche, Penzberg, Germany; pH 7.5) on ice for 30 minutes. Nuclei and cell debris were removed by centrifugation at 16,000 x g for 30 minutes; streptavidin-agarose beads (Pierce, Thermo Scientific, Rockford, Illinois, USA) were added

to the supernatant, and the samples were incubated with rotation at 4 °C for 1 hr. The bead-bound DISC complexes were washed five-times with lysis buffer and finally released by adding 100 µl of SDS sample buffer directly to the beads and heating at 95°C for 5 minutes. The agarose bead slurry was centrifuge-filtered through filter tips, and the samples were analysed by Western blotting.

Flow cytometry-based assays

The M30-FITC-based assay for quantifying effector caspase activity (cleavage of cytokeratin 18) was carried out according to the manufacturer's recommendations (ENZO LS). Briefly, at different time points after incubation, TRAIL cells were detached by trypsinization, washed with PBS and fixed in methanol at -20°C for 30 minutes. Then they were rinsed twice with ice-cold PBS containing 0.5% BSA and incubated with M30-FITC antibody (1 µg/ml) for 1 hr at room temperature. The cells were then washed twice with PBS and analyzed using a LSRII flow cytometer (BD Biosciences). Data were evaluated using FlowJo software (TreeStar, San Carlos, California, USA).

The determination of the cell surface expression of death receptors: adherent cells were released by treatment with PBS containing 10 mM EDTA and, after washing with ice-cold PBS, were incubated in blocking solution (PBS with 20% human serum, 1% gelatin and 0.1% azide) on ice for 15 min. Cells were then washed once with wash buffer (PBS containing 1% gelatine and 0.1% azide), incubated with the appropriate primary monoclonal antibodies, washed twice with ice-cold wash buffer and finally incubated with the secondary goat anti-mouse antibody coupled to phycoerythrin (IgG1-PE) (SouthernBiotech, Birmingham, Alabama, USA). All incubations were performed on ice. After two final washes, the surface expression of the receptors on living cells (Hoechst 33258 negative) was analysed.

For the quantification of cleaved caspase-8 (p43/p41 and p18 fragments), we followed the protocol from the manufacturer (Cell Signalling). In brief, TRAIL-treated cells were harvested by trypsinisation, washed and fixed in 3% formaldehyde at 37°C for 10 minutes. Ice-cold methanol was added to a final concentration of 90%, and the cells were incubated on ice for 30 min. After two washings with PBS, non-specific interactions were blocked by a 10 min incubation in blocking/staining buffer (0.5% bovine serum albumin in 1xPBS), then the cells were incubated with a rabbit monoclonal antibody against cleaved caspase-8 (Asp391) (18C8) for 30 min followed by incubation with Alexa 488-conjugated goat anti-rabbit IgG. Samples were finally diluted in PBS and analysed by flow cytometry.

Confocal microscopy

Cells grown on cover slips were washed with PBS and fixed with 3% paraformaldehyde in PBS at room temperature for 15 min, then permeabilized for 5 min in 0.3% Triton X-100/PBS and finally rinsed twice with PBS. Non-specific binding was blocked by 1% BSA/PBS, followed by 15 min incubation with the primary antibodies, diluted to 2 μg/ml in 1% BSA. After three washes with PBS, the slides were incubated with the appropriate fluorescently-labeled secondary antibody in the dark for 30 minutes. The secondary antibodies – Alexa 488- and 594-conjugated anti-rabbit IgG and Alexa 488- and 594-conjugated anti-mouse IgG – were supplied by Molecular Probes and diluted to 5 μg/ml. The slides were then washed twice with PBS, and DAPI (1.25 μM in PBS) was added to the third wash. Finally, after the last wash the slides were mounted in ProLong Gold anti-fade reagent (Invitrogen, Life Technologies, Carlsbad, California, USA).

Lysosensor (LysoSensor™ Green DND-189, Invitrogen) was used according to the manufacturer's recommendations. LysoSensor-treated cells were visualized alive. Alexa 647-labeled TRAIL was added directly to the cells and incubated at 37°C according to

experimental needs. Cells were mounted in ProLong Gold antifade reagent (Invitrogen), and the cell membrane-permeable DNA dye Hoechst 33540 (Invitrogen) was then used for DNA staining. Cells mounted on slides were analyzed by confocal microscopy using a Leica TCS SP5.

Quantification of sphingolipids by mass spectrometry

A cell pellet was transferred from an Eppendorf tube into a degreased glass tube (13 x 100 mm) using 1.5 ml of methanol. After adding 0.75 ml of chloroform, the contents were dispersed by using a bath sonicator for 5 min. at room temperature. The extraction process continued in a heating block at 48°C overnight. After cooling, 80 µl of 1 M KOH in methanol was added and, after brief sonication, the extract was incubated in a water bath for 2 h at 37°C. After cooling and sonication, 8 µl of glacial acetic acid was added to bring the extract to neutral pH. This slight hydrolysis removed most of the glycero- and phosphor-lipids that can interfere with or mask a low abundance of the sphingolipids of interest during mass spectrometric analysis (sphingolipids remain unaffected by this hydrolysis). The solvents were removed by nitrogen drying at room temperature, and the residue was reconstituted by 300 µl of a 3:1 methanol/chloroform mixture. An appropriately diluted aliquot of the cell extract was prepared using mobile phase solvents RA/RB 60/40 for HPLC-tandem MS analysis.

Sphingolipids of interest were separated by reverse-phase HPLC and detected by tandem mass spectrometry. The HPLC conditions were as follows: DEVELOSIL C30 column 150x2.1 mm, flow rate 0.7 ml/min., gradient mobile phases RA/RB (RB=methanol, RA= methanol/water 60/40), starting ratio RA/RB 60/40, total time 42 min, liquid chromatograph Agilent 1200 (Agilent Technologies, Santa Clara, California, USA). The tandem mass spectrometry (tandem MS on a TripleQuand 6410 mass spectrometer with ESI,

Agilent) conditions were as follows: ESI in positive mode, drying nitrogen flow 10 lt/min, fragmentor voltage and collision energy voltage optimized for each sphingolipid multiple reaction monitoring (MRM) scan mode. The amount of each sphingolipid species in the cell extract was based on the corresponding chromatographic peak area (using the same volume of cell extract and analysed aliquot for all samples). The following chemicals were used: methanol (HPLC grade, Avantor, Central Valey, Pennsilvania, USA), chloroform, formic acid, ammonium formate, acetic acid and potassium hydroxide (analytical grade, MS grade, Sigma-Aldrich).

SiRNA- or shRNA-mediated downregulation of gene expression

SiRNA targeting Rab7 (siGENOME MQ-010388-00-0002 – pool of four different siRNAs) and the non-targeting control siRNA (AGGUCGAACUACGGGUCAA) were purchased from Dharmacon (Thermo Scientific). DLD-1 cells were transfected with these siRNAs at final 100 nM concentration using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. Two days after transfection, cells were analysed for Rab7 expression and used in the experiments.

Recombinant pLKO1 lentoviruses expressing shRNAs for cFLIP were purchased from Sigma-Aldrich (see Suppl. Table 2). Two days after transduction, mixed cultures of DLD1 or Colo206F cells expressing the corresponding shRNA were selected for 4 days using medium containing puromycin (Enzo LS) at a concentration of 3 μg/ml. Out of five tested shRNAs, the two best performing ones (80-90% downregulation of mRNA expression) were chosen for further experiments. Mock-transduced cells were prepared in the same way using a non-silencing pLKO1 lentivirus.

Statistical analysis

Data where applicable are presented as means ± sd (standard deviation) for the given number (n) of independent experiments. Student's unpaired *t*-test or one-way ANOVA for multiple comparisons were used to determine significant differences between the experimental groups. Values of *p<0.05 were considered as significant, **p<0.01 as very significant and *** p<0.001 as extremely significant.

Acknowledgements

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Titles of Supplementary material

Supplementary Figure S1

V-ATPase inhibitors or chloroquine suppress to various extent TRAIL-induced apoptotic signalling in a number of human cancer cell lines

Supplementary Figure S2

The inhibitory effect of bafilomycin A1 is fast and affects the proximal TRAIL-induced activation of caspase-8.

Supplementary Figure S3

Effect of concanamycin A on the distribution and trafficking of TRAIL-containing endosomes in DLD-1 cells.

Supplementary Figure S4

Downregulation of Rab7 GTPase has no effect on the activation of proximal TRAIL-induced apoptotic signalling.

Supplementary Figure S5

C18 sphingosine did not reverse the BafA1-mediated suppression of TRAIL-induced apoptotic signalling.

Supplementary Figure S6

Bafilomycin A1 treatment does not alter the cell surface expression of death receptors or the kinetics of their TRAIL-induced internalization in DLD-1 and Colo206F cells.

Supplementary Figure S7

Effect of suppressed endosomal acidification on the cleavage/processing of several apoptosis-related proteins and TRAIL-induced pro-survival signalling in DLD-1 and Colo206F cells.

Supplementary Table S1

Sequences of short inhibitory RNAs in the shRNA-expressing lentiviral pLKO1-based vectors used for downregulation of cFLIP in DLD-1 cells.

Figure 1

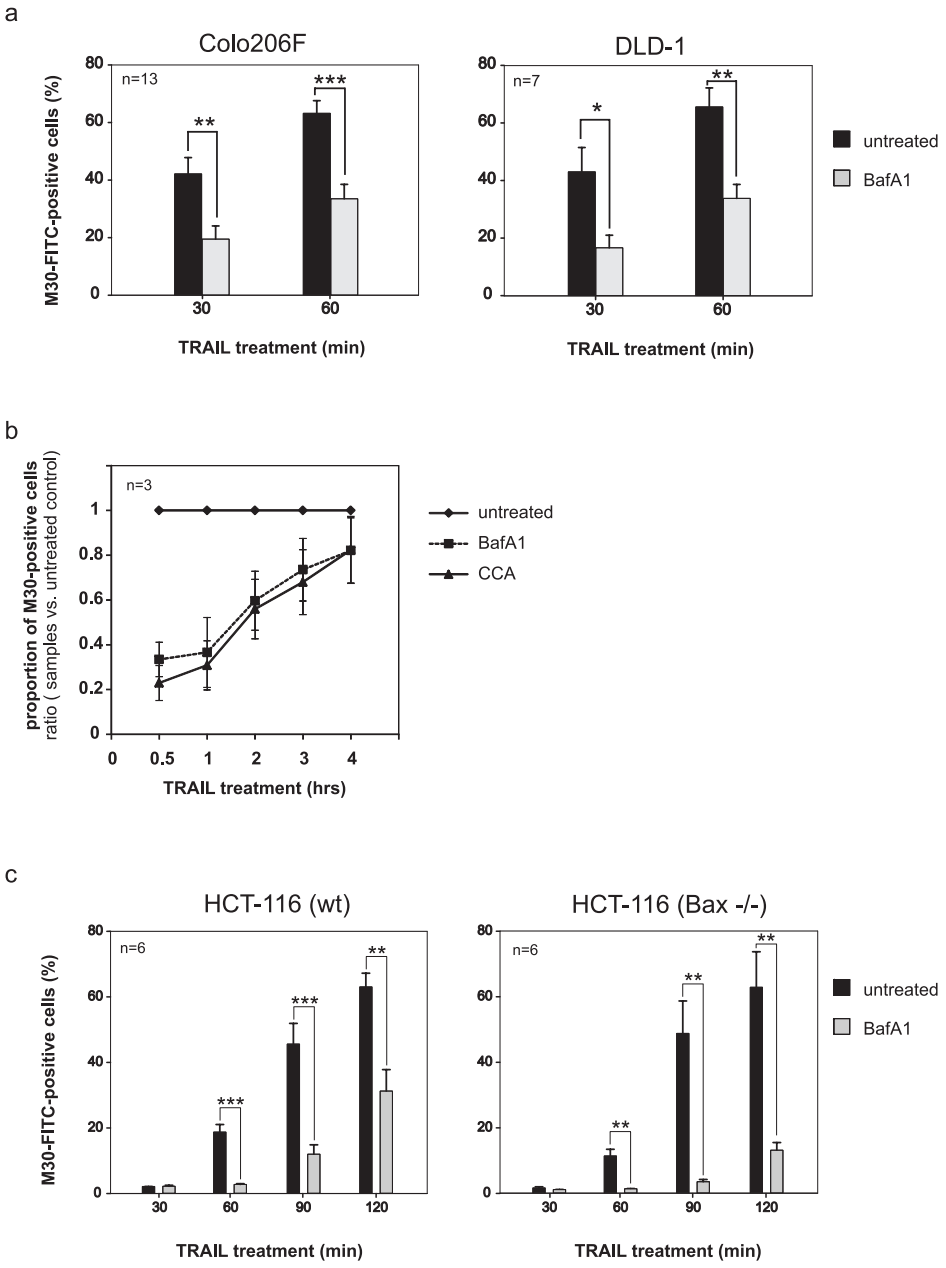


Figure 1

Inhibition of V-ATPase significantly but transiently suppresses TRAIL-triggered, mitochondrial amplification-independent activation of caspases

a) Colo206F cells and DLD-1, either untreated or pre-treated for 1 hour with bafilomycin A1 (BafA1, 20 nM), were incubated with recombinant TRAIL (200 ng/ml) for 30 or 60 min, and the fraction of cells with the cleaved substrate of activated caspases - cytokeratin 18 (CK18) - was quantified by M30-FITC staining and flow cytometry. The number of independent experiments (n), standard deviations and statistical significances are shown (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.005$).

b) Colo206F cells were pre-incubated with inhibitors of vesicular acidification (20 nM final concentrations) for 1 hr and then treated with TRAIL (200ng/ml) for up to 4 hrs. The data show the relative activities of caspases (M30-FITC-positive cells) at distinct time points in cells pre-treated with inhibitors compared to the activities in control cells (treated with TRAIL but without V-ATPase inhibitor). Data with standard deviations represent a set of three independent experiments.

c) HCT-116^{wt} and HCT-116$^{Bax-/-}$ cells, either untreated or pre-treated for 1 hour with bafilomycin A1 (BafA1, 20 nM), were incubated with recombinant TRAIL (200 ng/ml) for the indicated time periods, and the fraction of cells with activated caspases was quantified by M30-FITC staining and flow cytometry. The number of independent experiments (n) and statistical significances are shown (** = $P \leq 0.01$, *** = $P \leq 0.005$).

Figure 2

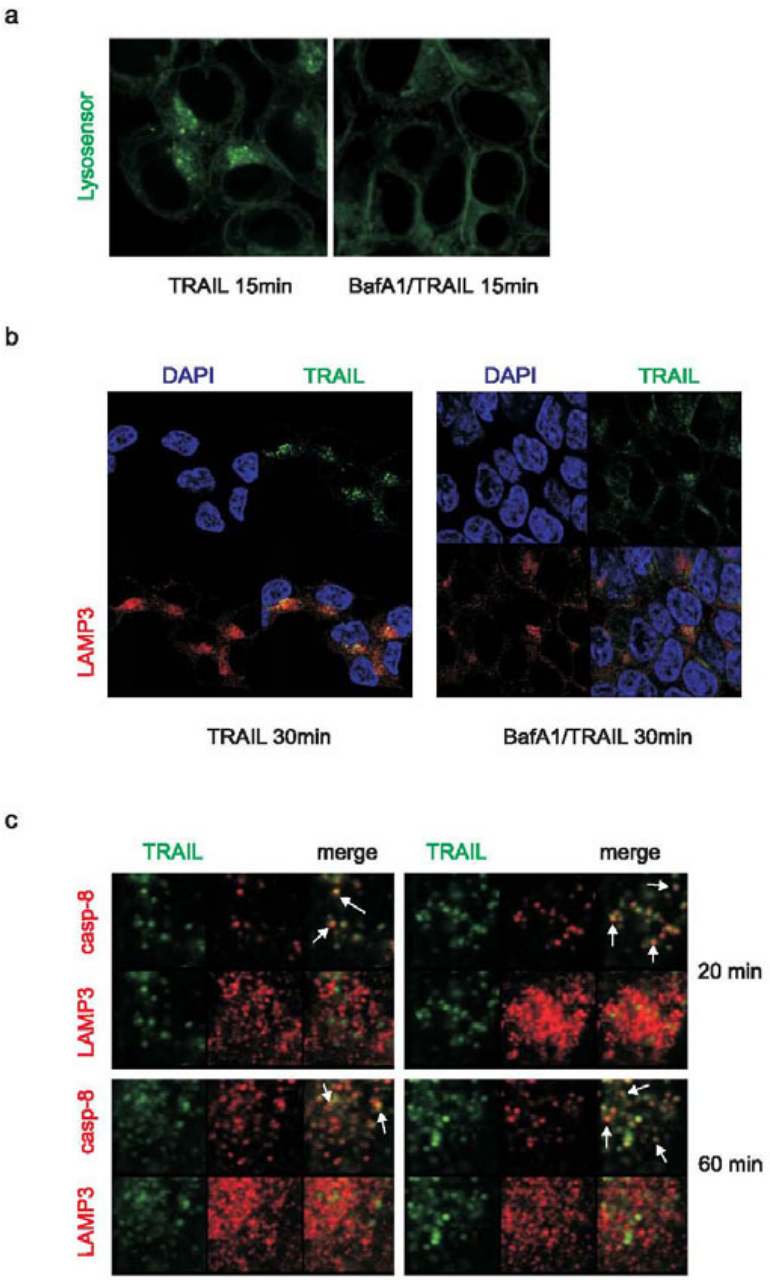


Figure 2

Pattern and distribution of lysosomes and TRAIL-containing receptosomes is affected by the inhibition of V-ATPase in a time-dependent manner.

a) HCT-116 cells were incubated for 30 minutes with LysoSensor DND157 (10 µg/ml), then for an additional 15 minutes with TRAIL (200 ng/ml) alone or in combination with bafilomycin A1 (BafA1, 20 nM), and the acidic compartments were visualized by confocal microscopy.

b) HCT-116 cells were grown on cover glasses and incubated with fluorescent Alexa 647-labeled TRAIL (1 µg/ml) for 30 min alone or in combination with bafilomycin A1 (BafA1, 20 nM). The cells were fixed in 3% formaldehyde and permeabilizated by 90% ice-cold methanol. The lysosomal marker LAMP3 was immune-stained and its cellular localization was visualized by confocal microscopy using a Leica SP5.

c) HCT-116 cells were grown on cover glasses and incubated with fluorescent Alexa 647-labeled TRAIL (1 µg/ml) for 20 or 60 min. The cells were fixed in 3% formaldehyde and permeabilizated by 90% ice-cold methanol. Cleaved caspase-8 (Asp391) and the lysosomal marker LAMP3 were immune-stained and their cellular localization was analysed by confocal microscopy using a Leica SP5. Two representative images for each duration of TRAIL treatment are shown.

Figure 3

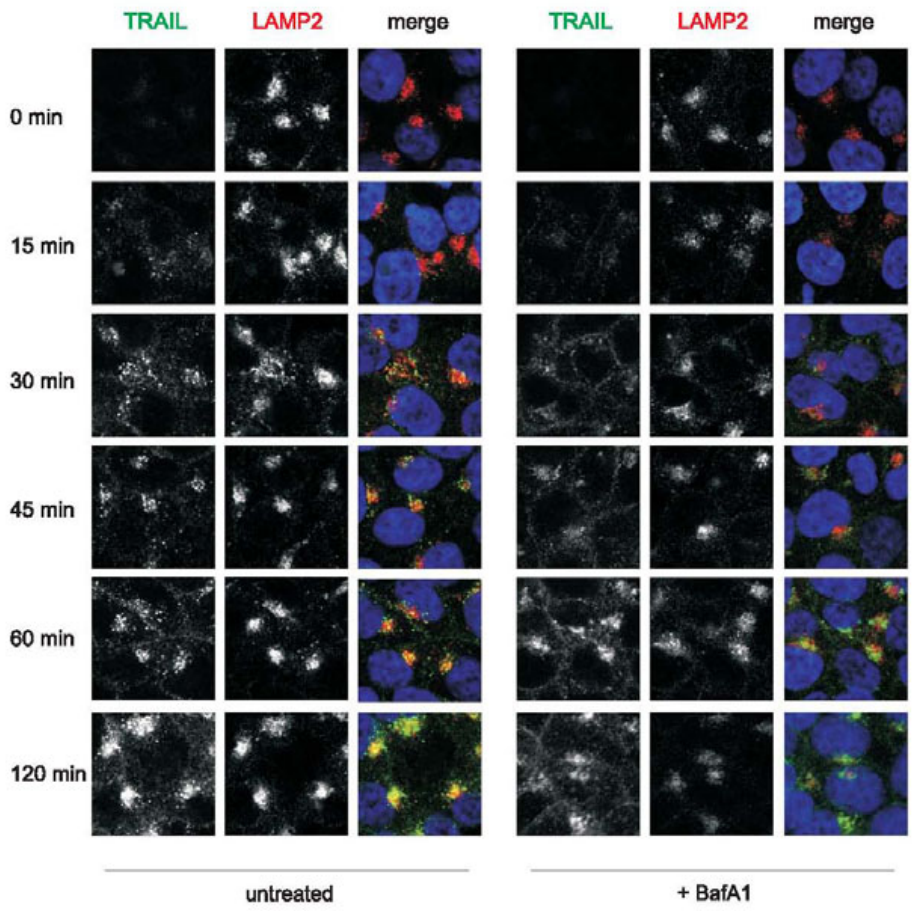


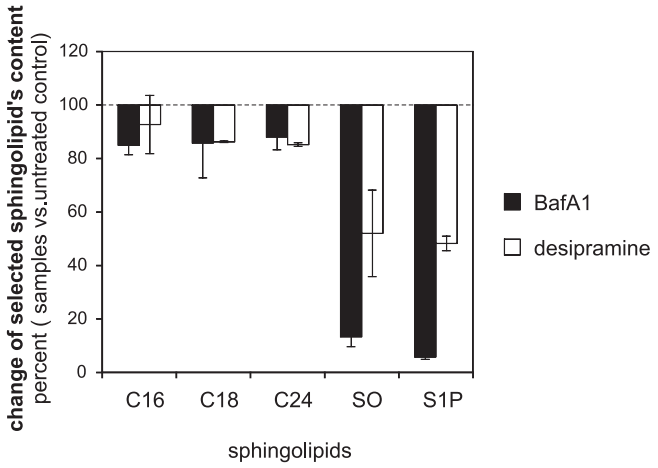
Figure 3

The blocking of endosomal acidification changes the pattern and kinetics of TRAIL-receptor complex trafficking within the first hour of the treatment..

HCT-116 cells grown on cover glasses were either untreated or pre-treated with bafilomycin A1 (BafA1, 20 nM), then incubated with fluorescent Alexa 647-conjugated TRAIL (1 µg/ml, green colour) for 0 to 120 min. Cells were fixed in 3% paraformaldehyde, permeabilizated by 0.1% Triton X, stained with an antibody against lysosomal protein LAMP2 (red signal) and the nuclei visualized by Hoechst 33540 (blue signal). Confocal microscopy was performed using a Leica SP5 microscope. The data shown are representative of two independent experiments.

Figure 4

a



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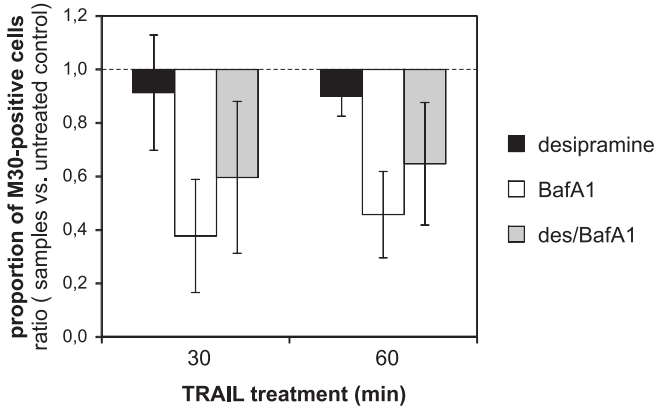


Figure 4

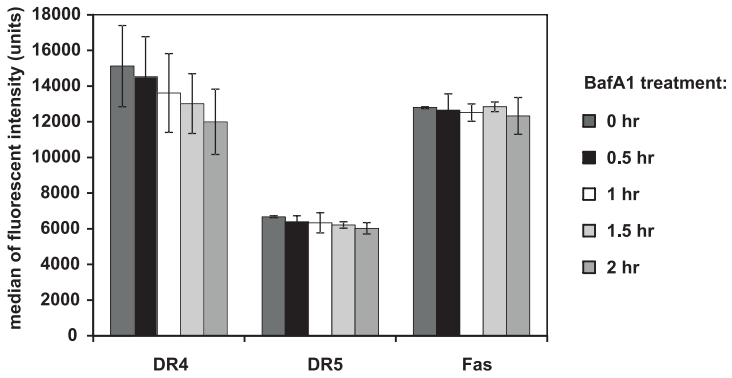
Inhibition of V-ATPase or ASM leads to a drop in the levels of cellular sphingolipids and variably affects the TRAIL-induced activation of caspases.

a) Colo206F cells were either untreated or treated with bafilomycin A1 (BafA1, 20 nM) or desipramine (20 uM) for 1 hr. Cells were trypsinized and counted, then equal numbers of cells per sample were subjected to HPLC-MS analysis of cellular sphingolipids. The relative levels of sphingolipids in treated vs. untreated cells are shown. C16 – ceramide 16, C18 – ceramide 18, C24 – ceramide 24, SO – C18 sphingosine, S1P – C18 sphingosine-1-phosphate. The data represent the means and standard deviations of four experiments.

b) Colo206F cells were either left untreated or pre-treated with bafilomycin A1 (BafA1, 20 nM), desipramine (20 uM) or their combination for 1hr. The cells were then incubated with recombinant TRAIL (200 ng/ml) for 30 or 60 min, and the fraction of cells with TRAIL-activated caspases was quantified by M30-FITC staining and flow cytometry. The relative ratio of cells with cleaved CK18 in pre-treated cells vs. non-pre-treated cells is shown. The data show averages means and standard deviations from three independent experiments.

Figure 5

a



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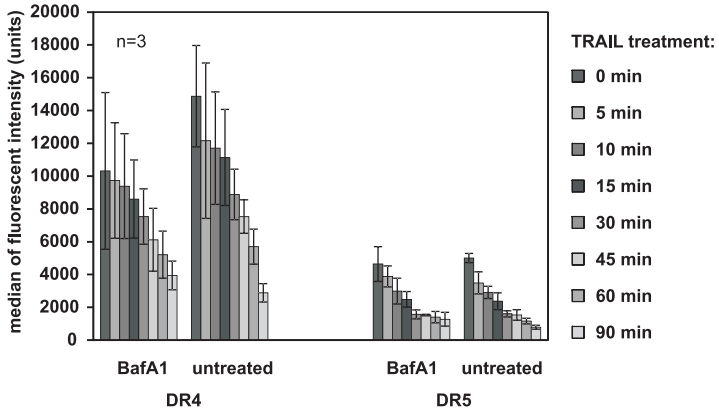


Figure 5

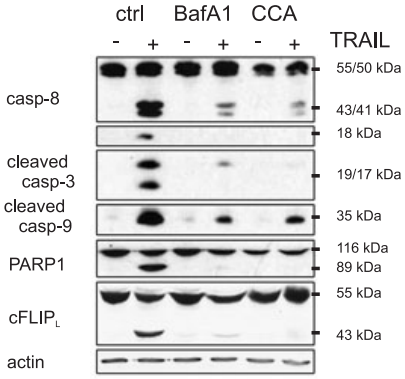
BafA1 treatment does not alter the cell surface expression of death receptors or the kinetics of their TRAIL-induced internalization.

a) HCT-116 cells were treated with bafilomycin A1 (BafA1, 20nM) for the indicated time periods, and the surface expression of the death receptors DR4, DR5 and Fas was determined by antibody staining and flow cytometry. The medians and standard deviations from three independent experiments are shown.

b) TRAIL was added to control (untreated) and bafilomycin A1- (BafA1, 20 nM) pre-treated HCT-116 cells, and at the indicated time points after TRAIL addition, the cell surface expression of the TRAIL receptors DR4 and DR5 was analyzed by antibody staining and flow cytometry. Medians and standard deviations from three independent experiments are presented.

