Universitat Autònoma de Barcelona

Vall d'Hebron Institut de Recerca VHIR

PhD Thesis

Priming neuroblastoma for cisplatin and etoposide drug therapy:

Role of NF-κB in TNFα-induced expression of Fas

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I dedicate this thesis to my parents

my brother and sister

my woman

my family

the Boyz

Knorrie

AMS

BCN

and

Dr. Pepper

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“Ze kunnen beter over je fiets lullen
dan over je lul fietsen.”
Translation: “They can better talk (dick) about your bike,
than bike over your dick.”

Herman Brood
Dutch musician, painter, actor, and poet (1946-2001)

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Abstract

"It's more fun to experience things when you don't know what's going to happen."

Louis C. K.
American comedian, actor, and writer (Born in 1967)

Abstract

Neuroblastoma (NB) is a pediatric solid tumor that accounts for ~15% of all cancer-related deaths in infants. High-risk NBs are hallmarked by a high degree of heterogeneity and aggressiveness, which results in poor patient outcome. Despite the improvement of standard therapies in the last twenty years, five-year survival rates are still below 50%, which impels the development of new treatment strategies for this condition.

Activation of death receptors (DRs) has been proposed as an alternative to standard chemo- and radio-therapies for various types of cancer. In NB, this approach has been largely disregarded, possibly due to the silencing of caspase-8 in 50-70% of the cases. Nevertheless, a significant group of NB patients could benefit from treatment that induces cell death through DR activation. Characterization of DR signaling (especially Fas and TNFR1) and their regulation in NB has been limitedly studied, but is a prerequisite for assessing their therapeutic relevance.

Given that the cytokine TNFα has been described to induce Fas expression in various types of cancer, we addressed whether TNFα and FasL co-treatment could be a valid therapeutic strategy in NB.

For the purpose of the study, TNFR1- and Fas-mediated signaling and cell death induction was characterized in a set of eight clinically representative NB cell lines. TNFα treatment was shown to induce Fas expression through NF-κB-mediated transcription of *FAS* and primed for FasL-induced cell death. Moreover, TNFα treatment enhanced the cytotoxic effects caused by DNA-damaging agents (i.e. cisplatin and etoposide) through caspase-8 activation. Further characterization revealed that the high degree of heterogeneity between NBs is also visible at the levels of Fas expression and modulation thereof by TNFα. TNFα-mediated priming for FasL-, cisplatin-, and etoposide-induced cell death was only observed for NBs that induced TNFα-mediated Fas expression.

In conclusion, our findings reveal that TNFα primes NB for FasL-induced cell death through the NF-κB-mediated induction of Fas expression. Moreover, TNFα pre-treatment enhanced cisplatin- and etoposide-induced cell death. These findings unveil a novel mechanism that could improve the efficacy of treatment regimens currently used for the eradication of NB tumors.

Resumen

El neuroblastoma (NB) es un cáncer pediátrico que representa ~15% de muertes en cánceres infantiles. Los NBs de alto riesgo se caracterizan por una gran heterogeneidad e agresividad, que conlleva un mal pronóstico para el paciente. A pesar de las mejoras alcanzadas con las estrategias estándar en los últimos 20 años, la prevalencia de supervivencia después de cinco años continua por debajo del 50%. Esta situación pone de manifiesto la necesidad del desarrollo de nuevas estrategias para afrontar este problema.

La activación de los receptores de muerte (DR) se ha propuesto como una alternativa a los tratamientos clásicos de quimio- y radio-terapia en distintos tipos de cáncer. En el caso de los NBs, esta aproximación fue descartada por el hecho de que entre un 50 y 70% de ellos no presentan expresión de caspasa-8. A pesar de ello, un grupo significativo de pacientes de NB se podrían beneficiar de un tratamiento que promoviera la muerte a través de la activación de los DR. Se conoce poco de la vía de señalización activada por los DR (especialmente Fas y TNFR1) y de su regulación en NBs, por ello consideramos básico su estudio antes de testar su posible relevancia terapéutica.

Dado a que se ha descrito que la citoquina TNFα induce la expresión de Fas en diferentes tipos de cáncer, nosotros decidimos abordar el co-tratamiento de TNFα con FasL como estrategia terapéutica para el tratamiento de NB.

Para llevar a cabo nuestro estudio, caracterizamos la señalización intracelular y la inducción de muerte a través de TNFR1 y Fas en ocho líneas celulares clínicamente representativas de NB. Observamos que el tratamiento con TNFα induce la expresión de Fas a través de la activación de la vía NF- κB e sensibiliza a la muerte inducida por FasL. A demás, el tratamiento con TNFα promueve la citotoxicidad de agentes genotóxicos, como cisplatino y etoposido, a través de la activación de la caspasa-8. La caracterización más en detalle que realizamos nos llevó a la conclusión que la heterogeneidad presente en neuroblastomas también se hace patente en los niveles de expresión de Fas y en su modulación por TNFα. La sensibilización a la muerte inducida por FasL, cisplatino o etoposido mediada por TNFα solo se podía observar en aquellos NBs donde TNFα era capaz de inducir la expresión de Fas.

En conclusión, nuestros resultados evidencian que TNFα sensibiliza NBs a la muerte inducida por FasL a través de la inducción de la transcripción de *FAS* mediada por NF- κB. A demás, el pre-tratamiento con TNFα incrementa la muerte inducida por cisplatino y etoposido. Nuestros resultados revelan un nuevo mecanismo que pude mejorar los tratamientos que actualmente se utilizan para la erradicación de los NBs.

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"The most merciful thing in the world...
is the inability of the human mind to
correlate all its contents."

H. P. Lovecraft
American writer, editor, novelist, and poet (1890 –1937)

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Abbreviations

"The military never uses a full word if they can create an abbreviation."

Philip Hammond
British politician (Born in 1955)

(v/v)	(volume/volume)
(w/v)	(weight/volume)
A1	BCL2-related protein A1
Ala	Alanine (A)
ANOVA	Analysis of variance
APAF1	Apoptotic Protease-Activating Factor 1
APO-1/2/3	Apoptosis Antigen-1/2/3
APO-1L	Apoptosis Antigen-1 Ligand
APS	Ammonium Persulfate
Arg	Arginine (R)
Asp	Aspartic acid (D)
BAD	BCL-2-Associated Agonist Of Cell Death
BAK	BCL-2-Antagonist/Killer
BAX	BCL-2-Associated X Protein
BCL-2	B-Cell CLL/Lymphoma 2
BCL-W	BCL-2-Like 2
BCL-X_L	B-cell Lymphoma-Extra Large
BH3	BCL-2 homology domain 3
BID	BH3 Interacting Domain Death Agonist
BIK	BCL2-Interacting Killer
BIM	Bcl-2 Interacting Mediator Of Cell Death
BIR	Baculovirus Inhibitior of Apoptosis Protein Repeat
BMF	Bcl2 Modifying Factor
CARD	Caspase Activation and Recruitment Domain
CASP8	Caspase-8
CD	Cluster of Differentiation
cDNA	Complementary DNA
c-FLIP	Cellular FLICE-Like Inhibitory Protein
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese Hamster Ovary
cIAP	Celular Inhibitor Of Apoptosis Protein
CMV	Cytomegalovirus
CRD	Cysteine-Rich Domain
CTL	Cytotoxic T Lymphocyte
CYLD	Cylindromatosis (Turban Tumor Syndrome)
Cys	Cysteine (C)
DcR	Decoy Receptor
DD	Death Domain
DED	Death-Effector Domain
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DN	Dominant-Negative
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DPBS	Dulbecco's Phosphate-Buffered Saline

DR	Death Receptor
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
E12.5	Embryonic day 12.5
ECL	Enhanced Chemiluminescence
EDA1	Ectodysplasin A
EDAR	Ectodysplasin A Receptor
EDTA	Ethylenediaminetetraacetic Acid
EF-1a	Elongation Factor-1 Alpha
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Endoplasmatic Reticulum
ERK	Extracellular Signal-Regulated Kinase
EtOH	Ethanol
FACS	Fluorescence-Activated Cell Sorting
FADD	Fas-Associated protein with Death Domain
FAIM	Fas Apoptotic Inhibitory Molecule
FasL	Fas Ligand
FBSi	Heat-Inactivated Fetal Bovine Serum
Fc	Fragment crystallizable region
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GD2	Glycolipid Disialoganglioside
GFP	Green Fluorescent Protein
Gln	Glutamine (Q)
Glu	Glutamic acid (E)
Gly	Glycine (G)
HDAC	Histone Deacetylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
His	Histidine (H)
HIV	Human Immunodeficiency Virus
HRK	Harakiri, BCL2 Interacting Protein
HRP	Horseradish Peroxidase
HTRA2	HtrA Serine Peptidase 2
IAP	Inhibitor Of Apoptosis Protein
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IMS	Intermembrane space
INRG	The International Neuroblastoma Risk Group
IP	Immunoprecipitation
IRP2	Iron Regulatory Protein 2
IκB	Inhibitor of Kappa B
JNK	c-Jun N-Terminal Kinase
kDa	Kilo Dalton
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog

LARD	Lymphocyte-Associated Receptor Of Death
Leu	Leucine (L)
LFG	Lifeguard
lpr	Lymphoproliferation spontaneous mutation
LPS	Lipopolysaccharide
MAP3K	MAP kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid Cell Leukemia 1
MDM2	Mouse Double Minute 2
MEFs	Mouse Embryonic Fibroblasts
MEM	Minimal Essential Medium
MeOH	Methanol
mFasL	Membrane-bound Fas Ligand
MLKL	Mixed Lineage Kinase Domain-Like
MOMP	Mitochondrial Outer Membrane Permeabilization
mRNA	Messenger RNA
mTNFα	Membrane-bound TNFα
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYCN	V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog
NADH	Reduced Nicotinamide Adenine Dinucleotide
NB	Neuroblastoma
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NGFR	Nerve Growth Factor Receptor
NHS	N-hydroxysulfosuccinimide
NK	Natural Killer
NMDA	N-methyl-D-aspartate
NOXA	(Latin for damage)
NP-40	Nonidet P-40
OD	Optical Density
OPG	Osteoprotegerin
p75NTR	p75 Neurotrophin Receptor
PBS	Phosphate-Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium Iodide
PI3K	Phosphoinositide-3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLAD	Pre-Ligand Binding Assembly Domain
PMA	Phorbol 12-myristate 13-acetate
PUMA	P53 Up-Regulated Modulator Of Apoptosis
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction

RAS	Rat Sarcoma Viral Oncogene
RelA/B	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A/B
RING	Really Interesting New Gene
RIP1/3	Receptor-Interacting Protein 1/3
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room Temperature
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Ser	Serine (S)
sFasL	Soluble Fas Ligand
SHANK	SH3 and multiple ankyrin repeat domains
SHC	Src Homology 2 Domain Containing
Smac	Second Mitochondria-derived Activator of Caspases
SR	Super Repressor
Src	(short for sarcoma)
STAT1/3	Signal Transducer And Activator Of Transcription 1/3
sTNFa	Soluble Tumor Necrosis Factor alpha
SV40	Simian vacuolating Virus 40
TAB1/2	TAK1-binding proteins 1/2
TAD	Transcriptional Activation Domain
TAK1	Transforming Growth Factor-Beta-Activated Kinase 1
TBS	Tris-Buffered Saline
TEMED	Tetramethylethylenediamine
TGFβ	Transforming Growth Factor-Beta
TL1A	TNF Ligand-Related Molecule 1A
TM	Transmembrane
TNFR	Tumor Necrosis Factor Receptor
TNFα	Tumor Necrosis Factor Alpha
TRADD	TNFR1-Associated Death Domain Protein
TRAF2/5	TNF Receptor-Associated Factor 2/5
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAMP	TNF Receptor-Related Apoptosis-Mediating Protein
TRICK2	TRAIL Receptor Inducer of Cell Killing 2
Trp	Tryptophan (W)
TWEAK	Tumor Necrosis Factor-like Weak Inducer of Apoptosis
TWEAKR	Tumor Necrosis Factor-like Weak Inducer of Apoptosis Receptor
Tyr	Tyrosine (Y)
UT	Untreated
UV	Ultraviolet
Val	Valine (V)
VEGF	Vascular Endothelial Growth Factor
XIAP	X-Linked Inhibitor Of Apoptosis Protein
YY1	Yin And Yang 1

Introduction

"Too Weird to Live, Too Rare to Die!"

Hunter S. Thompson
American journalist and author (1937-2005)

1. Introduction

1.1 Cell death and survival

The balance between specific internal and external cellular signals decides the cell's fate; cell death or cell survival. Both outcomes are equally important for the homeostasis of an organism. In the human body, about one million cells, which is equivalent to approximately 1.2kg, are estimated to die daily in a controlled manner [1, 2]. The human body uses this mechanism of controlled cell death for the removal of old and defective cells and recycles these cells for the production of building blocks for the generation of new cells [3]. However, deregulation of the balance between cell death and cell survival can give rise to malignant cell growth, which is the case in cancer. Or on the other hand, malignant cell death, such as in the cases of liver cirrhosis, immunodeficiency as seen in HIV infection, and neuro-degeneration as seen in Alzheimer's and Parkinson's disease [4, 5].

Naturally occurring cell death was first described in 1842 by Karl Vogt, while studying the tadpole of the midwife toad [6]. He observed that during metamorphosis, the vertebrate was formed after removal of the notochord through naturally occurring cell death. Until the mid-1960s, it was assumed that cell death was no more than a passive and predestined end of a cell's life [7, 8]. However, this hypothesis was replaced after the discovery of endogenous expressed genes that regulate developmental cell death and introduced the term programmed cell death (PCD).

1.1.1 Types of cell death

The presentation of images obtained by Kerr, Wyllie and Currie through electron microscopy, contributed in the 1970s to a better understanding and definition of different types of cell death [9]. Their images outlined structural hallmarks, specific for different types of cell death, which gave rise to the terms apoptosis and necrosis. In time, the terms autophagic cell death and necroptosis were added to the classification of PCD (Figure 1.1) [10, 11].

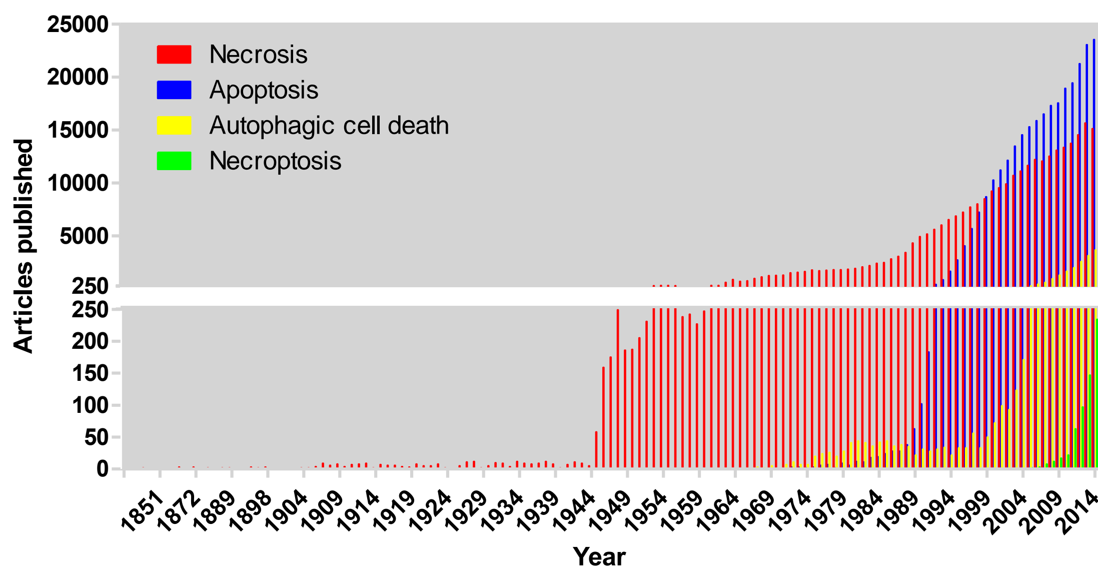


Figure 1.1: Expansion of our understanding and definition of programmed cell death. Articles published per year on programmed cell death topics. Data derived from PubMed

1.1.1.1 Apoptosis

Apoptosis is the most prevalent and most studied form of programmed cell death [3, 12]. It is characterized by clear morphological hallmarks, such as cytoplasmic shrinkage, nuclear condensation, DNA fragmentation, membrane "blebbing", and phagocytosis by another cell. In addition, the core machinery of the apoptotic pathway is well conserved between species and displays a central role for caspases. Apoptosis is necessary for the homeostasis of an organism, playing an important role in the removal of old and defective cells and shaping organs and body structures during development (Figure 1.2 and 1.4).

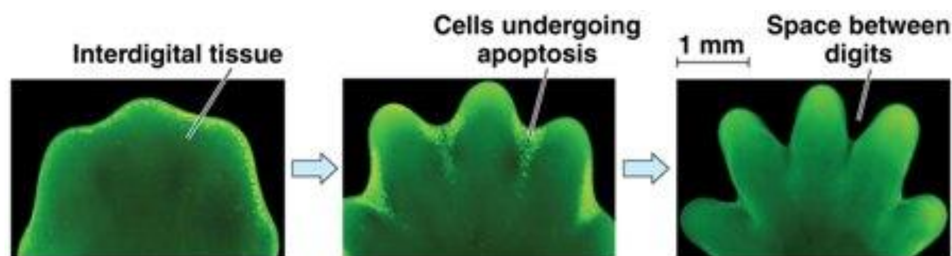


Figure 1.2: Apoptotic shaping of the limbs during development. Acridine Orange staining of the mouse forelimb footplate indicates apoptosis necessary for the shaping of the limb between embryonic days E12.5 and E14.5. Figure adapted from [13]

1.1.1.2 Autophagic Cell Death

Autophagic cell death depends on the formation of autophagosomes, which are double membrane layered spherical structures (Figure 1.3) [3, 12]. These structures, which originate from the endoplasmatic reticulum or mitochondria, contain sequestered cytoplasmic proteins and/or organelles and can fuse with lysosomes, thereby targeting the content of the autophagosome for degradation (Figure 1.3). Although this process is a survival mechanism against stress conditions, such a pathogen infection and mitochondrial damage, excessive autophagy can result in cell death and is therefore termed PCD. Controversially, this form of PCD is still under debate whether autophagy is the cause of cell death or whether in dying cells autophagy is a mechanism to promote cell survival.

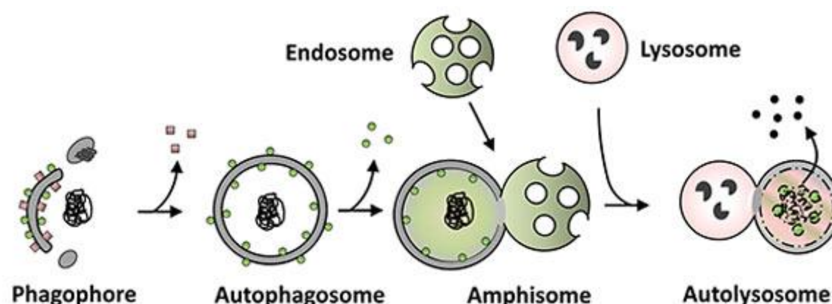


Figure 1.3: Step-wise formation of the autolysosome. Autolysosome protein and organelle engulfment by the phagophore membrane initiates the formation of a double-membrane vesicle, called the autophagosome. Further maturation, through fusion with endosomes and lysosomes induces the formation of the autolysosome. Here, the content of the cytoplasmic proteins and organelles are degraded and subsequently recycled back to the cytosol, where they can be reused by the cell. Figure derived from [14]

1.1.1.3 Necrosis

Necrosis is a form of uncontrolled premature cell death through autolysis, predominately induced by thermal and mechanistic effects, loss of blood vessels, and ischemia [3, 12]. In contrast to programmed cell death, necrosis is non-beneficial for the organism and can be damaging or fatal for the surrounding cells. Through the disruption of the plasma membrane and release of intracellular proteins and organelles, necrosis can induce a pro-inflammatory response which prevents phagocytosis of the dying cell, thereby impeding the recycling of cellular components (Figure 1.4).

1.1.1.4 Necroptosis

Necroptosis is the most recent added term of PCD, although it might have been observed previously since it is described to be a programmed form of necrosis [3, 12]. Necroptosis shares morphological hallmarks with necrosis, which are swelling of the organelles and cells, and bursting of the plasma membrane which leads to release of the intracellular contents (Figure 1.4). However, unlike necrosis, necroptosis is induced in a regulated manner through the activation of signaling pathways, which are activated when caspases are inhibited or downregulated.

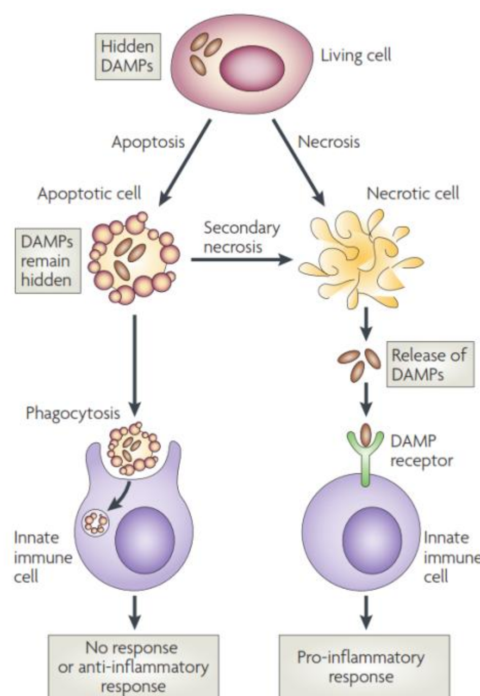


Figure 1.4: Mechanism responsible for the pro-inflammatory response observed in necrosis and necroptosis. Healthy cell contain certain intracellular molecules that are able to stimulate the immune system, causing a pro-inflammatory response. These so-called damage-associated molecular patterns (DAMPs) are not released upon apoptosis, since here the membrane integrity is conserved and the apoptotic bodies are removed by phagocytosis. However, in necrosis/necroptosis the intracellular content of the cell is released into the cytoplasm through swelling of the cell and rupture of the cytoplasmic membrane. Hereby, DAMPs are released and induce a pro-inflammatory response. Figure derived from [15]

1.2 The apoptotic machinery

Apoptosis is induced by intracellular or extracellular signals that activate two distinct apoptotic pathways; the intrinsic and extrinsic apoptotic pathway, respectively (Figure 1.8) [3, 12]. These apoptotic pathways rely on the activation of caspases, which induce the cell death through the regulated breakdown of proteins, DNA, and cellular organelles.

Our understanding of the apoptotic machinery has originated from studies in the flatworm *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster*, and the house mouse *Mus musculus* (Figure 1.5). These studies highlight a high degree of conservation between species and the essential role for various conserved functional homologous proteins, such as BH3-only-like, BCL-2-like, APAF1-like, IAP-like, IAP-antagonist-like, and caspase-like proteins **(functions and abbreviations explained below)**.

In *C. elegans* the cell fate and number of cells are predetermined [16]. During development, the organism deletes 131 cells out of the 1090 somatic cells through apoptosis. *C. elegans* exhibit a straightforward apoptotic pathway in which apoptotic stimuli induce the inhibition of CED-9 (a BCL-2-like protein) through the binding of EGL-1 (a BCL-2 homology domain 3 (BH3)-only-like protein) (Figure 1.5). Thereby, EGL-1 releases CED-4 (an APAF1-like protein) from the CED9-CED-4 inhibitory complex and mediates the activation of CED-3 (a caspase-9-like protein), which in turn induces apoptosis. As a consequence, knockdown of the pro-apoptotic proteins EGL-1, CED-4, and/or CED-9 fully inhibits apoptosis in *C. elegans* and results in the survival of the 131 somatic cells during development.

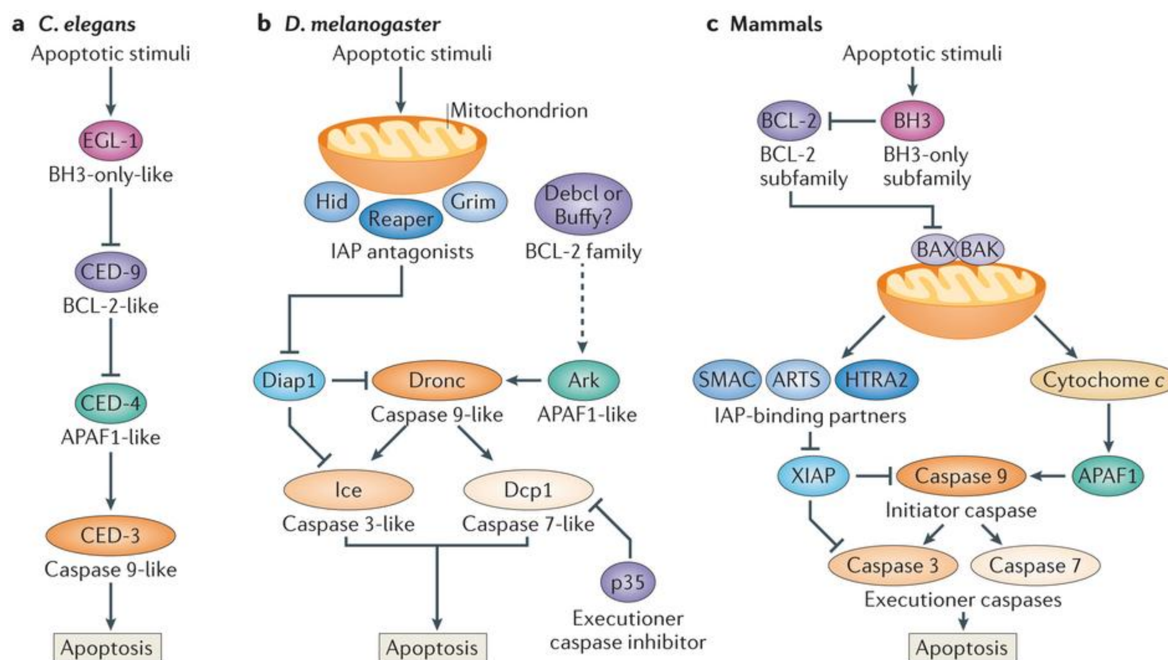


Figure 1.5: Conservation of the apoptotic machinery between the species. Studies between species on proteins of the apoptotic machinery indicate high conservation of the core apoptotic proteins; BH3-only-like, BCL-2-like, APAF1-like, IAP-like, IAP-antagonist-like, and caspase-like proteins. Homologous proteins are indicted by color. Figure derived from [3]

In contrast to *C. elegans*, the regulation of apoptosis in *D. melanogaster* and mammals is more complex. In these species the numbers of cells and cell fate are not pre-determined and can be influenced through extracellular signals and environmental factors. In both species, the mitochondria perform a central role. In *D. melanogaster*, apoptotic stimuli induce the release of the inhibitor of apoptosis (IAP) antagonists; Reaper, Head involution defective (Hid), and Grim (Figure 1.5) [17]. These IAP antagonists induce apoptosis through the release of the caspase-like proteins Ice and Dcp1 (Decapping protein 1), by mediating the degradation of the IAP-like protein Death-associated IAP (Diap1). In addition, the degradation of Diap1 mediates the liberation of the caspase-like protein Dronc (Death regulator Nedd2-like caspase), thereby enabling it to bind to the APAF1-like protein Ark (APAF1-related-killer) (Figure 1.5). Subsequently, Dronc and Ark form the apoptosome that induces apoptosis through the activation of Ice and Dcp1.

Mammals show a similar apoptotic machinery compared to *D. melanogaster* [18]. Upon apoptotic insult, the mitochondria release cytochrome c which drives apoptosis through the formation of the apoptosome by recruiting APAF1 (Apoptotic Protease-Activating Factor 1) and caspase-9, thereby activating caspase-9 and subsequently caspase-3 and -7 (Figure 1.5 and Box 1). Like *D. melanogaster*, mammals release IAP antagonists (Smac, ARTS, and HTRA2) from their mitochondria upon apoptotic insult, thereby further promoting apoptosis through inactivation of the caspase-3 and -9 inhibitor XIAP (X-Linked Inhibitor Of Apoptosis Protein) (Figure 1.5). However, unlike *D. melanogaster*, mammals have a more complex regulation of mitochondrial-induced apoptosis. Here, a highly regulated interplay between pro-apoptotic BH-3-only proteins and anti-apoptotic BCL-2-family proteins decides the activation of BCL-2-Antagonist/Killer (BAK) and BCL-2-Associated X Protein (BAX)-induced mitochondrial release of cytochrome c and other pro-apoptotic proteins (Figure 1.5).

1.2.1 Caspases

Caspases (cysteine-aspartic proteases) are a family of highly specific cysteine-proteases that contain a catalytic cysteine residue in their active site [19, 20]. Caspases cleave their substrates by hydrolyzing peptide bonds after the aspartic acid residue in the X-X-X-Asp-Gly/Ser/Ala peptide motif. Caspases are present as inactive forms called pro-caspases and are composed of a pro-domain, a small subunit (p10), and large subunit (p20), with aspartic acid cleavage sites located at their junctions. Dimerization, subsequent proteolytic cleavage, and removal of their pro-domain results in the “maturation” and activation of these apoptotic caspases (Figure 1.7) [19-21]. Caspase maturation induces the formation of the active site, which is present on the large domain and consists of a conserved pentapeptide QACXG (Gln-Ala-Cys-X-Gly, where X is Arg, Gln, or Asp), with the catalytic cysteine as a vital amino acid for substrate degradation [22, 23]. In addition, the catalytic site includes a Ser-His-Gly (SHG) sequence which is found upstream of the Gln-Ala-Cys-X-Gly pentapeptide. Here, the His residue is critical for caspase-activity.

In humans, caspases are classified in two groups; the pro-inflammatory caspases (caspase-1, -4, -5, and -12) and apoptotic caspases (caspase-2, -3, -6, -7, -8, -9, and -10) (Figure 1.6) [21]. The apoptotic caspases can be divided in two categories; the initiator caspases (caspase-2, -8, -9, and 10) and effector caspases (caspase-3, -6, and -7) (Figure 1.6 and 1.7) [21]. Recently, caspase-14 and -16 were added to the human repertoire of caspases, but these have not been classified as pro-inflammatory nor apoptotic caspases [24, 25].

As their name implies, the initiator caspases initiate the apoptotic pathway and their activity is necessary for the activation of the effector caspases [19-21]. Initiator caspases contain a pro-domain that facilitates the recruitment to specific proteins that induce their activation (Figure 1.6). In the case of caspase-8/10 a Death-Effector Domain (DED) that facilitates the recruitment to other proteins that contain a DED, such as and FADD (Fas-Associated protein with Death Domain) and in the case of caspase-2/9 a Caspase Activation and Recruitment Domain (CARD) that facilitates the recruitment to other CARD-containing proteins, such as APAF1 and RAIDD (RIP-Associated Protein With A Death Domain). After pro-domain mediated recruitment, the initiator caspases are activated through homo-dimerization and auto-proteolytic cleavage, which results in the active caspase-form, called the mature caspase (Figure 1.7). The initiator caspases have limited substrates, i.e. auto-cleavage, effector caspases, and BID (BH3-interacting domain death agonist) [21].

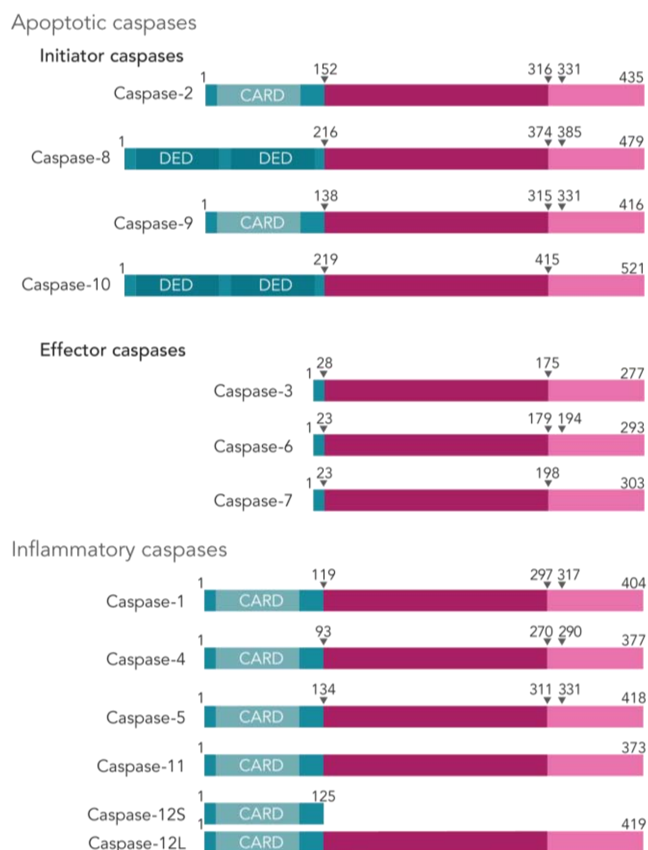


Figure 1.6: Mammalian caspase classification, domains, and cleavage sites. Mammalian caspases can be divided in two categories; the apoptotic and inflammatory caspases. All caspase contain a pro-peptide (marked in blue), however only the pro-peptide of the inflammatory and initiator caspases contain a characterized domain. Cleavage at the aspartic acid residues indicated with the triangles, results in removal of the pro-peptide and formation of the large subunit (marked in purple) and small subunit (marked in pink). Figure adapted from [26]

In contrast to the initiator caspases, the effector caspases lack a pro-domain (Figure 1.7) and are present as inactive dimers that upon proteolytic cleavage by the initiator caspases become activated through intermolecular rearrangements. These rearrangements result in the formation of the mature and active effector caspase (Figure 1.7). During apoptosis, the effector caspases are the main responsible for inducing the apoptotic phenotype through the cleavage of hundreds of substrates, amongst which are regulators of DNA processing (e.g. ICAD (Inhibitor Of Caspase-Activated DNase) and PARP (Poly (ADP-Ribose) Polymerase)), cell cycle regulators (e.g. Retinoblastoma protein(pRb) and p21), and structural proteins (e.g. α-tubulin and actin) [21].

The effector caspases cleave the X-X-X-Asp-Gly/Ser/Ala peptide motif less or more efficiently depending on the amino acid sequence [21, 27-29]. Asp-Glu-Val-Asp (DEVD) is cleaved highly efficient, whereas Trp-Glu-His-Asp (WEHD) is cleaved much less efficiently. In addition, caspase-substrate specificity overlaps between the different effector caspases, although there does exists a substrate preference. Asp-Glu-Val-Asp (DEVD) is cleaved efficiently by caspase-3 and -7, whereas the Leu-Glu-His-Asp (LEHD) sequence is cleaved more efficiently by caspase-3 than caspase-7. Nevertheless, the effector caspases have their own specific substrates; e.g. cytoskeletal protein fodrin [30], the filament proteins lamin A/C [31], and the chaperone protein p23 [32], are specific substrates for caspase-3, -6, and -7, respectively.

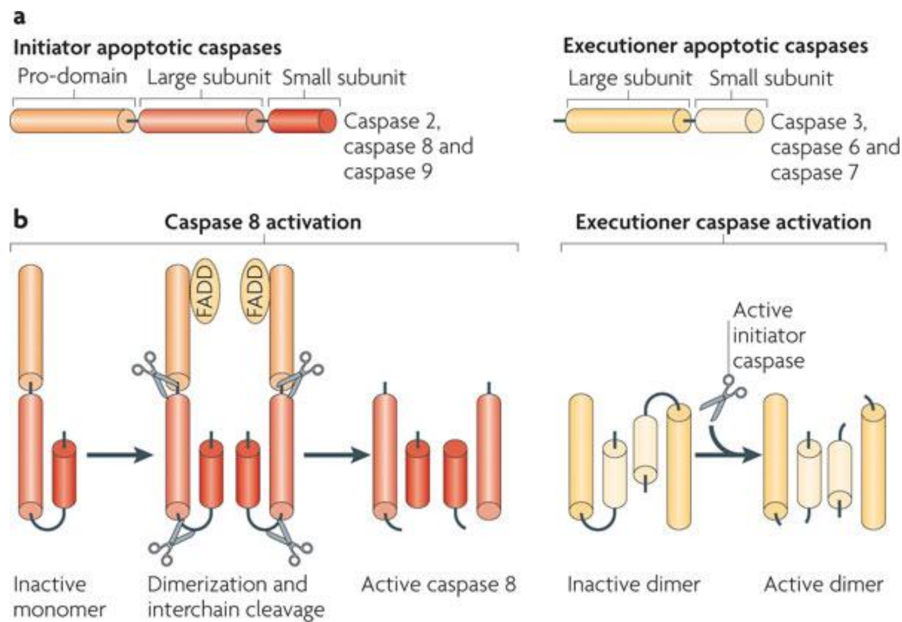


Figure 1.7: Difference and similarities between initiator and effector caspase cleavage and activation. The recruitment of initiator caspases by adapter proteins containing a CARD or DED domain induces the homo-dimerization of the initiator caspases. Through auto-proteolytic cleavage, the initiator caspases convert to active caspases. Executioner caspases pre-associate in inactive homodimers. Proteolytic cleavage of the inactive dimers by initiator caspases results in the formation of an active executioner caspase dimer. Figure derived from [33]

1.2.2 Intrinsic apoptotic pathway

Activation of the intrinsic apoptotic pathway can be initiated by various insults, such as DNA damage or growth factor starvation [33]. Although the stimuli are different, the mechanism of activation is alike and relies on the induction of mitochondrial outer membrane permeabilization (MOMP) (Figure 1.8). The induction of MOMP is tightly regulated by BCL-2 (B-Cell CLL/Lymphoma 2) family members, from which BAK and BAX are responsible for mediating the disruption of the mitochondrial outer membrane [34-39]. BAK/BAX-mediated MOMP leads to the cytoplasmic release of pro-apoptotic cytochrome c, which resides in the mitochondrial inner membrane space (IMS) [40, 41]. Once in the cytoplasm, cytochrome c drives the activation of caspase-3 and -7 through the formation of the apoptosome (Box 1). In addition to cytochrome c, other IMS residing pro-apoptotic proteins have been identified that are able induce apoptosis or promote cytochrome c-induced apoptosis, upon their release from the mitochondria (Box 2).

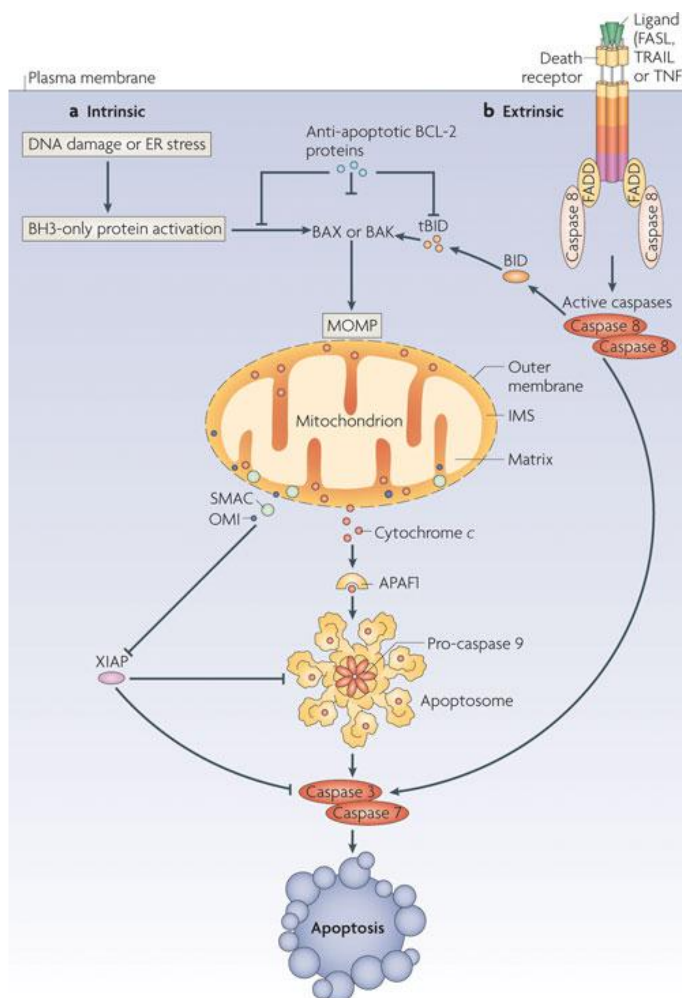
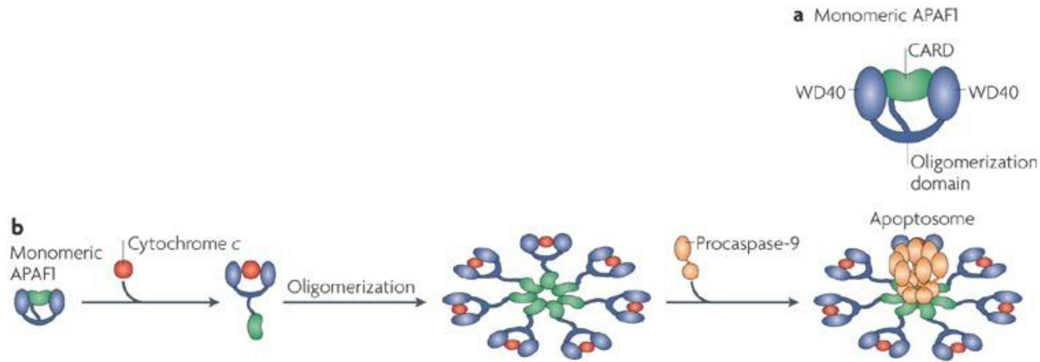


Figure 1.8: The intrinsic and extrinsic apoptotic pathway. The intrinsic apoptotic pathway is activated by intrinsic signals, such as DNA damage or growth factor starvation. These signals induce mitochondrial outer membrane permeabilization (MOMP) through activation of BCL-2 homology domain 3 (BH3)-only proteins, thereby activating the pro-apoptotic BAK and BAX proteins, which induce mitochondrial membrane permeabilization. MOMP causes the release of pro-apoptotic proteins from the mitochondrial inner membrane space (IMS), which activate the caspase-9-dependent pathway. The extrinsic apoptotic pathway is activated by death ligands. These ligands bind and activate DR on the cytoplasmic membrane, thereby inducing the recruitment of caspase-8 and -10. These initiator caspases are able to induce apoptosis through activation of effector caspases, or in certain circumstances, activate the intrinsic apoptotic pathway through cleavage of BID, resulting in truncated BID (tBID). In turn, tBID is able to activate BAK and BAX. Figure derived from [33]

Box 1: The apoptosome



The apoptosome is constituted through the interaction between APAF1 and cytochrome c released from the mitochondria, thereby producing a platform for the binding, dimerization, and activation of casapase-9 [42]. In turn, caspase-9 is able to induce apoptosis through the cleavage and activation of the effector caspases 3 and 7. Figure derived from [43]

Box 2: Mitochondrial-released apoptotic proteins

Cytochrome C

Cytochrome C is a heme protein that resides in the IMS [44]. It is part of the complex III and its main function is the transfer of electrons. Upon MOMP, it is released into the cytosol where it binds with APAF1 and subsequently with caspase-9, thereby forming the apoptosome (Box 1). This signaling complex is vital for the signal transduction of the intrinsic apoptotic pathway.

Smac/DIABLO

Second mitochondria-derived activator of caspase (Smac), or it human ortholog, direct IAP-binding protein with low pI (DIABLO), is released after MOMP and is thereafter able to bind and inhibit the IAP proteins cIAP1, cIAP2, and XIAP [45-47]. Thereby, it sequesters caspase-inhibitors and promotes the activation of caspase-3 and -7.

OMI/HTRA2 (HtrA Serine Peptidase 2)

Upon apoptotic insult, the serine protease OMI is released from the mitochondria into the cytoplasm [48]. There, it contributes to apoptosis through mediating the degradation of cIAP1, cIAP2, and XIAP, thus promoting caspase-activation. In addition, OMI has been shown to contribute to apoptosis in a caspase-independent manner through mediating the degradation of cytoskeletal proteins, such as actin, tubulin, and vimentin.

Apoptosis-inducing factor (AIF)

AIF is a flavin adenine dinucleotide-containing, NADH-dependent oxidoreductase that upon apoptotic insult translocates to the nucleus where it induces DNA degradation and chromatin condensation in a caspase-independent manner [49].

Endonuclease G (ENDOG)

ENDOG is an endonuclease that resides within the mitochondria. Upon apoptotic signal-induced release, it is able to translocate to the nucleus, where it mediates DNA fragmentation without sequence specificity [5, 50, 51].

Apoptosis-related protein in the TGF-beta signaling pathway (ARTS)

ARTS is a splice variant of the mammalian gene *Septin4* that upon apoptotic signaling is released from the mitochondria into the cytoplasm where it is able promote apoptosis through binding and inhibiting XIAP [52, 53]

1.2.2.1 B-cell lymphoma-2 (BCL-2) family members

Activation and inhibition of MOMP is regulated by proteins from the highly conserved BCL-2 family, which can be divided in three groups; the anti-apoptotic multidomain members, the pro-apoptotic multidomain members, and the pro-apoptotic BCL-2 homology domain 3 (BH3)-only members (Figure 1.9) [54]. Together, they regulate, through interactions on the mitochondrial outer membrane and in the cytosol, the threshold that has to be overcome to induce mitochondrial-mediated apoptosis.

- Proteins from the anti-apoptotic multidomain group (BCL-2, BCL-X_L, BCL-W, MCL1, and A1) contain four BCL-2 homology domains (BH domain) and, except for A1, a C-terminal transmembrane (TM) domain that enables the proteins localization to the mitochondria and endoplasmatic reticulum [55]. These proteins exert their anti-apoptotic effect through the binding of pro-apoptotic members of the BCL-2 family.
- The proteins from the pro-apoptotic multidomain group (BAX and BAK) mediate the induction of MOMP. They are structurally similar to their anti-apoptotic counter parts [55]. However, upon activation they undergo significant conformational changes. Like there anti-apoptotic homologues, they contain a TM domain. Nevertheless, in healthy cells, only BAK resides in the mitochondria [56]. BAX is primary cytosolic, and translocates to the mitochondrial membrane after cytotoxic signal-induced conformational changes [57].
- Like their name implies, the BH3-only proteins (BAD, BIK, BID, BMF, HRK, BIM, NOXA, and PUMA) only contain a BH3 domain and in certain cases (BIK, HRK, and BIM) a TM domain. These proteins are induced transcriptionally or post-transcriptionally by cytotoxic stress signals and are able to regulate MOMP through distinct pathways [58-60]. Some BH3-only proteins are able to directly activate BAK and BAX, whereas others neutralize the anti-apoptotic BCL-2 proteins (Figure 1.10) [61-67].

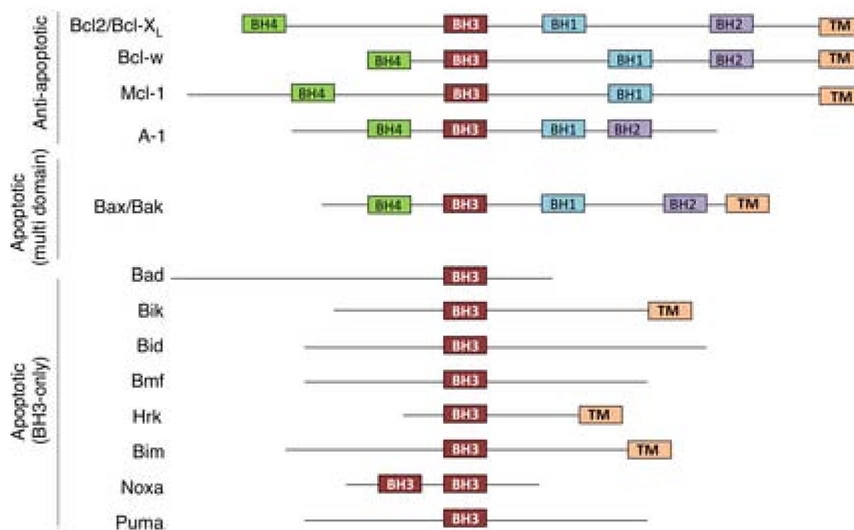


Figure 1.9: Classification and domain organization of BCL-2 family proteins. BCL-2 family members are divided in three categories; the anti-apoptotic and pro-apoptotic multi-domain proteins and the pro-apoptotic BH3-only proteins. The multi-domain proteins contain four BCL-2 homology domains (BH), whereas the BH3-only proteins only contain one BH domain. In addition, the BCL-2 family members can contain transmembrane domains (TM). Figure derived from [68]

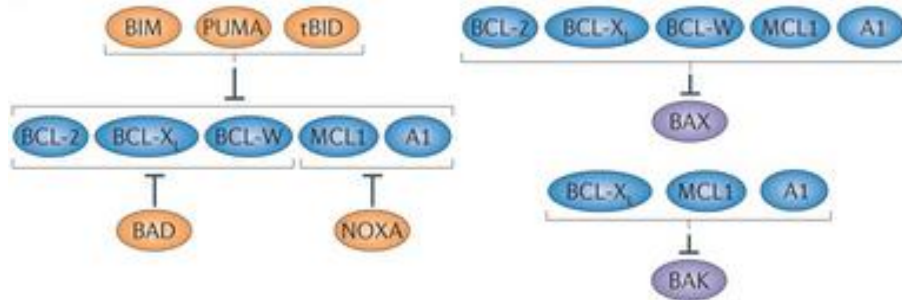


Figure 1.10: Hierarchy of BCL-2 family members. Whereas some BH3-only proteins are able to inhibit all anti-apoptotic BCL-2 family members, other BH3-only proteins (BAD and NOXA) have specificity for the anti-apoptotic BCL-2 family members. In addition, all anti-apoptotic BCL-2 family members are able to inhibit BAX activity, whereas only BCL-X_L, MCL1, and A1 are able to inhibit BAK activity. Figure adapted from [54]

Due to difficulty of detecting specific BCL-2 family member complexes, as a result of their transient nature, and because of the complexity of the discovered interactions between the BCL-2 family members, three BAK/BAX activation models have been described; the direct, the indirect, and the unified model (Figure 1.11).

- The direct activation model describes that certain BH3-only proteins (particularly truncated BID (tBID), BIM, and perhaps PUMA) are able to directly bind and activate the pro-apoptotic BAK and BAX proteins (Figure 1.11) [58, 61, 63-65, 69]. These proteins are therefore called “the activator BH3-only proteins”. The so-called “sensitizer BH3-only proteins”, BAD, NOXA, BIK, BMF, and HRK, are unable to activate BAK/BAX directly, but aid in the process through liberating “the activator BH3-only proteins” from their bound anti-apoptotic BCL-2 family members (Figure 1.10 and 1.11). The identification and classification of which BH3-only proteins are “activator BH3-only proteins” is still ongoing and remains debatable.
- The indirect activation model describes that the anti-apoptotic BCL-2 family members directly bind and inhibit BAK/BAX (Figure 1.10 and 1.11) [62, 66, 67]. Here, BAK/BAX can be spontaneously activated at low rate or by unknown modifications, but are inhibited by the anti-apoptotic BCL-2 family members. Full BAK/BAX activation is achieved through neutralization of the anti-apoptotic BCL-2 family members, which is mediated by the BH3-only proteins.
- Recently, Llambi *et al.* unified the above models (the direct and indirect) in the “unified model” (Figure 1.11) [60]. This model of BAK/BAX activation describes the anti-apoptotic BCL-2 family members-mediated inhibition of BAK/BAX activation through sequestering BH3-only proteins (Mode 1). In addition, the anti-apoptotic BCL-2 family members are also able to inhibit activated BAK/BAX (Mode 2). Activation of BAK/BAX occurs through BH3-only protein-mediated conformational changes in BAK/BAX.

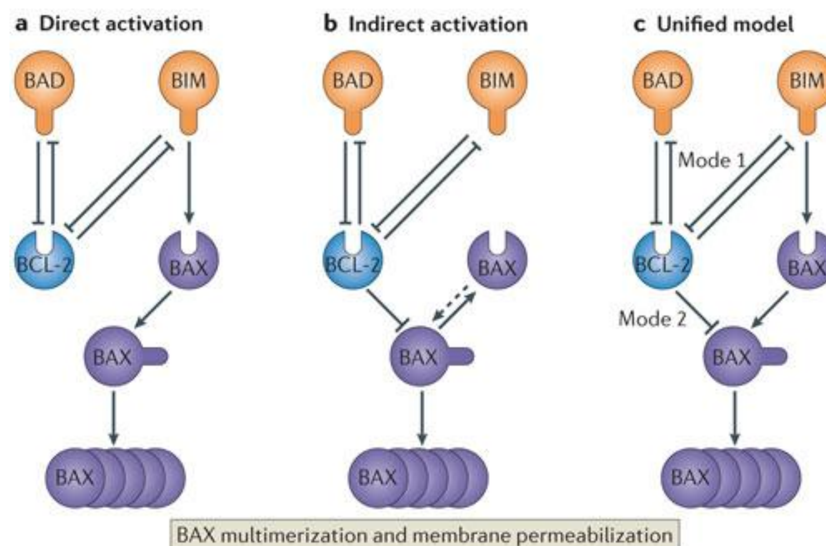


Figure 1.11: Models for BCL-2 family member-mediated induction of apoptosis. Three models have been proposed for the induction of MOMP by BCL-2 family members; the direct, indirect, and unified model. In the direct model, BAK/BAX is activated by BH3-only proteins. In the indirect model, the BH3-only proteins release BAK/BAX from their suppression by the anti-apoptotic BCL-2 family members. Recently, both models were unified in the unified model. Figure derived from [54]

1.2.2.2 BAK/BAX activation

So far, two distinct BAX activation sites have been proposed; a canonical hydrophobic groove and an alternative site on the opposite site of BAX [63, 70]. For BAK, the hydrophobic groove has been identified as the sole activation site [71-74]. In accordance to the unified model, activation of BAK/BAX by “activator BH3-only proteins” leads to the exposure of their BH3 domain [70]. In turn, the “activator BH3-only proteins” are disengaged and the BAK/BAX BH3 domain can be inserted in the hydrophobic groove of a neighboring BAK/BAX protein, thereby mediating the homo-oligomerization that induces pore formation and membrane permeabilization [39, 75]. The exact BAK/BAX structure and the mechanism mediating the permeabilization of the mitochondrial outer membrane are still poorly understood and remain to be characterized. Various models of membrane permeabilization have been proposed, such as BAK/BAX-mediated channel formation, BAK/BAX-induced formation of lipidic pores, and BAK/BAX-mediated promotion of mitochondrial fragmentation [70, 76, 77].

1.2.3 Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is activated by extracellular signals called death ligands, which activate death receptors (DRs) on the cytoplasmic membrane (Figure 1.8) [78-80]. These death receptors are able to bind adapter proteins and thereby activate the apoptotic pathway through recruitment and activation of caspase-8 and -10, which in turn are able to activate the effector caspases.