Figure 6

a



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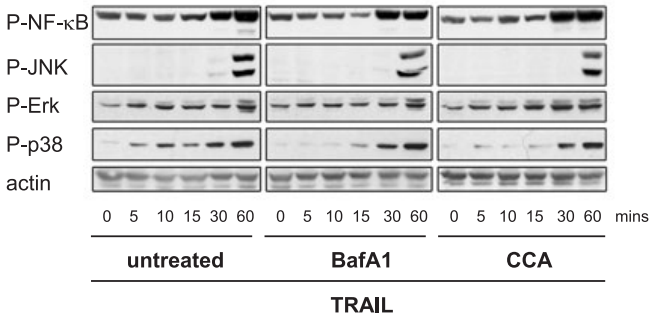


Figure 6

V-ATPase inhibitors suppress the cleavage/processing of several apoptosis-related proteins but do not affect TRAIL-induced pro-survival signalling.

a) Colo206F cells were either untreated (ctrl) or pre-treated with bafilomycin A1 (BafA1, 20 nM) or concanamycin A (CCA, 20nM) and incubated with recombinant TRAIL (200 ng/ml) for 60 minutes. The cells were then lysed in SDS lysis buffer, and the expression and status of the selected proteins were analyzed by Western blotting.

b) HCT-116 cells pre-treated with bafilomycin A1 (BafA1, 20 nM) or concanamycin (CCA, 20 nM) and control (untreated) cells were simultaneously treated with recombinant TRAIL (200 ng/ml) in a time-dependent manner for 0 to 60 min. The cell lysates were analyzed by Western blotting using the specified antibodies.

Figure 7

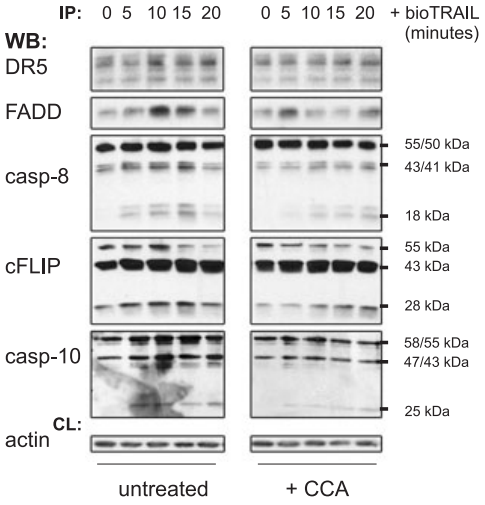


Figure 7

Inhibitors of endosomal acidification attenuate the DISC-mediated activation of the initiator caspases.

Colo206F cells, either untreated or pre-treated (1 hr) with concanamycin A (CCA, 20 nM), were incubated (0 - 30 min) with biotinylated TRAIL (bio TRAIL, 1 µg/ml) for the times indicated. The TRAIL receptor complexes were then isolated by streptavidin-agarose beads, and the captured protein complexes were analyzed by Western blotting with the indicated antibodies (IP – immunoprecipitation, CL – cell lysate).

Figure 8

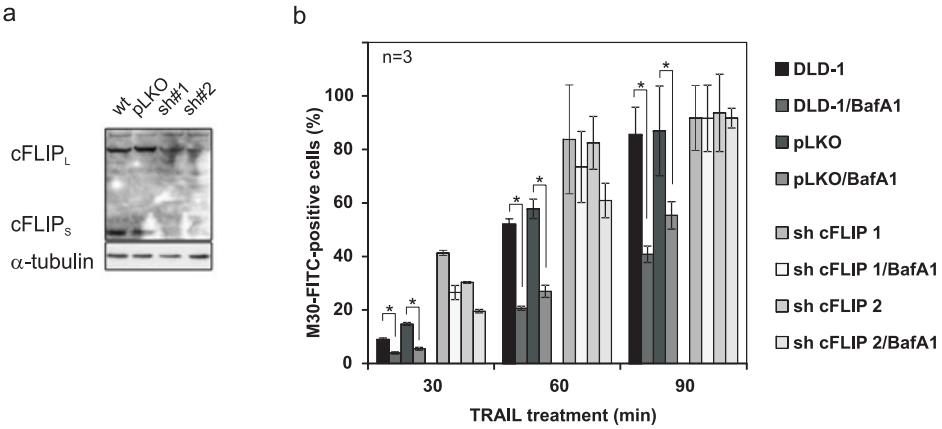


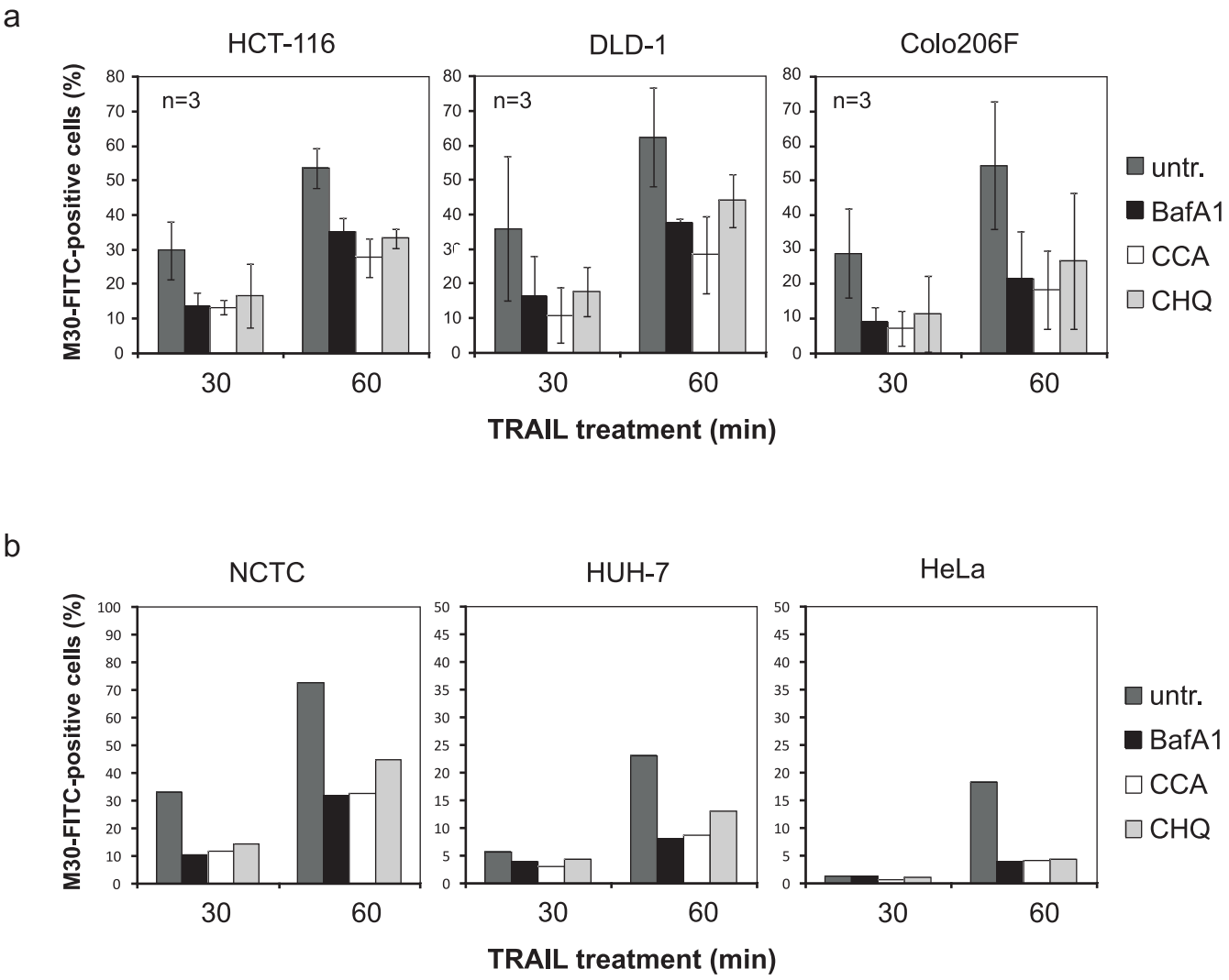
Figure 8

Downregulation of cFLIP largely blunts the inhibitory effect of V-ATPase inhibitors on TRAIL-induced apoptotic signalling.

a) Western blot analysis of $cFLIP_{L/S}$ expression in DLD-1 cells with shRNA-mediated suppression of cFLIP (2 different RNA hairpins, sh cFLIP 1 and 2) and in control samples (wt and cells transduced with non-silencing shRNA – NS).

b) DLD-1 cells with shRNA-mediated suppression of $cFLIP_{L/S}$ levels (as in Fig. 6a) were left untreated or pre-treated with bafilomycin A1 (BafA1, 20 nM) for 1 hr. The cells were then incubated with TRAIL (200ng/ml) for 30 to 90 min, stained with M30-FITC and analyzed by flow cytometry. The number of independent experiments (n), standard deviations and statistical significances are shown (* = $P \leq 0.05$).

Suppl. Figure S1



Supplementary Figure S1

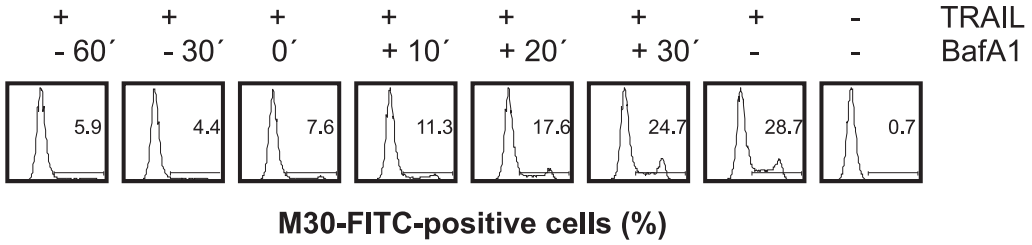
V-ATPase inhibitors or chloroquine suppress to various extents TRAIL-induced apoptotic signalling in a number of human cancer cell lines

a) HCT116, DLD-1 and Colo206F cells, either untreated or pre-treated for 1 hour with bafilomycin A1 (BafA1, 20 nM), concanamycin A (CCA, 20 nM) or chloroquine (CHQ, 500 μM), were incubated with recombinant TRAIL (200 ng/ml) for 30 or 60 min, and the fraction of cells with cleaved CK18 was quantified by M30-FITC staining and flow cytometry. Average values from three independent experiments are shown with standard deviations.

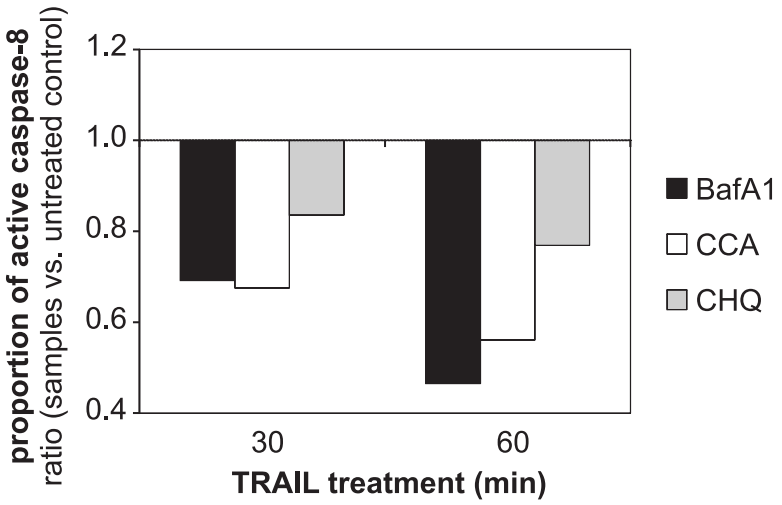
b) Cell lines of different tissue origin (NCTC – transformed keratinocytes, HUH7 – hepatocarcinoma, HeLa – cervical carcinoma) were treated and evaluated as specified in a). The percentages represent the population of apoptotic M30-positive cells in the cell culture. Average results from two independent experiments are shown.

Suppl. Figure S2

a



b



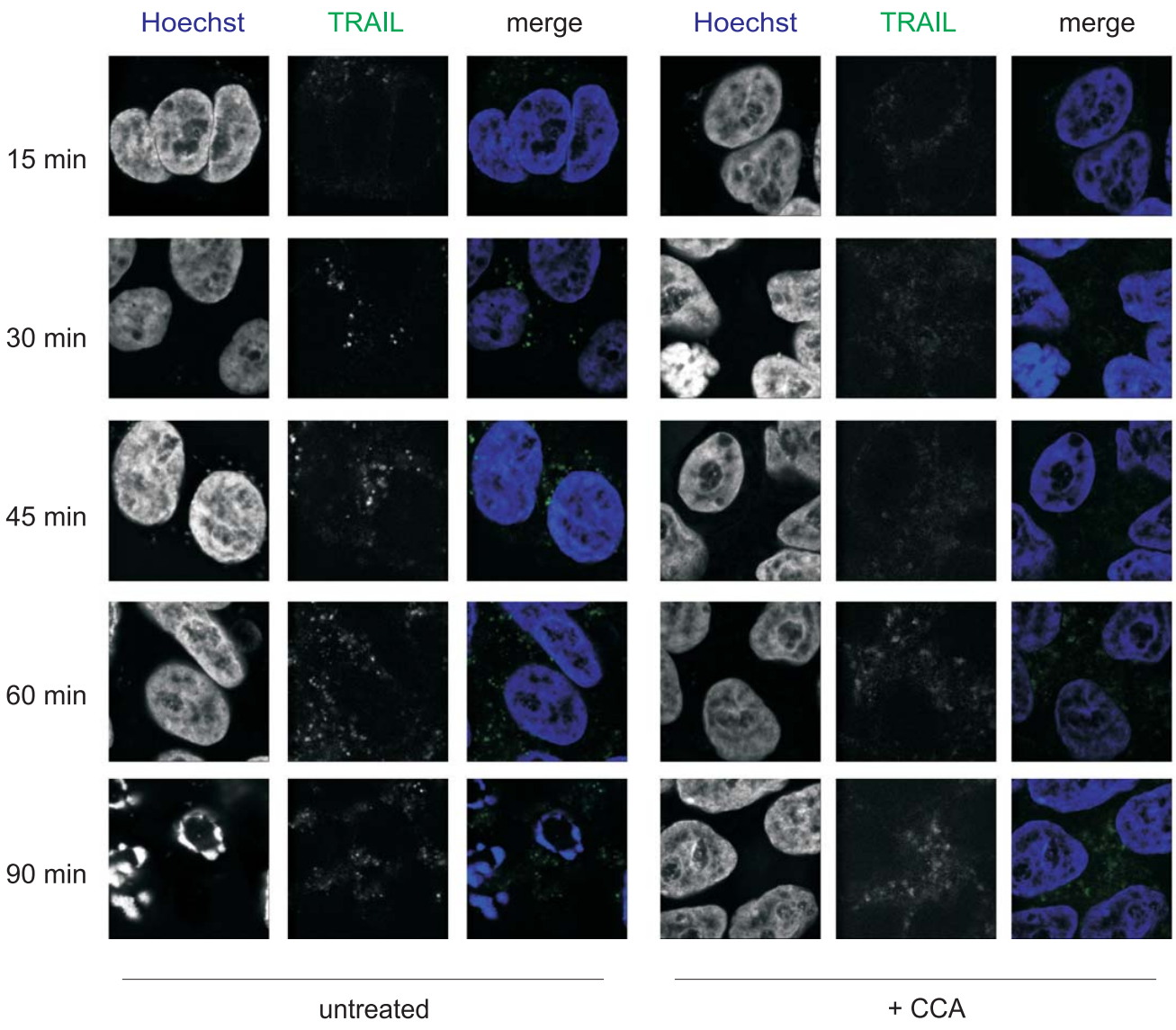
Supplementary Figure S2

The inhibitory effect of bafilomycin A1 is fast and affects the proximal TRAIL-induced activation of caspase-8.

a) Colo206F cells were incubated with bafilomycin A1 (BafA1, 20 nM) applied at various time points prior to (negative numbers) or after (positive numbers) adding TRAIL (200 ng/ml). Representative histograms of M30-FITC-stained cells from three independent experiments are shown (the numbers indicate the percentage of M30-FITC-positive apoptotic cells).

b) DLD-1 cells were pre-treated with the indicated reagents for 1 hr, then treated with TRAIL (200 ng/ml) for 30 and 60 min, harvested and stained for cleaved caspase-8 by a specific antibody according to the manufacture's recommendation. Cells with cleaved caspase-8 were then quantified by flow cytometry. Results from pre-treated samples are plotted as a relative ratio to the results from TRAIL-treated control cells (set to 1). Averages from 2 independent experiments are shown.

Suppl. Figure S3



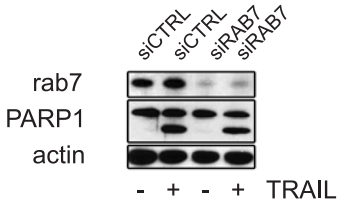
Supplementary Figure S3

Effect of concanamycin A on the distribution and trafficking of TRAIL-containing endosomes in DLD-1 cells.

DLD-1 cells grown on cover glasses were either untreated or pre-treated with concanamycin A (CCA, 20 nM), then incubated with fluorescent Alexa 647-conjugated TRAIL (1µg/ml, green colour) for 0 to 90 min. Cells were fixed in 3% paraformaldehyde and the nuclei visualized by Hoechst 33540 (blue signal). Confocal microscopy was performed using a Leica SP5 microscope. The shown data are representative of two independent experiments.

Suppl. Figure S4

a



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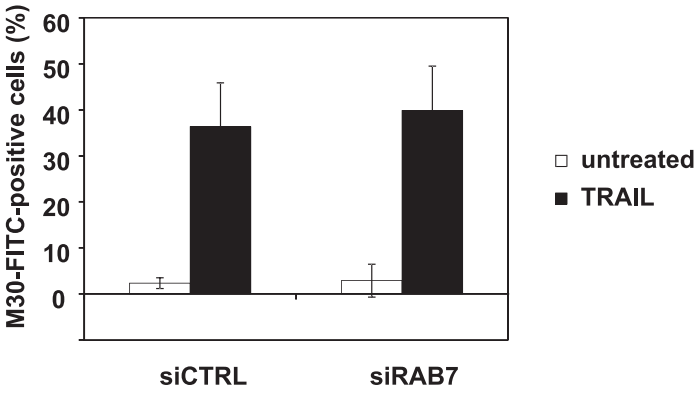
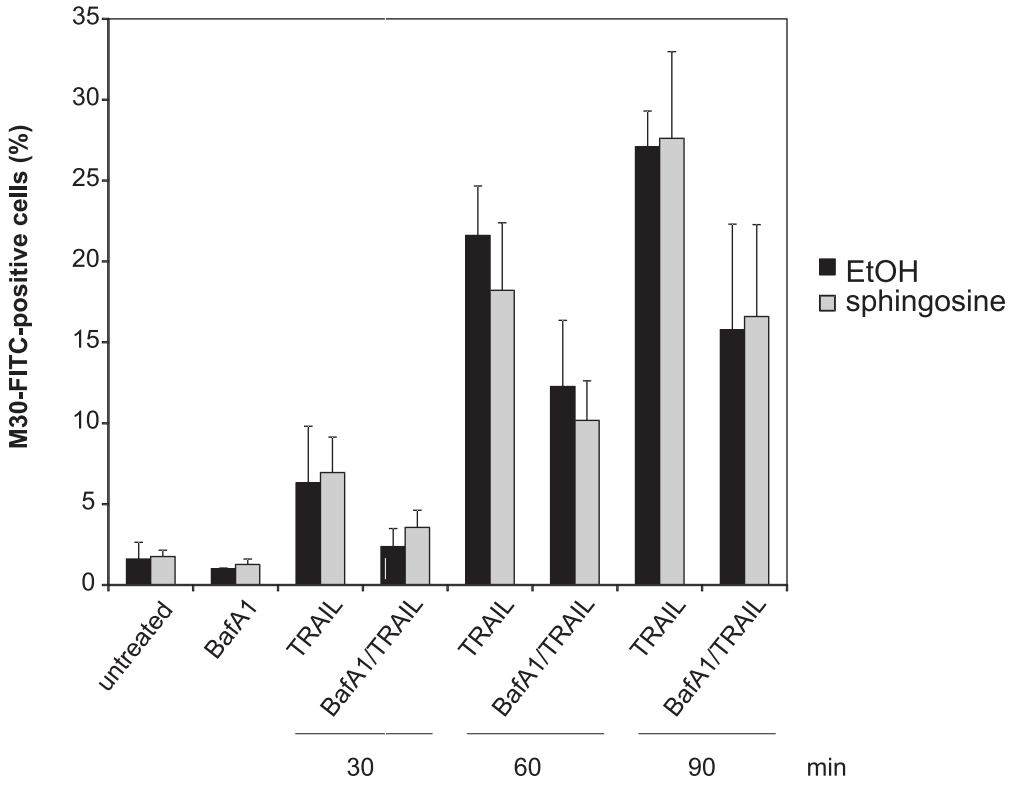


Supplementary Figure S4

Downregulation of Rab7 GTPase has no effect on the activation of proximal TRAIL-induced apoptotic signalling.

DLD-1 cells were transfected with Rab7 or control siRNAs and treated with TRAIL for 1 hr. TRAIL-induced pro-apoptotic signalling was determined by Western blotting with anti-PARP antibody (a) and flow cytometry using M30-FITC staining (b).

Suppl. Figure S5

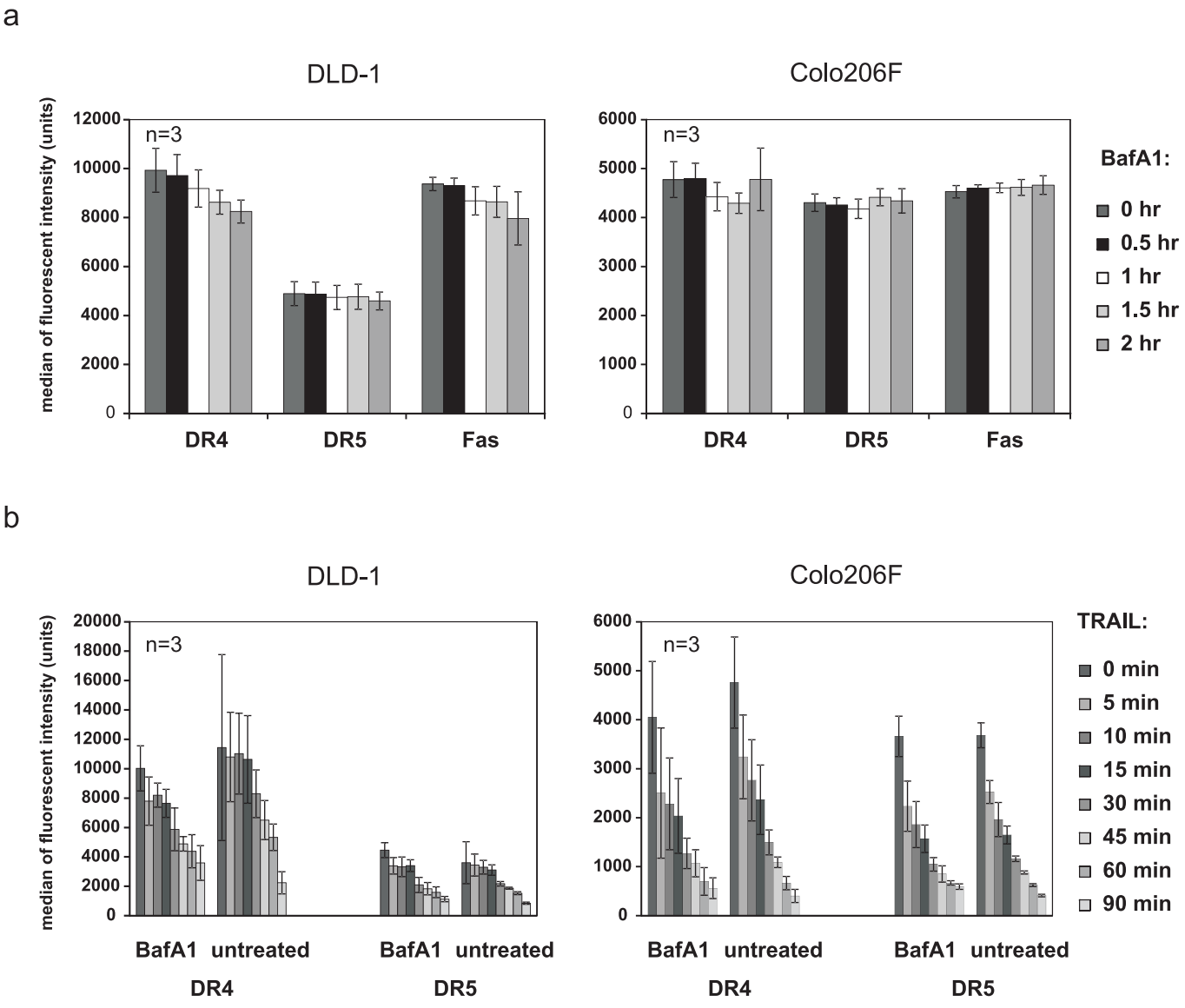


Supplementary Figure S5

C18 sphingosine did not reverse the BafA1-mediated suppression of TRAIL-induced apoptotic signalling.

Colo206F cells were pre-treated with bafilomycin A1 (BafA1, 20 nM) for 1 hr in the presence or absence of 5 mM C18 sphingosine and then with TRAIL (200ng/ml) for 30-90 min. The cells were then harvested, stained with M30-FITC and analyzed by flow cytometry.