In addition, there exists a crosstalk between the extrinsic and intrinsic apoptotic pathway which occurs when caspase-8 or -10 cleaves the BCL-2 family member BID, resulting in truncated BID (tBID), which is able to induce MOMP (Figure 1.8) [81-83]. Cell types can be characterized by this event; type I cells (or type I apoptosis) display direct caspase-8/10-induced activation of effector caspases, whereas type II cells (or type II apoptosis) induce apoptosis through the cleavage of BID and activation of the mitochondrial/intrinsic apoptotic pathway.

1.2.3.1 Death receptors

Death receptors are death domain containing members of the tumor necrosis factor superfamily [78-80]. Up to date, eight members have been identified to belong to the death receptor subfamily (Table 1.1). These receptors are type-I transmembrane proteins that contain a C-terminal intracellular tail which holds an ~80 residues containing cytoplasmic region known as the death domain (DD) [84, 85]. In addition, the receptors contain a membrane-spanning region and an extracellular N-terminal domain that consist of 2-4 cysteine-rich domains (CRDs) that mediate ligand binding [86].

Table 1.1: Classification of death receptors, decoy receptors, and their cognate ligands.

Receptor	Cognate Ligand	Decoy receptor (DcR)
TNFR1 (DR1, CD120a, p55 and p60)	TNFα	
Fas (DR2, APO-1 and CD95)	FasL (CD95L, APO-1L)	DcR3
DR3 (APO-3, TWEAKR, LARD, TRAMP and WSL1)	TL1A (APO-3L, TWEAK)	DcR3
DR4 (TRAIL-R1, APO-2)	TRAIL (APO-2L)	DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), OPG
DR5 (TRAIL-R2, KILLER and TRICK2)	TRAIL (APO-2L)	DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), OPG
DR6	?	
Nerve Growth Factor Receptor (NGFR, p75NTR)	NGF	
Ectodysplasin A receptor (EDAR)	EDA1	

Death receptors are activated by binding of their cognate ligands, named death ligands (Table 1.1). These cytokines belong to the TNF protein family and are type-II transmembrane proteins [87]. Certain death ligands can also be present in soluble form through proteolytic cleavage. This release of the death ligands lowers their apoptosis-inducing capacity and is therefore proposed to be mechanism for death ligand inactivation. Moreover, soluble death ligand can inhibit membrane-bound death ligand-induced cell death through competitive binding, thereby further increasing their inhibitory potential.

Competing for ligand binding are the so-called decoy receptors (Table 1.1), these receptors share a high homology with their cognate death receptors but lack a death domain [78-80]. Decoy receptors are present as soluble or membrane-attached receptors and, due to the lack of their death domain, are unable to form signaling complexes

Induction of death receptor signaling requires ligand binding and oligomerization/trimerization of the receptor [78-80]. This induces juxtaposing of the intracellular domains and recruitment of adapter proteins to their death domain [88]. The trimerization of the death receptor can be induced by ligand binding, however it has been shown that the receptors can be present as preassembled oligomers on the cell surface [89]. This ligand-independent oligomerization depends on interactions between the first CRD on the extracellular tail of the receptors [90-92]. This domain, which is named the preligand assembly domain (PLAD), is not directly involved, but its presence is crucial for ligand binding.

Death receptor induced signal transduction can results in various outcomes;

(1) Induction of apoptosis through the activation of the caspase-cascade
(2) Pro-survival signaling through the activation of the NF-κB transcription factor and MAPK pathways, amongst which are c-Jun N-Terminal Kinase (JNK), Extracellular Signal-Regulated Kinase (ERK), Phosphoinositide-3-Kinase (PI3K), and p38
(3) Necroptosis through the recruitment of RIP1 (Receptor-Interacting Protein 1) and subsequent activation of RIP3

1.2.3.2 Death receptor recruited adapter proteins

The death domain is not restricted to death receptors, as it is present on ~30 cytoplasmic proteins [93]. However, out of the ~30 cytoplasmic proteins only nine have been shown to interact with death receptors, with FADD, TRADD, and RIP1 being the best characterized.

1.2.3.2.1 Fas Associated Death Domain containing protein (FADD)

FADD, a 28kDa cytoplasmic protein, contains a Death Domain (DD) on its C-terminal tail and has been shown to interact with the DD of the death receptors Fas, TNFR1, DR3, DR4 and DR5 [93]. FADD is the only DD-domain containing protein that also harbors a Death-Effector Domain (DED) domain, thereby making it the key adapter protein for DR-induced apoptosis [94, 95]. The DED domain on the N-terminal tail facilitates the recruitment of other DED-containing proteins, such as caspase-8, caspase-10 and c-FLIP, thereby activating the extrinsic, caspase-dependent, apoptotic pathway.

Nevertheless, the cellular functions of FADD are more complex than only mediating DR-induced apoptosis. FADD is essential for embryonic development, since knockout mice are not viable and embryos die at day E12.5 *in utero* [96]. In addition to DR-induced apoptosis, FADD has also been shown to be involved in cell survival, cell proliferation, cell cycle progression, and is able to induce cell death independently of DRs (Box 3) [95]. Therefore, tight control of FADD activity is necessary, which is achieved through regulation of FADD phosphorylation and subcellular localization in the cytoplasm and nucleus [97, 98]. Furthermore, FADD can be contained in microvesicles and released to the extracellular fluid through adenosine receptor activation [99]. These aspects make FADD a multi-functional protein, thought to be involved in inflammation, chemoresistance, and tumorigenesis.

1.2.3.2.2 TNF Receptor associated Death Domain protein (TRADD)

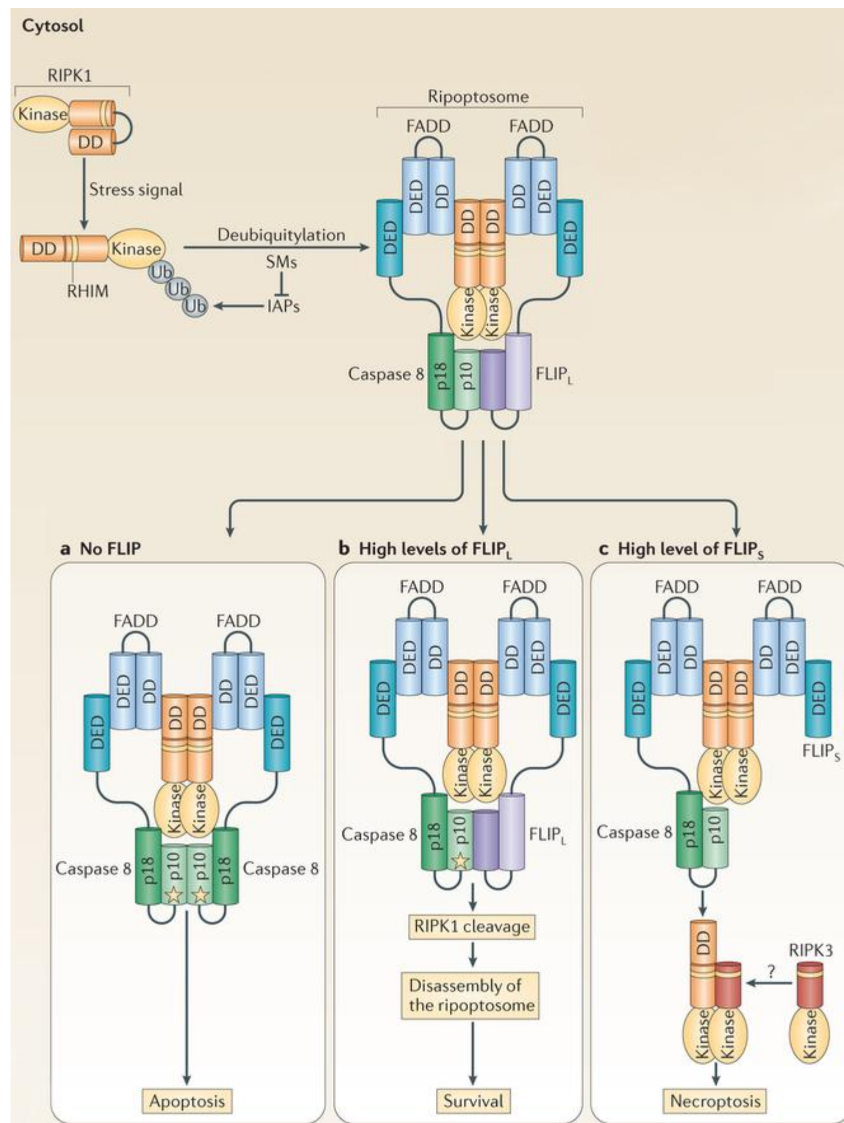
The 34 kDa cytoplasmic protein TRADD contains a DD on its C-terminus that is able to associate with the DDs of RIP1 and all DRs except for EDAR [93]. Moreover, TRADD contains an N-terminus that can recruit the TNF Receptor-Associated Factor 2 and 5 (TRAF2 and TRAF5) [100]. In contrast to FADD, which predominately promotes DR-induced apoptosis, TRADD is mainly involved in DR-induced non-apoptotic signaling. Upon DR-induced recruitment, TRADD is able to bind TRAF2/5, RIP1, and cellular inhibitor of apoptosis proteins 1 and/or 2 (cIAP1 and cIAP2), thereby inducing activation of signaling cascades that can lead to cell survival, proliferation and in certain cases cell death [101].

1.2.3.2.3 Receptor Interacting Protein 1 (RIP1)

RIP1 is a 76 kDa protein that is able to directly bind TNFR1, Fas, DR4, and DR5, however with lower affinity for DRs than FADD and TRADD [93, 102, 103]. As a consequence, in the presence of TRADD and FADD, upon DR activation, RIP1 is recruited to the DDs of these adapter proteins. However, in cases where TRADD expression is low, such as in MEFs, RIP1 is able to bind DRs without the support of TRADD [104, 105]. Besides the DD and a RIP Homotypic Interaction Motif (RHIM) on its C-terminal tail, RIP1 also contains a serine/threonine kinase domain on its N-terminus involved in the phosphorylation of proteins, amongst which is RIP3 [106]. RIP1 is a key protein in regulating the DR-induced outcome such as NF-κB and MAPK signaling and plays a significant role in the induction of necroptosis.

Box 3: The ripoptosome

The ripoptosome is a large cytoplasmic protein complex that forms independently of the DR and mitochondrial apoptotic pathways and is able to induce apoptosis through caspase-8 activation [107]. Known stimuli that are able to induce ripoptosome formation are IAP antagonists known as Smac mimetics and the genotoxic stressors etoposide and possibly cisplatin [108-112]. Inhibition of IAPs leads to an accumulation of RIP1 which induces the spontaneous formation of the ripoptosome. The complex comprises RIP1, FADD, and caspase-8, and is inhibited by c-FLIP$_L$, cIAP1, cIAP2, and XIAP [107]. Inhibition of the complex by c-FLIP$_S$, the pan-caspase-inhibitor zVAD, and caspase-8 or FADD knockout leads to recruitment of RIP3 and results in formation of the necrosome (Box 4). Figure adapted from [113]



1.3 Inhibition of DR-induced apoptosis

Apart from the anti-apoptotic BCL-2 family members, various endogenous proteins are able to inhibit apoptosis induced by death receptors and/or are able to shift their signaling from apoptosis to pro-survival signaling (Figure 1.12) [114]. In many occasions, DR stimulation is not sufficient for induction of cell death, especially in the case of TNFR1, presumably due to the endogenous expression or the DR-mediated induction of these anti-apoptotic proteins [115, 116]. Overturn of this inhibition requires the down-regulation of the anti-apoptotic proteins, which can be achieved through knockout or inhibition of the anti-apoptotic proteins, or by treatment with inhibitors of protein expression, such as actinomycin D and cycloheximide [115-122]. Known inhibitors of DR-mediated apoptosis are; c-FLIPs, cIAPs, XIAP, FAIM, Lifeguard, A20, Fas-Associated Phosphatase-1 (FAP-1), Phosphoprotein Enriched In Astrocytes 15 (PEA-15), and Bruton Agammaglobulinemia Tyrosine Kinase (BTK) [114].

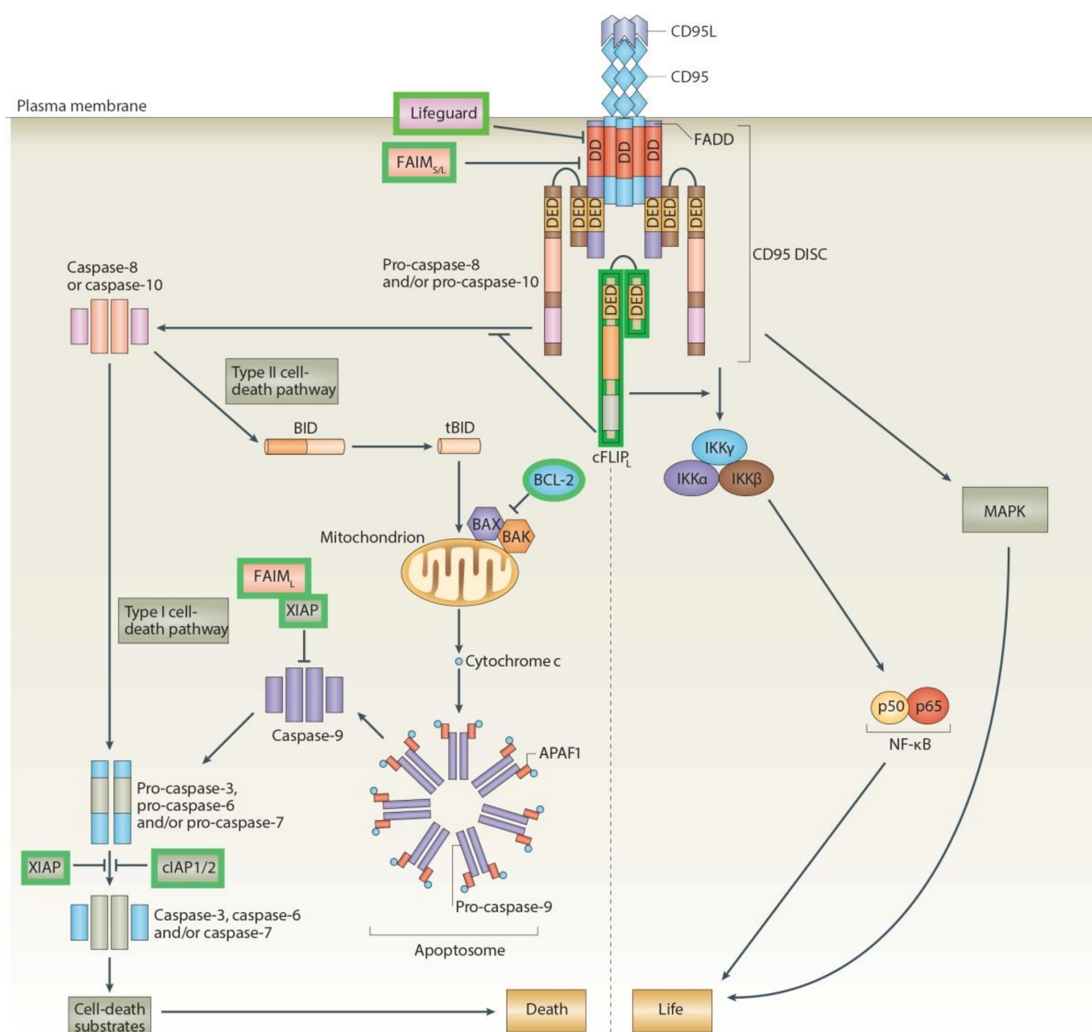


Figure 1.12: Inhibition of DR-induced apoptosis. DR-induced apoptosis can be inhibited at various stages of the extrinsic and intrinsic apoptotic pathway, through the inhibition of adapter protein recruitment, caspase activation, or MOMP induction. Proteins that inhibit the apoptotic pathway are marked in green. Figure adapted from [123]

1.3.1 Cellular FLICE-like inhibitory protein (c-FLIP)

The catalytically inactive caspase-8 homologue c-FLIP is a cytosolic anti-apoptotic protein that plays an important role in death receptor signaling [124, 125]. Although 11 splice variants of c-FLIP have been identified, the expression of only three splice variants of the protein has been detected; the Raji isoform (c-$FLIP_R$), the short isoform (c-$FLIP_S$), and the long isoform (c-$FLIP_L$). All three isoforms contain two DED motifs on the C-terminus that mediate their recruitment to death receptors. There, they exert their anti-apoptotic activity through competing for death receptor binding and inhibiting DR-mediated caspase-8 and -10 recruitment and activation (Figure 1.12 and 1.13).

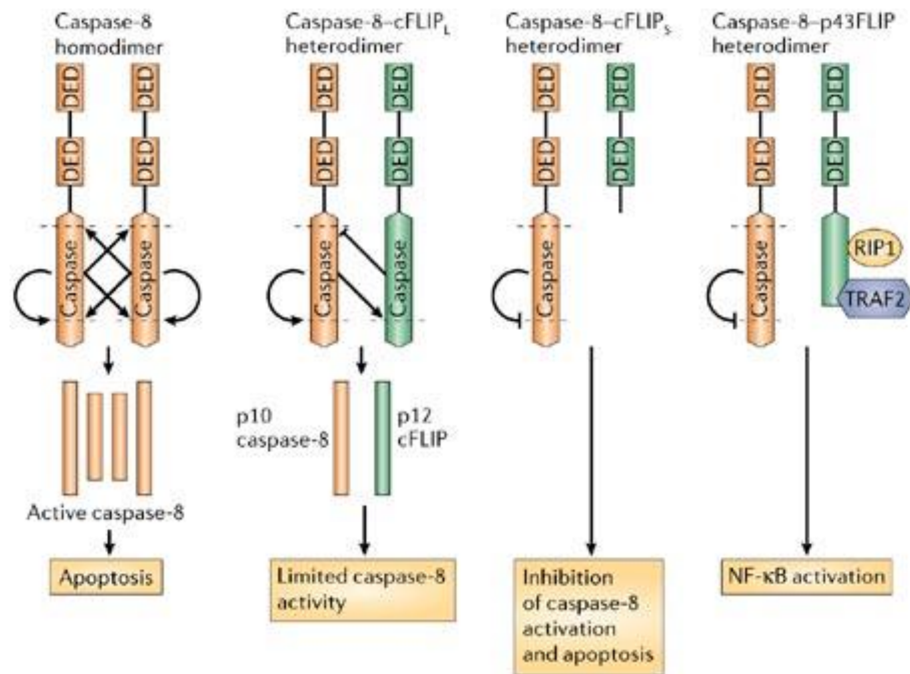


Figure 1.13: c-FLIP-mediated regulation of caspase-8-induced outcome. Caspase-8 is able to form homodimers, but also heterodimers with c-FLIP isoforms. Depending on the dimer formation, caspase-8 can be inhibited, induce apoptosis, of NF-κB activation. Figure derived from [126]

Like the other two isoforms, the long c-FLIP isoform (c-$FLIP_L$) contains two DED motifs on its C-terminus [124, 125]. However, on its N-terminus it harbors a catalytically inactive pseudo-caspase-domain (Figure 1.13). Upon death receptor activation, c-$FLIP_L$ is able to hetero-dimerize with caspase-8 and -10, thereby inhibiting caspase-activation. However, when c-$FLIP_S$ or c-$FLIP_R$ are highly expressed or when strong receptor activation is induced, c-$FLIP_L$ is able to promote caspase-8 and -10 activation [127-129]. In addition, caspase-8 mediated cleavage of c-$FLIP_L$ at the Asp-376 residue can results in the formation of an active caspase-8-p43FLIP hetero-dimer that is able to induce NF-κB activation (Figure 1.12 and 1.13) [130, 131]. Moreover, in response to FasL and TNFα stimulation, c-$FLIP_L$ has been shown to activate the ERK1/2 pathway through binding and activation of the serine/threonine-protein kinase Raf-1 [130, 132].

1.3.2 Inhibitors of apoptosis (IAPs)

The antagonists of DR-mediated apoptosis that belong to the inhibitors of apoptosis (IAP) family are characterized by their Baculovirus IAP Protein Repeat (BIR) domains (Figure 1.14) [133-136]. The BIR domain is a 70-80 amino acid zinc-binding region that is involved in the binding of other proteins and which is necessary for their anti-apoptotic activity. So far, 8 human proteins have been identified to belong to the IAP family [137].

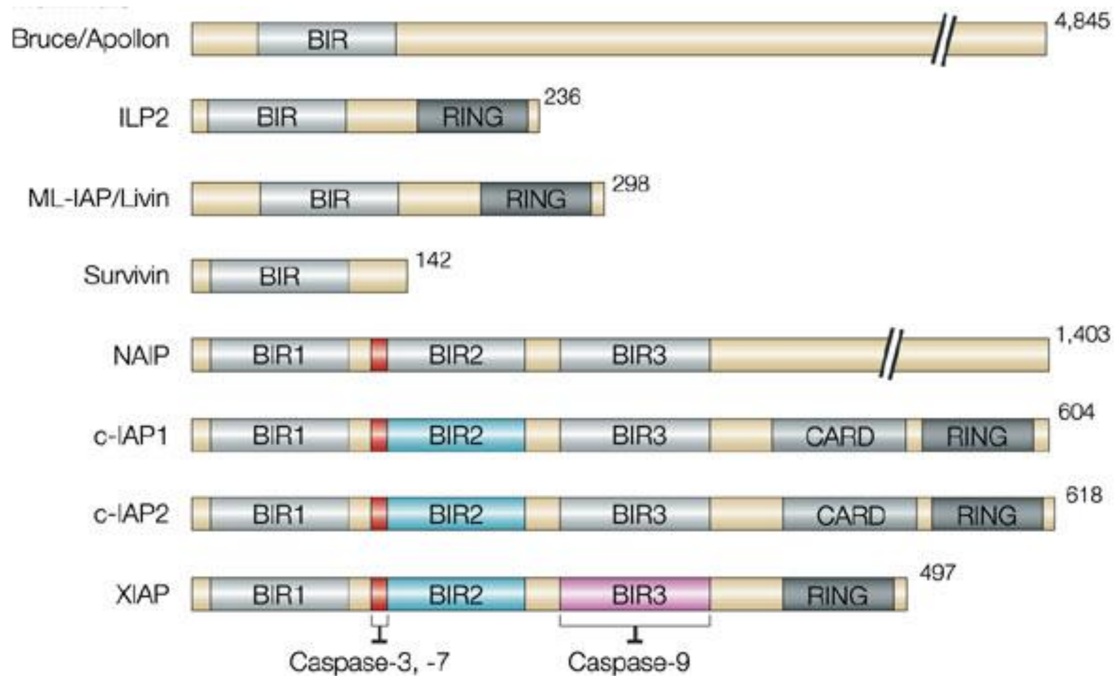


Figure 1.14: Classification and domain organization of IAP family proteins. IAP proteins are characterized by their baculovirus IAP repeat (BIR) domains. These domains are involved in protein binding and the inhibition of caspases. IAP proteins can contain E3 ligase activity through their Really Interesting New Gene (RING) domains, and certain IAP proteins (cIAP1 and cIAP2) have been shown to contain a CARD domain. However, up to date no activity or binding proteins have been described for this latter domain. Figure adapted from [138]

1.3.2.1 cIAP1 and cIAP2

Cellular inhibitors of apoptosis 1 and 2 (cIAP1 and cIAP2) are cytosolic proteins that contain three N-terminal BIR domains, a CARD domain, and RING domain on their C-terminal (Figure 1.14) [137]. The RING domain has been shown to be involved in RIP1 ubiquitination, whereas up to date no binding partners have been identified for the CARD domain [139, 140]. Both cIAP1 and cIAP2 play a vital role in the TNFR1-mediated signal transduction towards NF-κB. Knockdown of cIAP1 and cIAP2 results in full abrogation of the TNFR1-mediated ubiquitination of RIP1 and activation of NF-κB [141, 142]. Furthermore, TNFα induces apoptosis in cIAP1/2 knockout MEFs. In addition to regulating NF-κB activity, cIAP1/2 are able to inhibit the active effector caspase-3 and -7 by interactions through their BIR2 domain and the preceding linker (Figure 1.12 and 1.14) [143, 144]. However, this interaction does not take place at the active site of the caspase. Since cIAP-caspase binding only results in a weak caspase inhibition, it is debated wether cIAP1/2 have significant caspase-inhibitory potential [143]. Nevertheless, cIAP1/2 play a substantial role in the inhibition of DR-induced apoptosis by promoting NF-κB signaling [139, 140]. Inhibition of cIAP1/2 by Smac or through administration of Smac mimetics (inhibitors of cIAPs that promotes their degradation) results in sensitization to DR-induced cell death [122]. However, this effect is presumed to be attributed to the inhibition of XIAP.

1.3.2.2 XIAP

The X-chromosome-linked inhibitor of apoptosis (XIAP) is a potent cytosolic inhibitor of active caspase-3, -7, and -9 (Figure 1.12) [145, 146]. Moreover, XIAP is able to bind caspase-9 when present in the apoptosome. XIAP contains three N-terminal BIR domains and a C-terminal RING domain with E3 ligase activity (Figure 1.14) [137]. The second BIR domain, together with the preceding linker, is responsible for caspase-3 and -7 binding and inhibition [147]. The third BIR domain binds and inhibits caspase-9. The mitochondrial protein Smac that is released following MOMP induction is able to inhibit XIAP [46, 47]. Smac binds with higher affinity to the third BIR domain than to the second BIR domain [148]. Therefore, Smac predominately inhibits the XIAP-mediated inhibition of caspase-9. The RING domain of XIAP is involved in auto-ubiquitination, thereby targeting the protein for proteasomal degradation [149]. The DR antagonist $FAIM_L$ is able to inhibit this process, thereby stabilizing XIAP and promoting inhibition of apoptosis [150].

Apart from anti-apoptotic activity, XIAP is able to promote NF-κB activation and this is dependent on its RING domain [151-153]. Although there is some conflicting data, it has been shown that XIAP's first BIR domain is able to interact with TAK1-binding proteins 1 (TAB1) and that XIAP promotes the ubiquitination and activation of Transforming Growth Factor-Beta-Activated Kinase 1 (TAK1).

1.3.3 Fas Apoptotic Inhibitory Molecule (FAIM)

To date, two FAIM isoforms have been identified; the short ($FAIM_S$, 20 kDa) and the long isoform ($FAIM_L$, 22 kDa) [154, 155]. These cytosolic proteins do not contain previously characterized domains, but both isoforms have been shown to contain DR antagonist activity (Figure 1.12) [150, 156-160]. The short FAIM isoform is ubiquitously expressed and was first identified to inhibit Fas-induced apoptosis in B-lymphocytes [154, 159]. Its mode of action is proposed to depend on its ability to regulate c-$FLIP_L$ expression [161]. On the other hand, FAIM-L expression is restricted to neurons and is able to suppress TNFR1 and Fas-induced apoptosis [155, 160]. Recently, $FAIM_L$ was identified to be able to stabilize XIAP expression through inhibiting its proteasomal degradation induced by XIAP-mediated auto-ubiquitination [150]. Thereby, $FAIM_L$ increases the XIAP-mediated inhibition of caspases and protects from FasL-induced apoptosis. In addition, $FAIM_L$ is able to directly bind Fas and inhibit apoptosis by preventing the activation of caspase-8 [160].

Remarkably, in neurons, $FAIM_s$ does not protect from DR-induced apoptosis but promotes Nerve Growth Factor (NGF)-induced NF-κB activation, resulting in increased neurite outgrowth [162]. Its NF-κB promoting ability was also confirmed in B-lymphocytes where it increases CD40-induced NF-κB activation [163]. Also $FAIM_L$ has been proposed to promote NF-κB activation. However, a detailed characterization of its mode of action has still to be described. In Alzheimer patients, $FAIM_L$ levels were observed to be reduced and, *in vitro*, Amyloid-β was shown to induce $FAIM_L$ reduction in primary cortical neurons [164]. Moreover, Carriba *et al.* showed that $FAIM_L$ was necessary for TNFα-induced protection form Amyloid-β-induced cell death in cortical neurons, suggesting a role for $FAIM_L$ in TNFR1-mediated pro-survival signaling, possibly induced by NF-κB activation.

1.3.4 Lifeguard (LFG/FAIM2/*TMBIM2*)

The DR antagonist Lifeguard (LFG, 35 kDa) is a membrane protein which is expressed in most cells, but predominately in neurons [165, 166]. It is able to directly interact with Fas, without displacing FADD, and has been shown to inhibit Fas-mediated apoptosis (Figure 1.12) [166-169]. TNFR1-mediated signaling was shown not to be affected by LFG expression in HeLa cells [166].

LFG belongs to the family of Transmembrane BAX Inhibitor Motif –containing proteins (TMBIMs) [170]. These proteins contain 6 transmembrane domains and are involved in calcium homeostasis by regulating calcium efflux from the Endoplasmatic Reticulum (ER) [171]. Calcium efflux from the ER upon Fas stimulation has been shown to contribute to FasL-induced apoptosis [172]. This mechanism relies on the caspase-8-mediated cleavage of B-Cell Receptor-Associated Protein 31 (BAP31) and the formation of micropores between the ER and mitochondria, thereby promoting MOMP [173]. LFG is able interact with BCL-X_L, thereby inhibiting FasL-

induced calcium efflux from the ER and inhibiting apoptosis (Urresti *et al.,* article submitted for publication [174]).

In cancer, various roles have been attributed to LFG. In breast cancer models, LFG was proposed to contribute to cancer progression due to its high expression in malignant breast cancer cell lines [175, 176]. On the contrary, in neuroblastoma cell lines, LFG down-regulation promotes tumor aggressiveness [177]. Here, MYCN (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog) mediated repression of LFG expression modulates the expression of genes involved in cell adhesion and cell cycle regulation, resulting in sphere formation, reduced cell adhesion, and increased tumor cell migration. It was shown *in vivo* that silencing LFG increases the xenografts metastatic potential. Moreover, neuroblastoma patients with low expression of LFG show a marked decrease in 5-year survival rates.

1.4 Fas/CD95/APO-1

The Fas receptor is a 45 kDa prototypical death receptor with 3 cysteine rich-domain (CRDs) that is mainly expressed at the plasma membrane where it pre-associates in homotrimers [92, 178-181]. It is activated by its physiological ligand FasL which predominately binds at the second and third CRD [182]. FasL can be either membrane-bound (mFasL, 45 kDa) or soluble (sFasL, 27 kDa) and preassembles in homotrimers [183]. Soluble FasL is produced through metalloproteinase-mediated cleavage of mFasL and has been shown to lower the cell death-inducing potential at least 1000-fold [184, 185]. Therefore, it is thought that membrane-bound FasL is the main responsible for FasL-induced apoptosis under physiological conditions.

The Fas death receptor is widely expressed throughout the human body, with high levels in liver, heart, kidney, pancreas, colon, thymus, lymphoid tissue, and mature T cells [186-188]. In contrast, the expression of its cognate ligand, FasL, is highly restricted. FasL is predominately found in cells of the immune system, such as natural killer (NK) cells, NK-T cells, and activated T-cells [189]. There, it plays an important role in activation-induced cell death (AICD), a process for the elimination of T cells after immune responses [190]. Data from studies with Fas/FasL knockout and mutant mice highlight the importance of the Fas/FasL pathway for maintaining the homeostasis of the immune system [191-195]. The Fas/FasL pathways is activated to delete pathogen-infected cells and useless or autoreactive T cells. As a consequence, mutations in the *FAS* and *FASL* genes have been shown to promote the development of auto-immune diseases due to lymphoproliferation. In addition, cytotoxic T lymphocytes (CTLs) and NK cells are able to engage the Fas/FasL pathway to eliminate cancer cells [196-203].

1.4.1 Fas-mediated apoptosis

Upon FasL-induced receptor activation, Fas organizes in lipid rafts and forms high-order aggregates [204-206]. It recruits the FADD adapter protein to its Death Domain (DD) and thereby forms the platform for pro-caspase-8/10 recruitment and death inducing signaling complex (DISC) formation (Figure 1.15) [183, 207-210]. Thereby, caspase-8/10 becomes activated and initiates the apoptotic signaling pathway. In type I cells, where the effector caspases are directly activated by caspase-8/10, efficient DISC formation requires clathrin-mediated Fas internalization (Figure 1.15) [183, 204, 205, 207, 209]. This step is where FADD and caspase-8/10 are predominately recruited. As a consequence, blocking the internalization impairs DISC formation and apoptosis, and induces Fas-mediated NF-κB and ERK signaling [209]. On the other hand, type II cells, where caspase-8 induces MOMP through cleavage of BID, show slower and reduced DISC formation and caspase-8 activation [207].

XIAP has been shown to dictate whether a cell undergoes type I or type II apoptosis [83, 211]. In response to Fas activation, the inhibitor of the effector caspase-3 and -7 and the initiator caspase-9 is upregulated in type I cells but downregulated in type II cells. Moreover, in type II cells, knockdown of XIAP shifts the apoptotic phenotype to that of a type I cell. Although the compelling evidence for the regulation of the apoptotic phenotype, the regulation of XIAP in the different cell types remains to be elucidated. Another factor proposed to regulate if cells display type I or type II apoptosis is the level of Fas expression [212]. Meng *et al.* showed that in type I and type II cells, there is no clear difference in XIAP, FADD, caspase-8, and c-FLIP expression levels. However, type I cells display higher mRNA and cell surface levels of Fas compared to type II cells. Moreover, type II cells could be converted to type I cells, and vice versa, through overexpression or downregulation of Fas, respectively.

Lipid rafts exert a controlling role in Fas-mediated apoptosis as they mediate the redistribution and clustering of Fas [183, 204-210]. In addition, they promote the recruitment of DISC proteins. The redistribution of Fas in lipid rafts is able to convert type II cells to type I cells [213]. Our understanding of the influence of lipid rafts on Fas-mediated apoptosis has been further enriched by the group of Dr. Faustino Mollinedo, who showed that pharmacological induced formation of lipid raft is sufficient to induce Fas-mediated apoptosis without the need for FasL [214-216].

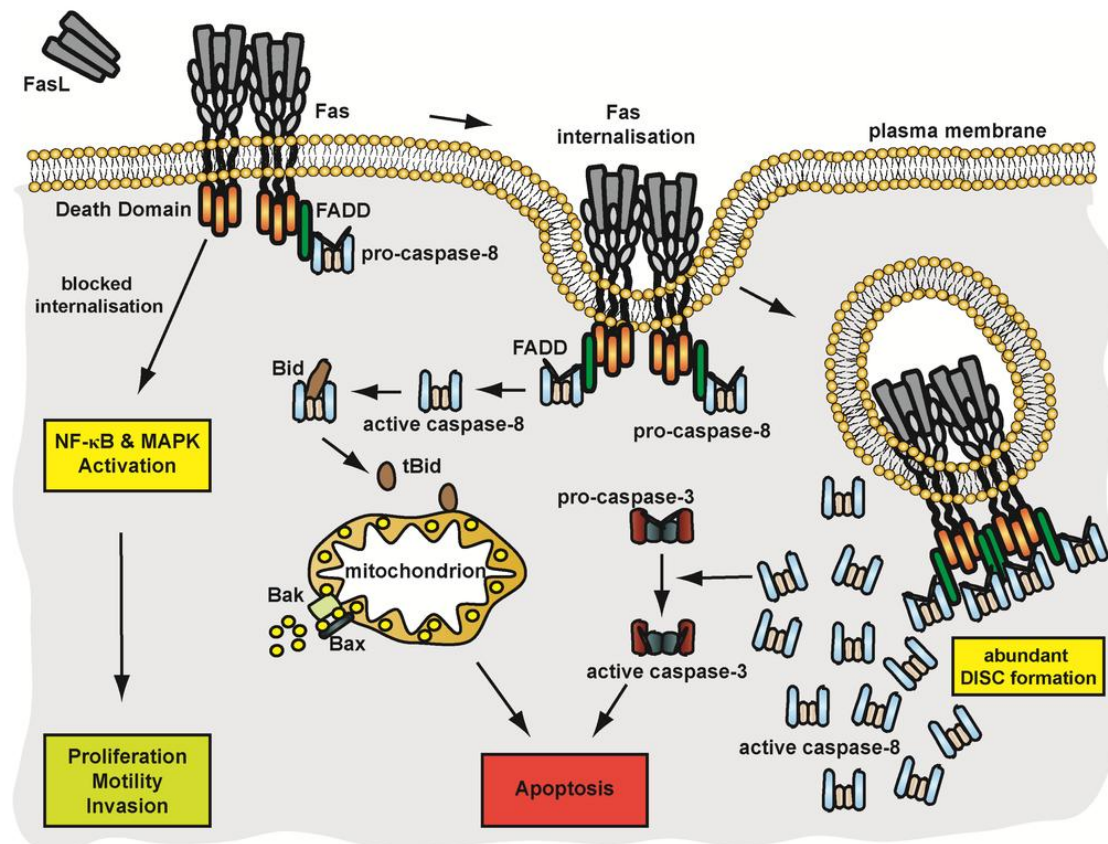


Figure 1.15: Fas mediated induction of apoptosis and NF-κB and MAPK pathway activation. FasL-induced activation of Fas induces the recruitment of the adapter protein FADD andcaspase-8/10. Subsequent internalization induces abundant DISC formation in type I cells and direct effector caspase activation. In type II cells, little caspase-8 is activated and apoptosis is induced through activation of the intrinsic apoptotic pathway by cleavage of BID, resulting in truncated BID (tBID). Under certain circumstances Fas stimulation can induce NF-κB and MAPK pathway activation. Figure derived from [217]

1.4.2 Non-apoptotic roles of Fas

In addition to cell death induction, Fas is able to promote inflammation, cell proliferation and cell survival through the activation of the NF-κB and MAPK pathways (Figure 1.15) [78, 218]. Moreover, inhibition of cIAP2 and knockdown of TRAF2 have been shown to promote Fas-induced necroptosis through the recruitment of RIP1 [219, 220].

Early findings described that Fas stimulation increased the proliferation of CD3-activated primary T cells [221]. Later, it was discovered that FasL could induce apoptosis or proliferation in a dose-dependent manner [222, 223]. Stimulation of primary human naive $CD4^+$ T cells with high doses of FasL resulted in apoptosis, whereas low concentrations promoted T cell receptor-induced NF-κB and MAPK activation that induced T cell proliferation. In the nervous system, where Fas and FasL are widely expressed, FasL has been shown to play a role in neuronal development, growth, regeneration, and differentiation [224-226]. In primary dorsal root ganglion neurons, FasL induces neurite outgrowth through ERK activation [186], whereas in adult neural stem

cells, FasL favors neuronal differentiation by activating the PI3K pathway [227]. Treatment with FasL also accelerates the recovery from sciatic nerve injury *in vivo.* Furthermore, activation of the Fas/FasL pathway promotes neuronal branching after a traumatic brain injury [228].

Further elucidation of the wide range of signaling pathways that can be induced by Fas activation was achieved through investigation of the role of the Fas/FasL pathway in inducing an inflammatory response. A large selection of cytokines and chemokines can be induced by Fas [229, 230]. Characterization of the pathways involved in the FasL-induced inflammatory response indicated roles for caspase-8 and c-FLIP$_L$ in regulating the activation of NF-κB and MAPK pathways. The caspase-8-p43FLIP hetero-dimer was shown to be necessary for NF-κB and MAPK pathway activation.

1.4.3 Regulation of Fas signaling outcome

Since the outcome of Fas signaling is differential depending on the cell type, various mechanisms have been identified to account for the final outcome.

One clear factor is FasL, which can be present as a membrane bound (mFasL) or soluble form (sFasL). In retinal ganglion cells, sFasL administration was shown to inhibit the mFasL-induced neurotoxicity [231]. Moreover, *in vivo*, sFasL rescued lymphoma cells from mFasL-induced apoptosis and inhibited the pro-inflammatory response [232]. RIP1 was identified as a possible regulator of the difference between sFasL and mFasL induced signaling [233]. Treatment with mFasL and crosslinked sFasL indicated that RIP1 is efficiently recruited to mFasL-activated Fas, but much less efficiently to sFasL-activated Fas. Therefore, it was proposed that less RIP1 recruitment promotes Fas-mediated non-apoptotic signaling. Nevertheless, many cell types respond in a similar manner when treated with sFasL or mFasL. Lastly, Fas expression levels have been shown to regulate the outcome of Fas activation, as was shown by assessing fibroblasts with high and low Fas expression levels. FasL stimulation of fibroblasts with low Fas expression induced proliferation, whereas FasL stimulation of fibroblast with high Fas expression induced apoptosis [234]. These data suggest that induction of Fas expression might shift the FasL-induced response from proliferation to apoptosis.

Post-translational Fas modifications also have been shown to regulate Fas outcome. Palmitoylation of Fas is necessary for localization and clustering in lipid rafts and inhibits its lysosomal degradation [204, 205]. Studies with Fas mutants that impede palmitoylation revealed that loss of palmitoylation induced a clear reduction in lipid raft translocation, internalization, and apoptosis. S-glutathionylation of murine Fas at Cys-294 also promotes Fas clustering and localization in lipid rafts, thereby sensitizing for FasL-induced cell death [235]. Phosphorylation of Fas at Tyr-232 and -291 in the DD increases the cell surface exposure of Fas and promotes oligomerization upon FasL binding [236]. On the other hand, Fas-Associated Phosphatase 1 (FAP-1)-mediated dephosphorylation inhibits cell surface Fas exposure and desensitizes for FasL-induced apoptosis [237]. Also the post-translational modification sialylation has been shown to play a role in Fas-induced apoptosis. The Golgi glycosyltransferase, ST6Gal-1 (ST6 Beta-Galactosamide Alpha-2,6-Sialyltranferase 1), is able to inhibit FasL-induced recruitment of FADD through sialylation of Fas [238]. Thereby, it impedes caspase-8 recruitment and inhibits the apoptotic pathway. Lastly, two N-glycosylation sites have been identified on Fas [239, 240]. Loss of glycosylation did not affect its DISC formation potential but did result in a reduction in capsase-8 activation.

Also DISC-interacting proteins have been observed to determine the Fas outcome. Overexpression of c-FLIP$_L$ has a significant influence on blocking apoptosis [125, 127-130, 230]. In addition, it is shown to promote FasL-induced activation of survival pathways such as NF-κB and MAPK through the recruitment of TRAF1, TRAF2, RIP1 and Raf-1. However, in other studies, knockdown of total c-FLIP was shown to increase FasL-induced NF-κB activation, whereas overexpression of c-FLIP resulted in inhibition of RIP1 recruitment and inhibition of NF-κB activation. Why in certain models c-FLIP inhibits apoptosis whereas in other models c-FLIP inhibits pro-survival signaling remains to be clarified. It has been proposed that these effects are regulated by the level of FasL stimulation or the ratio between caspase-8 and c-FLIP [127, 241]. One study showed that when treating

with low concentrations of FasL, c-FLIP inhibits apoptosis. However, when treated with high doses of FasL, c-FLIP promoted apoptosis. On the other hand, MAPK activation requires the activation of caspase-8 and thus c-FLIP can inhibit MAPK signaling through inhibition of caspase-8 [242-245]. Therefore, c-FLIP is proposed to be able to shift FasL-induced signaling from NF-κB to MAPK activation. These data are contradictory to findings by Kataoka *et al.* who described that c-FLIP-L promotes FasL-induced activation of the NF-κB and ERK pathway [130]. Dependence on c-FLIP-L was later confirmed for TNFα-induced ERK activation [132].

1.5 Tumor Necrosis Factor Receptors (TNFRs)

The well characterized cytokine TNFα is able to activate two TNF receptors, i.e. TNFR1 and TNFR2 [246-250]. Both receptors show a similar extracellular domain with 4 CRDs, however their intracellular domains are remarkably different. Whereas the death receptor TNFR1 contains a DD on its intracellular tail, the TNFR2 does not and is therefore not classified as a death receptor [93]. Nevertheless, TNFR1 and TNFR2 share similar signaling pathways, with slight differences, that can result in the activation of the MAPK pathways and NF-κB.

1.5.1 Tumor Necrosis Factor α (TNFα)

TNFα is initially expressed as a 26kDa trimeric type II transmembrane protein (mTNFα), however proteolytic cleavage by the metalloproteinase TNF-converting enzyme (TACE) results in release of an extracellular soluble form of TNFα (sTNFα, 17 kDa) [251-255]. Both membrane-bound and soluble TNFα are able to homotrimerize and bind the TNF receptors. However, whereas TNFR1 can be activated by both sTNFα and mTNFα binding, TNFR2 is only activated by mTNFα [246, 247].

The proinflammatory cytokine TNFα is widely expressed, with studies indicating its presence on activated NK and T cells, monocytes, macrophages, microglia, and various non-immune cells such as fibroblasts and endothelial cells [256, 257]. After its discovery it was named tumor necrosis factor alpha (TNFα), due to its cytotoxic ability to induce necrosis in the mouse fibrosarcoma cell line L929 [258, 259]. Later, more TNFα-induced outcomes were identified, such as inflammation, proliferation, differentiation, cell survival, and cell death [78, 248].

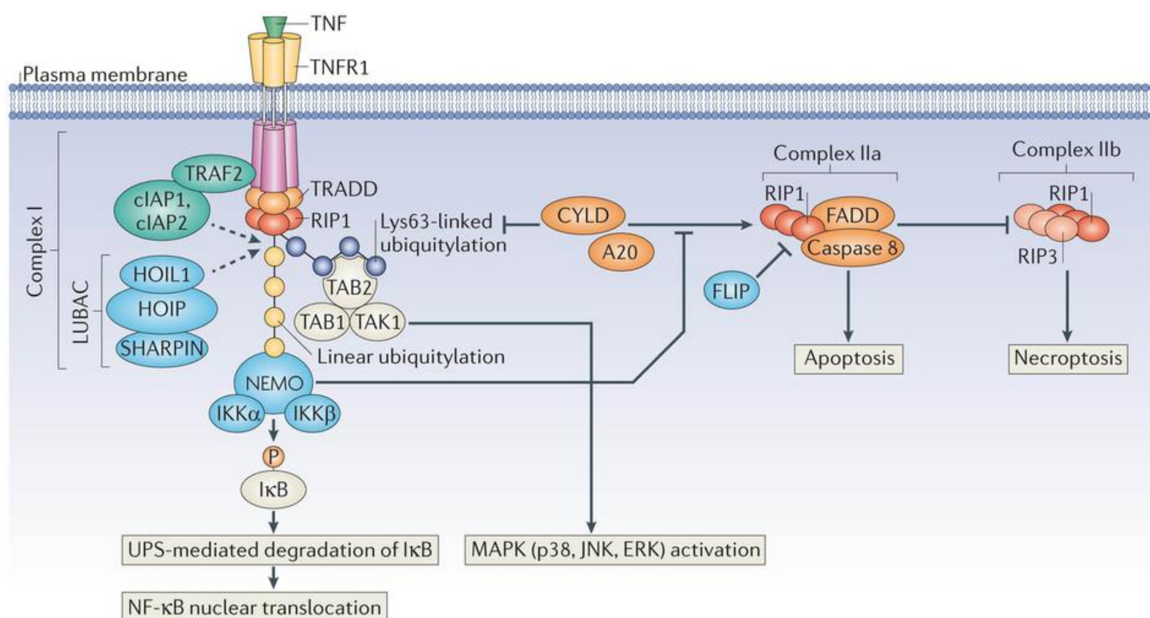


Figure 1.16: Differential outcomes of TNFR1-mediated signaling. TNFR1 stimulation leads to complex I formation and NF-κB and MAPK pathway activation. RIP1 de-ubiquitination by CYLD or A20 results in complex IIa formation which can induce apoptosis. However, this pathway is blocked through the NF-κB-mediated upregulation of c-FLIP. Inhibition of the NF-κB pathway or downregulation of anti-apoptotic proteins is therefore necessary for complex IIa-mediated apoptosis. Under certain circumstances were caspase-8 activity is lacking, complex IIa formation can lead to necroptosis through complex IIb (Necrosome) formation. Figure derived from [260]

1.5.2 TNFR1 and TNFR2

The TNFR1 death receptor is a ubiquitously expressed type I transmembrane protein and has been shown to preassemble in homotrimers through PLAD interactions [89, 90, 261]. Activation of TNFR1 can result in the formation of various complexes with different outcomes, i.e. complex I, IIa, and IIb (Figure 1.16) [78, 248]. Whereas, complex I induces pro-inflammatory signaling and the induction of predominately pro-survival genes, complex IIa and complex IIb induce apoptosis and necroptosis, respectively. In most cell types, TNFα-induced activation results in complex I formation and activation of NF-κB and MAPK pathways, which leads to the transcription of pro-survival genes that inhibit complex II induced cell death [117, 118, 132]. However, when complex I-mediated signaling is impeded, TNFR1 activation will result in complex II-mediated cell death [262]. Known stimuli that promote complex II mediated cell death are; inhibition of NF-κB and MAPK activation, downregulation of anti-apoptotic proteins, or inhibition of protein synthesis. Like Fas, TNFR1 can be post-transcriptionally modified by phosphorylation [263, 264]. The MAPK ERK2 is able to phosphorylate TNFR1 at the intracellular tail close to the membrane region. By doing so, ERK2 is able to terminate TNFR1 signaling through inducing the TNFR1 translocation from the plasma membrane to the Endoplasmatic Reticulum (ER), thereby inhibiting the TNFR1-activated apoptotic pathway.

In contrast to the ubiquitous expression of TNFR1, TNFR2 expression is restricted to immune cells, endothelial cells, and neurons [265]. Following activation, TNFR2 is able bind TRAF1/2, thereby mediating the recruitment of cIAP1/2 and inducing their ubiquitination [266-268]. This results in a TNFR1-like signaling that activates MAPK and NF-κB pathways. Due to the lower affinity of TRAF2 to TNFR2 compared to TRADD, expression of TNFR2 has been proposed as a mechanism for TNFR1 inhibition [269-271]. Nevertheless, knockout studies have indicated roles for TNFR2 in protecting neurons from apoptosis and generation of Cytotoxic T Lymphocyte (CTL) responses [272, 273]. Marchetti *et al.* showed that TNFα stimulation of cortical neurons from TNFR1 knockout mice induced persistent NF-κB activity, which was necessary for protection from glutamate excitotoxic stress-induced apoptosis [273]. On the other hand TNFα stimulation of cortical neurons from TNFR2 knockout mice did not result in protection of glutamate induced cell death. The role of TNFR2 in CTL response generation was elucidated by studies from Kafrouni *et al.* who described that TNFR2-deficient but not TNFR1-deficient CTLs showed delayed antiviral-immune responses [272]. Altogether these data indicate a critical role for TNFR2 in the regulation of specific cellular responses, which oppose the notion that TNFR2 expression operates as an inhibitory mechanism for TNFR1 activation.

1.5.2.1 Complex I

The TNFR1-recuited complex I consists of various proteins, some of which function as adapter proteins, whereas others contain E3 ubiquitin ligase activity which is necessary for the recruitment of other complex I proteins (Figure 1.17). Upon TNFα stimulation, TNFR1 translocates to lipid rafts and rapidly recruits TRADD which functions as the adapter protein for the binding of RIP1 and TRAF2/5 [103, 104, 274-277]. However, in certain cases where there are low levels of TRADD, RIP1 is directly recruited to the receptor [104]. In turn, TRAF2/5 is able to recruit cIAP1/2 (Figure 1.17) [104, 278]. Both TRAF2/5 and cIAP1/2 are E3 ubiquitin ligases that induce K63 ubiquitination of RIP1 and cIAP1/2, but it is proposed that cIAP1 and cIAP2 are the main mediators of RIP1 ubiquitination [279-281]. The K63 ubiquitination of RIP1 forms a platform for the recruitment of the TAK1/TAB complex (TGFβ-activated kinase 1 (TAK1) and TAK1-binding proteins *(TAB1/2/3)*) (Figure 1.17) [282-284]. In addition, the K63 ubiquitination of cIAP1/2 forms the platform for the recruitment of the linear ubiquitin chain assembly complex (LUBAC) that consists of three proteins; Heme-Oxidized IRP2 Ubiquitin Ligase 1 (HOIL1), HOIL1-Interacting Protein (HOIP), and SHANK-associated RH domain-interacting protein (SHARPIN) [285-291]. Recruitment of LUBAC stabilizes the TNFR1 complex I through linear ubiquitination of RIP1. In turn, the linear ubiquitination of RIP1 recruits the IKK complex that consists of IκB kinases α and β (IKKα and IKKβ) and NF-κB essential modulator (NEMO). Recruitment of the various protein complexes results in TAK/TAB-mediated phosphorylation and activation of the IKK complex. Upon activation, the IKK complex phosphorylates the NF-κB inhibitor IκBα (Inhibitor of Kappa B Alpha) that sequesters NF-κB in the cytosol thereby impeding its nuclear translocation (Figure 1.17) [292-295]. Phosphorylation of IκBα targets

the protein for K48 ubiquitin-mediated degradation by the proteasome and induces the release of NF-κB, which thereafter is able to translocate to the nucleus were it induces gene transcription. Apart from activating the NF-κB pathway, the MAP3K TAK1 and Raf-1 are able to mediate the activation of the MAPK pathways, resulting in JNK, ERK, and p38 activation [296].

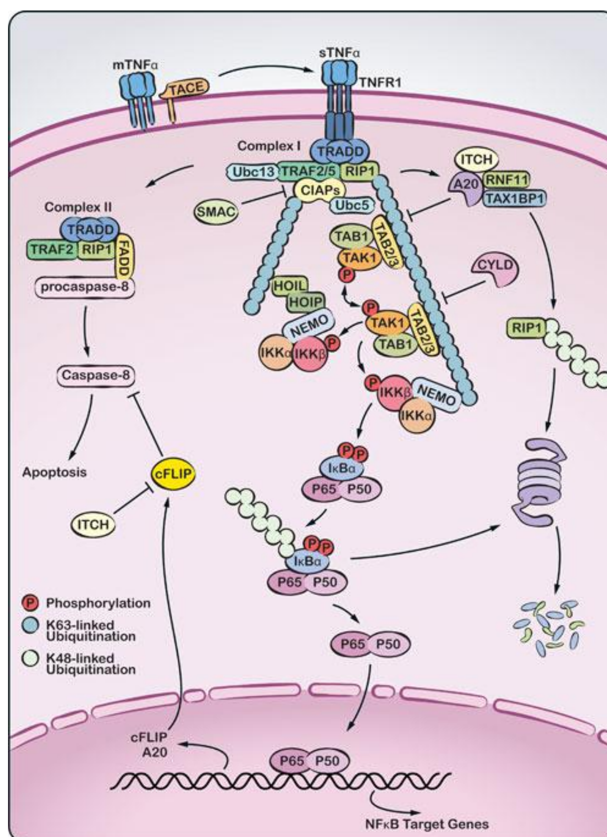


Figure 1.17: TNFR1 complex I recruited proteins and their ubiquitination and phosphorylation. TNFα stimulation of TNFR1 induces the recruitment of a large set of proteins with E3 ligase and kinase activity. Recruitment of these proteins is necessary for the ubiquitination of cIAPs and RIP1, thereby forming platforms for further protein recruitment, such as the TAK/TAB and IKK complex. Recruitment of these protein complexes results IKK-mediated phosphorylation of IκBα, thereby activating the NF-κB pathway. Figure derived from [297]

1.5.2.2 Complex IIa

TNFR1 internalization after TNFα stimulation is able to induce the formation of complex IIa (Figure 1.16) [298, 299]. TRADD, together with RIP1, and TRAF2/5, is able to dissociate from the receptor and recruits FADD, which functions as an adapter protein for caspase-8/10 binding [262]. Thereby, complex IIa is able to activate the caspase-pathway and induce apoptosis. However, in most cells, the NF-κB-induced expression of c-FLIP$_L$ inhibits complex IIa activity through inhibiting of caspase-8/10 activity (Figure 1.16) [262]. Formation of complex IIa depends on the ubiquitination state of RIP1 [282, 300-304]. Lack of ubiquitination, mediated by A20- and CYLD-induced de-ubiquitination, or through the inhibition or depletion of cIAP1/2, has been shown to drive complex IIa formation [304-306].