Suppl. Figure S6



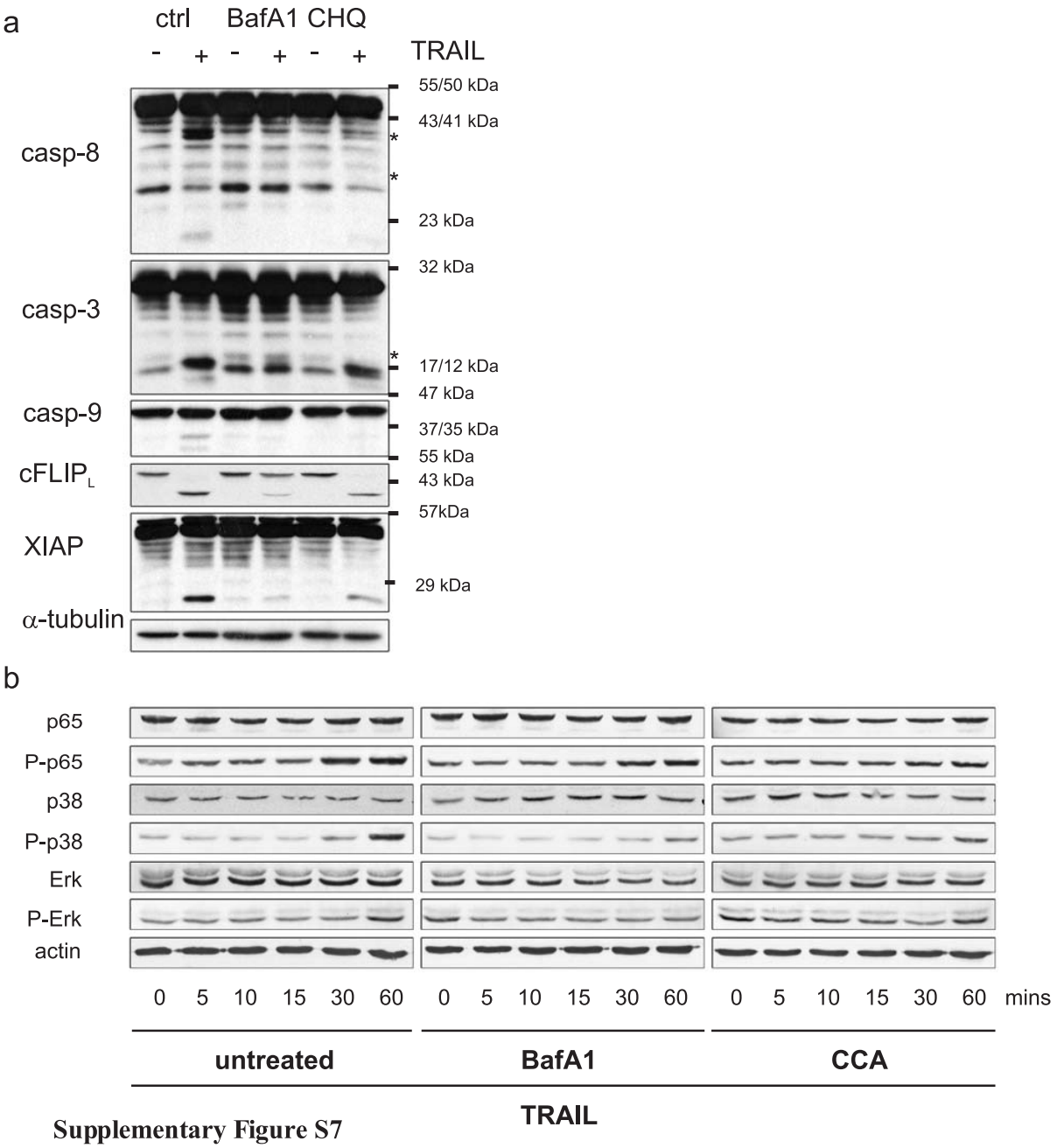
Supplementary Figure S6

Bafilomycin A1 treatment does not alter the cell surface expression of death receptors or the kinetics of their TRAIL-induced internalization in DLD-1 and Colo206F cells.

a) Cells were treated with bafilomycin A1 (BafA1, 20nM) for the indicated time periods, and the surface expression of the death receptors DR4, DR5 and Fas was determined by antibody staining and flow cytometry. The medians and standard deviations from three independent experiments are shown.

b) TRAIL was added to control (untreated) and bafilomycin A1- (BAfA1, 20 nM) pre-treated cells, and at the indicated time points after TRAIL addition, the cell surface expression of the TRAIL receptors DR4 and DR5 was analyzed by antibody staining and flow cytometry. Average values and standard deviations from three independent experiments are presented.

Suppl. Figure S7



Supplementary Figure S7

Effect of suppressed endosomal acidification on the cleavage/processing of several apoptosis-related proteins and TRAIL-induced pro-survival signalling in DLD-1 and Colo206F cells.

a) DLD-1 cells were either untreated (ctrl) or pre-treated with BafA1 (20 nM) or chloroquine (CHQ, 40 nM) and incubated with recombinant TRAIL (200 ng/ml) for 4 minutes. The cells were then lysed in SDS lysis buffer, and the expression and status of the selected proteins were analyzed by Western blotting (* - nonspecific bands).

b) Colo206F cells pre-treated with bafilomycin A1 (BafA1, 20 nM) or concanamycin (CCA, 20 nM) and control (untreated) cells were simultaneously treated with recombinant TRAIL (200 ng/ml) in a time-dependent manner for 0 to 60 min. The cell lysates were analyzed by Western blotting using the specified antibodies.

Supplementary Table S1

Sequences of short inhibitory RNAs in the shRNA-expressing lentiviral pLKO1-based vectors used for downregulation of cFLIP in DLD-1 cells.

No.	shRNA	Sequence (5'->3')
1	Non-silencing/non-targeting	CAACAAGATGAAGAGCACCAA
2	cFLIP1	CCTCACCTTGTTTCGGACTA
3	cFLIP2	GCTCCATAATGGGAGAAGTAA

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ORIGINAL PAPER

Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane

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Abstract TRAIL, a ligand of the TNFα family, induces upon binding to its pro-death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 the apoptosis of cancer cells. Activated receptors incite the formation of the Death-Inducing Signaling Complex followed by the activation of the downstream apoptotic signaling. TRAIL-induced apoptosis is regulated at multiple levels, one of them being the presence and relative number of TRAIL pro- and anti-apoptotic receptors on the cytoplasmic membrane. In a yeast two-hybrid search for proteins that interact with the intracellular part (ICP) of DR4, we picked ARAP1, an adapter protein with ArfGAP and RhoGAP activities. In yeast, DR4(ICP) interacts with the alternatively spliced ARAP1 lacking 11 amino acids from the PH5 domain. Transfected ARAP1 co-precipitates with DR4 and co-localizes with it in the endoplasmic reticulum/Golgi, at the cytoplasmic membrane and in early endosomes of TRAIL-treated cells. ARAP1 knockdown significantly compromises the localization of DR4 at the cell surface of several tumor cell lines and slows down their TRAIL-induced death. ARAP1 overexpressed in HEL cells does not affect their TRAIL-induced apoptosis or the membrane localization of DR4, but it enhances the cell-surface presentation of phosphatidyl serine. Our data indicate that ARAP1 is likely involved in the regulation of the cell-specific trafficking of DR4 and might thus affect the efficacy of TRAIL-induced apoptosis.

Keywords Apoptosis · TRAIL · Interaction · Receptor · Trafficking · Signaling

Abbreviations

TNF	Tumor necrosis factor
TRAIL/Apo2L	TNF-related apoptosis-inducing ligand
Arf	ADP-ribosylation factor small GTPase
ARAP1	ArfGAP, RhoGAP, Ankyrin repeats and pleckstrin homology (PH) domains containing protein
DD	Death domain
DISC	Death-inducing signaling complex
ER	Endoplasmic reticulum
GAP	GTPase-activating protein
IAP	Inhibitor of apoptosis
MFI	Median fluorescence intensity
MMP	Mitochondrial membrane permeabilization
PARP	Poly-ADP ribose polymerase
SiRNA	Small inhibitory RNA
TGN	Trans-Golgi network

Introduction

TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the tumor necrosis factor (TNF) family and is mainly expressed on the surface of hematopoietic cells (B-lymphocytes, antigen-activated T cells, NK cells and monocytes), though its mRNA has also been detected in many other tissues such as spleen, lung and prostate [1, 2].

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TRAIL preferentially induces the apoptosis of malignant cells rather than normal cells [3, 4] and interacts with five different receptors: apoptosis-inducing receptors DR4/TRAIL-R1 and DR5/TRAIL-R2, decoy receptors DcR1/TRAIL-R3 without the intracellular part and DcR2/TRAIL-R4 with a truncated death domain, and osteoprogeterin (OPG) [5–9]. Death receptors DR4 and DR5 are expressed on most, if not all, normal somatic cells; nevertheless, on these cells their engagement with TRAIL usually does not trigger apoptosis [1, 5, 6]. The combination of the lower expression of pro-apoptotic receptors and the increased expression of decoy receptors and of intracellular anti-apoptotic proteins such as FLIP or Mcl-1 provides protection for healthy human cells against TRAIL-induced apoptosis. However, the sensitivity of normal human cells to TRAIL-induced apoptosis is greatly enhanced by their transformation, viral infection, or other pathological processes [10].

The intracellular part of the death receptors contains an approximately 80 amino acid long, conserved domain called the death domain (DD) [11]. This structural motif is essential for the formation of the Death-Inducing Signaling Complex (DISC) and the induction of apoptosis [12]. The basic TRAIL DISC is composed of the ligand, the receptor(s), the adapter protein FADD, and the initiator pro-caspase-8 or -10 and is formed shortly after TRAIL binding to its receptors [13]. Pro-caspase-8, recruited to the DISC via FADD, is rapidly activated by auto-proteolytic cleavage; its activated pro-domains persist in the DISC and cleave downstream effector proteins [14]. Active caspase-8 can directly activate the executioner caspases (-3, -6, -7) in so-called type I signaling. Caspase-8-mediated cleavage of Bid is required for the translocation of truncated Bid (tBid) to the mitochondria and the activation of type II mitochondrial apoptotic signaling [15, 16]. Besides the expression and cell-surface localization of pro- and anti-apoptotic TRAIL receptors, TRAIL-induced apoptosis of human tumor cells is regulated by the inhibition of DISC assembly, ineffective downstream signal transduction (Bid processing and translocation, Bax/Bak activation), or by the suppression of the amplification loop at or downstream of the mitochondria (inhibition of MMP or activated effector caspases via IAPs) [10, 17, 18].

The first and essential condition for the productive initiation of TRAIL-induced apoptosis is the expression and proper cellular localization of the proapoptotic TRAIL receptors DR4 and DR5. They are predominantly localized at the cell surface and in the ER/TGN system [19]. A similar pattern of localization was also described for TNFR1 and CD95 [20–22]. Their transport from the TGN to the cellular membrane is apparently mediated by cargo-transport vesicles composed of Arf GTP-binding proteins, coat proteins such as COPI or clathrin, GGA and tetrameric adaptor proteins (APs), and transported receptors [23]. Among Arf proteins, Arf1 is required for the recruitment of coat proteins from the cytosol onto the membranes of the TGN [24]. Arf-protein cargo-related activity is regulated by a family of Arf GAP proteins such as ArfGAP1 or AZAP proteins that activate GTP hydrolysis and thus induce Arf dissociation from the coated vehicles [25]. Arf GAPs, however, not only serve as catalysts of Arf release and recycling, but they also represent a group of coat proteins that can interact with cargo proteins (e.g., transported receptors). Arf GAP1 interacts with the ERD2 receptor via its C-terminal domain, while ACAP1, a member of the AZAP family, binds to the cytoplasmic tail of the human transferrin receptor and promotes its recycling [26, 27].

Here we present evidence that ARAP1/centaurin δ2, a member of the AZAP family [28], can interact with DR4 both in yeast and in mammalian cells. siRNA-mediated downregulation of ARAP1 markedly and specifically reduced the cell-surface localization of DR4 in several human cell lines and attenuated the kinetics of their TRAIL-induced apoptosis. Our data thus indicate that ARAP1 could be involved in the regulation of DR4 cellular trafficking.

Materials and methods

Cells, reagents and antibodies

Human immortalized keratinocytes NCTC were provided by Dr. L. LoMuzio, MG-63 and Saos-2 by Dr. P. Hozak, human colon carcinoma cells HCT-116, DLD-1, HT29 and the erythroleukemia cell line HEL (human erythroleukemia) were purchased from ATCC and HEK293FT from Invitrogen. Human recombinant TRAIL (amino acids 95–281) was prepared in our laboratory as described previously [29]. Mouse monoclonal antibodies against the extracellular parts of DR4, DR5, DR6 and against the C-terminal part of ARAP1 (amino acids 1,084 to 1,372) were prepared in our laboratory. DR4 blocking antibody DR4-02 was purchased from Exbio. The following commercially available antibodies were obtained from the indicated suppliers: mouse monoclonal anti-Myc tag (Roche Molecular Biochemicals), rabbit polyclonal anti-caspase-8 (BD Biosciences), rabbit polyclonal anti-DR4, DR5, Bid, PARP and Rab5A (Santa Cruz). Rabbit polyclonal anti-ARAP1 antibody was kindly provided by Dr. P.A. Randazzo. Anti-TRAIL receptor-1 to -4 flow cytometry set and rabbit polyclonal anti-DR4 and DR5 antibodies were purchased from Alexis. Secondary Alexa-488- and Alexa-594-conjugated goat anti-rabbit IgG and Alexa-488- and -594-conjugated goat anti-mouse IgG antibodies were supplied by Molecular Probes. FITC-conjugated anti-M30 antibody was purchased from Roche

Molecular Biochemicals. Mouse monoclonal anti-CD43 and anti-β1-integrin antibodies were kindly provided by Dr. V. Horejsi and anti-α-tubulin antibody by Dr. P. Draber. Anti-DR4, DR5, DR6 and CD43 (MEM-59) antibodies used for immunoprecipitations were coupled to activated CNBr Sepharose (GE Healthcare) according to the manufacturer's protocol.

Plasmids

KIAA0782 plasmid encoding ARAP1 cDNA was supplied by Kazusa DNA Research Institute (Chiba, Japan). The cDNA was subcloned into the *Bam*HI and *Bgl*II sites of pKMyc-C3 (modified pCDNA3 with a Myc tag upstream of the MCS). Full-length ARAP1 (containing the SAM domain and lacking exon 30) was prepared by PCR-assisted subcloning from NCTC cDNA. pCDNA3-based DR6 and CD43 expression plasmids were prepared in our laboratory. pCR-DR4 and pCR-DR5 were kindly provided by Dr. J. Tschopp. Deletion mutants of DR4 were prepared by PCR using the primers shown in the Supplementary Table 1 and subcloned into the *Eco*RI and *Bam*HI sites of pCR3 plasmid. DR4-ICP was prepared by PCR (for primers—see Suppl. Table 1) and cloned into the *Eco*RI and *Bam*HI sites of pGBKT7. All PCR products were verified by sequencing.

Two-hybrid cDNA library screening

The yeast two-hybrid screening for DR4(ICP)-interacting proteins was performed using the Matchmaker 3 system (Clontech), according to the manufacturer's protocol. AH109 yeasts transformed with pGBKT7-DR4-ICP were mated with Y187 cells containing HA-tagged human cDNA libraries (leukocyte or bone marrow) cloned into the pACT2 plasmid (Clontech). Upon auxotrophic selection on SD/agar plates lacking tryptophan, leucine, adenine, and histidine (SD-WLAH), the surviving colonies were transferred to SD/agar plates lacking tryptophan, leucine (SD-WL) plates, and plates containing 5-brome-4-chloro-3-indolyl-β-D-galactosid (X-Gal). Blue colonies were then grown in the selective WLAH medium and analyzed by Western blotting with anti-HA antibody. Clones with a molecular weight above 35 kDa (i.e., with an insert longer than 80–100 amino acids) were sequenced and checked for the specificity of their interaction with the bait.

Cell culture and transfection

HEK293FT, HCT116, MG-63 and NCTC cells were cultured in Dublecco's modified Eagle's medium (DMEM), and Saos-2 in RPMI medium containing 10% fetal calf serum (FCS). HEL cells grown in RPMI medium with 10% FCS were transfected with the pKMyc-C3 ARAP1 (Δexon 30) expression plasmid or with the empty vector (mock transfection), and ARAP1-expressing clones were selected using geneticin G418 (Alexis). Transfection of all the cell lines was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Immunoprecipitation and Western blotting

Cells for immunoprecipitation were washed twice with ice-cold PBS and lysed on ice in 1 ml of the lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 mM sodium orthovanadate, and protease inhibitors (Complete, Roche), pH 7.5. Cell lysates were centrifuged at 4°C and 13,000 rpm for 30 min, and the supernatants were immunoprecipitated with the CNBr Sepharose-coupled antibodies at 4°C for 4 h. The immunoprecipitated complexes were washed five times with the lysis buffer and eluted from the immunosorbent with SDS sample buffer. Proteins in the immunocomplexes or the cell lysates were separated by SDS PAGE and transferred onto nitrocellulose membranes (Amersham). Membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween (PBS/Tween), incubated with specific primary antibodies and, after washes, with the appropriate peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (BioRad). After three washes with PBS/Tween, the bound secondary antibodies were visualized by enhanced chemoluminescence (ECL, Pierce).

Confocal microscopy

NCTC cells were cultured on coverslips in a 24-well culture dish, transfected with the corresponding plasmids and 24 h later treated with 200 ng/ml TRAIL. At given time points after adding TRAIL, the cells were washed with ice-cold PBS, fixed with methanol at -20°C for 5 min and permeabilized with acetone. Fixed cells were incubated in blocking buffer (PBS with 1% bovine serum albumin) for 20 min, then with the primary antibodies for an additional 30 min, washed three times with PBS, and further incubated with the appropriate secondary antibodies at room temperature for 30 min. The coverslips were mounted on slides using Mowiol mounting medium and analyzed by confocal microscopy (Leica TCS SP confocal laser scanning microscope).

Down-regulation of ARAP1 expression

A mix of or individual ARAP1 siRNAs (siGenome, Dharmacon) or control Luciferase siRNAs CAAGCUGA

CCCUGAAGUUCdTdT (Dharmacon) were used for Lipofectamine RNAiMax (Invitrogen)-mediated transfection of the analyzed cell lines. The sequences of selected ARAP1 siRNA were: oligo #1 GAAUAAGCUGUACGUGGCCdTdT, oligo #2 CUUCGUGGCUUCAAGAAU AdTdT. Cells were cultured in 24-well culture dishes and transfected at 40–50% confluence. Fifty-two hours later, ARAP1 downregulation in siARAP1 transfected cells was confirmed by Western blotting, and the cells were used in follow-up experiments (analysis of the surface expression of receptors, TRAIL-induced apoptosis, etc.).

Flow cytometry and apoptosis assays

Adherent cells were released by treatment with PBS containing 10 mM EDTA and, after washing with ice-cold PBS, were incubated in blocking solution (PBS with 20% human serum, 1% gelatin and 0.1% azide) on ice for 15 min. Cells were then washed once with wash buffer (PBS containing 1% gelatine and 0.1% azide), incubated with the appropriate primary monoclonal antibodies, washed twice with ice-cold wash buffer and finally incubated with the secondary goat anti-mouse antibody coupled to phycoerythrin (IgG1-PE) (SouthernBiotech). All incubations were performed on ice. After two final washes, the surface expression of the receptors on living cells (Hoechst 33258 negative) was analyzed using a LSRII flow cytometer (BD Biosciences). M30 Cytodeath assay (Roche) of caspase-3-cleaved cytokeratine 18 or Annexin V-FITC/Hoechst 33258 (Alexis) staining were used for the assessment and quantification of TRAIL-induced apoptosis according to the manufacturer's protocols. M30-FITC or Annexin V-FITC stained cells were analyzed by flow cytometry using a LSRII flow cytometer.

Results

Rho and Arf GAP adapter protein ARAP1 lacking exon 30 specifically interacts with the intracellular part of DR4/TRAIL-R1

Activation of TRAIL-induced apoptosis ultimately depends on the presence and status of TRAIL's proapoptotic receptors (cell surface expression, mutations, competition of decoy receptors) and on the kinetics and efficacy of DISC formation/activation (type I vs. type II, activation inhibitors such as FLIP) [31, 32]. Most of these critical regulatory events are mediated via protein–protein interactions. In order to search for new proteins that interact with TRAIL-R1/DR4 and could affect its signaling, we performed a yeast two-hybrid (Y2H) screening of several cDNA expression libraries using the intracellular part of DR4 (ICP, amino acids 246–445) as a bait. Two fusion proteins specifically interacting with DR4(ICP) contained a C-terminal part of ARAP1 (centaurin δ2), an adapter protein with multiple domains and Arf and Rho GAP activities ([28] and Fig. 1a). A comparison of the interacting C-terminal part of ARAP1 (ARAP1-C) with the Genebank database revealed that this ARAP1 clone does not contain the very short exon 30 (translated to amino acids 1,319–1,329) that maps into the fifth pleckstrin homology (PH) domain (Fig. 1a). Interaction analysis in yeasts uncovered that the presence of exon 30 in the ARAP1 C-terminus abolished its interaction with the intracellular part of DR4 (Fig. 1b). Similarly as the C-terminal part of ARAP1 (FL), the corresponding C-termini of its relatives ARAP2 and ARAP3 also did not bind to the intracellular part of DR4 in Y2H (not shown).

The specific interaction of the intracellular part of DR4 with the exon 30-less splice variant of ARAP1 posed the question of the relevance of ARAP1Δexon30 expression in human cell lines. We therefore employed quantitative real-time PCR to analyze cDNAs from several human tumor-derived cell lines as well as from different primary cells for the relative expression of ARAP1 and ARAP1Δexon30 using exon-30 flanking primers. The data from this analysis showed that the Δexon 30-less splice variant is the predominantly expressed form in a majority of the tested cell lines, most notably in NCTC, DLD-1 and HEK293 cells as well as in primary fibroblasts, colon epithelial and T cells (Suppl. Fig. 1a). The specificity of the RT-qPCR analysis of ARAP1 splice variant expression was confirmed by ApaI cleavage of the PCR-amplified 3′-end of ARAP1. The *Apa*I cleavage site resides in exon 30, and only a minor part of the ARAP1 3′ end amplified from NCTC cDNA was cleaved by this enzyme, verifying that the major ARAP1 splice variant in NCTC cells does not contain exon 30 (Suppl. Fig. 1b).

ARAP1 interacts and co-localizes with DR4 in human cells

Uncovering ARAP1 as a new DR4-interacting protein with a potential regulatory function prompted us to examine their interaction in human cells as well as the possible functional consequences of this interaction. First of all we examined whether transiently expressed ARAP1 C-terminus (ARAP1-CΔexon30) and ARAP1Δexon30 (ARAP1 full length lacking exon30) can interact with co-transfected DR4 or other death (DR5 and DR6) and control (CD43) receptors in HEK293FT cells. Myc-tagged ARAP1-CΔexon30 co-immunoprecipitated with DR4 and, to lesser extent, DR5 (Fig. 2b, lanes 2 and 8) while it did not

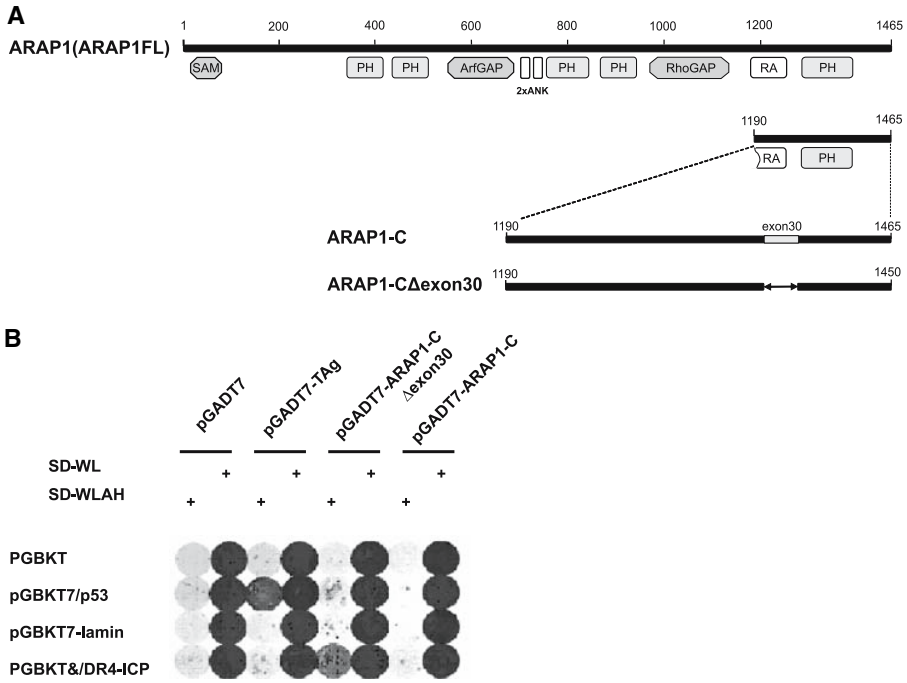

Fig. 1 ARAP1 (Δexon30) associates with DR4(ICP) in yeasts. (**a**) Schematic illustration of human ARAP1 (ARAP1 transcription variant 3, NM_001040118.1). Full-length ARAP1 (1465 amino acids) contains the N-terminal sterile α motif (SAM) domain, Arf GAP and Rho GAP domains, 5 pleckstrin homology domains (PH), two ankyrin repeats (ANK) and a Ras-associating domain (RA). The C-terminus of ARAP1, ARAP1-CΔexon30 (exon 30-lacking splice variant), was pulled out from the human leukocyte cDNA library by yeast two hybrid screening for DR4(ICP)-interacting proteins. (**b**) Intracellular part of DR4, DR4(ICP), interacts in yeasts only with the ARAP1-CΔexon30 variant. AH109 yeasts containing pGBKT7 bait plasmids were mated with Y187 yeasts transformed with the appropriate pGADT7 plasmids. Mated yeasts were grown first on a plate with the mating medium (SD-WL—every second column), then transferred onto a plate with the selection medium (SD-WLAH) to test the interaction (every first column). The mating couple pGBKT7-p53 and pGADT7-Tag was used as a positive control for mating and pGBKT7-lamin was used as a negative control

interact with DR6 or CD43 (Fig. 2b, lanes 10 and 12). Ectopically expressed full-length ARAP1Δexon30 co-immunoprecipitated with DR4 as well, albeit less efficiently than its C-terminal part (Fig. 2b, lane 14).

For mapping the ARAP1-interacting region in the intracellular part of DR4, we prepared a set of its deletion mutants (Fig. 2a) and analyzed their interaction with the C-terminal part of ARAP1. Removal of last 22 amino acids from the DR4 C-terminus (DR4-ΔC) compromised its interaction with ARAP1-CΔexon30 (Fig. 2b, compare lanes 2 and 3). A DR4 deletion mutant lacking the death domain and C-terminal extension (DR4-ΔDDC) failed to interact with ARAP1-CΔexon30 indicating a requirement for the death domain for DR4-ARAP1 interaction (Fig. 2b, lane 4). In our first set of experiments, we also prepared a DR4 deletion mutant that, in addition to the membrane proximal part, also contained the first α-helix and turn region from DR4 DD (DR4ΔxDDC). The strong interaction of this DR4 mutant with ARAP1-CΔexon30 suggested that this part of the DR4 DD could contain the ARAP1 interaction motif (Fig. 2b, lane 6). A comparison of first α-helices in the death domains of several human death receptors revealed that the predicted turn region between the first and second α-helix (C-terminus of the DR4ΔxDDC mutant) contains, in DR4 and DR5, conserved phenylalanine and aspartate (Suppl. Fig. 2a). Their substitution with the corresponding amino acids from TNFR1 (proline and leucine) in mutant DR4-FD/PL led to the severe suppression of ARAP1-CΔexon30 co-immunoprecipitation with DR4 (Fig. 2b, lane 7).