1.5.2.3 Complex IIb (Necrosome)

After complex IIa formation, RIP3 is recruited to the complex and degraded by c-FLIP$_L$-caspase-8 hetero- and caspase-8 homodimer-mediated proteolytic cleavage [262, 302-304, 307, 308]. However, lack of caspase-activity allows the binding of RIP3 and thereby the formation complex IIb, also called the necrosome (Figure 1.16 and 1.18) [3, 106, 309, 310].

The necrosome is the protein complex involved in the induction of necroptosis (Figure 1.18) [3, 106, 309, 310]. Its formation is induced through RIP1 activation by various DRs (through FADD-mediated recruitment), Toll-like receptors, and intracellular stimuli. In healthy cells, activated RIP1 is degraded by caspase-8, leading to termination of RIP1 signaling [262, 302-304, 307, 308]. However, in cells were caspase-8 is lacking or inhibited, RIP1 is able to recruit and activate RIP3. This dimerization leads to activation of the Mixed Lineage Kinase Domain-Like (MLKL) protein [311-314]. In turn, MLKL translocates to the membranes (cytoplasmic, mitochondrial, lysosomes, Golgi, and ER) where it forms pores and induces membrane rupture [311, 313, 315-317]. Thereby, MLKL induces cell death proposed to be mediated by the influx of Na^+ and Ca^{2+}, however whether this influx is the sole inducer of the necroptotic cell death is still under debate. Expression of caspase-8 and c-FLIP$_L$ has been shown to inhibit necroptosis. Additionally, necroptosis can be inhibited through the administration of small-molecule inhibitors, such as Necrostatin-1 (Nec-1) for RIP1 or Necrosulfonamide (NSA) for MLKL inhibition (Figure 1.18) [11, 312].

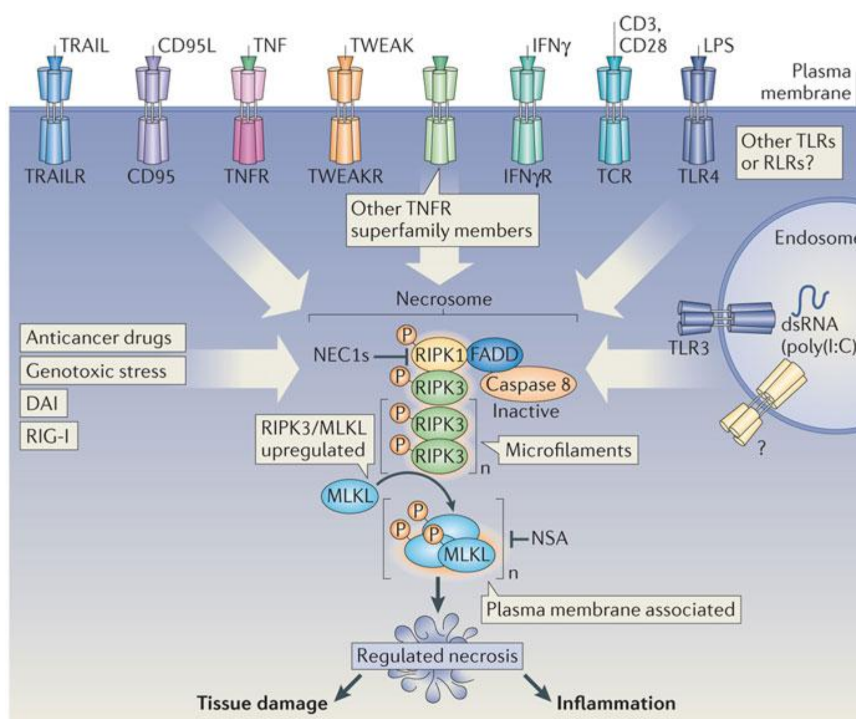


Figure 1.18: Various types of signals are able to induce necroptosis through necrosome formation. The necrosome has been shown to be induced by various kinds of signals. Activated RIP1 can recruit RIP3, however caspase-8 subsequently induces the degradation of RIP3, thereby inhibiting necrosome formation. When caspase-8 activity is lacking, RIP3 is recruited to the complex and activated by RIP1. This induces the subsequent phosphorylation and activation of MLKL, which following activation, is able to induce necroptosis through membrane rupture. Necrostatin-1 (NEC1s) and or Necrosulfonamide (NSA) are known inhibitors of necroptosis. Figure derived from [318]

1.6 Nuclear Factor κB (NF-κB)

The transcription factor NF-κB was first identified in 1986 and described as a nuclear factor that binds the enhancer element of the immunoglobulin kappa light-chain of activated B cells, hence NF-κB [319]. The transcription factor is present as hetero or homo dimers through combination of interaction between five NF-κB family members; p65 (RelA), RelB, c-Rel, p100 (NF-κB1), and p105 (NF-κB2) (Figure 1.19) [320-324]. Both p100 and p105 are present as pro-forms and their proteolytic cleavage results in the formation of p50 and p52, respectively. All NF-κB family members contain a Rel homology domain (RHD) which is necessary for dimerization and the binding to κB-response elements (5'-GGGRNYYYCC-3', where R is a purine, Y is a pyrimidine and N is any nucleic acid) [320-324]. In addition, p65, RelB, and c-Rel contain a C-terminal Transcriptional Activation Domain (TAD) necessary for the induction of gene transcription (Figure 1.19). The p52 and p50 hetero and homo dimers do not contain a TAD and as a consequence lack transcriptional activity and therefore act as transcriptional repressors [320-324]. Without stimuli, NF-κB is retained in the cytosol through binding of inhibitors of NF-κB (IκBs), with p100 and p105 having intrinsic IκB activity (Figure 1.19). These proteins are characterized by ankyrin repeats (ANK), which are able to bind the DNA binding domains of NF-κB and inhibit their transcriptional activity. Nevertheless, the equilibrium between IκB and NF-κB binding does allow for low levels of NF-κB shuttling between the cytosol and nucleus, which is proposed to explain their low basal transcriptional activity [325, 326].

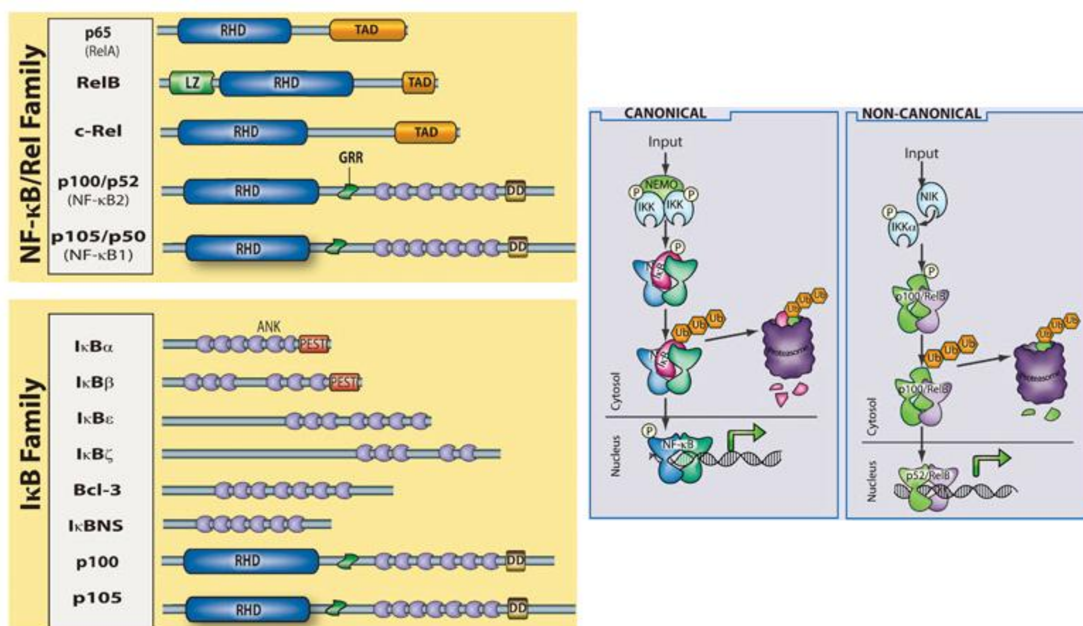


Figure 1.19: NF-κB activation and classification and domain organization of the mammalian NF-κB and IκB protein families. The NF-κB family proteins are characterized by their Rel homology domain (RHD) which is necessary for dimerization and DNA binding. In addition they contain a transactivation domain (TAD) necessary for the induction of gene transcription. The IκB family proteins are characterized by ankyrin repeats (ANK). These domains bind the DNA binding domains of the NF-κB family members and inhibit their transcriptional activity. The activation of NFkB can be achieved by two different pathways; the canonical and non-canonical pathway. In the canonical pathway, the IKK complex induces the phosphorylation of IκBα, whereas in the non-canonical pathway NIK activates IKKα which thereafter induces the phosphorylation of p100. Figure adapted from [327]

1.6.1 Canonical and non-canonical pathway

Two receptor-induced pathways have been described to activate NF-κB; the classical or canonical pathway and the alternative or non-canonical pathway (Figure 1.19) [320-324]. Known receptors that activate the canonical pathway are TNFR1, TNFR2, Toll-like receptors (TLRs), Interleukin-1 receptor and antigen receptors. Their stimulation leads to the activation of the IKK complex that is able to phosphorylate IκBα [292-295]. Thereby, IκBα is targeted for K48 ubiquitination and proteasomal degradation, thus releasing NF-κB and allowing for its translocation to the nucleus.

The non-canonical pathway is activated by receptors, such as CD40, TNFR2, B Cell-Activating Factor Receptor (BAFFR), and receptor activator for nuclear factor κB (RANK) [320-324]. The activation of the non-canonical pathway relies on receptor-induced activation of the NF-κB-inducing kinase (NIK). Following activation, NIK is able to phosphorylate predominately IKKα, thereby activating the IKK protein which in turn is able to phosphorylate p100 [328-330]. The IKK-mediated phosphorylation of p100 results in its K48 ubiquitination, which causes the protein to be partially degraded and the formation of p52. Generally, p100 is bound to RelB and therefore the proteolytic processing of p100 results in a transcriptionally active RelB/p52 NF-κB complex [331, 332].

1.6.2 Regulation of NF-κB outcome

NF-κB is able to induce the transcription of over a 1000 estimated genes, depending on their accessibility due to epigenetic regulation such as, DNA methylation and histone modification [333, 334]. Other factors that regulate the NF-κB outcome are dimer formation, repressor and activator interactions, chaperone biding, and post-transcriptional modifications [320-324, 335]. Known modifications to regulate NF-κB outcome are phosphorylation, acetylation, ubiquitination, and methylation. These modifications are able stimulate or inhibit the transcriptional activity, stabilize or destabilize the complex, and regulate nuclear translocation.

The longevity of the NF-κB-mediated gene induction is also regulated through a negative feedback loop. NF-κB induces various proteins that are able to terminate the NF-κB signaling, e.g. A20, IκBα, IκBε, p100 and p105 [336-341]. In the case of TNFR1 signaling, the induction of A20 mediates the K63 de-ubiquitination of RIP1 and its subsequent K48 ubiquitination and proteasomal degradation, thereby terminating the TNFR1-induced NF-κB and MAPK signaling [304, 306]. On the other hand, the NF-κB-mediated expression of IκBα, IκBε, p100 and p105 induces the sequestering and inhibition of the NF-κB dimers and targets the complex for translocation from the nucleus to the cytosol [320-324].

1.6.3 NF-κB and apoptosis

NF-κB is classically described as a mediator of anti-apoptotic responses [320-324]. The anti-apoptotic nature of NF-κB is displayed by its ability to induce the expression a large set of anti-apoptotic proteins, amongst which are c-FLIPs, cIAPs, XIAP, TRAFs, and BCL-2 anti-apoptotic proteins [333]. Through the upregulation of c-FLIPs, TRAFs, and cIAPs, NF-κB modulates the extrinsic apoptotic pathway by promoting the activation of the NF-κB and MAPK pathways, thereby inhibiting the apoptotic pathway [78, 83, 124, 218, 248]. On the other hand, upregulation of BCL-2 anti-apoptotic proteins and XIAP inhibits both the intrinsic and extrinsic apoptotic pathway; BCL-2 anti-apoptotic proteins through the inhibition of MOMP, whereas XIAP inhibits apoptosis by inhibiting caspase-3, -7, and -9 activation [33, 83, 137].

Although NF-κB plays a strong role in inhibiting apoptosis, various reports have described the NF-κB-mediated upregulation of pro-apoptotic proteins, indicating a dual role for NF-κB in the regulation of apoptosis [320-324]. Amongst these pro-apoptotic proteins are the death receptors Fas, DR4, DR5, and DR6, the death ligands FasL and TNF-Related Apoptosis-Inducing Ligand (TRAIL), the pro-apoptotic BCL-2 family members, BAX and BIM, and the tumor suppressors p53 and MYC [333]. Upregulation of death receptors and death ligands potentiates or sensitizes to apoptosis induced by the extrinsic apoptotic pathway, whereas BIM and BAX induction is able to induce MOMP-induced apoptosis [33, 78, 83, 124, 218, 248]. The NF-κB-mediated expression of p53 and MYC has been shown to play a role in NMDA-induced apoptosis in rat striatum [342].

Apart from inducing gene transcription, NF-κB is also able to repress the gene transcription of known anti-apoptotic NF-κB target genes, such as *TRAF*s*, cIAPs, XIAP, c-FLIP, A20,* and *BCL-X*$_L$ [343-345]. Thereby, NF-κB has been described to sensitize cells to cell death induced by cytotoxic agents and death ligands.

These studies demonstrate that the outcome of NF-κB activation is cell type- and stimuli-specific. Moreover, regulation of NF-κB interactions and post-transcriptional modifications are likely to play a role in the final outcome. Due to its pro-survival nature, NF-κB has been proposed as a target for cancer therapy, with the reasoning that by inhibiting NF-κB the expression of anti-apoptotic proteins will be downregulated [321-323]. However, the data on its pro-apoptotic role raises the question whether specific inhibition of its pro-survival function, without affecting NF-κB's pro-apoptotic function, would be more beneficial. Certain studies have indicated that the pro-apoptotic function of NF-κB is necessary for cell death induced by chemotherapy. In the case of doxorubicin treatment, a DNA-intercalating agent, NF-κB inhibition was shown to reduce apoptosis [346-348]. Moreover, the topoisomerase inhibitor etoposide induces cell death through the NF-κB-mediated upregulation of TRAIL [347].

1.7 Neuroblastoma

Neuroblastoma (NB) is a solid pediatric tumor that arises from neuronal crest cells [349-352]. It is the most common type of extracranial solid tumor in infants, which account for 15% of all of cancer-related deaths in children. NBs show a high degree of heterogeneity that becomes evident in the clinic. NB patients are observed to have cases that range from spontaneous regression to cases with rapid development of highly aggressive metastatic type of tumors that in many cases do not respond to the currently used treatment. In the latter cases, the 5-year survival rate is below 50%, whereas patients with low or intermediate-risk NB show high survival rates (Table 1.4).

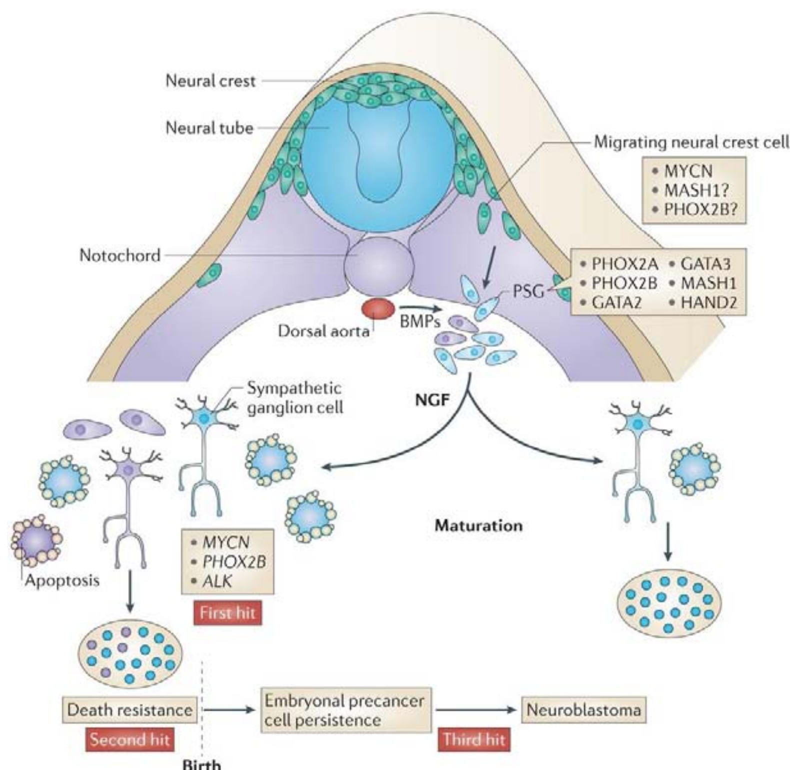


Figure 1.20: Neuroblastoma development from neural crest cells. During development, neuronal crest cells migrate away from the neuronal tube after which they differentiate, in a NGF-dependent manner, into neuronal structures. Genetic aberrations in the *MYCN*, *PHOX2B*, and/or *ALK* gene can lead to the formation of malignant cells. Figure derived from [353]

The development of NB starts from within the neuronal ganglia of the peripheral sympathetic nervous system (Figure 1.20) [349-352]. During embryogenesis, neuronal crest cells migrate away from the neuronal tube and differentiate into neuronal structures in response to NGF. In cases where the *MYCN* transcription factor gene is amplified or Anaplastic Lymphoma Kinase (*ALK*) and Paired-like Homeobox 2B (*PHOX2B*) are mutated, the differentiation of neuronal crest cells might be impaired and thus malignant transformation can occur. Subsequent evasion of cell death, along with increased in cell survival and cell proliferation may finally results in the formation of NB.

The International Neuroblastoma Staging System was introduced in 1988 to classify patients and correlate them with their outcome [354] (Table 1.2). Recently, a new staging system was introduced by the International

Neuroblastoma Risk Group (INRG), due to the developments made in the understanding of NB [355]. This system, entitled INGR Staging System (INRGSS), describes four risk groups; the very low, low, intermediate, and high risk (Table 1.3). NB patients are categorized through scoring of different parameters; stage of the NB, age of the patient, histology, the grade of tumor differentiation, *MYCN* amplification, 11q aberration, and ploidy. Depending on the risk group, NB patients have a low or high five-year event-free survival rate (Table 1.4).

Table 1.2: Definition of NB stage

Stage	Definition
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
L2	Locoregional tumor with presence of one or more image-defined risk factors
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow

Table 1.3: INGR Staging System

INRG stage	Age (months)	Histologic category	Grade of tumor, differentiation	*MYCN*	11q aberration	Ploidy	Pretreatment risk group
L1/L2	Any	GN maturing or GNB intermixed					A Very low
L1	Any	Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	<18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
L2	>18	GN nodular; Neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	<18			NA		Hyperdiploid	F Low
	<12			NA		Diploid	I Intermediate
	12 to <18			NA		Diploid	J Intermediate
	<18			Amp			O High
	≥18						P High
MS	<18	Any	Any	NA	No		C Very low
					Yes		Q High
				Amp			R High
Abbreviations: GN, ganglioneuroma; GNB, ganglioneuroblastoma; NA, nonamplified; Amp, amplified							

Table 1.4: Five-year event-free survival per risk group

Pretreatment risk group	Five-year event-free survival (EFS)
Very low	>85%
Low	75% to ≤85%
Intermediate	≥50% to ≤75%
High	<50%

As observed in Table 1.3, all patients with *MYCN* amplified tumors (~20% of all NB patients) are categorized in the high-risk group. The transcription factor MYCN has been shown to regulate protein expression through direct protein-protein interaction or the induction or repression of gene transcription [356-359]. Its expression regulates cell survival, migration, and differentiation, necessary for the migration and maturation of the neuronal crest cell [349-352]. However, deregulation of its expression through gene amplification or overexpression results in malignant cell formation with aggressive phenotypes. Although a clear number is missing, MYCN is believed to regulate the expression of over 200 genes [356]. For the complete MYC transcription factor family (MYC, MYCN, and MYCL), more than 1400 MYC target genes have been identified in the human genome [360].

Depending on the risk group, NB patients are treated with various strategies [361]. Children in the low risk groups don’t normally need intensive treatment or no treatment at all, since in certain cases the tumor regresses by itself [361]. However, when treatment is needed, the patients are subjected to surgery and, if the surgery is insufficient, chemotherapy. A common chemotherapy regimen, called COJEC, consists of the administration of a combination of cytotoxic drugs, such as carboplatin, cyclophosphamide, doxorubicin, cisplatin, and etoposide. Patients with an intermediate-risk NB are treated with surgery [361]. However, the surgical removal of the tumor is rarely enough to eradicate the whole tumor. Therefore, in addition patients are treated with chemotherapy before and after the surgery. If observed that the treatment was still not sufficient to eradicate the tumor, the patients are subjected to radiotherapy. High-risk NB patients are treated in a similar manner as intermediate risk patients, however more intense chemotherapy regimens are used [361]. In the follow-up after surgery, chemotherapy, and radiation therapy, the patients are again subjected to high-dose myeloablative chemotherapy that will require an autologous hematopoietic stem cell transplant. To lower the change of recurrence, thereafter the patients are treated with 13-cis-retinoic acid to differentiate the remaining cancer cells and immunotherapy (e.g. anti-GD2 or IL-2) to activate the patient’s immune system to recognize and destroy the cancer cells.

Owing to the low survival rate of high-risk group NB patients, there is an urgent need for the development of new treatment strategies or methods to improve the clinical outcome of currently used therapy regimens. In this thesis we assessed the targeting of death receptors (DRs) as a possible approach for NB therapy.

1.8 Death receptors in cancer

Due to their cell death-inducing potential, the targeting of DRs as a therapeutic approach in cancer therapy has been extensively studied [100, 362-367]. Albeit these studies, up to date, no DR ligand treatment with cell death inducing activity has reached the pharmaceutical market. The use of DR ligands has been shown to have various drawbacks, such as unspecific cell targeting and lack of cell death induction in cancer cells. In addition, activation of death receptors on cancer cells can promote tumorigenesis and metastasis.

1.8.1 Fas

Given that FasL is one of the few molecules that immune cells use to kill cancer cells and due to its strong apoptosis-inducing capability, Fas has been proposed to be a possible target for cancer therapy [94, 196-203, 365]. Early findings by Trauth *et al.* in 1989 showed that the injection of the agonistic Fas antibody APO-1 induced the rapid regression of a human B cell tumor xenograft in a nu/nu mouse model [179]. Furthermore, Rensing-Ehl *et al.* showed that local administration of recombinant FasL induced cell death in lymphoma cells [368]. These findings paved the way for further investigation of targeting Fas for the treatment of cancer, however with little success.

The first obstacle arose from the findings that the systemic administration of recombinant FasL or agonistic antibodies induced severe side effects in mice, such as hepatotoxicity and liver failure which led to subsequent death [368, 369]. Later studies highlighted a role for Fas in the promotion of tumor formation and metastasis. It was discovered that most cancer cells express both Fas and FasL, but are resistant to FasL-ligand induced cell death [370]. This led to believe that cancer cells lack the expression of different proteins that promote Fas-mediated apoptosis, such as caspase-8 [371-379], or on the other hand, express proteins that inhibit Fas-mediated apoptosis, such as the death receptor antagonist c-FLIP [380-389]. Indeed it was shown that reconstitution of caspase-8 and downregulation of c-FLIP sensitizes cancer cells to FasL-induced cell death. Moreover, it was discovered that Src-family kinases are able to phosphorylate caspase-8, thereby inactivating its pro-apoptotic function and promoting protumor activity [390-392].

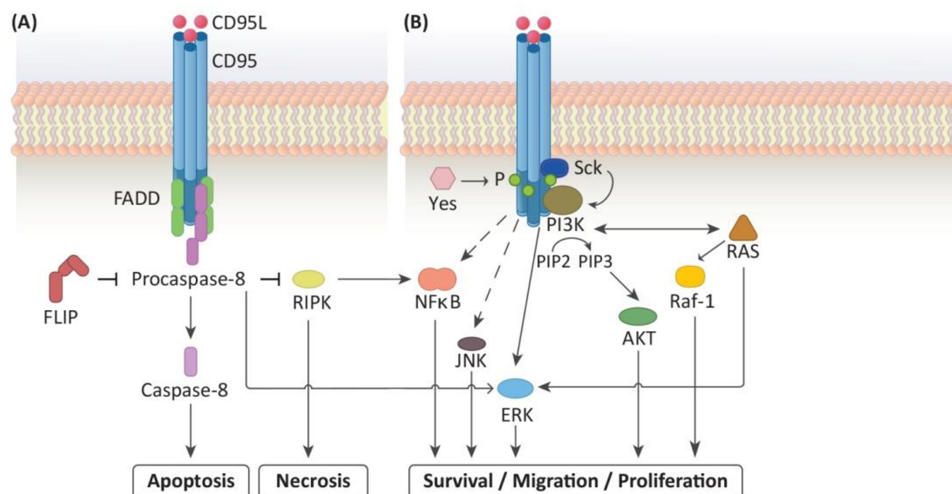


Figure 1.21: Fas-activated pathways in cancer. Fas is able to induce apoptosis, however, in cancer, Fas activation can induce different outcomes. Various studies have linked kinase proteins and transcription factors to Fas-mediated induction of tumor survival, migration, and proliferation. These pathways rely on Fas phosphorylation or direct protein-protein interactions. Figure adapted from [364]

Due to post-transcriptional modulation and the inhibition of the Fas-activated caspase-dependent pathways in cancer cells, Fas stimulation leads to the activation of the NF-κB, JNK, ERK, AKT, and Rat Sarcoma Viral Oncogene (RAS) pathways (Figure 1.21). In 2004, the group of Dr. Marcus E Peter targeted 22 Fas resistant cancer cell lines with FasL [370]. The set of 22 cell lines consisted of ovary, breast, kidney, skin, and lung cancers. They observed that *in vitro* treatment of these cell lines increased motility and invasiveness through the activation of NF-κB, ERK1/2 and caspase-8 dependent pathways. It was observed that in glioblastoma, the Src-family kinase Yes induces the phosphorylation of Fas, thereby promoting the Fas-mediated recruitment of PI3K and the activation of the AKT pathway, which resulted in increased tumor cell migration and invasion [393]. Another factor that was discovered to change the Fas outcome is KRAS. In colorectal cancer cells, KRAS is able to change Fas into an invasion-inducing receptor through promoting Fas-mediated Raf-1 signaling [394]. Moreover, cancer-produced FasL was shown to induce constitutive Fas stimulation that induced cancer proliferation through JNK activation [395]. Lastly, the group of Dr. Ana Martin-Villalba recently showed that Fas stimulation induces metastasis through PI3K and MAPK/ERK pathway activation via the recruitment of the SHC-related adaptor protein, Sck [396].

Following the discovery that Fas promotes cancer cell proliferation, the group of Dr. Marcus E Peter performed studies in which they deleted the expression of Fas and FasL on cancer cells. Here, they showed that Fas deletion resulted in decreased JNK activation and cancer cell proliferation [395]. Moreover, the silencing of FasL and Fas resulted in cancer cell death [397]. This process was entitled DICE (Death Induced by CD95/CD95L (Fas/FasL) Elimination) and was shown to preferentially occur in cancer cells and not in normal tissues. However, a clear characterization of the pathway that induces DICE has still to be described, despite several publications on DICE and the observations that the cell death is independent of caspase-8, RIP1/MLKL, and p53, and is not inhibited by BCL-X_L expression [397]. So far, it is known that DICE leads to double stranded DNA breaks and mitochondrial ROS production. The DICE-induced cell death represents a form of necrotic cell death with signs of apoptosis [397].

Noteworthy, the Fas/FasL pathway has also been proposed to play a role in immune surveillance evasion by tumors [398-400]. Here, the tumor cells express FasL or induce the expression of FasL on the tumor endothelial cells, thereby counterattacking against T-lymphocytes infiltration.

Although it is well established that in cancer the Fas/FasL signaling pathway plays a significant role in promoting tumorigenesis, there are several studies that show a beneficial role for Fas expression or FasL treatment. Data from 1997 by Arai *et al.* showed that *FASL* gene transfer, through infection with an adenovirus, induced tumor regression in a renal epithelial carcinoma xenograft mouse model [401]. Surprisingly, also a Fas negative colon carcinoma cell xenograft mouse model showed tumor regression upon *FASL* gene therapy. In the first case, the effect was attributed to Fas-mediated killing of the cells, whereas in the latter the tumor reduction was mediated by inflammatory cells. Lee *et al.* showed that, in the same renal epithelial carcinoma xenograft mouse model, Fas overexpression induces a delay in tumor progression [402]. Here, the effect was only observed in interferon-γ positive ($IFN\gamma^{+/+}$) mice and not in $IFN\gamma^{-/-}$ mice, indicating a role for IFNγ, possibly expressed by immune cells. For glioblastoma, survival was increased when intracranial implanted tumors in rats were transfected with Fas [403]. Recently, Eisele *et al.* showed the benefit of administrating a recombinant form of FasL capable of forming hexameric structures (APO010) [404]. In this study, administration of APO010 to mice bearing human glioma xenografts resulted in Fas-mediated caspase-dependent killing of the tumor cells, which prolonged the survival of tumor-bearing mice. In melanomas, a inverse correlation was found for the expression of Fas and FasL; melanomas with low Fas expression levels displayed high FasL expression levels, and vice versa [405]. When using a melanoma mouse xenograft model that displayed high FasL and low Fas expression, *FAS* gene transfer by liposomes marked a significant decrease in tumor progression. Lastly, Listopad *et al.* showed that FasL expression is necessary for the eradication of tumor stroma [406]. In a xenograft mouse model, where they injected cells previously isolated from a sporadic carcinoma from the gastrointestinal tract, IFNγ-regulated Fas expression was necessary for tumor rejection.

Moreover, adoptive T-cell therapy with CTLs negative for IFNγ or FasL could not prevent relapse after CTLs-induced regression of the tumor. Strikingly, no relapse was observed when tumor-bearing mice were treated with CTLs that were positive for IFNγ or FasL expression.

Altogether, these studies indicate that Fas and FasL expression by tumor cells can improve tumor eradication. However, these observed Fas/FasL effects are likely to be cell type specific. Thus, extensive studies should be performed to determine which types of tumors can be treated with FasL and this will shed light on the Fas/FasL pro- and anti-apoptotic paradox.

1.8.2 Tumor Necrosis Factor Receptors (TNFRs)

Although named after its tumor necrosis inducing ability and early studies suggested a high potential for the TNFR ligand TNFα in cancer treatment [258, 259], more recent studies have shown TNFα to be a poor cell death inducer and a strong inducer of pro-survival signaling [78, 248]. Nevertheless, TNFα is currently used in the clinic for the treatment of sarcomas, metastatic melanomas, and liver metastases of colorectal tumors [407-411]. Due to the severe side effects when treating systemically, patients are treated locally with TNFα using isolated limb perfusions. Moreover, the treatments with TNFα are in combination with the alkylating agent melphalan or the DNA intercalating agent doxorubicin. TNFα is thought to increase intratumoral concentrations of the chemotherapeutics by inducing early vascular destruction [412-415]. However, recently this concept was cast in doubt by a study that was unable to observe significant changes in tumor oxygenation [416].

Despite its use for cancer treatment, high plasma levels of TNFα are associated with a poor patient prognosis [417-420]. In mice, knockout of TNFα or its receptors, or sequestering TNFα with a soluble form of TNFR1 or an antibody was observed to inhibit tumor formation and growth in response to various cancer-inducing treatments [421-427]. In addition, various studies have indicated that TNFα contains metastasis-inducing activity [428-434]. Treatment with a TNFα-sequestering antibody or soluble receptor resulted in decreased metastasis in a melanoma and a pancreatic ductal adenocarcinoma xenograft mouse model [429, 430, 433]. The other way around, TNFα administration had the opposite effect and increased metastasis. Additionally, TNFα treatment of cancer cell lines before or at the time of injection in mice increased the tumor's aggressiveness and metastatic potential of mice-implanted fibrosarcoma and colorectal adenocarcinoma [428, 432].

Besides exerting a direct effect on tumor homeostasis, various studies have highlighted a role for TNFα in the regulation of tumor immunosurveillance. Tumor rejection was observed to be inhibited in TNFα$^{-/-}$ mice when implanted with fibroscarcoma cells [435]. These effects were attributed to the lack of natural killer (NK)- or lymphokine-activated killer-mediated cytotoxicity. In addition, these results were confirmed by administration of a TNFα sequestering antibody, which induced similar effects. In another study, *TNFα* gene therapy by adenoviral infection of a glioma xenograft mouse model resulted in increased intratumoral infiltration with CD4^{+} and CD8^{+} T cells and prolonged survival [436]. Using a lung carcinoma xenograft mouse model, Prevost-Blondel *et al.* provided evidence for TNFα-dependence in tumor elimination by CD8^{+} T cells [437]. Recently, Braumüller *et al.* published a paper describing the role of T-helper-1-cell-released cytokines in inducing tumor senescence [438]. Here, TNFα and IFNγ directly induced a permanent growth arrest in cancers induced by the expression of the SV40 large T antigen.

Like Fas and FasL in cancer, TNFα and TNFRs have tumor promoting or inhibiting activity, with indications that the tumoral context and the mode of action are important for the final treatment outcome. The success story of the usage of TNFα in combination with chemotherapeutics for the treatment of specific types of tumors opens doors for the development of new treatment strategies. Hopefully, findings from *in vivo* models can soon be translated to the clinic and used for TNFα-mediated induction of an anti-tumoral immune response or a direct TNFα-induced anti-tumoral activity.

1.8.3 Fas and TNFR signaling in NB

NBs are frequently found unsusceptible to DR-induced apoptosis through the silencing of caspase-8 or overexpression of anti-apoptotic proteins. Various studies have described caspase-8 to be downregulated in 50-70% of all NB [372-374, 439-441]. Some of these studies have attributed this effect to *MYCN* amplification, whereas others did not find a correlation. The downregulation indicates that caspase-8 may play a role in NB tumorigenesis, possibly through the inhibition of death receptor induced apoptosis. In addition, loss of caspase-8 decreases the sensitivity of NB against chemotherapeutic drugs and increases their metastatic potential [374, 442-445]. The NB mouse model, Th-MYCN, displayed enhanced bone marrow metastasis in 37% of the cases when caspase-8 was knocked down [446, 447].

The loss of caspase-8 has been shown to be regulated by hypermethylation of CpG islands within the *CASP8* promoter [373, 439, 448]. Various studies showed that caspase-8 expression could be reconstituted through IFNγ treatment, which surprisingly induces caspase-8 expression through an unknown mechanism that does not rely on the modification of *CASP8* promoter hypermethylation [379, 439, 449-451]. Lack of caspase-8 expression has been described to desensitize cancer cells to death receptor-induced cell death. However, reconstitution of caspase-8 expression through IFNγ treatment is able to restore DR-induced apoptotic pathway-mediated killing of the cells.

In addition to caspase-8 silencing, overexpression of anti-apoptotic BCL-2 family members and inhibitors of DR-activated apoptotic pathways have been described to inhibit DR-induced apoptosis in NB and correlate with poor prognosis. From the BCL-2 family, BCL-2, BCL-X_L, and MCL1, have been found to inhibit DR-induced apoptosis in NB [17-26].In addition, c-FLIPL expression is able to inhibit Fas-induced apoptosis [28, 30], whereas Survivin inhibited apoptosis induced by activation of the TRAIL death receptors [29, 31].

1.8.3.1 Fas

Fas expression is frequently observed on most NB tumors and NB cell lines [452-457]. On the other hand, certain studies indicated weak or no Fas expression on NB and found a correlation with poor prognosis. Due to the caspase-8 silencing, cells are often resistant to FasL-induced cell death [372, 458]. Moreover, the Fas/FasL pathway has been implicated in chemotherapeutic-induced NB cell death [458, 459]. Etoposide and cisplatin have been shown to induce Fas and FasL expression, thereby activating the Fas/FasL pathway. Resistance to FasL-induced cell death correlated with a cross-resistance to chemotherapy.

1.8.3.1 TNFRs

Little is known about the expression of TNFRs on NB. Retinoic acid-induced differentiation of the SK-N-BE(2) NB cell line induced TNFR1 expression and promoted TNFα-induced cell death [460]. On the other hand, TNFα treatment of undifferentiated cells induced proliferation. This proliferation was attributed to TNFR2 expression. Lastly, a study performed on NB samples isolated from patients before these patients received treatment, showed that TNFR1 was expressed on all tumors that responded to chemotherapy, whereas the expression was found on 57% of the tumors that recurred after the treatment with chemotherapy regimens [461]. Suggesting that lack of TNFR1 expression indicates a poor prognosis.

Hypothesis and objectives

"Per evitar fer i desfer, convé saber cap on anem."

Translation: "To avoid doing and undoing, one should know where we are going."

Joan X. Comella

Catalan biologist (Born in 1963)

2. Hypothesis and objectives

High-risk neuroblastoma (NB) patients continue to have a poor prognosis despite the advances in standard treatments and the addition of immunotherapies (i.e. anti-GD2, IL2) with 5-year survival rates still below 50% [349-352]. To improve the clinical outcome of these patients, new treatment strategies or methods to increase the efficacy of currently used treatment regimens are highly demanded. Targeting death receptors (DRs) has been proposed as a possible therapeutic approach for many types of cancer [100, 362-367]; however, in NBs, this therapeutic setting has been largely disregarded due to the silencing of caspase-8 in 50-70% of all human NBs [372-374, 439-441]. Nevertheless, a significant group of NBs are found positive for casaspe-8 expression and therefore, these patients could benefit from DR-induced cell death therapy.

Cytokines, such as TNFα, have been described to upregulate Fas expression in various types of cancer, thereby sensitizing cancer cells to Fas-mediated cell death [462-464]. In addition, activation of the Fas/FasL apoptotic pathway assists in chemotherapy-induced cell death, i.e. cisplatin and etoposide, both of which are part of common NB chemotherapy regimens [458, 459].

Owing to the need for new NB treatment strategies, the limited characterization of DRs (especially TNFR1 and Fas) in NB, and the implication of the Fas/FasL apoptotic pathway in chemotherapy-induced cell death, we proposed the following hypothesis and objectives;

Hypothesis

TNFα- and/or FasL-mediated NB cell killing could be a potential new strategy to treat NB tumours.

Objectives

The main objectives of this thesis are:

- Characterize Fas and TNFR1 expression and signalling in NB
- Study the effects of TNFα, FasL, and TNFα/FasL combination treatment on NB cell viability
- Explore the possibility of TNFα-induced sensitization for cisplatin- and etoposide-induced cell death

Material and methods

“Don’t think about your errors or failures; otherwise, you'll never do a thing.”

Bill Murray
American actor and comedian (Born in 1950)

3. Material and methods

3.1 Reagents

The following reagents and working concentrations were used for this study. Unless indicated otherwise, reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Reagent	Description	Working concentration	Supplier
Ligands			
Fc:hFasL	Fc-tagged cytokine	100ng/ml or 1ng/ml (Tet21N)	Dr. Pascal Schneider
IFNγ	Cytokine	100ng/ml	Biotrend
IL-2	Cytokine	200U/ml	Biotrend
SuperFasLigand	FLAG-tagged cytokine	100ng/ml	Enzo Life Sciences
TNFα	Cytokine	100ng/ml	Biotrend
TRAIL	Cytokine	100ng/ml	Biotrend
DNA binding or DNA synthesis inhibiting agents			
Cisplatin	DNA crosslinking agent	0.05-50µM	Sigma-Aldrich
Etoposide	Topoisomerase II inhibitor	0.05-50µM	Sigma-Aldrich
Inhibitors			
Actinomycin D	Transcription inhibitor	20nM	Sigma-Aldrich
BAY 11–7082	IKKα inhibitor	10µM or 25µM	Merck Millipore
Cycloheximide	Translation inhibitor	1µg/ml	Sigma-Aldrich
hFas-Fc	Soluble Fas receptor	2x diluted conditioned medium	Dr. Pascal Schneider
LY294002	PI3K Inhibitor	20µM	Sigma-Aldrich
PD98059	MEK1 inhibitor	25µM	Merck Millipore
SP600125	JNK inhibitor	20µM	Merck Millipore
Caspase substrates			
Ac-DEVD-Afc	Caspase-3/7 substrate	25µM	Merck Millipore
Q-VD-OPH	Pan-caspase inhibitor	10µM	Merck Millipore
Z-IETD-Afc	Caspase-8 substrate	25µM	Merck Millipore
Z-IETD-FMK	Caspase-8 inhibitor	50µM	Merck Millipore
Stains			
Calcein AM	Cell-permeant dye	1µM	Merck Millipore
Hoechst 33342	Nucleic acid stain	0.05µg/ml	Sigma-Aldrich
MTT	Cell-permeant dye	0.5mg/ml	Sigma-Aldrich
Propidium iodide	DNA intercalating dye	0.5µg/ml	Sigma-Aldrich
Suppliers		**Headquarters**	
Biotrend		Köln, Germany	
Dr. Pascal Schneider		University of Lausanne, Epalinges, Switzerland	
Enzo Life Sciences		Farmingdale, NY, USA	
Merck Millipore		Billerica, MA, USA	
Sigma-Aldrich		St. Louis, MO, USA	

3.2 Cell culture

The following cell lines and their corresponding medium were used for this study. Cell cultures were maintained at 37°C in a humidified atmosphere with 95% air and 5% CO_2.

Cell line	Type	Supplier
DMEM + 10% (v/v) FBSi + 100U/ml penicillin + 100µg/ml streptomycin		
HEK293T	Renal epithelial cells	ATCC
IMR32	Neuroblastoma	ATCC
LAI-5S	Neuroblastoma	NCTC
SK-N-AS	Neuroblastoma	ATCC
SK-N-BE(2)	Neuroblastoma	NCTC
DMEM + 15% (v/v) FBSi + 100U/ml penicillin + 100µg/ml streptomycin		
SH-SY5Y	Neuroblastoma	ATCC
IMDM + 20% (v/v) FBSi + 100U/ml penicillin + 100µg/ml streptomycin		
CHLA90	Neuroblastoma	COG
SK-N-SH	Neuroblastoma	ATCC
RPMI 1640 + 10% (v/v) FBSi + 25mM HEPES + 200µg/ml geneticin (G418) + 0.5µg/ml amphotericin B + 10µg/ml hygromycin B + 100U/ml penicillin + 100µg/ml streptomycin		
Tet21N	Neuroblastoma (tetracycline-repressible MYCN expression)	Dr. Manfred Schwab
RPMI 1640 + 10% (v/v) FBSi + 200U/ml IL-2 + 100U/ml penicillin + 100µg/ml streptomycin		
NKL	Natural killer cells	Dr. Miguel López-Botet
Suppliers	**Description**	**Headquarters**
ATCC	American Type Tissue Collection	Manassas, VA, USA
NCTC	PHE's National Collection of Type Cultures	Salisbury, UK
COG	Children's Oncology Group Cell culture repository	Lubbock , TX, USA
Dr. Manfred Schwab		DKFZ, Heidelberg, Germany
Dr. Miguel López-Botet		UPF, Barcelona, Spain

Cell culture medium components		
Reagent	**Description**	**Supplier**
DMEM	Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific
IMDM	Iscove's Modified Dulbecco's Medium	Thermo Fisher Scientific
RPMI 1640	Roswell Park Memorial Institute 1640 Medium	Thermo Fisher Scientific
FBSi	Heat-inactivated fetal bovine serum	Thermo Fisher Scientific
Pen/Strep	Penicillin/ streptomycin (Antibiotics)	Thermo Fisher Scientific
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Buffering agent)	Thermo Fisher Scientific
Geneticin (G418)	Antibiotic (Selecting agent)	Sigma-Aldrich
Amphotericin B	Antibiotic	Sigma-Aldrich
Hygromycin B	Antibiotic (Selecting agent)	Sigma-Aldrich

3.2.1 Cryopreservation of cell lines

To maintain a continuous stock of cell lines, cells can be stored through cryopreservation. It is recommended to cryopreserve early passage numbers to avoid selection and introduction of cell line modifications (genotype and phenotype). For cryopreservation of cell lines, cultured cells are frozen gradually (-1°C/min), using propanol, and in the presence of DMSO to avoid formation of ice crystals that can perforate the cell membrane and kill the cells. Cryopreserved cell lines are stored in liquid nitrogen to abrogate all cellular processes.

For cryopreservation of cell lines, the following protocol was used;

Cryopreservation of cell lines	
Day 1	
Step	
1.	Harvest cells using Trypsin
2.	Centrifuge cells for 5min at 200g
3.	Aspirate medium
4.	Resuspend in 1ml cryopreservation medium
Work as quickly as possible to avoid DMSO-mediated toxicity	
5.	Aliquot 500µL in cryovials
6.	Store cryovials in Mr. Frosty Freezing Container (Thermo Fisher Scientific) and store at -80°C
Day 2	
7.	Transfer cryovials to liquid nitrogen tank for long-term storage

Cryopreservation medium	
Reagent	**Concentration**
FBSi	90% (v/v)
DMSO	10% (v/v)

3.2.2 Thawing of cryopreserved cell lines

To start cell cultures from cryopreserved cell lines, cell lines should be thawed and DMSO should be removed to avoid DMSO-mediated cytotoxicity. As a rule of thumb, the presence of more than 0.01% (v/v) DMSO is toxic for cell lines, but DMSO endurance differs between cell lines.

For thawing cryopreserved cell lines, the following protocol was used;

Thawing cryopreserved cell lines	
Step	
1.	Withdraw cryopreserved cell line from liquid nitrogen. Maintain on ice
2.	Thaw cryopreserved cell line in 37°C water bath while swirling
Work as quickly as possible to avoid DMSO-mediated toxicity	
3.	Dilute thawed cell line in 10ml of cell line corresponding medium at 37°C
4.	Centrifuge cell suspension for 5min at 200g
5.	Aspirate medium
6.	Resuspend cells in 5ml fresh medium
7.	Count cells using a neubauer chamber and Trypan Blue to assess cell viability
8.	Seed cells on cell culture plate suitable for the amount of viable cells.
9.	Add fresh medium if necessary

3.3 Cell transfection

Nucleic acid molecules are negatively charged, which prevents their cellular internalization due to electrostatic repulsion by the cellular membranes which carry a negative net charge. To overcome this repulsion, in this study, HEK293T cells were transfected with the soluble cationic lipid agent; Lipofectamine 2000 (Thermo Fisher Scientific). This transfection reagent forms liposomes that carry a positively charged surface which mediates the interaction with nucleic acids, thereby shielding their negative charge. After liposome-nucleic acid complex formation, the transfection complex interacts with the cell membrane and delivers the DNA/RNA into the cells through endocytosis. There, the DNA or RNA is expressed or induces expression silencing. In this thesis, transfection of HEK293T cells was used for the production of lentiviral particles and conditioned medium containing the soluble Fc-tagged Fas receptor (hFas-Fc).

For the transfection of HEK293T cells on 10cm cell culture plates the following protocol was used;

Lipofectamine 2000 transfection of HEK293T cells	
Day 1	
Step	
1.	Harvest, centrifuge, and resuspend HEK293T cells in DMEM + 10% (v/v) FBSi **without antibiotics**
2.	Seed 10ml of a 400 000 cells/ml cell suspension in a 10cm cell culture plate
Day 2	
1.	Per plate, add 30µL of Lipofectamine 2000 to 500µl of Opti-MEM I (Thermo Fisher Scientific)
2.	Incubate 5min
3.	Dilute 12µg of plasmid DNA in 500µl Opti-MEM I
4.	Mix Lipofectamine and DNA solutions
5.	Incubate 20min
6.	Add 1ml Lipofectamine-DNA mix to each cell culture plates
7.	Incubate 4-6h
8.	Change medium for 10ml DMEM + 10% (v/v) FBSi **without antibiotics**
Day 4	
1.	Use for the intended purpose (Collection of conditioned medium or characterization of expression)
For conditioned medium	
2	Centrifuge conditioned medium for 5min at 1000g to remove floating cells and debris
3.	Pass conditioned medium through a 45µm Whatman filter (GE Healthcare, Little Chalfont, UK)
4.	Aliquot conditioned medium and store at -80°C until further use

3.3.1 Plasmids

For this study the following overexpression plasmids were used

Expression plasmids			
Gene	**Vector**	**Promoter**	**Reference**
hFas-Fc	PCR3	CMV	[465]

Lentiviral expression plasmids			
Gene	**Vector**	**Promoter**	**Reference**
BCL-X$_L$	pEIGW	EF-1α	[120]
c-FLIP$_L$	pEIGW	EF-1α	[466]
SR-IκBα	pEIGW	EF-1α	[132]
TurboGFP	pGIPZ	CMV	GE Healthcare

3.4 Lentiviral production and transduction

In some cases were transfection methods cannot be used due to low efficiency or toxicity, lentiviral transduction is an effective way to induce expression or silencing of the protein of interest. Lentiviral particles are able to infect proliferating and differentiated cells. Up to date, three plasmids generations derived from the HIV-1 lentivirus have been designed for the production of lentiviral particles. Each generation increased the biosafety of lentiviral production through the removal of viral element or the distribution of the viral forming elements over various plasmids.

In this study, we used lentiviral plasmids from the 2nd generation designed by the Trono lab. These plasmids consist of a gene expression (pEIGW/pGIPZ), packaging (psPAX2), and envelope vector (pM2G).

Lentiviral production			
Vector	**Type**	**Description**	**Quantity used for transfection**
pEIGW/pGIPZ	Expression	Overexpression of gene or RNA of interest	12µg
psPAX2	Packaging	Contains *Gag*, *Pol*, *Rev*, and *Tat* genes	8µg
pM2G	Envelope	VSV-G-expressing envelope vector	4µg

Lentiviral particles were produced by transfecting HEK293T cells according to the transfection protocol using the indicated amounts of the three plasmids. Cells were allowed to generate lentiviral particles for 48h, after which the conditioned medium carrying the lentivirus was collected and passed through a 45µm Whatman filter. The lentivirus-bearing medium was aliquoted and stored at -80°C until further use.

For infection, lentivirus-bearing medium was thawed and added to the host cells in combination with 8µg/ml polybrene. This agent is able to increase transduction efficiency through shielding the electrostatic repulsion between the viral particles and the cell membrane, which both carry negative electrostatic charges. Host cells were used for experiments 72h after transduction and after confirming that the infection efficiency had reached ≥95%. Infection efficiency was assessed by direct counting of GFP-positive cells and, when needed, infection was repeated until an efficiency of ≥95% was reached.

The expression vectors pEIGW and pGIPZ contain various elements that flank the gene of interest and are necessary for viral particle formation and transgene inclusion into the genome. The packaging vector psPAX2 contains genes for various proteins necessary for the lentiviral formation and activity. Lastly, the envelope vector pM2G contains the Vesicular Somatitis Virus G Glycoprotein (VSV-G), which is necessary for viral penetration of the target cell’s plasma membrane. For a detailed description of all the viral elements see table 3.1.

Table 3.1: The role of lentiviral elements encoded by the lentiviral plasmids from the 2nd generation

pEIGW/pGIPZ Lentiviral elements		
Viral element	**Description**	**Role**
5' LTR	Long terminal repeat	Required for viral RNA (vRNA) transcription, integration, and gene expression steps
Ψ	Packaging signal	Targets vRNA for packaging into the viral nucleocapsid
RRE	Rev response element	Rev binding sequence. vRNA Export from the nucleus to cytoplasm for viral packaging.
pEF-1α/pCMV	Elongation factor 1α promoter Cytomegalovirus promoter	Initiation of GFP and Transgene mRNA transcription
EGFP/tGFP	Enhanced/Turbo Green fluorescent protein	Transfection/Transduction efficiency tracking
IRES	Internal ribosome entry site	Allows the expression of 2 genes from the same transcript
Transgene	Gene of interest	Induces transgene overexpression
WPRE	Woodchuck Hepatitis *Virus* (WHP) Posttranscriptional Regulatory Element	Enhances transgene expression through increasing nuclear export
3' LTR	Long terminal repeat	Terminates transcription, required for reverse transcription, and integration steps
psPAX2 Lentiviral elements		
Viral element	**Description**	**Role**
Gag	Polyprotein for matrix, capsid, and nucleocapsid components	Lentiviral packaging components
Pol	Precursor protein for reverse transcriptase and integrase	Mediate reverse transcription and integration of the DNA into the genome
Rev	Rev protein	Binds RRE within vRNA and mediates nuclear export
Tat	Trans-activator of transcription	Trans-activator that activates transcription from the 3' LTR promoter
pM2G Lentiviral elements		
Viral element	**Description**	**Role**
VSV-G	Vesicular Somatitis Virus G Glycoprotein	Envelope glycoprotein with broad tropism necessary for plasma membrane penetration

3.5 Cell death and viability assays

Cell death and viability can be characterized by the use of various assays. These assays assess hallmarks related to cell death and viability, such as death-induced changes in DNA, membrane integrity, protein activity/cleavage, protein release, and cellular morphology. A detailed characterization of the methods used for assessing specific types of cell death and viability and their advantages and disadvantages are described in reviews from the group of Dr. Guido Kroemer [12, 467, 468].

3.5.1 Hoechst staining

Hoechst staining is a fluorescent nucleic acid staining method which can be used for the characterization of apoptosis through assessing nuclear pyknosis; the irreversible condensation of chromatin in the nucleus. The most commonly used Hoechst stains are Hoechst 33258 and Hoechst 33342, which both are excited by UV light (optimal excitation 350nm) and emit blue/cyan fluorescent light (maximal emission 461nM). Both dyes do not significantly affect cell viability. However, Hoechst 33342 is more cell-permeant than Hoechst 33258, which allows for its use in nuclear staining of viable cells.