In comparison with ARAP1-CΔexon30, the interaction of full length ARAP1Δexon30 with DR4 was relatively weak. Thus, it was not very surprising that we were unable to co-immunoprecipitate the endogenous proteins from the whole cell lysates of several analyzed human cell lines (not shown). Only upon TRAIL treatment of NCTC cells did we observe the co-immunoprecipitation of the apparently processed form of ARAP1 with endogenous DR4 (Fig. 2c). The appearance of the ARAP1 processed form and its co-immunoprecipitation with DR4 was dependent on caspase activity (Suppl. Fig. 2b). Thus, it is likely that the interaction of the full-length (transfected or endogenous) proteins with one another is either weak or transient and

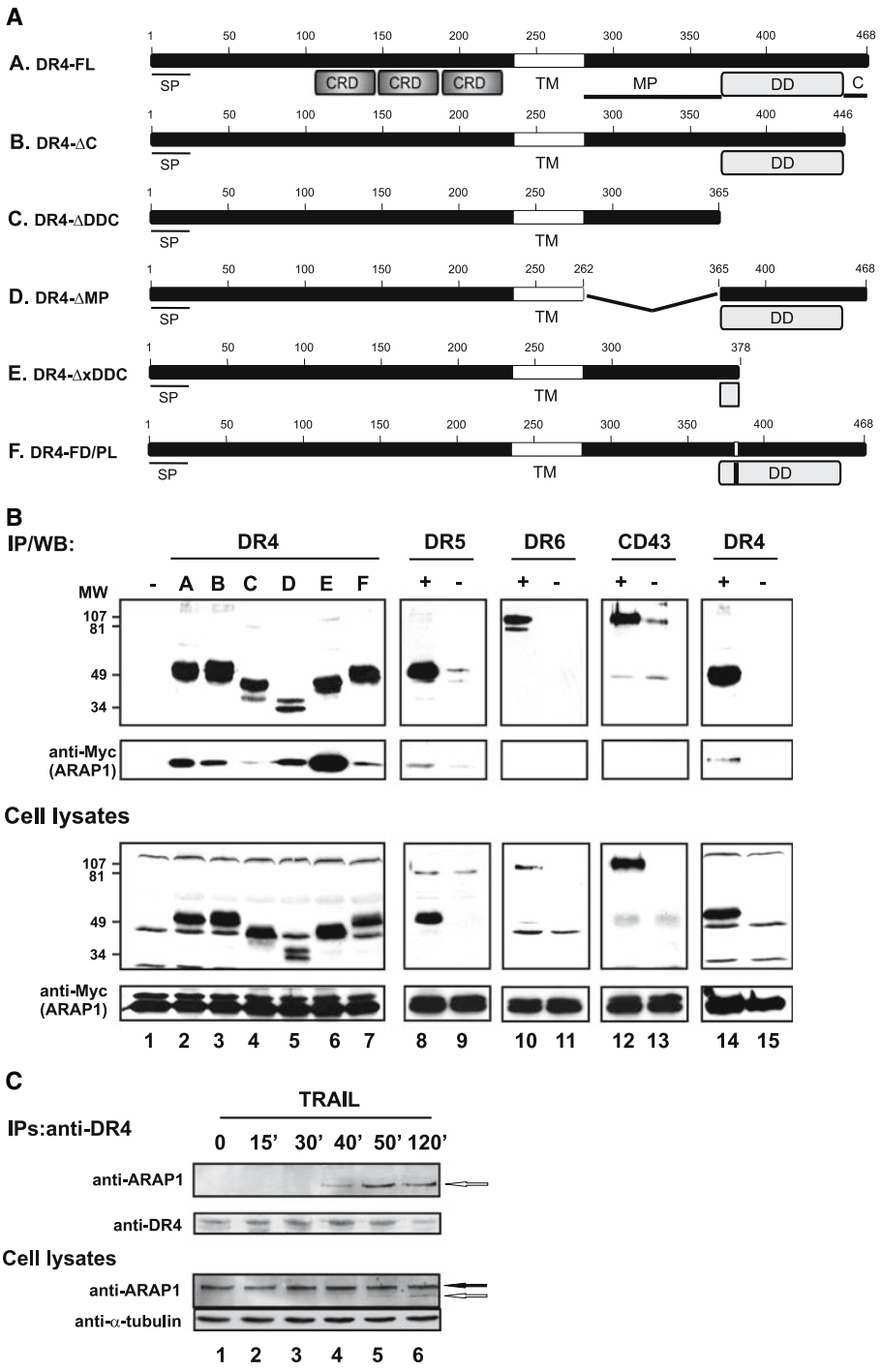

Fig. 2 ARAP1 interacts with DR4 in human cells. (**a**) Graphical representation of DR4 mutants (SP, Signal Peptide; CRD, Cysteine Rich Domain; TM, transmembrane domain; MP, membrane proximal region; DD, Death Domain; C, C terminus). (**b**) HEK293FT were transfected with pKMycARAP1-CΔexon30 either alone (lanes 1, 9, 11, 13) or together with the expression plasmids for DR4FL and mutants (A-F)—lanes 2–7, DR5 (lane 8), DR6 (lane 10), CD43 (lane 12); or with plasmid expressing ARAP1Δexon30 alone (lane 15) or together with DR4FL (lane 14). Twenty-four hours after transfection, the cells were lysed and the lysates were immunoprecipitated with the corresponding antibodies (anti-DR4, -DR5, -DR6 or -CD43) coupled to CNBr Sepharose. Immunoprecipitated complexes and the cell lysates were analyzed by Western blotting with the corresponding antibodies (WB, blotting antibody; IP, immunoprecipitating antibody). (**c**) NCTC cells were treated with TRAIL (200 ng/ml) and at the time points indicated in the figure harvested and lysed; DR4 complexes were immunoprecipitated with anti-DR4 Sepharose. Western blots of the immunoprecipitates and cell lysates were stained with anti-DR4, anti-ARAP1 or anti-a-tubulin (loading control). Arrows depict the position of ARAP1 (filled) or its processed form (empty)

could be stabilized by removing the inhibitory/destabilizing sequences.

An obvious requirement for the interaction of two proteins (or protein complexes) is their proximity within the cellular compartments. We co-transfected ARAP1Δexon30 together with DR4 into NCTC cells and analyzed their relative localization in untreated and TRAIL-treated cells (Fig. 3). In untreated cells transfected DR4 was localized at the membrane and in the ER/Golgi compartment surrounding the nuclei. ARAP1 was positioned in the ER/Golgi compartment in agreement with previously published data, but a significant fraction of

ARAP1 was also localized at the cellular membrane. At both sites ARAP1 apparently co-localized with DR4 (Fig 3a, 0′, arrowheads). Upon TRAIL treatment, both ARAP1 and DR4 changed their original positions and subsequently co-localized in early endosomes as detected with anti-Rab5 antibody (Fig. 3a, b, c, 60′, arrows).

Overexpression of ARAP1 led to enhanced phosphatidyl serine exposure but not to the acceleration of TRAIL-induced apoptosis in DR4-positive HEL cells

ARAP1 via its association with DR4 could affect either DR4-induced signaling or localization. Initially, we examined the effect of transient ARAP1 overexpression on DR4 membrane localization and TRAIL-induced apoptosis in HEK293FT cells. Neither DR4 surface expression nor TRAIL-induced apoptosis was significantly affected by overexpressed ARAP1 (not shown). However, HEK293FT cells express both TRAIL receptors DR4 and DR5, and thus an analysis of ARAP1's effect on DR4-induced signaling is affected by concurrent DR5 signaling.

We analyzed the cell surface expression of TRAIL receptors in a panel of human cell lines and discovered that the human erythroleukemia cell line HEL uniquely expresses only DR4 (Fig. 4a). We stably transfected HEL cells (which express low levels of ARAP1) either with ARAP1Δexon30 expression plasmid or with the empty vector. In selected mock transfected (2E, 3E) and

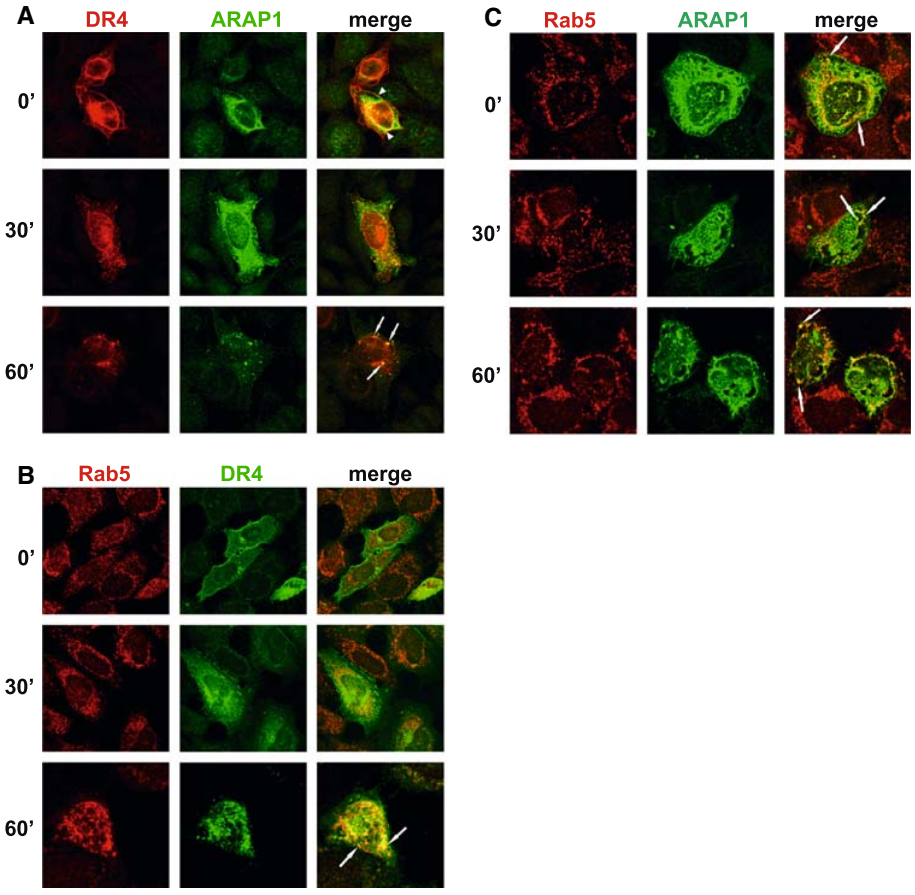

Fig. 3 ARAP1 co-localizes with DR4 at the cell membrane and in early endosomes. (**a**) NCTC cells grown in 24-well plates on coverslips were transfected with pKMyc-ARAP1Δexon30 and pCR-DR4FL. Twenty-four hours after transfection, the cells were treated with TRAIL (200 ng/ml) for 0, 30 or 60 min and fixed with methanol. Fixed cells were stained with rabbit polyclonal anti-DR4 (red) and mouse monoclonal anti-ARAP1 (green) antibodies and with appropriate secondary antibodies Alexa-594 anti-rabbit IgG and Alexa488 anti-mouse IgG. Arrowheads indicate ARAP1-DR4 co-localization at the plasma and intracellular membranes, while arrows show the co-localization of ARAP1 with DR4 in early endosomes after 60 min of TRAIL treatment. (**b**) and (**c**) NCTC cells were transfected either with pCR-DR4FL alone (**b**) or with pKMyc-ARAP1 Δexon30 alone (**c**), treated and fixed as above. The fixed cells were stained either with mouse monoclonal DR4 (**b**) or ARAP1 (**c**) antibodies in combination with rabbit polyclonal Rab5A antibody and with secondary antibodies Alexa488 anti-mouse IgG and Alexa594 anti-rabbit IgG. Arrows indicate the co-localization of either DR4 (green) with Rab5 (red) (**b**) or ARAP1 (green) with Rab5 (red) (**c**) in early endosomes

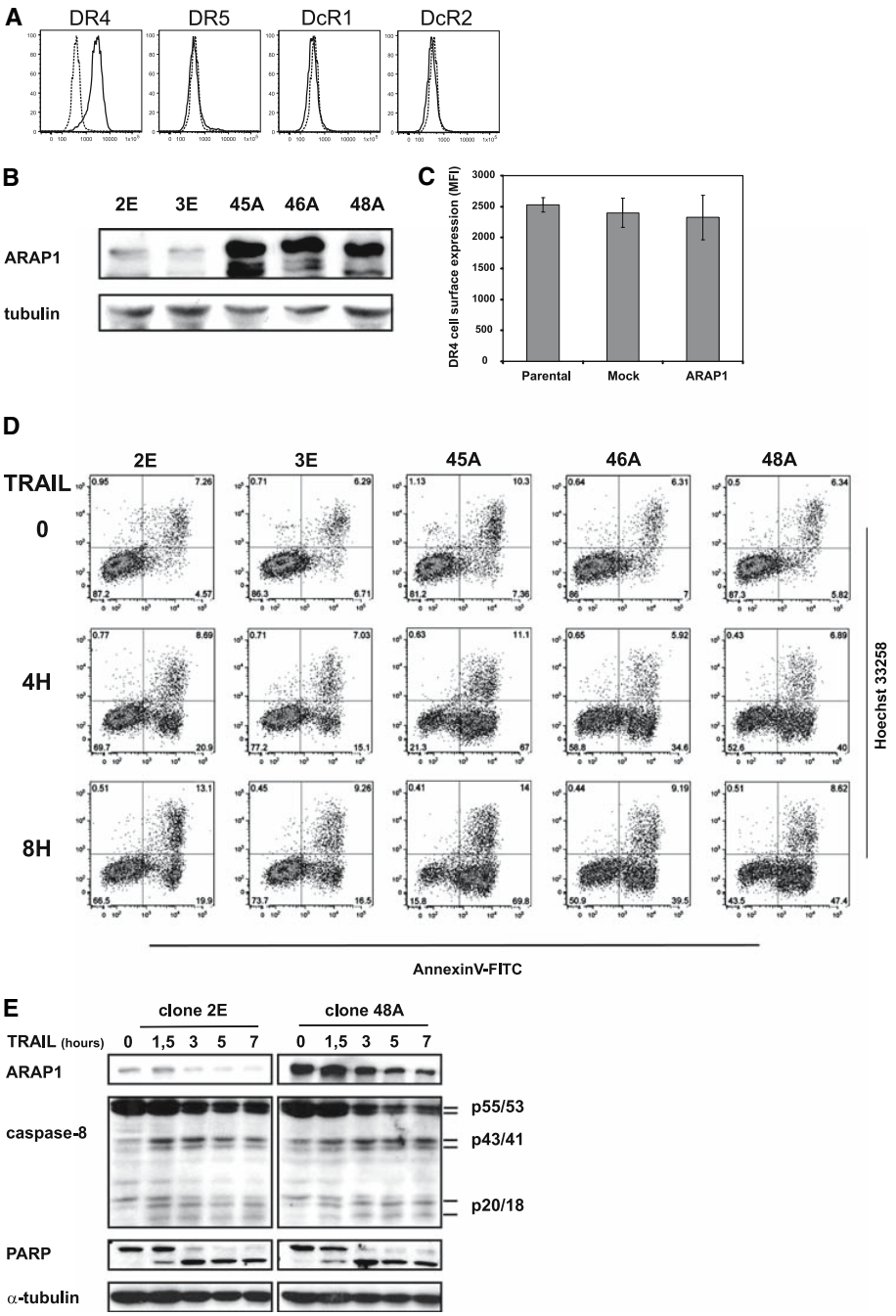

Fig. 4 ARAP1 enhances phosphatidyl serine exposure during TRAIL-induced apoptosis of HEL cells. (**a**) HEL cells were stained with anti-TRAIL receptor antibodies, and the cell surface expression of TRAIL receptors was analyzed by flow cytometry. (**b**) HEL cells were stably transfected either with an ARAP1-expressing plasmid or with the empty vector. Two control clones with the empty vector (2E, 3E) and three HEL clones expressing ARAP1 (45A, 46A, 48A) were analyzed by Western blotting using mouse monoclonal anti-ARAP1 antibody and anti-α-tubulin antibody as a loading control. HEL cells express a low level of endogenous ARAP1 (weak band in lines 2E and 3E). (**c**) Cell surface expression of DR4 in parental HEL cells, mock-transfected (mock) and ARAP1 clones. The average mean and standard deviation of DR4 cell surface expression represented as MFI of 4 independent analyses of parental HEL cells and 4 mock- and 5 ARAP1-transfected HEL clones are shown. (**d**) Selected clones were treated with TRAIL (200 ng/ml) for 4 or 8 h. After the treatment the cells were washed with PBS, stained with FITC-conjugated Annexin V and Hoechst 33258 and analyzed by flow cytometry. The values in the dot plots indicate the percentage of Annexin V- and/or Hoechst-positive cells. (**e**) Total cell lysates from clone 2E and clone 48A taken at the given time points were analyzed by Western blotting using the corresponding antibodies (α-tubulin antibody was used as a loading control)

ARAP1 expressing HEL clones (45A, 46A, 48A) (Fig. 4b), we analyzed the effect of ARAP1 expression on DR4 cell surface expression and TRAIL-induced apoptosis. Overexpressed ARAP1 did not significantly affect the cell surface expression of DR4 in selected HEL clones (Fig. 4b). An initial comparison of TRAIL-induced apoptosis between mock-transfected and ARAP1-expressing cells revealed that phosphatidyl serine exposure detected by Annexin V-FITC is significantly enhanced in ARAP1-expressing HEL clones (Fig. 4c). This observation suggested that ARAP1 overexpression could accelerate TRAIL-induced apoptosis. Nonetheless, biochemical analysis did not confirm these indications, and TRAIL-induced caspase-8 activation or PARP cleavage were comparable in mock-transfected and ARAP1 expressing cells (Fig. 4d). Thus, ARAP1 apparently affected only some cytoplasmic membrane-related and apoptosis-connected changes, such as phosphatidyl serine (PS) exposure,

without having a significant impact on downstream apoptotic signaling.

ARAP1 participates in the regulation of DR4 localization at the cytoplasmic membrane

From confocal microscopy showing ARAP1-DR4 co-localization at the plasma membrane (Fig. 3) and an apparent role for ARAP1 in enhancing PS exposure at the cell membrane (Fig. 4c), we hypothesized that ARAP1 could influence membrane-related events in DR4 signaling. As ARAP1 overexpression did not significantly affect TRAIL-induced apoptosis or DR4 plasma membrane expression, we undertook an opposite approach. Using siRNA we downregulated ARAP1 expression in a panel of human cell lines (Fig. 5a). ARAP1 knockdown in these cells did not affect total DR4 mRNA (not shown) and protein levels (Fig. 5a) but, in some of them (NCTC, Saos-2 and MG-63), did significantly suppress DR4 and, to a lesser extent, DR5 cell surface expression. The cell surface localization of other death receptors (TNFR1 or DR6) or CD29/β1integrin was not affected (Fig. 5b). Interestingly, ARAP1 knockdown in colon cancer-derived HCT116 or DLD1 cells did not reduce DR4 plasma membrane localization at all (Fig. 5b and not shown). ARAP1 siRNA-mediated cell surface downregulation of DR4 in tested cells was statistically significant (Fig. 5c and Suppl. Fig. 3) and was reproducibly observed with two independent ARAP1 siRNAs (Fig. 5d).

Downregulation of ARAP1 by siRNA decelerates TRAIL-induced apoptosis

Next, we asked whether and how ARAP1 siRNA-mediated downregulation of DR4 from the cell surface would affect TRAIL-induced apoptosis in these cells. NCTC (Fig. 6a) and MG-63 (Suppl. Fig. 4) cells transfected either with ARAP1 or with control (luciferase) siRNAs were treated with TRAIL. The kinetics of TRAIL-induced apoptosis was analyzed by M30-FITC staining (caspase-3-mediated cleavage of cytokeratin 18). In contrast to the control cells, the activation of caspases in NCTC cells with downregulated ARAP1 was significantly attenuated—45 min after adding TRAIL, only 24.5% of siARAP1-treated cells were M30-positive, compare to 55.6% of siLuciferase-treated cells (Fig. 5a, 45′). Two hours after the treatment, both ARAP1 and luciferase siRNA-transfected cells displayed comparable M30 staining (not shown). MG-63 cells also behaved similarly, though with less pronounced differences between control and ARAP1 siRNA transfected cells (Suppl. Fig. 4). To exclude the possibility of an off-target effect influencing the kinetics of apoptosis in ARAP1 siRNA-treated cells, we blocked DR4 signaling in NCTC and MG-63 cells with DR4-blocking mAb and examined the effect on the kinetics of TRAIL-induced apoptosis. In comparison with the control block of DR6, in both cell lines the blocking of DR4 signaling slowed down the kinetics of TRAIL-induced cell death to a similar extent as did ARAP1 siRNA (Fig. 6a and Suppl. Fig. 4).

In addition to the quantification of TRAIL-mediated apoptosis, we also analyzed the activation of TRAIL-induced signaling in NCTC cells by Western blotting (Fig. 5b). NCTC cells with downregulated ARAP1 (siARAP1, lanes 7–12) did not change their total level of DR4 (compare the DR4 signal in siLuciferase with siARAP1), but they showed a marked diminution of caspase-8 processing and Bid or PARP cleavage.

In conclusion, our data show that in some cells, ARAP1 could participate in the regulation of DR4 cell surface localization and thus could affect the kinetics and efficacy of TRAIL-induced apoptosis via this receptor.

Discussion

The foremost requirements for the initial steps of TRAIL-induced apoptosis are the cell surface availability of its proapoptototic receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 and productive DISC formation. In addition to DISC-associated proteins, such as procaspases-8/-10, FADD and FLIPs, several other proteins have been reported to interact with the intracellular parts of DR4/DR5 and affect TRAIL-induced signaling. RIP1 co-immunoprecipitates with DR4 and is likely involved in the TRAIL-induced activation of Jun kinases and necrotic cell death [33, 34]. Different splice variants of IG20 interact with the intracellular parts of TRAIL receptors and either enhance or suppress TRAIL apoptotic signaling [35, 36]. Our effort to uncover novel DR4-interacting proteins that could regulate or participate in DR4-dependent signaling resulted in the discovery of the Arf and Rho GAP protein ARAP1. ARAP1 specifically interacts with the intracellular part (ICP) of DR4 both in yeast and in human cells. In contrast to the published sequence of ARAP1, we have found that the C-terminal, a DR4-interacting region comprising part of RA and the last PH domain, contains an alternative exon 30 within the fifth PH domain. At least in yeast, DR4(ICP) interacts only with this exonless variant of ARAP1. As we have shown in Suppl. Fig. 1, this ARAP1 (Δexon 30) is preferentially expressed in the majority of the tested human cell lines and primary cells. Exon 30 codes for 11 amino acids and is localized at the C-terminus of the 3-phosphoinositide binding motif [28], and

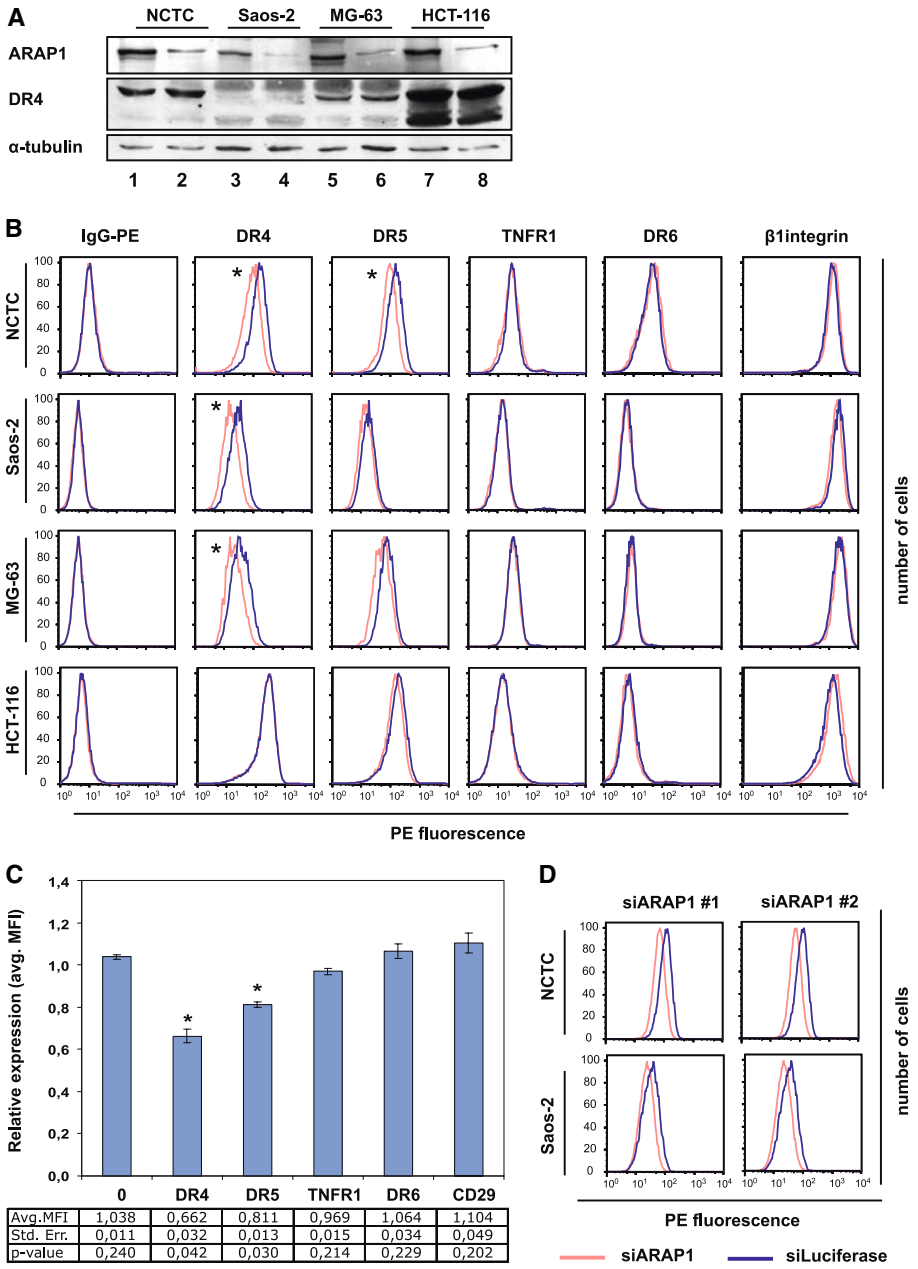

Fig. 5 ARAP1 knockdown compromises the cell surface localization of DR4. (**a**) Cell lysates from NCTC, Saos-2, MG-63 and HCT-116 cell lines transfected either with control luciferase siRNA (lanes 1, 3, 5, 7) or with ARAP1 siGENOME (lanes 2, 4, 6, 8) were stained on Western blots with antibodies against ARAP1, DR4 and α-tubulin (loading control). (**b**) Cells were grown in 24-well plates and transfected either with Luciferase siRNA (blue lines) or ARAP1 siGENOME (red lines). Fifty-two hours after transfection, the cells were harvested and stained with antibodies against DR4, DR5, TNFR1, DR6, β1 integrin or with the secondary GAM-PE antibody only (IgG-PE) and the living cells (Hoechst 33258 negative) were analyzed by flow cytometry. Representative histograms from at least four independent experiments are shown. (**c**) Relative representations of the averaged fluorescence medians of the cell surface expression (MFI siARAP1/MFI siLuc) of selected receptors in NCTC cells from four independent experiments. Standard Errors and statistical significances are shown (* = $P < 0.05$). (**d**) NCTC and Saos-2 cells were transfected with luciferase or two individual ARAP1 siRNAs and analyzed for DR4 cell surface expression as in (**b**)

thus its absence does not have to compromise the binding of phosphatidylinositols to the PH5 domain.