Upon binding to double stranded DNA at Adenosine-Thymidine rich regions, Hoechst dyes become highly fluorescent and allow for the detection of viable cells, mitotic cells, and death cells. Death cells can be identified through discriminating condensed and fragmented nuclei, which is a classical morphological hallmark of apoptosis. Nevertheless, care has to be taken in distinguishing mitotic cells from apoptotic cells (Figure 3.1).

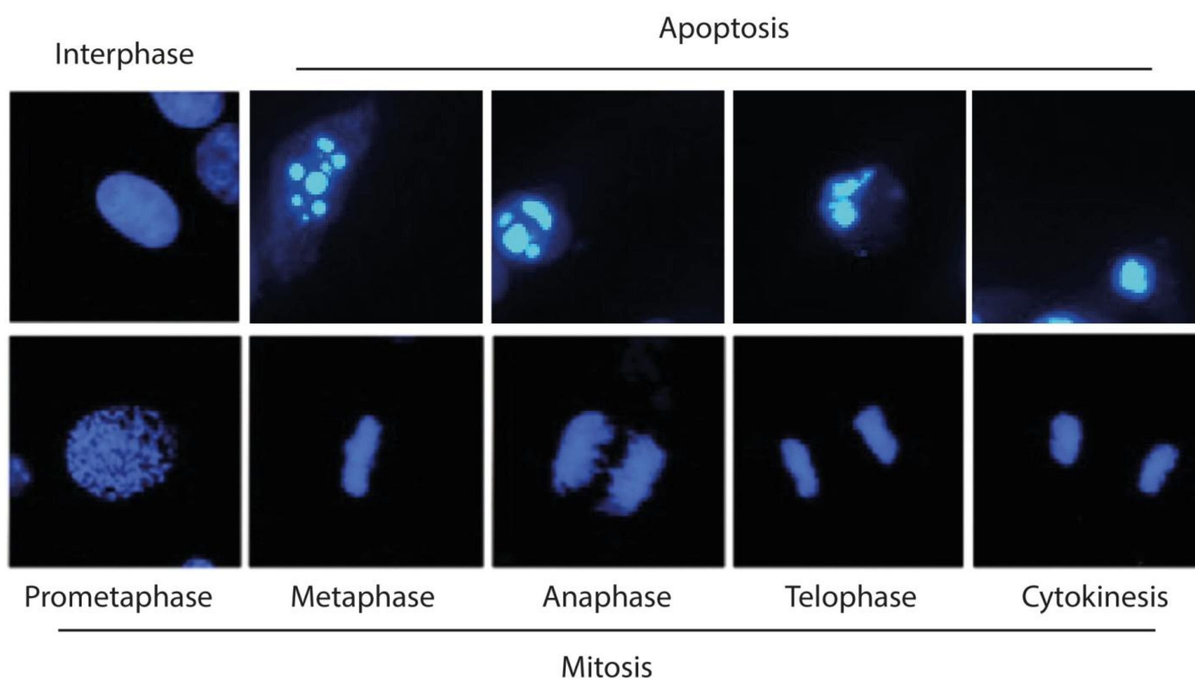


Figure 3.1: Identification of nuclear chromatin morphology. Hoechst staining of CHO cells and the identification of apoptosis and different stages of mitosis. Figure adapted from [469, 470]

For assessing cell death through discriminating nuclear apoptotic morphology cell were seeded on a 24wells or 48wells plate, treated for the indicated times, and the following Hoechst staining protocol was used;

Hoechst staining	
Step	
1.	Fix cells by directly adding paraformaldehyde (PFA) to the wells, reaching a final concentration of 2% (v/v) PFA
2.	Incubate 20min at room temperature (RT)
3.	Add Hoechst staining buffer, equal to 1/5 of the total volume in the well, to the cells. (Hoechst staining buffer contains Triton X-100 to increase staining efficiency)
4.	Incubate 20min at RT
5.	Use cells for fluorescent microscopy, using excitation with UV light and 20x magnification
6.	Assess cell death by counting at least 500 cells per condition in a blind testing

Hoechst staining buffer 6x		
Reagent	**Concentration**	**Final concentration**
Hoechst 33342	0.3µg/ml	0.05µg/ml
Triton X-100	0.6% (v/v)	0.1% (v/v)
PBS	1x	

3.5.2 Caspase activity

Activation of the apoptotic pathway is characterized by caspase activation, which can be measured using a caspase activity assay. Here, incubation of cell lysates with a specific caspase substrate that becomes fluorescent after digestion indicates the relative amount of caspase activation at a specific time point after treatment. Caspase activity shows temporal dynamics, thus requiring a previous characterization of stimuli-induced caspase activation at different time points after treatment. In this thesis, we assessed initiator caspase activity using the caspase-8 specific substrate Z-IETD-Afc, and effector caspase activity using the caspase-3/7 specific substrate Ac-DEVD-Afc. Activity of these caspases was titled IETDase and DEVDase activity, respectively, due to their enzymatic digestion of a specific caspase substrate.

To assess caspase activity, cells were seeded on a 35mm cell culture plate or 6wells plate, treated for the indicated times, and the following caspase activity assay protocol was used;

Caspase activity assay	
Step	
1.	Harvest cells by pipetting or using a scraper (Thermo Fisher Scientific).
2.	Centrifuge cells for 3min at 4°C and 500g
3.	Wash cells in 500µL ice-cold PBS
4.	Lyse cells for 30min in 100µL ice-cold 1x caspase activity buffer (add protease inhibitors before use)
5.	Remove insoluble fractions by centrifugation for 30min at 4°C and 16 000g
6.	Quantify protein concentration in the supernatant using the Lowry-based DC protein assay (Biorad, Hercules, CA, USA)
7.	In triplicate, add 10µg of protein to the wells of a black 96wells plate
8.	Add ddH_2O to reach a final volume of 50µL
9.	Add 50µl complete 1x caspase activity buffer, containing the specific fluorogenic caspase substrate, to the wells
10.	Incubate at 37°C for 1-24h, measure caspase activity after every hour if necessary
11.	Assess caspase activity in a fluorometer using 405nm excitation and 535nm emission wavelengths.

Caspase activity buffer 2x		
Reagent	**Concentration**	**Final concentration**
HEPES-NaOH, pH7.2	40mM	20mM
EDTA	10mM	5mM
NaCl	300mM	150mM
Sucrose	20% (w/v)	10%(w/v)
CHAPS	0.2% (w/v)	0.1% (w/v)
Igepal CA-630 (NP-40)	2% (v/v)	1% (v/v)
Added before use		
EDTA-free Complete protease inhibitor mixture (Roche, Basel, Switzerland)	2x	1x
Added before caspase activity assay		
DTT	20mM	10mM
Z-IETD-Afc	50µM	25µM
Ac-DEVD-Afc	50µM	25µM

3.5.3 Calcein AM staining

Viable cells harbor active esterases which can be used to assess cell viability. Calcein AM is a nonfluorescent cell-permeable dye that is converted to green-fluorescent calcein by intracellular esterases. On the other hand, dead cells lack esterase activity, thus do not convert calcein AM. These characteristics make calcein AM a useful dye for short-term labeling of cells and assessing cell viability by fluorometry, microscopy and flow cytometry.

To assess cell viability, cells were seeded on a 24wells or 96wells plates, treated for the indicated times, and the following calcein AM staining protocol was used;

Calcein AM staining assay	
Step	
1.	Wash cells with 250µL or 50µL DPBS (Thermo Fisher Scientific), for 24 and 96wells plates respectively **DPBS, instead of PBS, is used to avoid cell detachment over time.** **Cells are washed to remove medium, which contains esterases and increases background signals**
2.	Add calcein AM staining buffer to the wells, 250µL for a 24wells plate, 50µL for a 96wells plate
3.	Incubate for 1h at 37°C
4.	Assess cell viability in a fluorometer using 485nm excitation and 535nm emission wavelengths
5.	Untreated cells are normalized as 100% viability

Calcein AM staining buffer	
Reagent	**Concentration**
DPBS	1x
Calcein AM	1µM

3.5.4 MTT reduction

Mitochondrial function is vital for cell viability, and their functionality is associated with mitochondrial NADH dehydrogenases activity. The metabolic property of mitochondrial NADH dehydrogenases is used for assessing cell viability in the MTT reduction assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a soluble dye that is reduced to formazan by mitochondrial NADH dehydrogenases. Formazan has a purple color and is insoluble in aqueous solutions, thus after the assay DMSO is used to dissolve formazan. The level of formazan formation is indicative of mitochondrial activity and cell viability. Although, frequently used for assessing cell death, various other stimuli that do not induce cell death or regulate proliferation are able to affect mitochondrial activity. Thus, care has to be taken while interpreting the results obtained after performing a MTT reduction assay.

To assess cell viability, cells were seeded on a 96wells plates, treated for the indicated times with 50µL of reagent containing medium, and the following MTT reduction protocol was used;

MTT reduction assay	
Step	
1.	Add 50µL of MTT reduction buffer directly to the wells
2.	Incubate for 30min at 37°C
3.	Aspirate supernatant
4.	Add 100µL DMSO to the wells
5.	Gently shake plate until formazan in completely dissolved
6.	Assess cell viability in a spectrometer using absorbance at 590nm and 620nm as a reference
7.	Subtract OD620 from OD590
8.	Untreated cells are normalized as 100% viability

MTT reduction buffer		
Reagent	**Concentration**	**Final concentration**
MTT	1mg/ml	0.5mg/ml
DMEM	1x	-

3.5.5 Propidium iodide staining

Necrotic and late-apoptotic cells lose their membrane integrity, which allows for membrane impermeant dyes to enter the cell. Propidium iodide (PI) is such a dye that enters the cell when membrane integrity is lost. PI is a fluorescent molecule that intercalates between nucleic acids of double stranded DNA at unknown regions and when bound enhances its fluorescence. It is used to assess necrosis and late apoptosis and can be used for flow cytometry, microscopy and fluorometry.

To assess late apoptotic and necrotic cell death, pGIPZ-infected cells were seeded on a 24wells plates, treated, co-cultured with NKL cells, and the following PI staining protocol was used;

PI staining	
Step	
1.	Collect floating cells in an eppendorf tube
2.	Wash cells with 250µL PBS and add to the eppendorf tube
3.	Add 250µL dissociation buffer to the wells
4.	Incubate 5min at RT
5.	Collect cells in the eppendorf tube
6.	Centrifuge cells for 3min at 500g
7.	Wash cells with 500µL ice-cold PBS
8.	Resuspend cells in 500µL PI staining buffer
9.	Transfer cell suspension to flow cytometry tube
10.	Maintain on ice and protected from light until used for flow cytometry
11.	Assess cell death using a FACSCalibur flow cytometer
To select target cells	
12.	Gate GFP expression cells using the FL-1 channel
To assess cell death	
13.	Determine PI positive cells using the FL-3 channel

PI staining was assessed using a FACSCalibur flow cytometer (Becton Dickinson). Flow cytometry data was analyzed using the FlowJo VX software (Tree Star, San Carlos, CA, USA)

Cell dissociation buffer	
Reagent	**Concentration**
PBS	1x
EDTA	5mM

PI staining buffer	
Reagent	**Concentration**
Ice-cold PBS	1x
Propidium Iodide	0.5µg/ml

3.6 Analysis of mRNA expression levels (qRT-PCR)

Analysis of mRNA expression levels gives an indication of changes in the expression of the gene of interest, but does not always directly translate into changes at the protein level. The proper handling of mRNA samples is important for acquiring credible results, since mRNA samples are fragile. Samples of mRNA are vulnerable to degradation induced by ribonucleases (RNases) and temperature-related effects. Therefore, mRNA extraction and handling should be performed in a clean and quiet environment and samples should always be kept on ice, short-time storage at -20°C, and long-time storage at -80°C. The analysis of mRNA levels in this thesis was performed by qRT-PCR.

3.6.1 Sample storage for RNA extraction

For the preparation of mRNA samples, cell were seeded on a 6wells plate or 35mm cell culture plate, treated for the indicated times, and handled as described below;

Sample storage for mRNA extraction	
Step	
1.	Wash cells with 1ml ice-cold PBS
2.	Harvest cells by pipetting or using a scraper
3.	Centrifuge cells for 3min at 4°C and 500g
4.	Aspirate supernatant
5.	Flash freeze samples in liquid nitrogen (or dry-ice mixed with EtOH)
6.	Store samples at -80°C until further use

3.6.2 RNA extraction

For work performed in this thesis, RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

In brief, cells were subjected to lysis under denaturing conditions using a guanidine-isothiocyanate-based lysis buffer supplemented with β-mercaptoethanol. The denaturing conditions and β-mercaptoethanol induce a potent inhibition of RNases present in the samples. Thereafter, ethanol is added to the lysates to provide ideal RNA binding conditions and the lysates are loaded on silica-membrane containing columns which trap the RNA. The columns are washed to remove sample contaminant and RNA is efficiently eluted using ddH_2O free of RNases and DNases (deoxyribonucleases).

3.6.3 Conversion of RNA to cDNA

Before assessing mRNA expression levels, RNA samples were converted to complementary DNA (cDNA) through reverse transcription. This method generates single stranded minus (or antisense) strands of cDNA complementary to the RNA template. Single stranded cDNA is obtained through thermal degradation of RNA at the end of the protocol.

To obtain cDNA we used the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. This kit contains the necessary components for the conversion of RNA to cDNA, i.e. Moloney Murine Leukemia Virus Reverse Transcriptase (MuLV), RNase inhibitor protein, dNTPs, random octamers, and oligo dT-16. These latter two are primers that anneal at random regions or the poly A-tail of mRNA, respectively.

In brief, the following reverse transcriptase protocol was used;

Reverse transcriptase protocol				
Step				
1.	Use 1-2µg of RNA (in equal amounts between samples) with the High Capacity RNA-to-cDNA Kit			
2.	Load reaction mix into the thermal cycler using the following settings			
	Step 1	**Temperature**	**Time**	**Description**
	1.	37°C	60min	Primer annealing and reverse transcription
	2.	95°C	5min	Enzyme and RNA denaturation/inactivation/degradation
	3.	4°C	∞	Short-term storage
3.	Store cDNA samples at -20°C for short-term storage or -80°C for long-term storage			

3.6.4 Quantitative PCR

For the analysis of mRNA expression levels, cDNA samples were submitted to quantitative PCR using a TaqMan-based protocol. This protocol consists of a PCR analysis using predesigned and validated TaqMan primer/probe sets (Thermo Fisher Scientific) in combination with the corresponding TaqMan Universal PCR Master Mix (Thermo Fisher Scientific), which contains AmpliTaq DNA polymerase and dNTPs for DNA elongation. For the analysis of mRNA expression levels the following TaqMan primer/probe sets were used;

Taqman probes					
Target transcript	**Assay Design**	**Amplicon length**	**Reference**	**Detects # out of total transcript variants**	**Dye**
18S	Within single exon	61	Hs03928990_g1	1/1	FAM
BCL-2	Probe spans exons	81	Hs00608023_m1	1/1	FAM
Caspase-8	Probe spans exons	124	Hs01018151_m1	6/6	FAM
c-FLIP	Probe spans exons	59	Hs01116280_m1	7/8	FAM
c-FLIP$_L$	Probe spans exons	100	Hs01117851_m1	5/7	FAM
cIAP2	Probe spans exons	72	Hs00154109_m1	2/2	FAM
DcR3	Probe spans exons	98	Hs01548060_m1	1/1	FAM
FADD	Probe spans exons	112	Hs00538709_m1	1/1	FAM
FAIM$_L$	Probe spans exons	99	Hs00992098_m1	1/1	FAM
Fas	Probe spans exons	97	Hs00531110_m1	3/3	FAM
FasL	Probe spans exons	78	Hs00181225_m1	1/1	FAM
LFG	Probe spans exons	98	Hs00392342_m1	1/1	FAM
RIP1	Probe spans exons	83	Hs00169407_m1	1/1	FAM
TNFR1	Probe spans exons	150	Hs01042313_m1	1/1	FAM
XIAP	Probe spans exons	143	Hs01597783_m1	1/1	FAM

TaqMan primer/probe sets consist of forward and reverse primers in combination with a primer probe. These highly specific primers allow for the detection of low cDNA template copy numbers and restrict the detection of PCR amplification products other than the gene of interest. For every gene, various primer/probe sets are available which allows for the selection of primer/probe sets that detect specific splice variants or sets of splice variants. In addition, TaqMan primers and probes are designed in such a way that they bind within the same

exon, which allows for the detection of genomic DNA, or that the probe binds at an exon junction, thus impeding the detection of genomic DNA. For the analysis of mRNA expression levels, exon junction spanning probes are recommended to avoid the detection of genomic DNA contaminants in the cDNA samples. For a detailed explanation of the theory behind the TaqMan -based assay see figure 3.2.

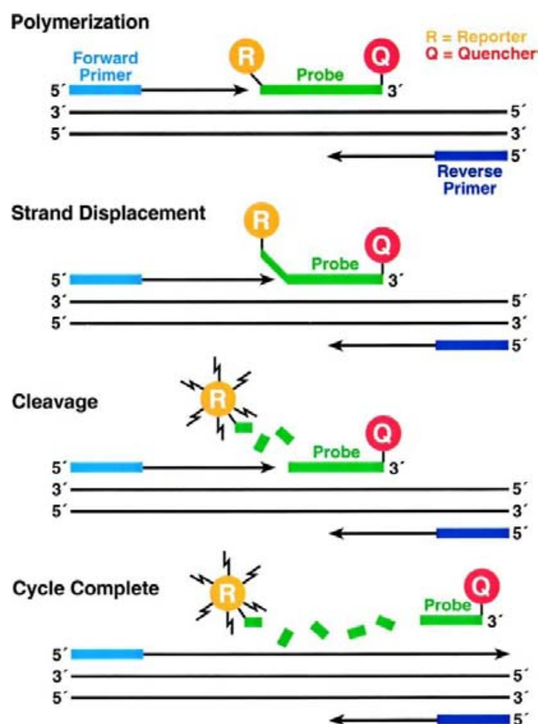


Figure 3.2: Description of the theory behind the TaqMan qRT-PCR method. Taqman primers and probes bind the cDNA template, and while the probe is intact the reporter dye is quenched by the quencher dye attached to the probe. During polymerization, the Taq polymerase enzyme displaces and cleaves the TaqMan probes, thereby releasing the quencher dye and allowing for reporter dye emission. Figure derived from [471]

For the analysis of mRNA expression levels, cDNA samples loaded in triplicate on a 384wells plate and were mixed with the TaqMan primer/probes sets and the corresponding TaqMan Universal PCR Master Mix according to the manufacturer's instructions using the following volumes;

Volumes used for TaqMan assay on a 384wells plate	
Reagent	**Volume**
cDNA (20x diluted)	4.5μL
TaqMan primer/probes	0.5μL
2x TaqMan Universal PCR Master Mix	5μL

Next, the samples were subjected to a PCR amplification protocol using a 7900HT Real-Time PCR System (Thermo Fisher Scientific) with the following setting;

PCR settings using a 7900HT Real-Time PCR System			
40 cycles			
Step	**Temperature**	**Time**	**Description**
1.	95°C	15sec	DNA denaturation
2.	60°C	1min	Primer annealing and DNA elongation

After completion of the PCR reaction, data was analyzed using the 7900HT Sequence Detection System 2.3 software (Thermo Fisher Scientific). Here, mRNA expression levels were quantified using Ct values, which indicate the cycle at which TaqMan probe-induced fluorescent signal reached a predetermined threshold. Lower Ct values correlate with fluorescent signal detection at earlier PCR cycles, which indicates the presence of higher cDNA copy numbers and correlate to higher mRNA expression levels. To eliminate loading errors, these data are normalized for cDNA copy numbers of house-keeping genes, such as the 18S ribosomal RNA, GAPDH, or actin, by substracting the Ct value of the housekeeping gene from the Ct values of the gene of interest

$$Ct_{(gene\ of\ interest)} - Ct_{(housekeeping\ gene)} = \Delta Ct$$

These ΔCt values can be used directly for absolute quantification, indicating the cycle at which the gene of interest was detected or can be used for relative quantification to compare the expression levels to control conditions, as measured in relative fold change. Relative quantification methodology has been described by Livak and Schmittgen [472] and relative expression data is obtained using the following formula;

$$2^{-(\Delta Ct_{(sample)} - \Delta Ct_{(control\ sample)})} = 2^{-\Delta\Delta \mathrm{CT}}$$

For the work performed in this thesis, we used 18S ribosomal RNA as the housekeeping gene for data normalization and the relative quantification method to compare treated samples to control conditions or to compare mRNA expression between cell lines.

3.7 Analysis of protein expression levels (Western Blot)

A common way to analyze protein expression is to lyse cells and subsequently assess protein expression levels by Western blot. An efficient extraction of proteins is necessary to ensure an accurate reflection of their levels and their physiological state, which can be affected by post-translational modifications such as phosphorylation, ubiquitination, and proteolytic cleavage. Depending on the purpose of the extraction, different protein extraction methods can be used which have their advantages and disadvantages.

3.7.1 Protein extraction under non-denaturing conditions

Protein extraction under non-denaturing conditions is a useful method to obtain samples which contain proteins in their native state. Lysis buffers containing mild nonionic detergents (NP-40 and Triton X-100) are able to solubilise membrane proteins and isolate cytoplasmic proteins, but will not lyse nuclear membranes and mitochondria. These nonionic detergent-based lysis buffers are recommended for assessing protein-protein interactions and protein activity. Since protein samples obtained through these methods contain proteins in their native state, amongst which are proteases and phosphatases, care has to be taken while handling these samples. To avoid protein degradation or loss of post-translational modifications, samples have to be maintained on ice at all time and the lysis buffer has to be supplemented with protease inhibitors, and when assessing protein phosphorylation supplemented with phosphatases inhibitors. Long-time storage of these cell lysates should be performed at -20°C.

For the analysis of expression levels of membrane and cytosolic proteins, cells were seeded on 35mm cell culture plates or 6wells plates and after the indicated treatments, the following protocol was used;

Protein extraction under non-denaturing conditions	
Step	
1.	Harvest cells by pipetting or using a scraper
2.	Centrifuge cells for 3min at 4°C and 500g
3.	Wash cells with 1ml ice-cold PBS
4.	Aspirate supernatant
5.	Lyse cells in 100µL ice-cold Triton lysis buffer (add phosphatase and/or protease inhibitors before use)
6.	Incubate 30min on ice
7.	Remove insoluble fractions by centrifugation for 30min at 4°C and 16 000g
8.	Store cell lysates at -20°C

Triton lysis buffer	
Reagent	**Concentration**
Tris-HCl pH7.4	50mM
NaCl	150mM
EDTA	1mM
Triton X-100	1% (v/v)
Added before use	
EDTA-free Complete protease inhibitor mixture	1x

3.7.2 Protein extraction under denaturing conditions

Protein extraction under denaturing conditions induces the near-complete to complete disruption of the cells, liberating cytosolic, membrane, nuclear, and mitochondrial proteins. The strong non-ionic detergents or chaotropic agents (SDS and guanidinium-thiocyanate, respecitively) in these lysis buffers mediate protein denaturation, which induces loss of protein activity and protein interactions. Due to their denaturing conditions, there is no need for the addition of phosphatases and/or protease inhibitors, since these proteins have been fully inactivated. Moreover, samples can be maintained at RT for short-time storage. A disadvantage of using strong ionic detergents is the release of genomic DNA from the nucleus. Upon release from the nucleus, genomic DNA aggregates and increases viscosity of the samples, which can impede proper pipetting. However, with some simple steps the genomic DNA aggregates can be removed from the sample.

For the analysis of protein phosphorylation, cells were seeded on 35mm cell culture plates or 6wells plates and after the indicated treatments, the following protocol was used;

Protein extraction under denaturing conditions	
Step	
1.	Aspirate medium
2.	Wash cells with 1ml ice-cold PBS
3.	Add 200µL boiling SET lysis buffer directly to the plates (lysis buffer will form a goo)
4.	Ascertain complete lysis of the samples by moving the goo around the plate using a pipette tip
5.	Collect samples
To remove goo/genomic DNA mediated viscosity	
6.	Incubate samples for 10min at 95°C
7.	Sonicate samples for 10sec
Maintain protein samples at RT, cooling samples will induce SDS precipitation	
8.	For long-time storage, store cell lysates at -20°C

SET buffer	
Reagent	**Concentration**
Tris-HCl pH7.4	10mM
NaCl	150mM
EDTA	1mM
SDS	1% (w/v)

3.7.3 Protein quantification

After protein extraction, protein concentrations have to be quantified to allow for comparison between samples. Various protein quantification kits are commercially available which are most commonly based on the Smith (Bicinchoninic Acid Assay, BCA), Bradford, or Lowry method. These methods rely on protein-induced conversion of reagents that can be measured by colorimetric techniques (absorbance). Depending on the method used, the assays require the samples to be within a specific protein and compatible reagents concentration range, which should be confirmed before submitting the samples to the assay. In cases where samples do not meet the specific manufacture's-indicated requirements, the samples should be diluted prior to assessing the protein concentrations or another protein quantification method should be chosen.

For work performed in this thesis, protein concentrations were assessed using the Lowry-based DC protein assay (Biorad). In this assay, protein quantification is obtained by measuring peptide bonds and the radical groups of tyrosine and tryptophan, and to a lesser extent cystine, cysteine, and histidine, under alkaline conditions. Divalent copper ions form complexes with peptide bonds which reduces the divalent copper ions to monovalent ions. Monovalent copper and the radical groups induce the reduction of the Folin reagent, thereby destabilizing the Folin reagent and inducing its reduction to molybdenum/tungsten blue (Figure 3.3).

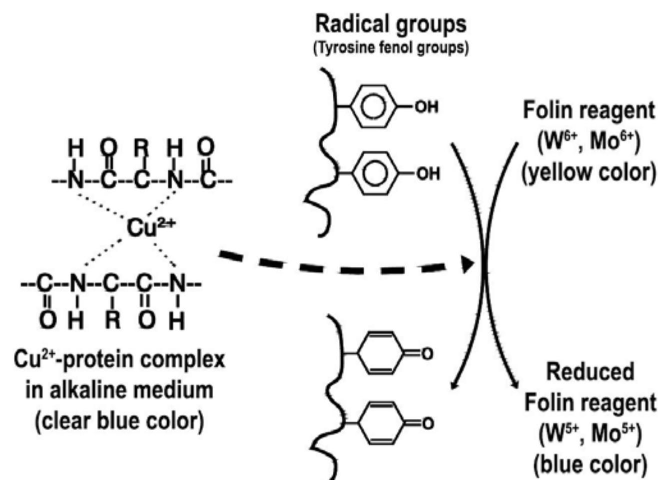


Figure 3.3: Description of the theory behind the Lowry protein quantification methods. Divalent copper ions bind peptide bonds and form protein complexes, thereby reducing the copper to monovalent ions. Together with the radical groups of amino-acids, the monovalent copper reduces the Folin reagent, resulting in the formation of a blue colored dye that can be measured by colorimtric techniques. Figure adapted from [473].

For assessing the protein concentrations after lysis, the following protocol was used;

Protein quantification using the Lowry-based DC protein assay	
Step	
1.	Load in triplicate 1µL of the cell lysates on a transparent 96wells plate
2.	Add 25µL of Reagent A/Reagent S mix to the wells (**100µL Reagent A + 2µL Reagent S)**
3.	Add 200µL of Reagent B to the wells
4.	Incubate 20min at RT and protected from light
5.	Measure protein concentrations in a spectrometer using absorbance between 650-750nm
6.	Calculate protein concentrations from OD values by comparing to OD values from known reference protein concentrations (BSA in ddH_2O)

3.7.4 Western blot

To analyze protein expression levels in cell lysates, Western blot is frequently used to separate proteins by size trough gel electrophoresis, thereby facilitating the detection of proteins and confirmation of their correct molecular weight. The detection of the proteins is realized by the use of protein-specific antibodies, whereas the use of a protein ladder facilitates the validation of the molecular weight of the protein.