DR4 apparently interacts with ARAP1 through the N-terminal part of its death domain. The death domain of TRAIL receptors is essential for FADD-mediated DISC formation, and it also mediates interaction with RIP [33]. As ARAP1 was not detected at early time points in DR4 immunoprecipitate (Fig. 2c and Suppl. Fig. 2), it seems to be unlikely that it could affect DISC assembly or caspase-8 activation. Though obviously weak and transient, ARAP1 interaction with DR4 and, to a lesser extent, DR5 is likely to be specific for these two death receptors as the apparent interaction motif in the first DR4 α-helix/turn region is conserved only between DR4 and DR5 (Fig. 2b and Suppl. Fig. 2a). This first DR4 α-helix also contains FxDxF motif, which is apparently recognized by α-ear and PHEar domains from clathrin endocytosis accessory proteins AP-2 and Necap, respectively [37, 38]. As this motif overlaps with or is in the proximity of ARAP1-interacting region, it seems to be plausible that ARAP1 could also participate in

regulation of the spontaneous or activated clathrin-dependent endocytosis of DR4. Removal of the C-terminus of DR4 or DR5 (the last 16 or 13 amino acids, respectively) compromised FADD binding to the truncated receptors and suppressed TRAIL-induced apoptosis [39]. This C-terminal truncation apparently affected the structure or orientation of their death domains and also negatively influenced the interaction between DR4 and ARAP1-C (Fig. 2b). In contrast to the published ARAP1 localization in Golgi [28], we detected both transfected and endogenous ARAP1 at the cell membrane where it co-localized with DR4 (Fig. 3a) or co-fractionated in light membrane fractions (Suppl. Fig. 5). DR4 and ARAP1 were also associated within the ER/Golgi compartment, but neither ARAP1 nor DR4 were present in Golgi membrane-containing fractions in a sucrose gradient (Suppl. Fig. 5). Miura et al. published incomplete ARAP1 cDNA lacking the N-terminal SAM domain, and thus this clear discrepancy in ARAP1 cellular localization is likely to be attributed to this missing SAM domain. Moreover, the PH domains of ARAP1 and its relative ARAP3 preferentially interact with plasma membrane bound PtIns(3,4,5)P_3 or PtIns(3,4)P_2 and not with Golgi-associated PtIns phosphates [28, 40]. Thus, it seems likely that transient interaction between DR4 and ARAP1 takes place at the plasma membrane or on the way to the plasma membrane and could affect DR4 localization and/or function.

Transient ARAP1Δexon30 overexpression did not affect either DR4 cell surface expression or TRAIL-induced apoptosis in HEK293FT or HeLa cells. Indeed, analysis of HEL (which express only DR4) clones producing ARAP1Δexon30 confirmed these data (no significant changes in DR4 expression or in the kinetics of TRAIL-induced apoptosis) with one notable exception. Cells with overexpressed ARAP1 significantly more efficiently presented phosphatidyl serine (PS) at their cell surface upon activation of TRAIL-induced apoptosis (Fig. 4). This phenomenon was not accompanied by their increased apoptosis and thus could be related either to ARAP1-mediated enhancement of scramblase or to more efficient suppression of floppase activities. Enhanced PS externalization also occurs independently of apoptosis and could be induced by the aggregation of GPI-anchored proteins or by low levels of tyrosine phosphatase CD45RB in T lymphocytes [41, 42]. ARAP1 overexpression in HeLa cells induced the formation of actin-containing peripheral blebs [28]. The ARAP1 relatives ARAP2/3 bear a high affinity for Arf6 and RhoA and participate in the regulation of focal adhesion and cell spreading [43–45]. Thus, overexpressed ARAP1 can somehow disturb plasma membrane homeostasis and modulate the activities of aminophospholipid-distributing enzymes.

In contrast to cells with overexpressed ARAP1, its siRNA-mediated down-regulation led in several cell lines to the decreased cell surface expression of DR4 as well as to a markedly slower initial phase of TRAIL-induced apoptosis (Figs. 5 and 6). The cell surface localization of non-interacting death receptors (DR6) or other receptors (β1-integrin, HLA-1) was almost or entirely unaffected. Though it was reported that DR4 and other death receptors reside primarily in the trans-Golgi network (TGN) [19], in NCTC cells either overexpressed or endogenous DR4 was predominantly localized in the endoplasmic reticulum and at the plasma membrane (Fig. 3, Suppl. Fig. 5 and not shown). Hence, ARAP1 down-regulation could disturb DR4 trafficking from the intracellular stores to the plasma membrane. We reproducibly observed a correlation between the loss of DR4 plasma membrane localization and its increased accumulation in the ER/Golgi-containing pellet in cells with downregulated ARAP1 (Suppl. Fig. 5). In addition to its Arf1 and RhoA GAP activities, ARAP1 can function as a vesicle coat protein and directly interact with and regulate the trafficking of cargo proteins such as DR4. This was proven for its relative from the AZAP family, ASAP1, which is directly involved together with ARAP3 in the trafficking of the EGF receptor [23, 46]. The other members of this family, AGAP1 and AGAP2, associate with coat-protein complexes AP-3 and AP-1, respectively, and specifically regulate the recycling of the transferrin receptor [47]. Therefore, direct interaction between ARAP1 and DR4 could play a role in establishing and/or stabilizing DR4-containing transport vesicles. Several other reports also point to the specific regulation of DR4 trafficking in normal and transformed cells. A failure in the transport of DR4 to the plasma membrane was behind the acquired resistance of SW480 colon adenocarcinoma cells to TRAIL-induced apoptosis [48]. SiRNA-mediated knockdown of a 72 kDa subunit of the SRP (signal recognition particle) complex suppressed the plasma membrane localization of DR4 but not its total cellular level. No other tested death receptors (DR5, TNFR1 or CD95) were affected by this silencing [49]. Why this trafficking regulation is specific only for DR4 remains to be answered.

The availability of other death receptors such as Fas/CD95 is also regulated at the TGN-plasma membrane trafficking step. p53 activation can transiently increase Fas transport from the Golgi complex to the cytoplasmic membrane [20], and Fas-interacting proteins FAP-1 and dynamin-2 could play an opposite role in this process [50]. In contrast to FAP-1, dynamin-2 enhances Fas transport from the TGN to the cell surface. The regulation of death receptor activities through their mobilization to the cell surface from the TGN appears to be a very effective way to respond rapidly to sudden changes in the environment or within the cell. Bile salts induce rapid Fas-mediated apoptosis of hepatocytes through the enhancement of Fas

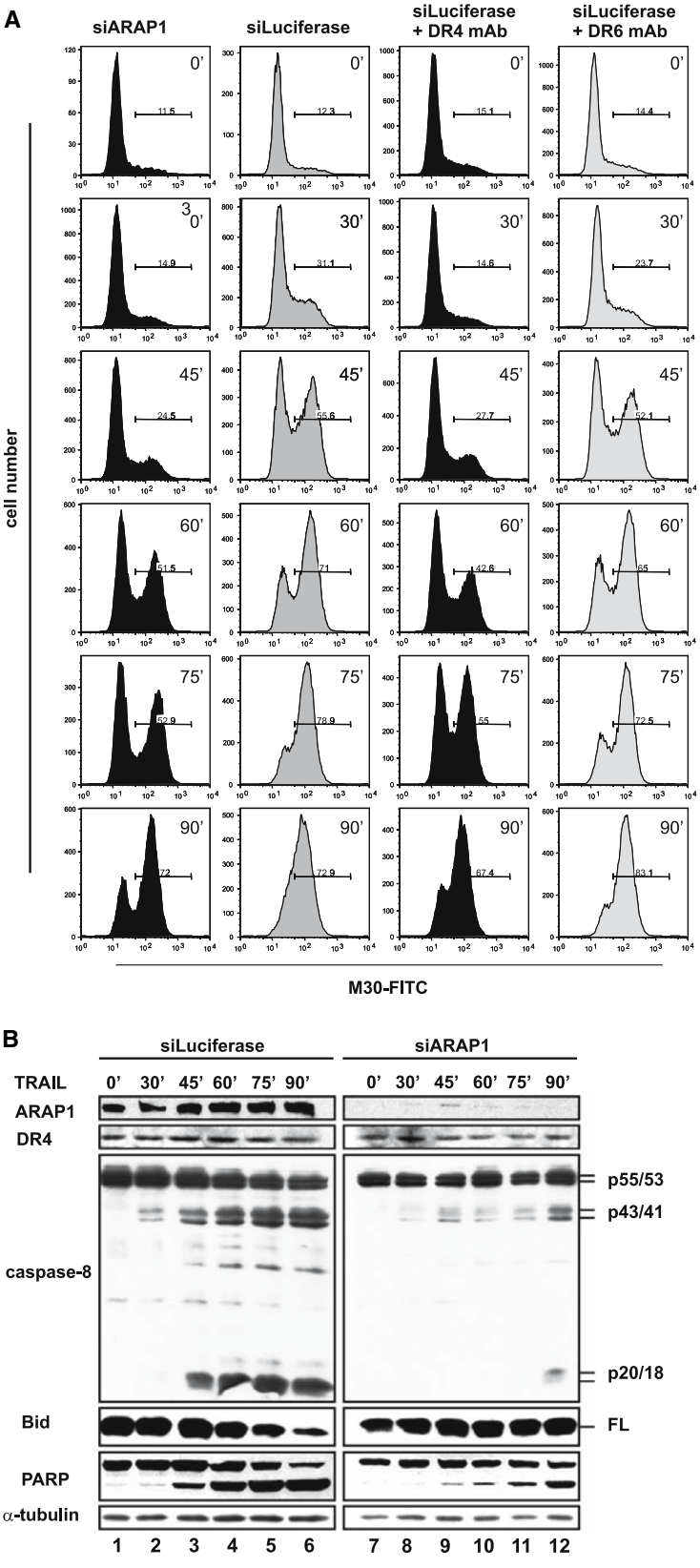

Fig. 6 Downregulation of ARAP1 decelerates TRAIL-induced apoptosis of NCTC cells. NCTC cells were grown in 24-well plates and transfected either with luciferase or with ARAP1 siRNA. Fifty-two hours post-transfection, some of the siLuc transfected cells were pre-incubated with blocking anti-DR4 or anti-DR6 monoclonal antibodies (at a final conc. of 10 μg/ml for 1 h), then the cells were treated with TRAIL (200 ng/ml) for 0, 30, 45, 60, 75, or 90 min, harvested and washed with ice-cold PBS. (**a**) Cells were fixed with ice-cold methanol, stained with FITC-conjugated anti-M30 antibody according to the manufacturer's instructions and analyzed by flow cytometry. The abscissa above the histograms with the indicated percentage represents the percentage of M30-positive, apoptotic cells. (**b**) Total cell lysates of luciferase or ARAP1 siRNA-treated NCTC cells taken at the indicated time points were analyzed by Western blotting using the corresponding antibodies (α-tubulin antibody was used as a loading control)

translocation from the Golgi to the cell membrane [51]. Mobilization of the intracellular stores of death receptors may also represent a self-destruction shortcut during malignant transformation. For example, malignant and normal epithelial cancer cells both express similar levels of DR4, but only transformed PC3 or JCA-1 cells have DR4 present at the cell surface and are sensitive to TRAIL-induced apoptosis [52].

In addition to possible ARAP1-mediated enhancement of DR4 transport to the plasma membrane, its co-localization with DR4 at this membrane suggests that ARAP1 could also affect DR4 plasma membrane stability or its correct localization. ARAP1 could be an important mediator of Arf or Rho GTPase-regulated trafficking of proapoptotic TRAIL receptors from the Golgi/trans-Golgi to the membrane and/or their endocytosis. The precise mechanism underlying ARAP1-affected DR4/DR5 trafficking is currently under investigation.

Conclusions

The Rho and Arf GAP adapter protein ARAP1 specifically interacts with the membrane proximal part of the death domain of DR4/TRAIL-R1 in both yeast and human cells. In contrast to the originally published sequence, DR4-interacting ARAP1 variant ARAP1Δexon30 lacks the alternatively spliced exon 30. Transfected ARAP1 co-precipitates with DR4 and co-localizes with it at the ER/Golgi and at the plasma membrane. Upon TRAIL treatment, internalized DR4 is endocytosed and subsequently co-localizes with ARAP1 in early endosomes. Endogenous DR4 and partially processed ARAP1 also co-immunoprecipitate from the lysate of TRAIL-treated NCTC cells. siRNA-mediated downregulation of ARAP1 markedly and specifically reduces the cell-surface localization of DR4 (and partly of DR5) in most of the tested human cell lines. The downregulation of the surface expression of DR4 results in slower TRAIL-induced apoptosis in two tested cell lines, comparable to the rate obtained by blocking the DR4 receptor with a monoclonal antibody. The stable overexpression of ARAP1 in HEL cells does not change the surface expression of DR4, but leads to more rapid exposure of phosphatidyl serine on the cell surface during TRAIL-induced apoptosis. ARAP1 could be an important regulator of DR4 trafficking from the ER/Golgi to the cellular membrane.

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Selective BRAFV600E Inhibitor PLX4720, Requires TRAIL Assistance to Overcome Oncogenic PIK3CA Resistance

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Abstract

Documented sensitivity of melanoma cells to PLX4720, a selective BRAFV600E inhibitor, is based on the presence of mutant $BRAF^{V600E}$ alone, while wt-BRAF or mutated KRAS result in cell proliferation. In colon cancer appearance of oncogenic alterations is complex , since BRAF, like KRAS mutations, tend to co-exist with those in PIK3CA and mutated PI3K has been shown to interfere with the successful application of MEK inhibitors. When PLX4720 was used to treat colon tumours, results were not encouraging and herein we attempt to understand the cause of this recorded resistance and discover rational therapeutic combinations to resensitize oncogene driven tumours to apoptosis. Treatment of two genetically different $BRAF^{V600E}$ mutant colon cancer cell lines with PLX4720 conferred complete resistance to cell death. Even though p-MAPK/ERK kinase (MEK) suppression was achieved, TRAIL, an apoptosis inducing agent, was used synergistically in order to achieve cell death by apoptosis in $RKO^{BRAFV600E/PIK3CAH1047}$ cells. In contrast, for the same level of apoptosis in $HT29^{BRAFV600E/PIK3CAP449T}$ cells, TRAIL was combined with 17-AAG, an Hsp90 inhibitor. For cells where PLX4720 was completely ineffective, 17-AAG was alternatively used to target mutant $BRAF^{V600E}$. TRAIL dependence on the constitutive activation of $BRAF^{V600E}$ is emphasised through the overexpression of $BRAF^{V600E}$ in the permissive genetic background of colon adenocarcinoma Caco-2 cells. Pharmacological suppression of the PI3K pathway further enhances the synergistic effect between TRAIL and PLX4720 in RKO cells, indicating the presence of $PIK3CA^{MT}$ as the inhibitory factor. Another rational combination includes 17-AAG synergism with TRAIL in a $BRAF^{V600E}$ mutant dependent manner to commit cells to apoptosis, through DR5 and the amplification of the apoptotic pathway. We have successfully utilised combinations of two chemically unrelated $BRAF^{V600E}$ inhibitors in combination with TRAIL in a $BRAF^{V600E}$ mutated background and provided insight for new anti-cancer strategies where the activated PI3KCA mutation oncogene should be suppressed.

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Introduction

For a long time it has been appreciated that presence of KRAS mutations highly correlates with colorectal cancer (CRC) progression and decreased patient survival. In addition, the more recently identified BRAF mutations in CRC, do not co-exist with those in KRAS and display a more potent transforming activity to be associated with progression to metastasis [1,2]. Notable among the apoptosis-inducing stresses are signalling imbalances resulting from elevated levels of oncogene signalling, as mentioned earlier, and DNA damage associated with hyper-proliferation. In contrast to KRAS mutations, those in BRAF have the ability to cause genomic rearrangements in colon cells that can potentially sensitize them to apoptosis a major advantage in cancer therapeutics, since deregulation of apoptosis can lead to growth advantage in cancer cells. Yet other research has revealed how apoptosis is attenuated in those tumours that succeed in progressing to states of high-grade malignancy and resistance to therapy [3,4]. The goal of most anti-tumour therapies, including chemotherapy, radiation or newer targeted therapies is to ultimately induce the death of tumour cells. Different chemotherapies induce death of tumour cells by different mechanisms. These include both apoptotic forms of cell death, as well as non-apoptotic mechanisms such as autophagy, necrosis and mitotic catastrophe [5]. However, the fraction of tumour cells that undergo non-apoptotic death are significantly increased if apoptosis-related mechanisms are inhibited [6]. Constitutive activation of MAPK has been found in many different tumor cell lines and primary tumors including colon cancer cells and tissues [7–9]. High expression and constitutive activation of PI3K is also found in gastric cancer and CRC [10]. Prominent among cell surface molecules capable of initiating and tightly controlling apoptosis in cancer cells is TRAIL, rendering it a promising anti-cancer agent [11,12].

TRAIL induces apoptosis via interacting with its death receptors (DRs), DR4 and DR5, which in turn results in death-inducing signalling complex (DISC) formation and caspase-8 processing [13]. Caspase-8 activation can then result in caspase-3 activation through the mitochondrial-independent pathway, and/or through the activation of Bid, a pro-apoptotic BH3-only Bcl-2 family member, which when cleaved induces the mitochondrial release of apoptogenic factors such as Bax and Bak through the mitochondrial-dependent pathway [14]. Despite the fact that during colorectal carcinogenesis a marked increase in sensitivity to

TRAIL has been reported, cells like HT29 and RKO remain partially resistant to TRAIL-induced apoptosis [15].

The MAPKs that are activated by phosphorylation may also act as important modulators of various apoptosis-inducing signals, while the protective effect of extracellular signal-related kinase 1/2 (ERK1/2) on DR-induced apoptosis has been described [16,17]. Deregulation of this pathway by RAS and more recently BRAF oncoproteins induce constitutive ERK1/2 activation, thereby promoting cell growth and survival. Manipulation of the MAPK signalling pathway could be a powerful means of treatment for tumours with BRAF mutations especially those resistant to TRAIL. Difluorophenyl-sulfonamine (PLX4720) targeting $BRAF^{V600E}$ cancer cell proliferation has recently been described as potent and selective among the many tested clinically [18]. However application and dosage of this inhibitor should be scrutinised since in can bind wild-type BRAF in KRAS mutant cells and activate the MAPK pathway through CRAF The mechanism of RAF regulation presents another strategy for its inhibition [19–21].

The protein chaperone Hsp90 is required for the conformational maturation of several key signalling proteins, including PI3K, p-Protein Kinase B (pAKT), NF-kB, CRAF and more recently $BRAF^{V600E}$ [22,23]. Inhibition of Hsp90 function with a geldanamycin derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), has been shown to effectively inhibit Hsp90 function of malignant cells *in vivo* due to its great affinity for the activated polymerized form of the molecular chaperone at tolerable doses [24]. Although CRAF and ARAF are degraded in cells that are exposed to 17-AAG, wild-type BRAF is not found in an Hsp90 complex, while $BRAF^{V600E}$ degradation leads to MAPK inhibition, cell-cycle arrest, and apoptosis with concomitant antitumour activity *in vivo* [22].

Here we compare the selective $BRAF^{V600E}$ inhibitor PLX4720 with the Hsp90 inhibitor 17-AAG, in colon cancer cells. Recorded resistance to PLX4720 is attributed to the activating PIK3CA mutations that coexist with $BRAF^{V600E}$. Treatment with 17-AAG is more responsive, potentially due to the multiple oncogenic proteins that are Hsp90 clients. Cell death by apoptosis at these conditions is significantly facilitated when TRAIL is concomitantly administered, as compared to the innate sensitivity to TRAIL in colon cancer cells bearing a single $BRAF^{V600E}$ mutation.

Materials and Methods

Growth Inhibition Studies and Cytoxicity Assays

The Caco-2, Colo205, HT29, RKO, DLD-1 and SW620 human colon adenoma-carcinoma cell lines were obtained from ATCC. The Caco-BR clones constitutively expressing active $BRAF^{V600E}$ proteins and the Caco-NEO9-empty vector clones have been previously described [1,25]. For growth inhibition and cytoxicity studies we used the sulforhodamine B (SRB) assay. Methodology is listed in supplementary materials and methods (File S1). For assessment and quantification of TRAIL-induced apoptosis, the ELISA cell death kit by Roche (Indianapolis, IN) was alternatively used according to the manufacturer's protocol that is highly sensitive and detects apoptotic nucleosomes in cell lysates.

For blocking experiments cells were pre-incubated for 15 minutes with 2 μg/ml of the respective blocking antibody against DR4 and DR5 and then stimulated with TRAIL with and without pretreatment with 17-AAG. Photographs were taken using a Nikon Eclipse T-200 (Tokyo, Japan) inverted phase-contrast microscope equipped with an Olympus digital camera (Olympus SP-51OU2, Hamburg, Germany).

Three-Dimensional Culture

For three-dimensional culture experiments, cells were grown in 24-well plates on 20% Matrigel that was allowed to set for 15 minutes at 37°C in order to form a gel of 1 mm thickness. The bottom later was then covered with 2×10^4 cells mixed 1:1 with 4% Matrigel in a total volume of 600 μl. Growth medium containing 2% matrigel was replaced every 2 days and the cells were left to grow for 12–14 days to allow development of extensive tubule network, after which treatment were applied for indicated incubation times. Photographs of the three-dimensional cultures were taken using a Nikon Eclipse T-200 inverted phase-contrast microscope equipped with an Olympus digital camera. The nuclei were stained with Hoechst No. 33342.

Immunoblotting and Immunoprecipitation

Whole cell lysates were prepared with Nonidet P-40 (NP-40) buffer containing protease inhibitors and were subject to Western blot analysis or immunoprecipitation studies. Methodology and antibody information is listed in supplementary materials and methods (File S1). For immunoprecipitation of the NP-40 insoluble fractions, pellets were resuspended in NP-40 lysis buffer and sonicated three times for 10 sec at 4°C using an MSE Soniprep150 to give the insoluble fraction.

Suppression of $BRAF^{V600E}$ and $PIK3CA^{H1047}$ expression by RNA interference

The small inhibitory duplex shRNA oligo was cloned into the *Hind*III and *Bgl*II sites in pSUPER (Oligoengine, Seattle, WA). The sense strand of the shRNA pSUPER $BRAF^{V600E}$ insert was BRAFmutshRNA: gatccccGCTACAGAGAAATCTCGATttcaagagaATCGAG-ATTTCTCTGTAGCtttttggaaa (Hingorani et al., 2003). BRAFmutshRNA or vector control (pSUPER) plasmids or siRNA- $PIK3CA^{H1047}$ (Darmacon) were transiently expressed into cells using lipofectamin (Sigma).

DISC Analysis

The ligand affinity precipitation was done using biotin-conjugated TRAIL (Bio-TRAIL) in combination with streptavidin-agarose beads. Methodology and antibody information is listed in supplementary materials and methods (File S1). Ligand affinity precipitates were washed 5 times with lysis buffer and the protein complexes were eluted from the beads by the addition of 30 μl SDS sample buffer and heating at 95°C for 15 minutes. Proteins were separated in SDS-PAGE and immunoblotted.

Immunofluorescence Microscopy

Immunostaining methodology is listed in supplementary materials and methods (File S1). Briefly, cells were incubated with indicated primary antibodies for 2 hours at room temperature, while the secondary antibody was applied to the cells for 1 hour also at room temperature. The nuclei were stained with Hoechst and visualized with a Leica TCS SPE confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany).

Flow cytometry and apoptosis assays

For immunostaining, cells were incubated with 50 μg/ml anti-DR4 or anti-DR5 on ice for 30 minutes. Surface expression of the receptors on living cells (Hoechst negative) was analyzed using a LSRII flow cytometer (BD Biosciences). M30 Cytodeath assay of caspase-3-cleaved cytokeratine 18 or Annexin V-FITC/ Hoechst staining were used for the assessment and quantification of TRAIL-induced apoptosis according to the manufacturer's protocols. Methodology is listed in supplementary materials and methods (File S1).

Semiquantitative RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Karlsruhe, Germany). The extracted total RNA (3 μg) was reverse transcribed into cDNA using the SuperScriptt II Reverse Transcriptase (Invitrogen) according to the manufacture. RT–PCR amplification was performed as previously described and intensity values were measured using Molecular Dynamics ImageQuant Software. All PCR products were normalized to GAPDH expression [26].

Results

A small therapeutic window for PLX4720 efficacy in BRAF mutant cells

To identify molecular modifiers in currently resistant cells that influence the sensitivity of BRAF mutant cells to TRAIL induced apoptosis, we recorded a panel of colon cancer cell lines to determine their response to the BRAF inhibitor PLX4720, which targets the mutated V600E form of BRAF. Given the frequent co-occurrence of KRAS/ BRAF and PI3K pathway lesions, two of the selected cell lines harbored both BRAF and PI3K pathway mutations. In response to PLX4720 no concentration dependence was observed for any of the cell lines tested and only a small inhibitory effect was achieved upon treatment with the highest concentration in HT29 and RKO cells (Figure 1A). Treatment with PLX4720 managed to inhibit the phosphorylation of BRAF kinase to a degree as compared to the increased pCRAF levels only in the $BRAF^{V600E}$ mutant cell lines (Colo205, RKO and HT29). Further downstream the MAPK pathway, the inhibitory effect was more pronounced with a sustained inhibition mainly on pMEK and pERK levels in cell lines harboring BRAF and PI3K pathway mutations. Sustained inhibitory effect on the MAPK pathway had an immediate effect on cyclin D1 target gene, all be it in a much shorter (1–4 hours) incubation period (Figure 1B, Figure S1). The inhibitory effect of PLX4720 on MEK pathway in shorter rather than longer incubation periods indicates a potentially small therapeutic window for treatment efficacy. As expected, cell lines that are wild-type for BRAF (Caco-2) or KRAS mutant (DLD-1) increased their proliferation rate by about 20% as compared to control untreated cell following treatment with PLX4720, independently of their doubling, that was also described by the increase of pMEK and pERK levels in these cells (Figure 1A–B).

Potent inhibition of cell growth in $BRAF^{MT}$ and activated $BRAF^{WT}$ cells using 17-AAG

Attenuation of the MAPK pathway through mutant BRAF inactivation by 17-AAG, which inhibits Hsp90 function, could underline reversal of observed TRAIL resistance. The same panel of colon cancer cell lines was monitored for their response to 17-AAG inhibitor. Significant cytotoxicity was observed in one BRAF mutant cell line (HT29), while considerable was the extent of cell toxicity in Colo205, RKO and SW620, cell lines with distinct genetic background. Nevertheless, sensitivity seemed to correlate to pATK levels rather than the PI3K mutation itself (Figure 2A, 1B). Regardless the co-occurrence of BRAF and PI3K pathway mutations in RKO and HT29 cells, basal pAKT levels were significantly increased only in the RKO cells indicating a diminished activating potential for the PIK3CA mutation (P449T) present in HT29 cells. Notably, sensitivity to 17-AAG was observed only in cell lines with moderate pAKT levels (HT29, DLD-1, Colo205 and SW620) (Figure 2A, 1B). Degradation of mutant BRAF in HT29 cells but also wild-type BRAF in DLD-1 cells, activated by co-expression with mutant KRAS, account for the inhibitory effect observed in cell growth (Figure 2B). To determine whether $BRAF^{V600E}$ is an Hsp90 client protein, we tested its sensitivity using as a control CRAF, a known Hsp90 client. CRAF expression was much more rapidly and efficiently degraded. As expected, the reduction in CRAF was accompanied by decreased ERK phosphorylation only in BRAF/ KARS mutant cells (Figure 2B). By testing whether BRAF and Hsp90 associate with each other, we established that both mutant BRAF and wild-type BRAF activated by the upstream mutant KRAS, are Hsp90 client proteins in HT29 and DLD-1 cells respectively. The kinase-specific Hsp90 co-chaperone p50cdc37 was also found to co-precipitate with mutant BRAF (Figure 2C, Figure S2). Surprisingly, as previously shown by Dias and colleagues, we also did not observe any binding of CRAF to Hsp90 in any cell line (data not shown) possibly for reasons previously discussed [27]. Taken together, either mutated or activated BRAF is more dependent on Hsp90-chaperone for its folding, stability and oncogenic activity than wild-type inactive protein.

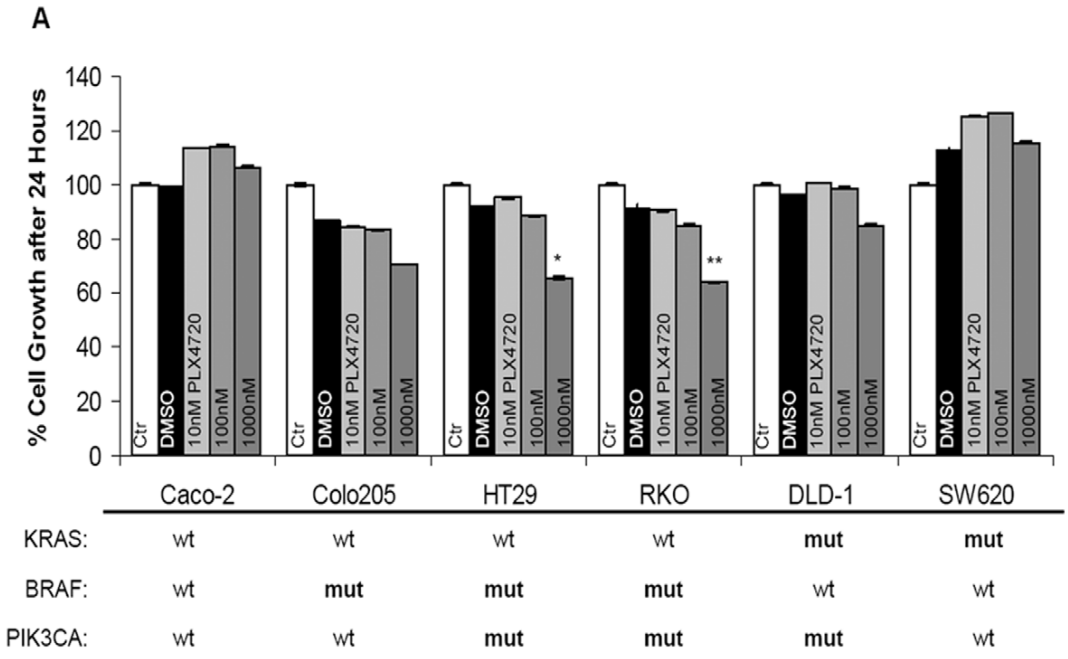
PLX4720 cooperates with TRAIL to commit cells to apoptosis

In an attempt to render resistant colon cancer cell more sensitive to RAF inhibitor treatment with PLX4720, TRAIL was used as a synergistic agent. Resistance to TRAIL treatment as a single agent appeared to strongly correlate with the PI3K pathway mutational status. TRAIL conferred limited cytotoxicity in BRAF mutant cells harboring activating mutations in PIK3CA whereas, extensive apoptosis was recorded in cells like Colo205 harboring a single BRAF mutation, at very small doses (10 ng/ml), comparable to the sensitive DLD-1 cells (Figure 3A). As expected, highly metastatic SW620 colon carcinoma cells were completely resistant to TRAIL as were also the intermediate adenocarcinoma Caco-2 cells. Treatment of cells with PLX4720 followed by co-concomitant administration with the lowest dose of TRAIL (10 ng/ml) induced increased cell death only in cells (RKO) harboring a BRAF mutation and high pAKT levels. The combination of PLX4720 with TRAIL was not beneficial to already sensitive to TRAIL cells, but was additive in cells harboring a BRAF mutation only (Colo205) (Figure 3B). Notably previously sensitive to TRAIL DLD-1 cells were rescued during the combined treatment of TRAIL with PLX4720 suggesting that the presence of wild-type BRAF may interfere with the apoptotic outcome. Conferred resistance to the therapeutic combination by wild-type BRAF was also confirmed in two independent and genetically similar colon cancer cell lines, DLD-1 and HCT116 (Figure S3), indicating only wild-type BRAF as the exclusionary factor for colon cancer treatment with PLX4720.

The combination efficacy of BRAF inhibition and TRAIL treatment was also applied to a three-dimensional (3D) cell culture system as an *in vitro* tumor model where a significant apoptotic effect was recorded. After two weeks of cell growth and tubule formation within the spheroid structures, PLX4720/ TRAIL induced caspase-3 activation and nuclear fragmentation was evident (Figure 3C). This provides further evidence that this combinatorial treatment can also be efficient *in vivo*.

Combined inhibition of BRAF and PIK3CA increases TRAIL induced apoptosis in RKO cells

The antiproliferative effect in response to the combined treatment involving the mutant BRAF inhibition and the induction of the apoptotic TRAIL pathway could be related to the PI3K mutational status. In order to answer this question, the molecular mechanism underlying this effect, was analyzed.



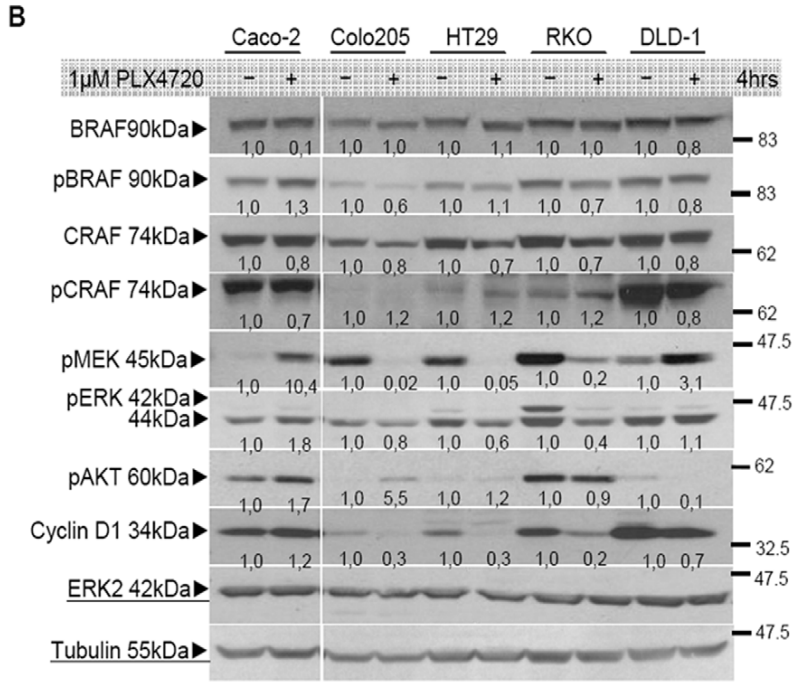


Figure 1. Presence of BRAF mutations are not selective for efficient PLX4720 treatment in colon cancer cells. (**A**) Cell survival for a panel of colon cancer cell lines treated with the selective for mutant BRAF pharmacologic inhibitor PLX4720. The values are the average of three independent experiments and are presented as fold change of the absorbance of treated/ untreated cells, for each condition. The number of viable cells was assayed by SRB 24 hours after treatment. Treatment conditions are provided along the top on each column. *$P<0.05$, **$P<0.01$ vs Ctr-untreated. (**B**) Total cell lysates harvested from cells treated for 4 hours with 1 μM PLX4720 were immunoblotted for the indicated antibodies. Proteins are quantified against α-tubulin and pERK levels against total ERK using the untreated condition of each cell line as a reference to the treated one.
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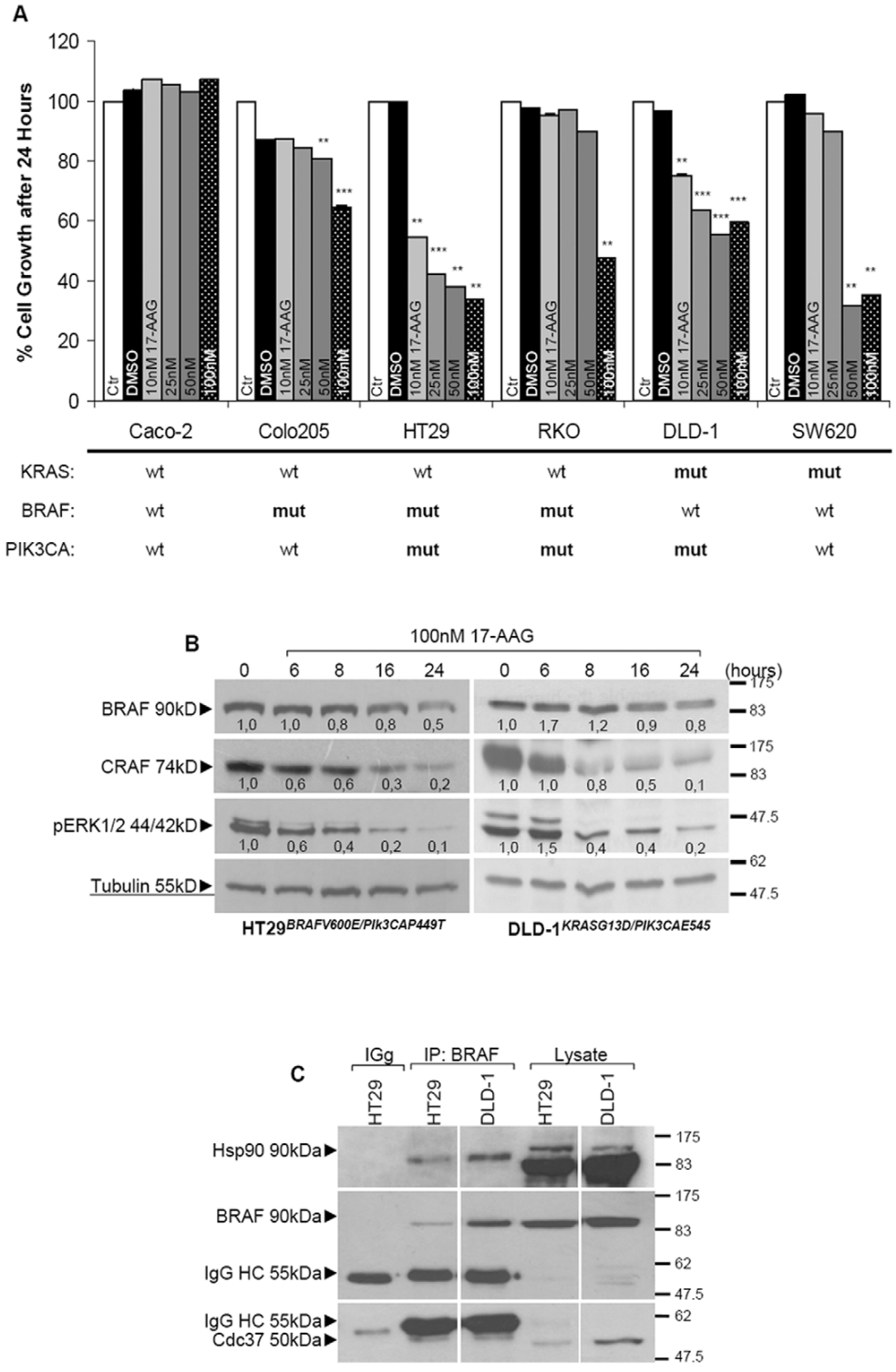

Figure 2. Presence of BRAF^{V600E}, an Hsp90 client protein, confers sensitivity to 17-AAG in colon cancer cells. (**A**) Cell survival for a panel of colon cancer cell lines treated with the Hsp90 pharmacologic inhibitor 17-AAG. The values are the average of three independent experiments and are presented as fold change of the absorbance of treated/ untreated cells, for each condition. The number of viable cells was assayed by SRB 24 hours after treatment. Treatment conditions are provided along the top on each column. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs Ctr-untreated. (**B**) Total cell lysates harvested from cells treated for indicated time points with 100 nM 17-AAG were immunoblotted for the indicated antibodies. Proteins are quantified against α-tubulin. (**C**) Total cell lysates from indicated cell lines were immunoprecipitated with BRAF and the complexes were immunoblotted with Hsp90, BRAF and Cdc37, which is right bellow the heavy chain (HC) of the antibody.
doi:10.1371/journal.pone.0021632.g002

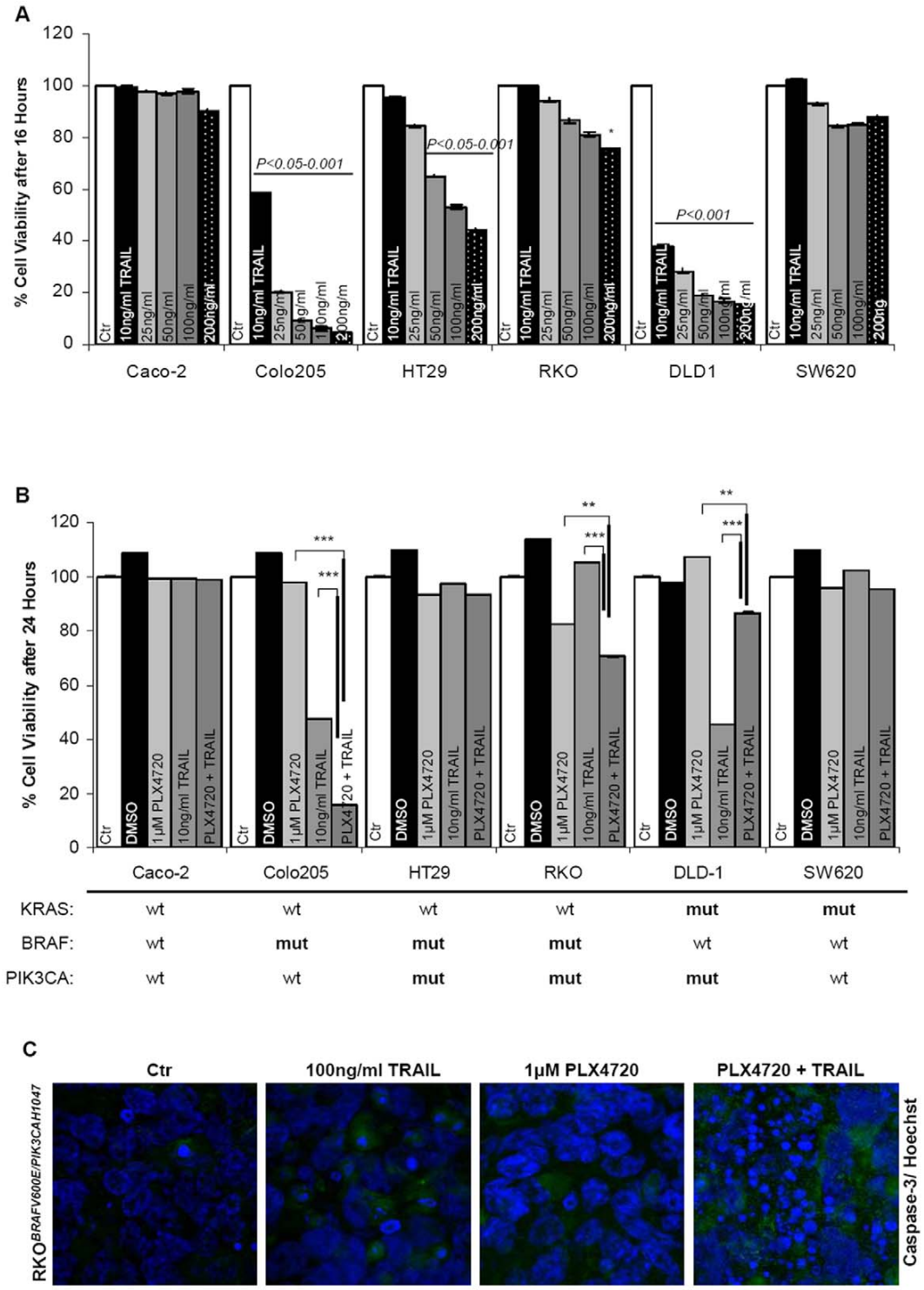

Figure 3. PLX4720 sensitises RKO cells to TRAIL-induced apoptosis. (**A**) Cytotoxic effects of the apoptotic agent TRAIL in a panel of colon cancer cell lines. The values are the average of three independent experiments and are presented as fold change of the absorbance of treated/ untreated cells, for each condition. The number of viable cells was assayed by SRB 16 hours after treatment. Treatment conditions are provided along the top on each column. *$P<0.05$, **$P<0.01$, ***$P<0.001$. (**B**) Combined treatment with 1 μM PLX4720 for 8 hours and concomitant administration of 10 ng/ml TRAIL for 16 hours in a panel of colon cancer cell lines. Cell viability assayed by SRB 24 hours after treatment. (**C**) Caspase-3 activation analysed by immunofluorescence 24 hours after induction with TRAIL/ PLX4720 alone or in combination, in a 3D culture of RKO cells. Representative confocal immunofluorescence images, double labelled with Hoechst (blue) and active caspase-3 (green). Original magnification 63x.
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Depletion of either mutant BRAF or PIK3CA, using PLX4720 or the PI-103 inhibitor, that block PI3K signaling downstream of p110a, did not induce any apoptotic response (Figure 4A, lanes 2–3) nor did their combination (Figure 4A, lane 5). When TRAIL was used alone, partial apoptosis was recorded (Figure 4A, lane 4). However, combining the inhibition of mutant BRAF with TRAIL treatment induced several markers of apoptosis including cleavage of Poly ADP (Adenosine Diphosphate)-Ribose Polymerase (PARP) and caspases-3 activation (Figure 4A, lane 6). Inhibition of PIK3CA and concomitant TRAIL treatment did not induce a significant apoptotic response (Figure 4A, lane 7). A more potent growth-inhibitory effect was elicited by the combination of the PI3KCA inhibition and the concomitant treatment with PLX4720 and TRAIL in RKO cells that resulted in increased apoptotic cell death (Figure 4A, lane 8). Replacement of the PIK3CA-p110a inhibitor (PI-103) with a siRNA against the $PIK3CA^{H1047}$ mutant in RKO, did not manage to increase apoptotic cell death any further as compared to PI-103 (Figure 4A, lanes 13–14). BRAF depletion using shRNA against mutant BRAF instead of PLX4720 likewise proved less efficient in co-treatment protocols involving TRAIL (Figure S4).

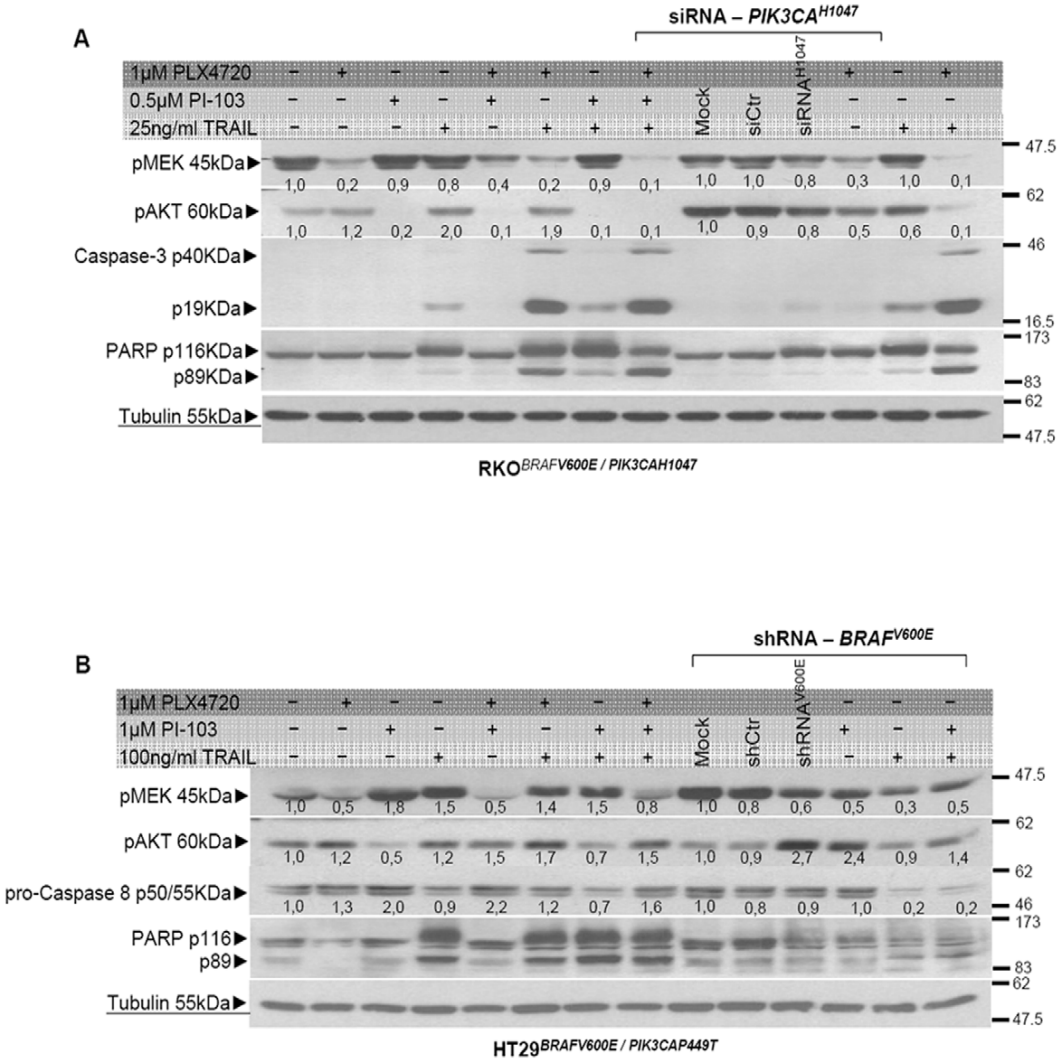

Figure 4. Inhibition of PIK3CA increases the combination efficacy of TRAIL with PLX4720 in RKO cells. (**A**) Cells left untreated or treated with either 1 μM PLX4720 or a PIK3CA pharmacologic inhibitor PI-103 (0.5 μM) for 24 hours. Combined treatment of PLX4720 for 8 hours with concomitant administration of 25 ng/ml TRAIL for 16 hours was performed along side with TRAIL treatment alone for 16 hours. Combined inhibition of mutant PI3K and BRAF using 0.5 μM PI-103 and 1 μM PLX4720 for 8 hours following concomitant administration of 25 ng/ml TRAIL for 16 hours. Alternative depletion of mutant PIK3CA by transient transfection of 1.6 pmol siRNA against the PIK3CAH1047 present in RKO cells in combination with all indicated parameters. Total cell lysates harvested from the indicated treatments were analyzed for the phosphorylation of downstream targets and induction of apoptotic markers. (**B**) Cells left untreated or treated with either 1 μM PLX4720 or 1 μM PI-103 for 24 hours. Combined treatment with PLX4720 for 8 hours and concomitant administration of 100 ng/ml TRAIL for 16 hours was performed along side with TRAIL treatment alone for 16 hours. Combined inhibition of mutant PI3K and BRAF using PI-103 and PLX4720 following concomitant administration of TRAIL and alternative depletion of mutant BRAF by transient transfection of 7 μg shRNA against the BRAFV600E present in HT29 cells in combination with all indicated parameters.

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Inhibition of PIK3CA increases TRAIL induced apoptosis in HT29 cells

When the same experimental approach was applied to HT29 cells, the combination of BRAF inhibition using PLX4720 and TRAIL treatment was not any more advantageous than TRAIL alone (Figure 4B, lanes 4 and 6). Depletion of PIK3CA using PI-103 and concomitant treatment with TRAIL, rendered cells slightly more prone to apoptosis, evident by increased PARP cleavage and the decrease in pro-caspase-8 protein expression (Figure 4B, lane 7). Nevertheless, the combination of the PI3KCA inhibitor and concomitant treatment with PLX4720 and TRAIL did not increase apoptotic cell death in HT29 cells that are genetically similar to the RKO cell line and sensitive to the combination just described (Figure 4B, lane 7). Even though, the shRNA against mutant BRAF in RKO cells was not very efficient, possible because of highly activated BRAF, its application in HT29 seems to be able to sequester pMEK about 2-fold (Figure 4B, lane 11) without any obvious impact in amplifying apoptosis for any of attempted combination treatments.

17-AAG overcomes TRAIL resistance in HT29 cells through enhanced apoptosis

Pretreatment of colon cancer cells with 17-AAG following treatment with TRAIL significantly sensitized HT29 to TRAIL and had a more mild effect on DLD-1 cells. As previously, the combination of 17-AAG with TRAIL was not beneficial to cells already sensitive to TRAIL (Colo205), nor seemed to be related to the mutational background of the cells (Figure 5A). The synergistic effect of 17-AAG and TRAIL significantly increased cell death as compared to each drug alone, while the sensitivity to 17-AAG did not correlate with the combination's cytotoxicity.

Sensitivity to pretreatment with 17-AAG appeared to be directly related to $BRAF^{V600E}$ protein degradation in HT29 cells (Figure 5B-upper panel). TRAIL alone has a sub-toxic effect on partially resistant HT29 cells, while their combinatorial treatment resulted in procaspase-8 processing and Bid cleavage leading to caspase-3 activation and PARP cleavage. This suggests amplification of the mitochondrial pathway to mediate apoptosis (Figure 5B-lower panel). Independent caspase-9 processing in the presence of 17-AAG alone, suggests a mechanism of activation dependent on degradation of either $BRAF^{V600E}$ or Hsp90 itself (Figure 5B). To determine whether this gained sensitivity to apoptosis was dependent on the mutant BRAF and not a non-specific effect of 17-AAG on other Hsp90 client proteins, attenuation of mutant BRAF in HT29 cells by $shRNA^{V600E}$ managed to decrease apoptotic cell death by 17-AAG (Figure 5C). Evidence of significantly impaired apoptosis was evaluated using the M30 CytoDEATH assay, which measures a cleaved fragment of cytokeratin 18 and Annexin V that measures apoptotic fractions (Figure 5C). Interaction between Hsp90 and mutant BRAF was impeded resulting to low BRAF/ pBRAF levels which appeared to interfere with the amplification of apoptotic cell death, previously attained by the combination of TRAIL with 17-AAG (Figure S5).

BRAFV600E incites rapid DISC formation and engagement of the apoptotic machinery in response to TRAIL

To further unravel the role of mutant BRAF as a single mutational event, previously established stable clones overexpressing the mutant protein in a permissive genetic background of Caco-2 cells were analyzed[1]. Significant cell death by apoptosis at the lowest dose of TRAIL was observed in Caco-2 clones overexpressing the mutant $BRAF^{V600E}$ (Caco-BR cells). The extent of apoptosis was comparable to that observed in Colo205 also bearing a single BRAF mutation, but also to that in DLD-1 cells (Figure 6A, Supporting Figure 6).

Analysis of the DISC complex formation in response to TRAIL within 30 minutes indicated that caspase-8 and FADD are recruited to the DISC far more efficiently in the Caco-BR13 and DLD-1 cells than in HT29 cells. In HT29 two mutational pathways are activated, the MAPK and the PI3K pathway, and may be antagonizing each other at the expense of remitting apoptosis (Figure 6B, Supporting Figure 7). Induction kinetics of apoptosis in Caco-BR cells showed that apoptosis was achieved within two hours with the lowest concentration of TRAIL, while only half time treatment with the highest dose was required for efficient induction of apoptosis, as indicated by several key apoptotic markers like proaspase-8 and Bid processing leading to PARP cleavage (Figure S8). Activation of Bax following expeditious mitochondrial release of proapoptotic Cytochrome C, activation of caspase-3 and chromatin condensation within 30 minutes of TRAIL induction was confirmed by immunostaining, correlated to DISC complex formation (Figure 6C).

PLX4720 requires TRAIL assistance to induce apoptotic cell death in $BRAF^{V600E}$ colon cancer cells

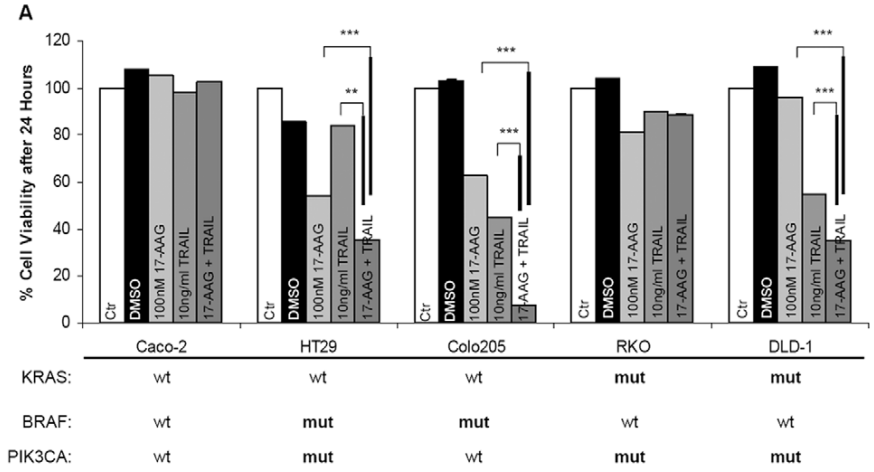
As previously observed the presence of mutant BRAF in colon cancer cells, does not predict cell sensitivity to PLX4720 nor did $BRAF^{V600E}$ overexpression in the mutational permissive background of Caco-2 cells (Figure 7A). Limited response to PLX4720 treatment is most likely due to the ambivalence overexpression and phosphorylation of CRAF in response to the mutant BRAF stable induction, but also due to the constitutive activation of MEK/ ERK phosphorylation (Figure S9). In order to facilitate cell death, TRAIL was administered in combination with PLX4720. Following exposure to low concentration of TRAIL, cells were sensitized to the combination whereas at higher concentration the advantage was lost due to the conferred sensitivity of $BRAF^{V600E}$ to TRAIL treated cells. Sensitivity of Caco-BR cells to TRAIL and conferred sensitivity following treatment with PLX4720 was also confirmed in 3D culture conditions. Caspase-3 activation and nuclear fragmentation indicated engagement to apoptotic cell death (Figure 7B).

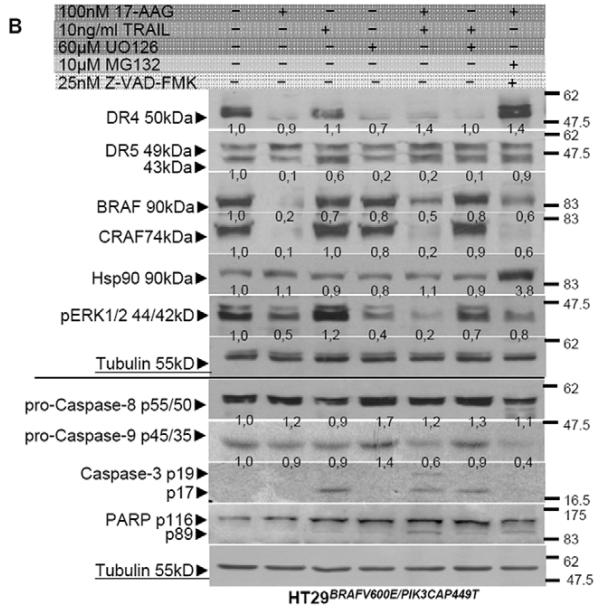
17-AAG requires longer incubation periods to induce cell death in the presence of dominant $BRAF^{V600E}$

The overexpression of mutant BRAF in colon adenocarcinoma cells had quite the opposite effect of the anticipated increase in sensitivity to the 17-AAG inhibitor. Significantly longer (4-day) treatment periods with 17-AAG were required to achieve an antiproliferative effect in Caco-BR cells (Figure 8A) comparable to that achieved in HT29 cells in 24 hours (Figure 2A). Partial degradation of mutant BRAF and insufficient MAPK pathway inhibition in Caco-BR cells overexpressing mutant $BRAF^{V600E}$, appear to preclude cell sensitivity to 17-AAG (Figure S10), regardless of the interaction between mutant BRAF and Hsp90 in the Caco-BR cells (Figure 8B).

Pathways for $BRAF^{V600E}$ sensitization of colon cells to TRAIL-induced apoptosis

Following mutant $BRAF^{V600E}$ overexpression, the phosphorylation of both BRAF/ CRAF kinases was significantly increased, which resulted in the downstream activation of MEK but not ERK signalling (Figure 9-upper panel, lanes 3–4). Detailed characterisation of this MAPK pathway activation and its cell tumourorigenic effects have been described elsewhere [1].





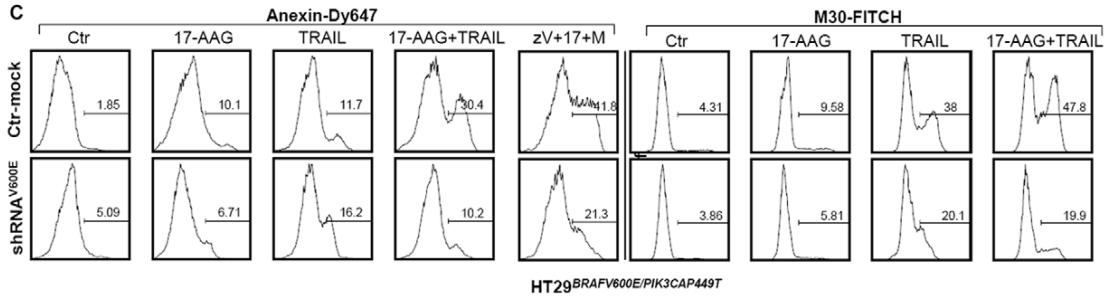


Figure 5. 17-AAG sensitises HT29 cells to TRAIL-induced apoptosis. (**A**) Cytotoxic effects of the combined treatment using 17-AAG and concomitant administration of TRAIL in a panel of colon cancer cell lines. The values are the average of three independent experiments and are presented as fold change of the absorbance of treated/ untreated cells, for each condition. The number of viable cells was assayed by SRB 24 hours after treatment. Treatment conditions are provided along the top on each column. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs Ctr-untreated. (**B**) Cells left untreated or treated with 100nM 17AAG for 24 hours. Combined treatment of 17-AAG for 8 hours and concomitant administration of 100 ng/ml TRAIL for 16 hours was performed along side with TRAIL treatment alone for 16 hours. Alternatively cells were pre-treated with 25 nM Z-VAD-FMK for 1 hour then 10 µM MG132 was added for another hour after which cell were treated with 100 nM 17-AAG for 22 hours. Finally cells were pre-treated

pretreated with 60 μM UO126 for 8 hours after which TRAIL was added for another 16 hours. (**C**) Depletion of mutant BRAF by transient transfection of 7 μg shRNA against the $BRAF^{V600E}$ present in HT29 cells in combination with all indicated parameters described above. Apoptotic cell death after 24 hours was assayed using the Annexin V and M30 CytoDEATH assay.
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Increased BRAF/ MEK phosphorylation in Caco-BR cells evoked the differential expression of some key apoptotic and anti-apoptotic molecules, altered in such way that facilitates apoptosis. Increased phosphorylation of the CRAF/ BRAF complex mainly observed in the TRAIL sensitive cell lines Caco-BR and DLD-1 cells correlated with DR4 and DR5 overexpression observed on the cell surface (Figure 9-lower panel, lanes 3-4 and 8; Figure S11). Pro-apoptotic proteins including Bad, Bid and Bax were also found overexpressed in Caco-BR and DLD-1 cells, while anti-apoptotic molecules like Bcl-2 remained unchanged (Figure 9-lower panel, lanes 3–4 and 8). Overexpressed Bad allows Bax to escape Bcl-2 control and drive apoptosis while Bid accumulation caused by $BRAF^{V600E}$ overexpression comprises the initial step towards the sensitization process. Moderate intrinsic pBRAF activity was found in HT29 and RKO cells regardless the presence of a BRAF mutation which was associated with potent activation of the MAPK pathway (Figure 9-upper panel, lanes 6–7). This observation raised the question whether sensitivity to TRAIL induced apoptosis was associated with the constitutive BRAF phosphorylation. Regardless activation of the CRAF/ BRAF complex and TRAIL receptor overexpression in RKO cells (Figure 9-upper panel, lane 7), resistance to TRAIL was recorded (Figure 3A), potentially due to the concomitant activating PIK3CA mutation. Towards this end, we hypothese that the sensitivity of DLD-1 cells to TRAIL is not affected by the concomitant presence of KRAS and PIK3CA mutations because of the constitutive activation of pBRAF in these cells (Figure 9). In contrast, Colo205 cells that did not overexpress the CRAF/ BRAF complex (Figure 9-upper panel, lane 5), but did express only marginally the TRAIL receptors and anti-apoptotic BID (Figure 9-lower panel, lane 5), were successfully committed to apoptosis possibly because of the dominant $BRAF^{V600E}$ presence (Figure 3A).

Ubiquitination of DR4 following treatment with 17-AAG affects its transcriptional regulation

Significant downregulation of DR4 on the cell surface was observed following treatment with 17-AAG alone or in combination with TRAIL in HT29 cells (Figure 5B-upper panel, lane 2; Figure S12). Immediately a question was raised, regarding the increased apoptosis attained under these conditions. Similar DR4 processing in the presence of 17-AAG was not observed in cells sensitive to 17-AAG inhibitor like DLD-1 and Caco-BR13 cells (Supporting Figures 13, 14). TRAIL alone did not cause any visible proteolysis to either of DRs nor to BRAF/ CRAF expression as compared to the significant degradation of RAF isoforms accompanied by decreased pERK in the presence of 17-AAG (Figure 5B-upper panel, lanes 2–3). The presence of the proteasome inhibitor MG132 managed to suppress 17-AAG-induced BRAF and DR4 degradation in HT29 cells, while CRAF was irreversibly degraded (Figure 5B-upper panel, lane 7). To determine whether DR4 downregulation resulted from decreased pERK, which accompanies BRAF/ CRAF degradation, HT29 cells were treated with the MEK inhibitor UO126 (Figure 5B-upper panel, lane 6) . Inhibition of MEK-ERK signaling resulted in DR4 degradation confirming our model and previous findings, regarding DR been regulated through a MEK-dependent pathway due to increased pERK in the presence of TRAIL and $KRAS^{G12V}$ [17].

By adopting the double inhibition strategy it was shown that $BRAF^{V600E}$ serves as an Hsp90 client, since a marked increase in RAF isoforms in the NP-40 insoluble fraction was observed (Figure 10A, lane 7). To our surprise DR4 was also detected in the same fraction suggesting that DR4 could also be an Hsp90 client protein targeted for degradation (Figure 10B, lane 7). Even though DR5 was not degraded during 17-AAG treatment, it was also found accumulated in the NP-40 insoluble fraction at all conditions (Figure 10B, lane 7). This is more likely to reflect DRs attached on the cell membrane and dragged into the insoluble. Immunoprecipitation of the insoluble fraction following the double inhibition treatment showed ubiquitin accumulation confirming our hypothesis that DR4 gets ubiquitinated upon treatment with 17-AAG, whereas attempts to co-immunoprecipitate Hsp90 and DR4/5, were unsuccessful (Figure 10B, lane 5). Furthermore, during the double inhibition treatment pERK activity remained suppressed in the cytosolic extracts, while DR4 was rescued. This should not negate MEK-ERK dependent regulation of DR4, since proteins found in an ubiquitinated state should have their signaling ability impaired, which is depicted by the selective mRNA downregulation of DR4 at these conditions (Figure S15). Nevertheless, regardless complete absence of DR4 in HT29 cells, apoptosis was amplified in the combined treatment via the DR5.

17-AAG sensitizes HT29 cells to TRAIL-induced apoptosis via DR5

To prove our hypothesis regarding DR5 being the mediator of apoptosis following treatment with 17-AAG, HT29 cells were pre-treated with a blocking antibody against DR4 or DR5 and then subjected to TRAIL treatment. Inhibition of cell death was achieved in the presence of the blocking Ab against DR5 but not against DR4, regardless TRAIL been administered alone or in combination with 17-AAG. In addition, inhibition of cell death was also achieved when both blocking antibodies were combined during pretreatment (Figure 11A-B). Data obtained so far suggest that increased apoptosis in HT29 cells after combined treatment of 17-AAG with TRAIL is mediated via DR5.

Discussion

Constitutive activation of signaling via EGFR, MAPK and PI3K pathways can promote uncontrolled cell growth, tumor cell survival as well as resistance to cytotoxic agents [28,29]. Inhibiting the source of activation, when due to activating mutations (KRAS, BRAF, PI3K), is a complex task. Inhibition of mutant $BRAF^{V600E}$ using the selective PLX4720 inhibitor, induces apoptosis in melanoma cells [18]. In contrast, the clinical activity seen with PLX4032 in metastatic CRC was more modest suggesting the biology behind this type of cancer is more heterogeneous [30]. Initiation and progression of CRC proceeds through a series of genetic alterations involving oncogenes and tumour suppressor genes, therefore prolonged and substantial suppression can only be achieved in combination therapy. In the present study we present the anti-proliferative effects of the pharmacological application of PLX4720 as a single agent or in combination with TRAIL, an inducer of apoptosis. Treatment resistance recorded after exposure to PLX4720, had not been anticipated considering studies reporting that BRAF mutant

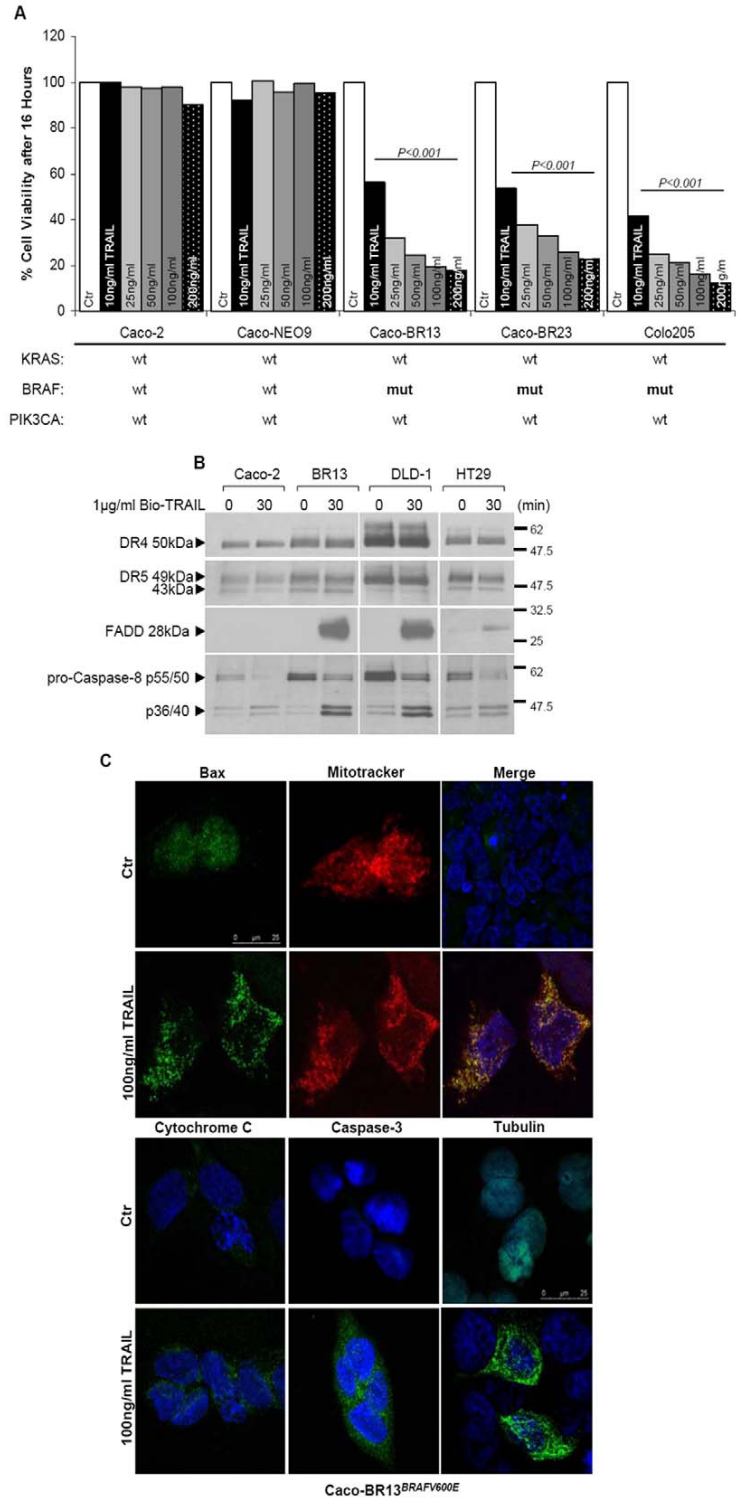

Figure 6. BRAF^{V600E} enhances DISC formation and apoptosis in response to TRAIL in colon cancer cells. (**A**) Cell survival for in Caco-BR and Colo205 cells treated with TRAIL. The values are the average of three independent experiments and are presented as fold change of the absorbance of treated/ untreated cells, for each condition. The cytotoxic effects of TRAIL were assayed by SRB. Treatment conditions are provided along the top on each column. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs Ctr-untreated. (**B**) DISC complex analysis of functional proteins bound to Bio-TRAIL at indicated time points. (**C**) Rapid induction of apoptosis within 30 minutes with respect to activation of apoptotic markers namely Tubulin, active caspase-3, cytochrome C and Bax were analysed by immunofluorescence after addition of TRAIL. Representative confocal immunofluorescence images, double labelled with Hoechst (blue), Mitotracker (Red) and indicated antibody (green). Original magnification 63x.
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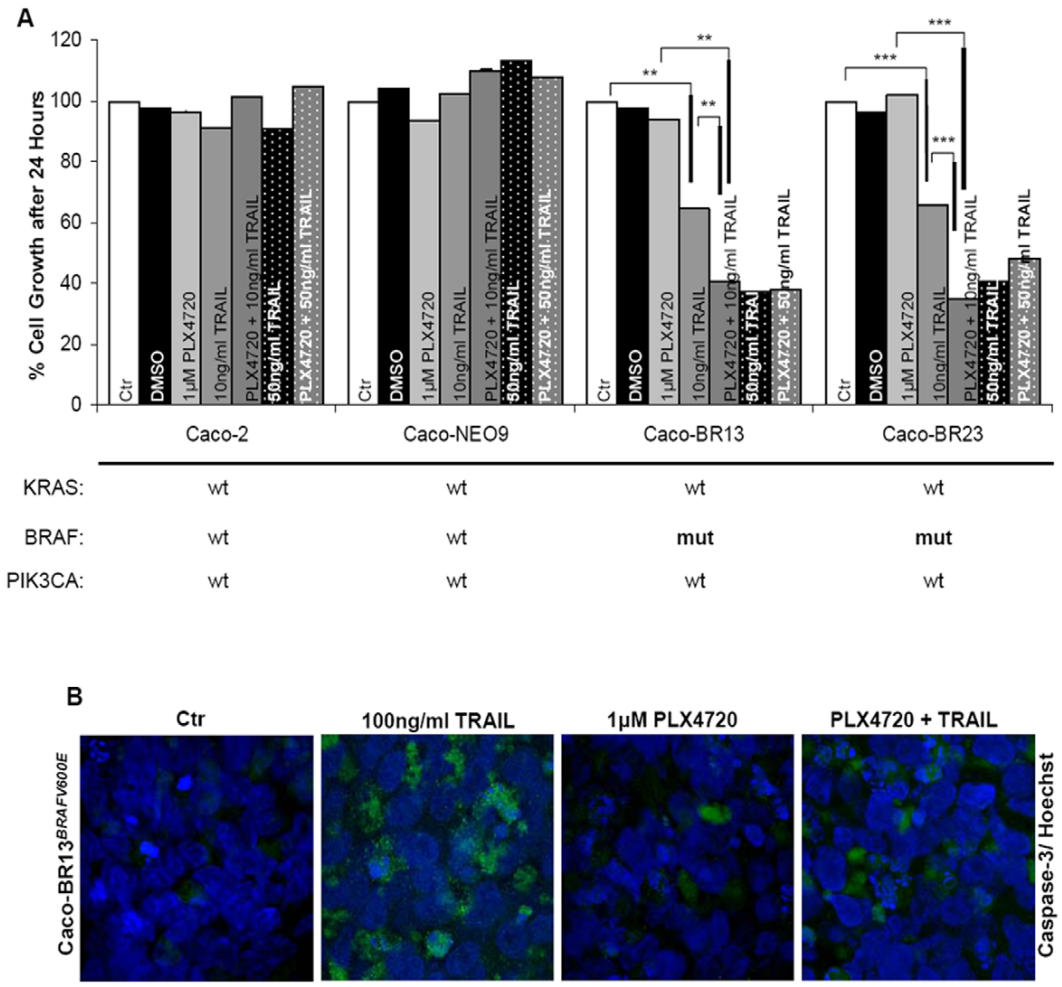

Figure 7. PLX4720 requires TRAIL synergy to induce an anti-proliferative effect. (**A**) Combined treatment with 1 μM PLX4720 for 8 hours and concomitant administration of a low (10 ng/ml) or higher (50 ng/ml) concetration of TRAIL for 16 hours in Caco-BR cells. The values are the average of three independent experiments and are presented as fold change of the absorbance of treated/ untreated cells, for each condition. The cytotoxic effects of TRAIL were assayed by SRB 24 hours after treatment. *$P<0.05$, **$P<0.01$, ***$P<0.001$. (**B**) Caspase-3 activation analysed by immunofluorescence 24 hours after induction with TRAIL/ PLX4720 alone or in combination, in a 3D culture of Caco-BR13 cells. Representative confocal immunofluorescence images, double labelled with Hoechst (blue) and active caspase-3 (green). Original magnification 63x.
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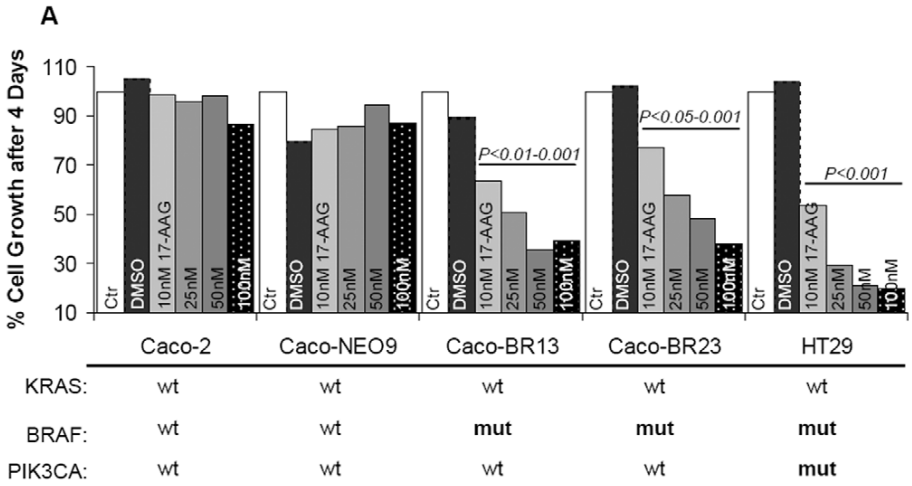
cancers are very sensitive to pharmacological MEK inhibitors and did not correlate to the mutational background of the cells [31]. In our study, even though inhibition of cell proliferation was not accomplished by PLX4720, successful inhibition of downstream targets pMEK/ pERK with a profound effect on cyclin D1 target gene was achieved in much shorter incubation periods indicating a small therapeutic window. This observation illustrates the need for supportive combinatorial treatment. The proliferative advantage previously described in cells bearing KRAS mutant and wild-type BRAF following treatment with PLX4720, was only observed for SW620 cells. In contrast, cell numbers of DLD-1 cells remained unchanged suggesting a possible implication of constitutive pBRAF in these cells. Towards this end, when PLX4720 was co-administered with TRAIL, in TRAIL sensitive DLD-1 cells, TRAIL induced apoptosis was abrogated indicating wild-type BRAF and not mutant KRAS, also present in DLD-1, as the exclusionary factor for colon cancer treatment with PLX4720.

Pharmacological application of an indirect $BRAF^{V600E}$ inhibitor, 17-AAG as a single agent was more efficient in colon cancer cells. The antiproliferative effect was not selective to a specific mutational background but to the levels of AKT phosphorylation. Treatment with 17-AAG was least effective in RKO cell line, where an increased phosphorylation of AKT is observed. Notably, recent studies have shown that $PIK3CA^{MT}$ and altered pAKT activity can be critical markers for optimal tumour treatment protocols [32,33]. Most encouraging was the selectively of 17-AAG towards SW620, a metastatic colon cancer cell line, both Fas and TRAIL-resistant [34]. Antiproliferative treatment with PLX4720 could not be implemented within the small therapeutic window predicted and the 17-AAG inhibitor could not eradicate tumour cells under short time exposure at low concentration, their combination with an apoptotic agent like TRAIL proved essential and could intensify the results on cancer cell death. Thus, whether the clinical goal is long-term exposure to low concentrations or intermittent administration of relatively higher doses, the present



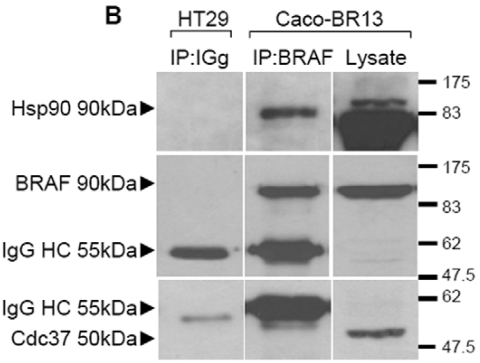


Figure 8. 17-AAG requires longer incubation periods to induce cell death. (**A**) Cell survival for Caco-BR and two colon cancer cell lines treated with 17-AAG. The values are the average of three independent experiments and are presented as fold change of the absorbance of treated/untreated cells, for each condition. The number of viable cells was assayed by SRB 4 days after treatment. Treatment conditions are provided along the top on each column. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs Ctr-untreated. (**B**) Total cell lysates of Caco-BR13 and HT29 cells were immunoprecipitated with BRAF and the complexes were immunoblotted with Hsp90, BRAF and Cdc37.
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study proposes rational drug combinations and their therapeutic application that appears to offer superior opportunities provided its tolerability in humans is acceptable, which will require phase I clinical evaluation.

In order to achieve rapid induction of apoptosis, combination of tested BRAF inhibitors with TRAIL in a concomitant approach was applied in cell lines exhibiting some degree of sensitivity to these inhibitors. When TRAIL was applied as a single agent conferred limited cytotoxity in colon cells harboring activating mutations in both the MAPK and PI3K pathways, whereas its effect was significantly increased in cells with a single BRAF mutation and accompanied by high pBRAF levels. If the presence of dominant $BRAF^{V600E}$ and high presentation of pBRAF serve as a sensitizing factor to TRAIL induced apoptosis, then the significantly activate wild-type BRAF in DLD-1 cell may also be a TRAIL sensitizer. The combination of PLX4720 with concomitant TRAIL induction managed to increase cell death by apoptosis in RKO cells but not in HT29, even though downstream targets like pMEK/ pERK levels were equally inhibited. Interestingly, the presence of PIK3CA mutation and the highly activated PI3K/Akt pathway in RKO cells does not inhibit the synergism between PLX4720 and TRAIL, which can be further exploited in the clinical application of these pharmacologic agents. The importance of this finding is further emphasized by the fact that pharmacologic MEK pathway inhibition of cell proliferation in BRAF mutant cancers may be significantly decreased by the presence of activating PIK3CA mutations [35].

In order to identify molecular modifiers that may influence cell sensitivity to apoptosis even further, the proposed role of mutant PIK3CA as an inhibitory factor was explored in our study [35]. Inhibitors of the PI3K/Akt signaling pathway gain considerable attention in the treatment of CRC, especially since PI3K/Akt pathway inhibition can modulate TRAIL induced apoptosis in HT29 cells [34]. Down-regulation of PI3K by siRNA may also sensitize colon cancer cells to TRAIL-induced apoptosis [36]. Herein, in order to improve cell response to BRAF inhibitor treatment, attenuation of the $PIK3CA^{H1047}$ that co-exist with $BRAF^{V600E}$ in the relevant colon cancer cell lines was performed. Deletion of PI3K using the PI-103 inhibitor alone or in combination with either PLX4720 or TRAIL ranged from inactive to moderate respectively and was only marginally

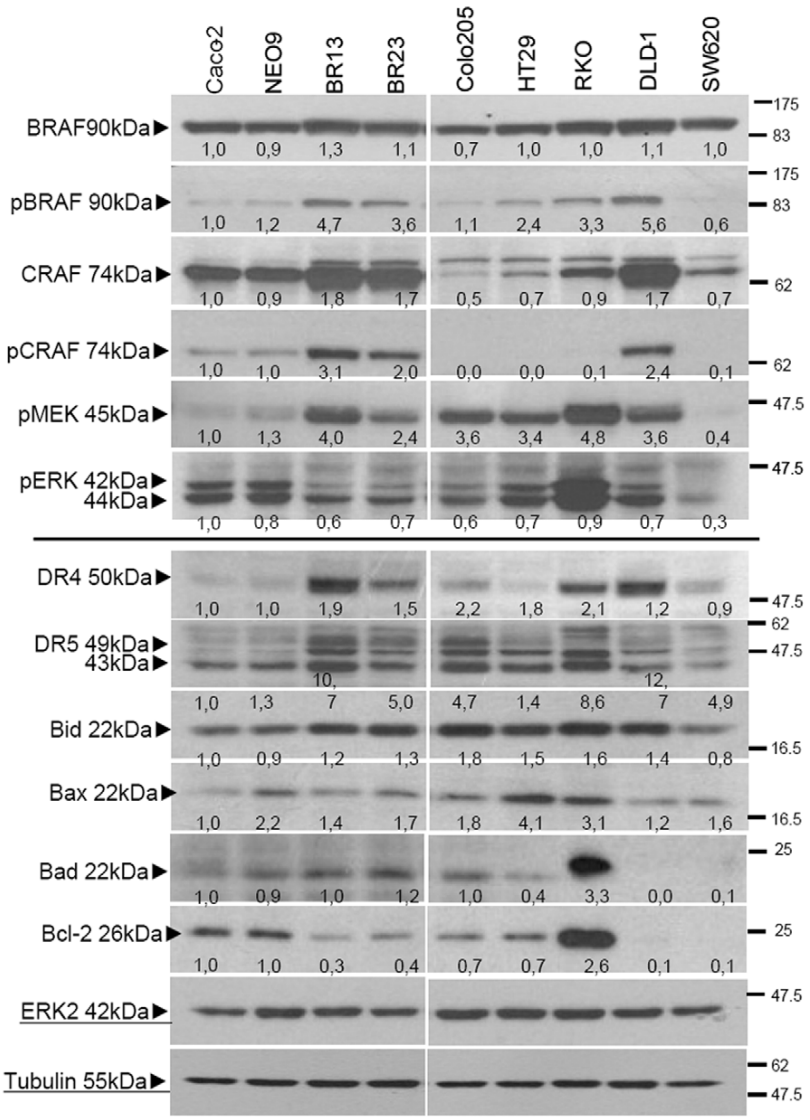

Figure 9. BRAF^{V600E} induces apoptosis related proteins in Caco-2 cells. (**A**) MAPK and apoptotic pathway protein expression analysis in indicated cell lines.
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increased when PI-103 was incorporated into the PLX4720/ TRAIL combination in RKO cells. In conclusion, the presence of PI3KCA mutation might not be interfering with the anti-proliferative action of the PLX4720/ TRAIL combination, but its suppression might offer an additional advantage towards apoptotic cell death. Replacing PI-103 inhibitor with an siRNA against PIK3CA^{H1047} in RKO cells contributed equally to the induction of apoptosis, illustrating once again the inhibitory role of PIK3CA^{H1047} as well as the benefits of using siRNA as a treatment alternative. The combination efficacy of BRAF inhibition and TRAIL treatment in the 2D cell culture system was also validated in three-dimensional spheroid culture conditions, a pre-clinical experimental model that may indicate the most promising combinations to be later exploited in the clinic.

In a similar approach the PI-103 inhibitor was also used in combination with TRAIL in HT29 cells. Increased sensitivity to apoptosis was evident but there were no signs of further synergism when incorporated into the PLX4720/ TRAIL combination. The PI3K inhibitor PI-103 has been shown to efficiently cooperate with TRAIL to synergistically induce apoptosis [37]. When PLX4720 was replaced by shRNA against BRAF^{V600E} in HT29 cells, there was not additive effect on apoptotic cell death for any of the treatment combinations, suggesting that the shRNA approach in a heterozygous mutant background is not very efficient. If our earlier hypothesis identifying activated-mutated BRAF as a sensitizing factor is valid, the significantly low pBRAF activity present in HT29 cells, as compared to RKO and DLD-1, may explain the semi-resistant response not only to TRAIL but also to the different combinations applied. In addition to the moderate pBRAF activation, we also noted that the PIK3CA^{P449T} activating mutation in HT29 is also of low activity which suggests that none of the MAPK or the PI3K pathways is dominant. A

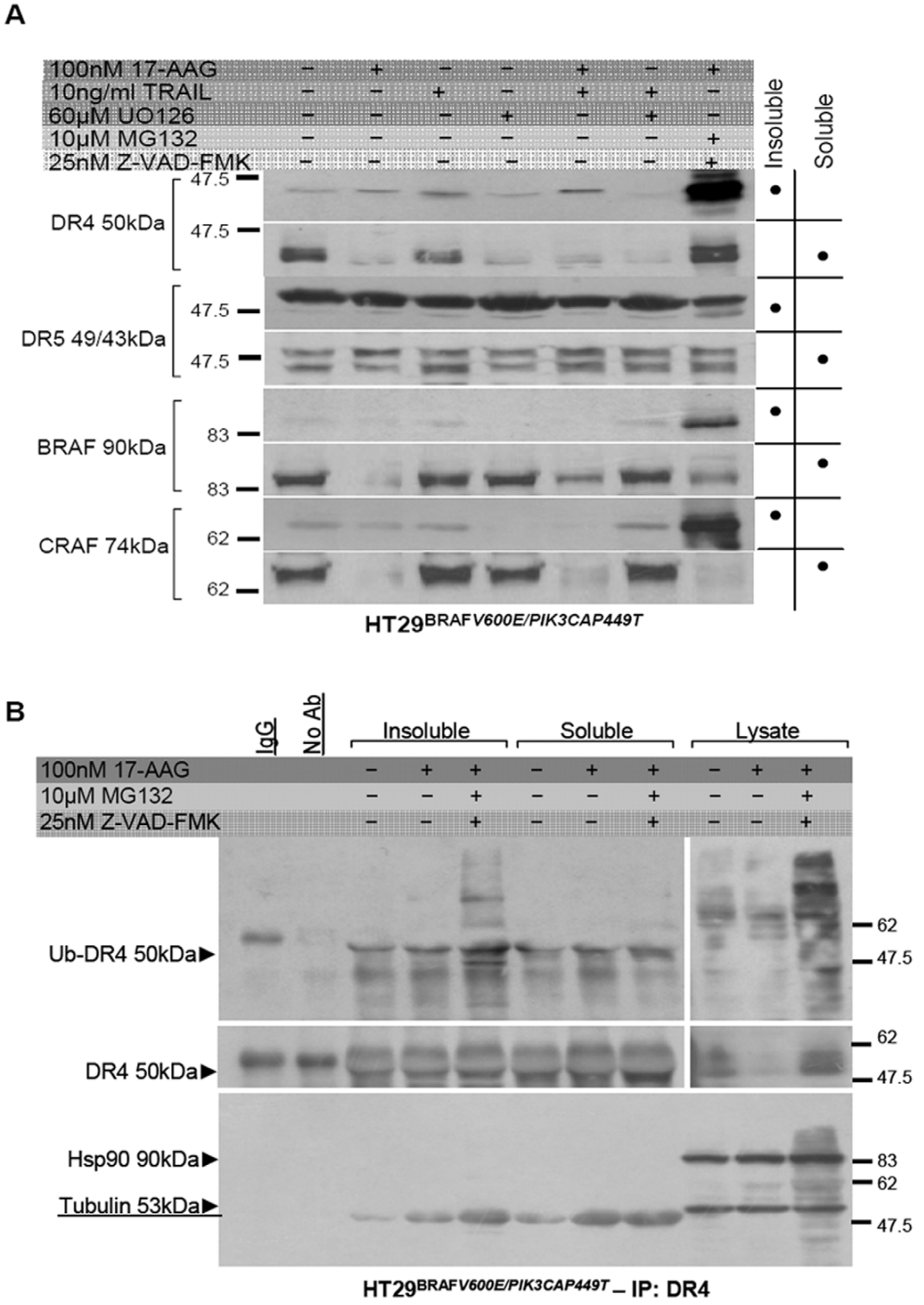

Figure 10. DR4 processing by 17-AAG in HT29 cells. (**A**) NP-40-insoluble fraction from HT29 cell treated as indicated were solubilized in 2% SDS buffer and immunoblotted for the indicated antibodies. The proteasome inhibitor MG132, abrogated 17-AAG-induced loss of $BRAF^{V600E}$ allowing the protected but ubiquitinated BRAF, CRAF and DR4 protein to accumulate in the NP-40 insoluble fraction. (**B**) NP-40 soluble and insoluble fractions were immunoprecipitated with DR4 and blotted with indicated antibodies.
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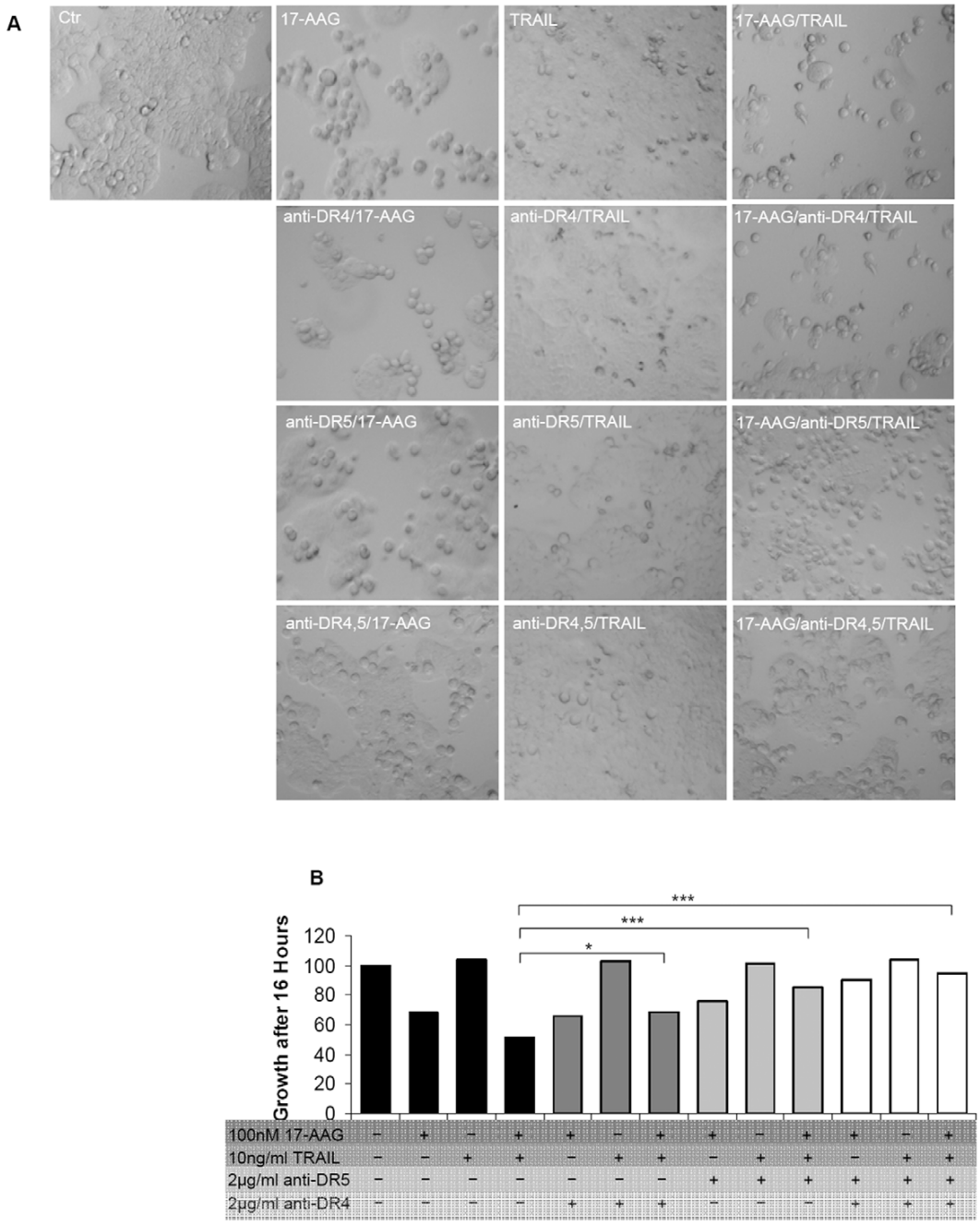

Figure 11. 17-AAG sensitizes HT29 cells to TRAIL-induced apoptosis via the DR5. (**A**) Effects of anti-DR4/DR5 blocking antibodies on TRAIL/17-AAG combination treatment in HT29 cells. Pre-treatment for 15 minutes with blocking antibody preceded all indicated treatments. Representative images, original magnification 30x. (**B**) Cell viability of HT29 cells assays by SRB. Cell viability is presented as fold change of the absorbance of treated/untreated cells, for each condition. *$P<0.05$, **$P<0.01$, ***$P<0.001$.
doi:10.1371/journal.pone.0021632.g011

simulated balance created by both pathways prevents the cell from shifting completely into the apoptotic pathway, achieving as a result mild sensitization to apoptosis.

The synergistic effect between TRAIL and 17-AAG led to a significantly apoptotic response in HT29 cells. Induction of apoptosis was most likely achieved through amplification of the mitochondrial pathway based on the extensive pro-caspase-9 processing in the combined treatment. It has been previously demonstrated that combination of TRAIL with certain signaling inhibitors results in enhancement of apoptosis through inhibition of important pro-survival pathway components like AKT [38,39]. Activated AKT kinase is known to be an Hsp90 client and can be targeted by 17-AAG [23]. Nevertheless, the anti-proliferative effect of 17-AAG alone or in combination with TRAIL observed

in HT29 and DLD- 1 cells relays entirely on the interaction between Hsp90 and BRAF either mutated or activated by $KRAS^{G13D}$ respectively, since depletion of mutant $BRAF^{V600E}$ in HT29 resulted to complete reversal of apoptotic cell death. Interaction between Hsp90 and mutant BRAF is impaired resulting to even lower pBRAF activity that interferes with the amplification of apoptotic cell death.

To further unravel the role of mutant BRAF as a single mutational event, a stable expression of $BRAF^{V600E}$ was performed in Caco-2 cells. Overexpression of the mutant protein significantly sensitized Caco-2 cells to TRAIL induced apoptosis, a response comparable to that of Colo205 cells also bearing a single BRAF mutation. Functional analysis of DRs and apoptosis kinetics revealed rapid DISC assembly and activation of the mitochondria-dependent pathway after induction with TRAIL in $BRAF^{V600E}$ transformed and high pBRAF presenting DLD-1 cells. Nevertheless, overexpression of mutant BRAF does not confer any sensitivity to the antiproliferative effect of PLX4720, unless a small concentration of TRAIL is added to achieve an additive effective response. Limited response to PLX4720 treatment is most likely due to the ambivalence overexpression and phosphorylation of CRAF in response to mutant BRAF stable induction. The successful combination of PLX4720 with TRAIL in cells overexpressing mutant BRAF was also confirmed in three-dimensional culture conditions. Efficacy of TRAIL in Caco-BR cells grown in spheroids holds great potential for future application, especially when apoptosis is engaged in microsatellite instable Caco-BR cells [1], potentially by circumventing their impaired DNA repair mechanism in addition to deregulated signaling pathways. The deregulate cell cycle Caco-BR cells are under not only because of their deregulation of their mitotic check point [1], but also because of the high levels of hyperphosphorylated cyclinD1, might be accounted for the very late response of the Caco-BR clones to 17-AAG treatment. The unstable cell cycle renders Caco-BR cells able to escape 17-AAG cell cycle targeted effects. On the other hand, HT29 cells have a more stable cell cycle that can be more efficiently targeted by 17-AAG, whereas DLD-1 cells harboring a KRAS mutation were also found partially sensitive to 17-AAG, possibly because of their high intrinsic pBRAF levels. Sensitivity of cancer cells to specific drugs can be regulated through the expression patterns of the BH3-only family members.

Overexpression of pro-apoptotic as compared to anti-apoptotic factors was recorded following $BRAF^{V600E}$ transformation and was comparable to high pBRAF presenting DLD-1 and Colo205 cells. Recorded upregulation in DR4/ 5 serve as the initial step towards the efficient DISC formation whereas Bid accumulation provides the link between terminal effector processes and signaling related alterations such as DNA integrity, cell attachment and microtubule function. Simultaneous downregulation of Bcl-2 ensures Bax translocation to the mitochondrial membrane and Cytochrome C release. Upregulation of pro-apoptotic proteins may explain how increased sensitivity of Caco-BR cells to TRAIL-induced apoptosis is achieved.

Previous studies describe the synergistic effect between TRAIL and 17-AAG and the inhibitory effect of 17-AAG on DR4. Although it has been well described how the ubiquitin system regulates the proximal steps of the DISC assembly and its components, ubiquitination of DR4 has not been observed before [40]. Despite ubiquitin dependent proteolysis of DR4 accompanied by an mRNA depended downregulation, lack of interaction between Hsp90 and DR4 suggests that DR4 is targeted by the ubiquitin aimed for HSp90. In this case internalization of DR4 must be more frequent as compared to DR5 or the protein turnover is increased. Moreover, significant suppression of pERK following BRAF degradation by 17-AAG has also contributed to a MEK dependent DR4 downregulation in the presence of attenuated $BRAF^{V600E}$ activity. Nevertheless, apoptosis was efficiently mediated through DR5 as determined by functional analysis using specific DR5 blocking antibodies. Considering occasions where inactivation of KILLER/ DR5 due to mutations [41], or absence of its cell surface expression because of improper transport to the cell surface [42], cells being treated with the proposed combination of TRAIL/ 17-AAG will manage to escape DR5 mediated apoptosis.

This study has analyzed in detail the role of BRAF/ KRAS/ PIK3CA mutation status of particular colorectal tumours on predicting efficient therapeutic treatments with the BRAF and the PI3K inhibitors as well as their rational combination with TRAIL. Cell death by apoptosis when TRAIL was concomitantly administered with PLX4720 or 17-AAG was significantly increased suggesting that when $BRAF^{V600E}$ or $PIK3CA^{H1047}$ exist as dominant mutational events may confer sensitivity to TRAIL.

Supporting Information

Figure S1 Suppression of BRAF phosphorylation within 16 hours and rapid inhibition of pERK within an hour of treatment with 1 and 10 μM of PLX4720 in RKO cells.
(TIF)

Figure S2 Complete experiment of immunoprecipitation of indicated cell lines with BRAF and the complexes subsequently immunoblotted first with Hsp90 and then with BRAF and Cdc37, which is right bellow the heavy chain (HC) of the antibody.
(TIF)

Figure S3 Combined treatment with 1 μM PLX4720 for 8 hours and concomitant administration of 10 ng/ml TRAIL for 16 hours in DLD-1 and HCT116 colon cancer cells. Cell viability assayed by SRB 24 hours after treatment. *$^{**}P<0.01$, $^{***}P<0.001$.*
(TIF)

Figure S4 Depletion of mutant BRAF by transient transfection using indicated shRNA against the $BRAF^{V600E}$ present in HT29 cells and subsequent treatment of cells with 100 ng/ml TRAIL. Total cell lysates harvested for the described treatments were immunoblotted with the indicate antibodies.
(TIF)

Figure S5 (B) Western blotting of HT29, HT-PS (empty vector), HTShBR-1, -3 and -5 stable clones. Expression levels of total and phosphorylated BRAF is shown accompanied by phosphorylation status of ERK1/2.
(TIF)

Figure S6 A dose response with TRAIL was performed for 16 hours in Caco-BR transformed cells and $BRAF^{V600E}$ mutant HT29 cells. The cytotoxic effects of TRAIL were measured using the apoptosis ELISA kit by Roche. Log percentage cell viability and fold change of the absorbance of treated/ untreated cells, for each condition are presented.
(TIF)

Figure S7 Cell lysates and flow through controls of the western blot analysis for the DISC immunoprecipitation.
(TIF)

Figure S8 Induction kinetics of apoptosis at indicated TRAIL concentrations and specific time points. Total cell lysates harvested from cells treated for indicated time points following treatment with 10 and 100 ng/ml TRAIL were immunoblotted for the indicated antibodies. Proteins are quantified against α-tubulin.
(TIF)

Figure S9 Total cell lysates harvested from in Caco-BR cells treated for 4 hours with indicated concentrations of PLX4720 were immunoblotted for the indicated target proteins. Proteins are quantified against α-tubulin.
(TIF)

Figure S10 Total cell lysates harvested from Caco-BR13 cells treated for indicated time points with 100 nM 17-AAG were immunoblotted for the indicated antibodies. Proteins are quantified against α-tubulin.
(TIF)

Figure S11 Cell surface expression of DR4 and DR5 analysed by means of flow cytometry following staining with antibodies against DR4, DR5 and the secondary GAM-PE antibody only (IgG-PE) that was used against DR4 and DR5 in living cells (Hoechst negative). Blue line indicates expression levels of DRs in parental Caco-2 cells. Representative histograms from at least three independent experiments are shown. Blue line indicates expression levels of DRs in parental Caco-2 cells.
(TIF)

Figure S12 DR expression on the cell surface of HT29 cells. Downregulation of the DR4 was assayed by means of flow cytometry following treatment with 100 nM 17-AAG alone or in combination with 10 ng/ml TRAIL for 24 hours. Nearly 40% of the DR4 was rescued in the presence of 10 μM MG132. Representative histograms from at least four independent experiments are shown. Blue line indicates expression levels of DRs in parental Caco-2 cells.
(TIF)

Figure S13 DR4 escapes 17-AAG dependent degradation in DLD-1 cells. Cells were left untreated or treated with 150 nM or 1 μM 17-AAG for 30 hours, or pre-treated with the 150 nM 17-AAG for 24 hours after which 10 ng/ml TRAIL was added for another 16 hours or 10 ng/ml TRAIL alone was added for 16 hours. Alternatively cells were pre-treated with 25 nM Z-VAD-FMK for 1 hour then 10 μM MG132 was added for another hour after which cell were treated with 150 nM 17-AAG for 24 hours. Protein extracts were separated into NP-40 soluble and NP-40 insoluble fractions and subjected to Western blot analysis.
(TIF)

Figure S14 Caco-BR13 cells were left untreated or treated with a low 150 nM and a high 1 μM concentration of 17-AAG for 30 hours, or pre-treated with the 1 μM 17-AAG for 24 hours. Alternatively cells were pre-treated with 25 nM Z-VAD-FMK for 1 hour then 10 μM MG132 was added for another hour after which cell were treated with 1 μM 17-AAG for 24 hours. Protein extracts were subjected to Western blot analysis. Picture shown is representative of three independent experiments.
(TIF)

Figure S15 mRNA extracts from HT29 cells treated as indicated were analysis by RT-PCR with regard to TRAIL receptor, DR4 and DR5, transcriptional activity.
(TIF)

File S1 Supplementary Materials and Methods.
(DOC)

Author Contributions

Conceived and designed the experiments: EO LA AP. Performed the experiments: EO MK VS. Analyzed the data: EO LA AP. Contributed reagents/materials/analysis tools: EO MK VS. Wrote the paper: EO AP.

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