3.7.4.1 Sample preparation

To compare protein expression levels between samples, cell lysates were quantified and used for sample preparation in Laemmli buffer (Table 3.2). This buffer contains various components which aid in sample loading and protein migration during electrophoresis.

For work performed in this thesis, samples were prepared to contain between 10-25µg of protein. Samples were always prepared in equal volume and quantity, and heated for 5min at 95°C to ascertain complete protein denaturation.

Table 3.2: Laemmli buffer components and their role in sample preparation

Laemmli buffer 1x		
Reagent	**Concentration**	**Description**
Tris-HCl pH6.8	60mM	Buffering agent to maintain pH
EDTA	4mM	Chelating agent that inhibits proteases and nucleases
To allow for protein separation by size and not by shape or charge		
DTT	100mM	Reducing agent that disrupts protein-protein disulphide bonds and denatures the proteins
SDS	2% (w/v)	Denatures and coats the proteins with a uniform negative charge
To allow for loading		
Glycerol	10% (v/v)	Increases the density of the sample, which aids in loading of the samples on the gel
Bromophenol blue	Traces	Serves as a indicator dye that visually aids when loading the samples on the gels and when tracking protein migration

3.7.4.2 Gel preparation and loading

Protein samples were separated by electrophoresis using SDS-polyacrylamide gels, which consist of a stacking and resolving gel. The stacking gel, with pH6.8, is the gel where the proteins samples are loaded and stacked together before they migrate into the resolving gel, whereas the resolving gel, pH8.3, is the gel where proteins are separated by size. Migration of the proteins is realized by running a current through the gel, with a cathode above and an anode below the gel inducing the migration of the negatively charged proteins towards the anode.

The pH of the stacking gel induces the formation of two migrations fronts. The running buffer contains glycine which, due to the pH, converts to a zwitterionic (neutrally charged) state when present in the stacking gel, thus inducing its slow migration. On the other hand, the Cl^- ions migrate more quickly and ahead of glycine through the stacking gel. This creates a small zone with a steep voltage gradient that induces the glycine to run along behind the Cl^- front. The negatively charged proteins have a migrating mobility that is in between the mobility of the Cl^- and glycine fronts, thereby allowing for their concentration between the two fronts. Once glycine reaches the resolving gel, it converts to a negatively charged state due to the pH8.8 and migrates more quickly than the proteins, thus producing a narrow band containing the proteins at the interface of the stacking and running gel. There, the negatively charged glycine leaves the proteins behind and thus starts the separation of the proteins according to size. The resolving gel has an increased polyacrylamide concentration compared to the stacking gel, which slows the migration of the proteins according to size, since low molecular weight

proteins move faster through the polyacrylamide pores than high molecular weight proteins. Adjustment of the polyacrylamide concentrations allows for the regulation of protein migration, with increasing or decreasing polyacrylamide concentrations proteins migrate slower or faster, respectively.

Polyacrylamide gels were prepared according to the following indications and electrophoresis was performed by running a current of 25mA/gel through the gels and using running buffer. For a detailed explanation of how SDS-polyacrylamide gels are formed see figure 3.4.

Stacking gel	
Reagent	**Concentration**
Acrylamide/Bis solution (37,5 : 1) (Biorad)	4% (w/v)
Tris-HCl pH6.8	280mM
SDS	0,1% (w/v)
APS and TEMED should be added last since they induce gel polymerization	
APS	0,05% (w/v)
TEMED (Biorad)	0,1% (v/v)

Resolving gel	
Reagent	**Concentration**
Acrylamide/Bis solution (37,5 : 1) (Biorad)	8-12% (w/v)
Tris-HCl pH8.8	375mM
SDS	0.1% (w/v)
APS and TEMED should be added last since they induce gel polymerization	
APS	0.05% (w/v)
TEMED (Biorad)	0.05% (v/v)

Running buffer	
Reagent	**Concentration**
Tris	25mM
Glycine	192mM
SDS	0.1% (w/v)
pH8.3	

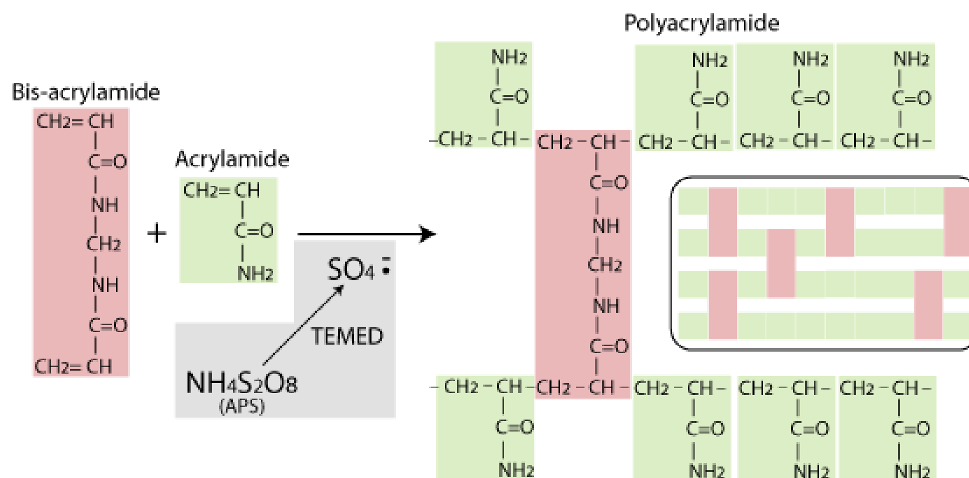


Figure 3.4: Polyacrylamide gels for western blot consist of a matrix formed from monomers of acrylamide and bisacrylamide. TEMED is a catalyst for free radical formation from Ammonium Persulfate (APS). The persulfate free radicals convert the acrylamide/bisacrylamide monomers to free radicals, thereby inducing their polymerization. Acrylamide/ bisacrylamide polymer elongation results in random cross linking of acrylamide/bisacrylamide monomers. In the absence of bisacrylamide, the acrylamide will form long polymers and not a porous gel. However, the presence of bisacrylamide induces cross-linking of the polymers and mediates pore formation. The resulting polyacrylamide gel is chemically inert and does not interact with proteins. Figure derived from [474]

3.7.4.3 Protein transfer to membranes

Once protein samples have been subjected to gel electrophoresis, proteins are transferred to membranes to allow for further handling. Two types of membranes are commonly used for immobilizing the proteins, which are polyvinylidine difluoride (PVDF) and nitrocellulose membranes. Depending on the properties of the protein of interest and on protein detection steps, one membrane is recommended over the other. For work performed in this thesis we used PVDF membranes and the following protocol;

Protein transfer from polyacrylamide gel to PVDF membrane			
Step			
Wear gloves at all time to avoid membrane-immobilization of proteins that were present on the hands of the handler.			
1.	Activate PVDF membrane in MeOH		
2.	Hydrate PVDF membrane in ddH_2O		
3.	Equilibrate PVDF membrane for 5min in Transfer buffer		
4.	Remove stacking gel from polyacrylamide gel		
5.	Mount transfer sandwich in the following order;		
PVDF membrane, polyacrylamide gel, and Whatman membranes have to be in tight contact to allow for efficient protein transfer, which can be obstructed when air bubbles are present.			
	1.	Sponge wet in transfer buffer	
	2.	3x Whatman membrane wet in transfer buffer	
	3.	Polyacrylamide resolving gel	
	4.	PVDF membrane previously activated, hydrated, and equilibrated	
	5.	3x Whatman membrane wet in transfer buffer	
	6.	Sponge wet in transfer buffer	
6.	Submerge transfer sandwich in ice-cold transfer buffer. Proteins will migrate towards the anode, thus the PVDF membrane should be facing this site.		
7.	Add ice block to the transfer buffer. **The applied current will increase the temperature of the transfer buffer and will accelerate protein migration. When using transfer times over 90min, change ice block after 90min.**		
8.	Transfer proteins from polyacrylamide gel to PVDF membrane by using a 100V current.		
	Depending on the size of the proteins, proteins have to be allowed to transfer for a certain time period. High molecular weight proteins migrate slower than low molecular weight proteins. For work performed in this thesis proteins were allowed to transfer for 90min.		

Transfer buffer	
Reagent	**Concentration**
Tris	25mM
Glycine	192mM
Methanol	20% (v/v)
pH8.3	

3.7.4.4 Protein detection

After immobilizing proteins on a PVDF membrane, the membranes can be used to assess protein levels by immunoblotting. The presence of the protein of interest is detected by incubating the membrane with primary antibodies against the protein of interest. The primary antibody is subsequently detected by using a host-corresponding secondary antibody against the first antibody. The secondary antibody is coupled to the Horseradish Peroxidase (HRP) enzyme, which allows for assessing protein levels by using HRP substrates. HRP catalyzes the oxidation of cyclic diacylhydrazides, such as luminol, in the presence of H_2O_2. Oxidation of luminol induces an excited state, which returns to the ground state by emitting light. Enhanced chemiluminescence (ECL) is a luminol-based method commonly used for the detection of immobilized proteins. Incubation of immunoblotted membranes with ECL allows for antigen detection by exposing the membrane to an autoradiography film or digital imaging system, such as ImageQuant.

After immobilizing proteins on a PVDF membrane the following protocol was used for antigen detection;

Immunoblotting	
Step	
1.	Quickly wash membrane with TBS-T
2.	Block membrane for 1h with 5% (w/v) skimmed milk in TBS-T under constant agitation, to avoid unspecific antibody binding
3.	Wash membrane thoroughly with TBS-T to remove skimmed milk **This reduces the chance of adding skimmed milk to the primary antibodies, thus prolonging their shelf-life**
4.	Incubate membrane under constant agitation with the primary antibody for 16h at 4°C or 1h at RT
5.	Quickly wash membrane 3x with TBS-T
6.	Incubate membrane for 1h with the host-corresponding secondary antibody diluted in 5% (w/v) skimmed milk in TBS-T under constant agitation
7.	Wash membrane 3x for 10min with TBS-T under constant agitation
8.	Incubate membrane for 1min with ECL
9.	Expose membrane to an autoradiography film in a dark room, and reveal the film or, use a digital imaging system for signal detection

TBS-T	
Reagent	**Concentration**
Tris	20mM
NaCl	150mM
Tween-20	0.1% (v/v)
pH8.0	

For the dilution of primary antibodies it is recommended to add 0.02% (w/v) sodium azide (NaN_3) to the TBS-T to avoid contamination with fungi and bacteria and thereby prolong their shelf-life.

The following primary antibodies were used for this study;

Primary antibodies								
Antibody	**Antigen Mw**	**Ref.**	**Working dilution**	**Dilution media**	**Storage once diluted**	**Source**	**Type**	**Supplier**
AKT	60 kDa	C-20	1:1000	TBS-T	4°C	Goat	Polyclonal	SCBT
BAK	28 kDa	#06-536	1:1000	TBS-T	4°C	Rabbit	Polyclonal	Merck-Millipore
BAX	22 kDa	Clone 6A7	1:1000	TBS-T	4°C	Mouse	Monoclonal IgG1κ	BD
BCL-2	26 kDa	Clone 124	1:1000	TBS-T	4°C	Mouse	Monoclonal IgG1κ	Dako
BCL-X_L	26 kDa	#610211	1:2000	TBS-T	4°C	Rabbit	Polyclonal	BD
Caspase-3	35 kDa	#9662	1:1000	5% (w/v) BSA in TBS-T	-20°C	Rabbit	Polyclonal	CST
Caspase-8	57 kDa	Clone 1C12	1:1000	5% (w/v) BSA in TBS-T	-20°C	Mouse	Monoclonal IgG1	CST
c-$FLIP_L$	55 kDa	Clone Dave-2	1:1000	TBS-T	4°C	Rat	Monoclonal IgG2a	Enzo
c-$FLIP_{S/L}$	26 kDa (c-$FLIP_S$) 55 kDa (c-$FLIP_L$)	H-202	1:1000	TBS-T	4°C	Rabbit	Polyclonal	SCBT
cIAP2	68 kDa	H-85	1:1000	TBS-T	4°C	Rabbit	Polyclonal	SCBT
ERK1/2	44-42 kDa	Clone 16/ERK	1:5000	TBS-T	4°C	Mouse	Monoclonal IgG2a	BD
FADD	27 kDa	S-18	1:1000	TBS-T	4°C	Goat	Polyclonal	SCBT
Fas	45 kDa	C-20	1:2000	TBS-T	4°C	Rabbit	Polyclonal	SCBT
IκBα	39 kDa	C-21	1:2000	TBS-T	4°C	Rabbit	Polyclonal	SCBT
JNK1/2	46-54 kDa	#9252	1:1000	TBS-T	4°C	Rabbit	Polyclonal	CST
p-AKT	60 kDa	#9271	1:1000	TBS-T	4°C	Rabbit	Polyclonal	CST
p-ERK1/2	44 kDa (ERK1) 42 kDa (ERK2)	#9101	1:2000	TBS-T	4°C	Rabbit	Polyclonal	CST
p-JNK1/2	46 kDa (JNK1) 54 kDa (JNK2)	Clone G9	1:1000	TBS-T	4°C	Mouse	Monoclonal IgG1	SCBT
XIAP	57 kDa	Clone 48	1:2000	TBS-T	4°C	Mouse	Monoclonal IgG1	BD
α-Tubulin	50 kDa	Clone B-5-1-2	1:50 000	TBS-T	4°C	Mouse	Monoclonal IgG1	Sigma-Aldrich

The following secondary antibodies were used for this study;

Secondary antibodies							
Antibody	**Ref.**	**Working dilution**	**Dilution media**	**Storage once diluted**	**Source**	**Type**	**Supplier**
Anti-Goat IgG HRP	A5420	1:10 000	TBS-T + 5% (w/v) skimmed milk	1x use	Rabbit	Polyclonal	Sigma-Aldrich
Anti-Mouse IgG HRP	A9044	1:20 000	TBS-T + 5% (w/v) skimmed milk	1x use	Rabbit	Polyclonal	Sigma-Aldrich
Anti-Rabbit IgG HRP	A0545	1:20 000	TBS-T + 5% (w/v) skimmed milk	1x use	Goat	Polyclonal	Sigma-Aldrich
Anti-Rat IgG HRP	A9037	1:5 000	TBS-T + 5% (w/v) skimmed milk	1x use	Goat	Polyclonal	Sigma-Aldrich

Suppliers	**Description**	**Headquarters**
BD	Becton Dickinson Biosciences	Franklin Lakes, NJ, USA
CST	Cell Signaling Technologies	Beverly, MA, USA
Dako	Agilent Technologies	Santa Clara, CA, USA
Enzo	Enzo Life Sciences	Farmingdale, NY, USA
Merck Millipore		Billerica, MA, USA
SCBT	Santa Cruz Biotechnology	Santa Cruz, CA, USA
Sigma-Aldrich		St. Louis, MO, USA

3.7.4.5 Membrane reprobing

After immunoblotting, the membranes can frequently be reused for reprobing of other proteins, as long as these proteins are of a different molecular weight. In cases where the detection of proteins of similar molecular weight is required, it is recommended to strip the membrane before immunoblotting with the next primary antibody. Stripping of the membrane releases bound proteins, such as antibodies and skimmed milk proteins, from the membrane, thereby reducing background signals when reprobing. Release of membrane-bound protein is usually accomplished by incubation in denaturing buffers, containing detergents, reducing agents, and/or low pH. Stripping the membrane might result in loss of immobilized proteins.

For work in this thesis, membrane stripping was performed according to the following instructions;

Membrane stripping	
Step	
1.	Quickly wash membrane with TBS-T
2.	Incubate membrane for 30min in stripping buffer under constant agitation
3.	Wash membrane thoroughly in ddH$_2$O
4.	Block membrane for 1h with 5% (w/v) skimmed milk in TBS-T under constant agitation, to avoid unspecific antibody binding
5.	Use for immunoblotting

Stripping buffer	
Reagent	**Concentration**
Glycine	2M
SDS	35mM
Tween-20	1% (v/v)
pH2.2	

Under conditions where reprobing is performed with a primary antibody from a different host species, inactivation of HRP of the secondary antibodies bound to the membrane will suffice to decrease background signals. For this purpose the membrane is incubated with sodium azide (NaN_3) which will irreversibly bind to HRP and inactivate the enzyme.

For work performed in this thesis, HRP inactivation of the secondary antibodies was performed according to the following instructions;

HRP inactivation	
Step	
1.	Quickly wash membrane with TBS-T
2.	Incubate membrane for 30min in TBS-T + 5% (w/v) skimmed milk + 0.1% (w/v) NaN_3
3.	Wash membrane thoroughly in TBS-T
4.	Use for immunoblotting

3.7.4.6 Membrane storage

Membranes with immobilized proteins can be stored for later reprobing. Short-time storage can be performed by maintaining the membrane submerged in TBS-T and at 4°C. For long-term storage it is recommended to dry the membrane and store the membrane in airtight sealed plastic at 4°C or even
-20°C. In addition, membrane stripping is recommended before the storage of dried membranes, since membrane drying can result in antibody immobilization on the membrane.

For work performed in this thesis, membrane storage was performed according to the following instructions;

Membrane storage		
Step		
1.	Quickly wash membrane with TBS-T	
2.	Incubate membrane for 30min in stripping buffer under constant agitation	
3.	Wash membrane thoroughly in ddH_2O	
4.	Dry membrane according to the options below	
	1.	Incubate membrane for 1min in MeOH
	2.	Leave membrane for 30min at 37°C
	3.	Leave membrane for ≥4h on workbench
5.	Store membrane in airtight sealed plastic at 4°C or even -20°C	
For reprobing after storage		
6.	Incubate membrane with MeOH for 1min	
7.	Hydrate membrane for 5min with ddH_2O	
8.	Block membrane for 1h with 5% (w/v) skimmed milk in TBS-T under constant agitation, to avoid unspecific antibody binding	
9.	Use for immunoblotting	

3.8 Characterization of cell surface protein levels

The cell surface exposure of various proteins, such as cell membrane receptors, is necessary for their function and signal transduction. Analysis of cell surface protein levels gives an indication of the cellular distribution of the protein of interest and gives insight in their contribution to activation of signaling pathways. Total protein levels obtained by cell lysis do not indicate the levels of cell surface exposure, thus other methods should be used to assess cell surface levels of the protein of interest.

3.8.1 Flow cytometry

Immunostaining of live or fixed cells by using a flouphore-tagged antibody against the protein of interest is a useful and quick method for assessing intracellular and cell surface protein expression. Care has to be taken when fixing the cells, since PFA is able to permeabilize the cytoplasmic membrane. Therefore, it is recommended to use live cells for cell surface protein immunostaining. Quantification of the amount of cell surface protein staining is performed by using flow cytometry and detecting the fluophore by using the corresponding detection channel. As a control, an isotype antibody is used to assess autofluorescence of the cells.

For assessing Fas expression, cells were seeded on 35mm cell culture plates, treated for the indicated times, and the following protocol and antibodies were used. Fas expression was assessed using a FACSCalibur flow cytometer (Becton Dickinson). Flow cytometry data was analyzed using the FlowJo VX software (Tree Star, San Carlos, CA, USA)

Analysis of cell surface expression using flow cytometry	
Step	
1.	Wash cells with 1ml PBS
2.	Dissociate cells by incubating for 5min at RT with 1ml cell dissociation buffer
3.	Collect cells in eppendorf tube
4.	Centrifuge cells for 3min at 500g at 4°C
5.	Wash cells 2x with 500µl ice-cold PBS
6.	Wash cells with 500µl ice-cold FACS buffer
7.	Incubate cells for 30min in 100µl ice-cold FACS buffer containing the fluophore-tagged antibodies **Shake cell suspension from time to time**
8.	Wash cells 2x with 500µl ice-cold FACS buffer
9.	Resuspend cells in 500µl ice-cold FACS buffer
10.	Transfer cell suspension to flow cytometry tube
11.	Assess cell surface expression using flow cytometry. **For PE-tagged antibodies use the FL-2 channel**

For assessing cell surface Fas expression, the following antibodies were used;

Flow cytometry antibodies						
Antibody	**Fluophore**	**Reference**	**Working dilution**	**Source**	**Type**	**Supplier**
Fas	PE	Clone DX2	1:100	Mouse	Monoclonal IgG1κ	BD Biosciences
Isotype	PE	Clone MOPC-21	1:100	Mouse	Monoclonal IgG1κ	BD Biosciences

Cell dissociation buffer	
Reagent	**Concentration**
PBS	1x
EDTA	5mM

FACS buffer	
Reagent	**Concentration**
PBS	1x
FBSi	2% (v/v)
Sodium azide	0.02% (w/v)

3.8.2 Cell surface biotinylation

Biotinylation of cell surface proteins is a useful method to asses cell surface protein levels or protein trafficking from and to the cell surface. The method relies on the use of the biotinylating reagent Sulfo-NHS-SS-Biotin, which due to its negative charge is unable to penetrate cell membranes, thus only labelling cell surface proteins. Sulfo-NHS-SS-Biotin contains a biotin group, a disulfide bond which can be used for releasing biotin from the protein using a reducing agent, such as DTT, and contains an N-hydroxysulfosuccinimide (NHS) ester that covalently binds with amine groups of proteins on the cell surface in alkaline buffers, thereby biotinylating the cell surface proteins (Figure 3.5).

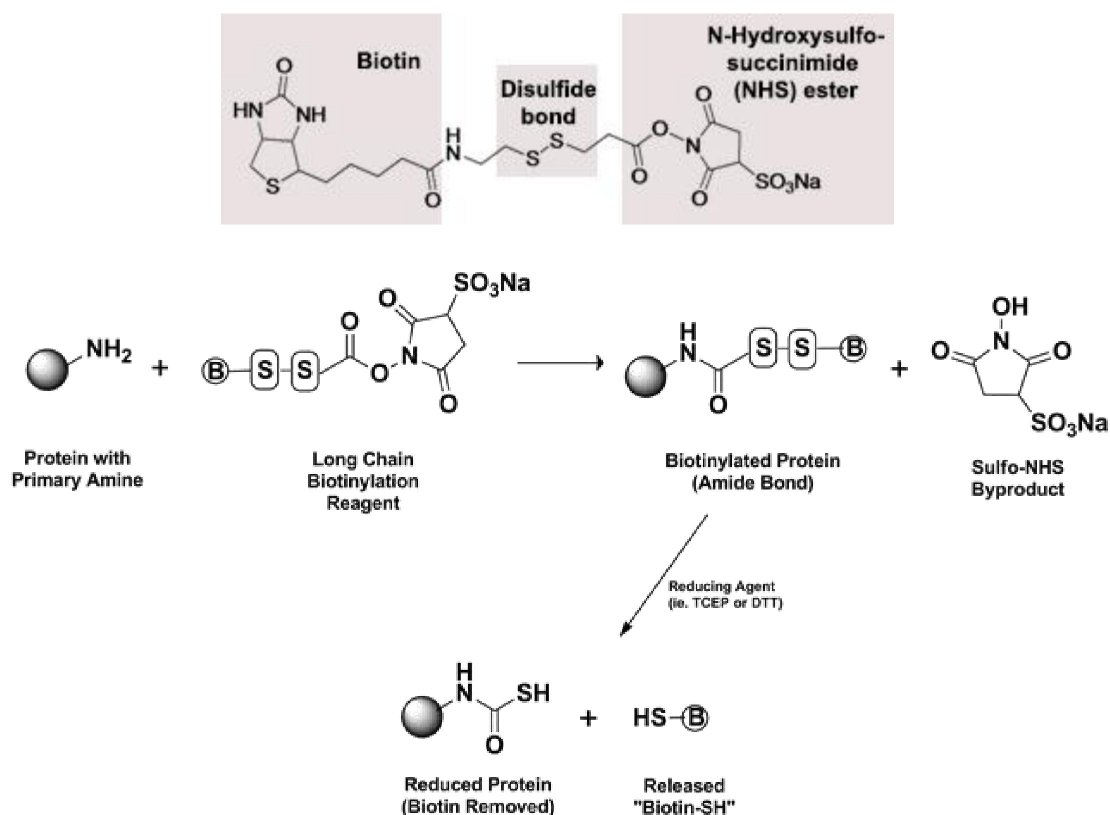


Figure 3.5: Characterization of Sulfo-NHS-SS-Biotin groups and description of the theory behind cell surface protein biotinylation. Sulfo-NHS-SS-Biotin binds amine groups of proteins on the cell surface through a covalent interaction with the NHS ester. After cell lysis, cell surface proteins are isolated by immuno-precipitating the attached biotin group. To release the proteins from the NeutrAvidin Agarose beads, reducing agents can be used to break the disulfide bond. Figure adapted from [475, 476]

To asses cell surface protein levels by biotinylation, cells were seeded on 60mm cell culture plates, treated for the indicated times and used according to the following adapted protocol for use with the Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific).

Cell surface biotinylation protocol using the Pierce Cell Surface Protein Isolation Kit	
Sulfo-NHS-SS-Biotin should be dissolved and handled quickly to avoid loss of activity due to hydrolysis of the NHS ester. In aqueous solutions hydrolysis competes with protein biotinylation.	
Step	
Sulfo-NHS-SS-Biotin stock preparation	
1.	Dissolve 1 vial (12mg) of Sulfo-NHS-SS-Biotin in 960ul of DMSO free of water to avoid hydrolysis
2.	Aliquot Sulfo-NHS-SS-Biotin in eppendorf tubes
3.	Store aliquots at -20°C, sealed with Parafilm and in the presence of silica gel
Biotinylation	
4.	Collect cell culture plates and put on ice
5.	Dilute 40µL Sulfo-NHS-SS-Biotin DMSO stock in 2ml ice-cold PBS per 60mm cell culture dish **Final concentration 412µM**
6.	Quickly wash cells 2x with 2ml ice-cold PBS
7.	Add 2ml of 412µM Sulfo-NHS-SS-Biotin diluted in PBS to every plate
8.	Incubate for 30min at 4°C while gently agitating
9.	Quench the reaction by adding 100µL of Quenching Solution to the plates. Mix by gentle shaking **When needed, home-made quenching solution can be used:** 50mM glycine in PBS
10.	Harvest cells by scraping and collect in falcon
11.	Wash plates with 2ml ice-cold TBS and add to the falcons
12.	Centrifuge for 3min at 500g and 4°C
13.	Aspirate supernatant
14.	Resuspend and wash cell pellet with 2mL ice-cold TBS
Lysis	
15.	Lyse cells using 1ml ice-cold Triton lysis buffer
When needed, cell lysates can be stored at -20°C for future use	
16.	Incubate for 30min at 4°C
17.	Remove insoluble fractions by centrifugation for 30min at 4°C and 16 000g
18.	Quantify protein concentration
19.	Adjust protein concentrations to obtain equal concentrations in all samples **Prepare protein sample in Laemmli buffer to be used as input/cell lysate**
Isolation of labeled proteins	
20.	Per sample, add 25µl of NeutrAvidin Agarose slurry to an eppendorf tube
21.	Wash 3x with 250µl TBS (or wash buffer), using a tabletop centrifuge
22.	Use a insulin syringe to remove all TBS
23.	Add 1ml cell lysate to the NeutrAvidin Agarose beads.
24.	Incubate for 1h at RT on an end-over-end rotator
25.	Centrifuge samples using a tabletop centrifuge
26.	Remove supernatant (store if needed for loading flow through)
27.	Wash NeutrAvidin Agarose beads 3x with 250µl TBS (or wash buffer) + protease inhibitors
Protein elution	
28.	Remove all TBS (or wash buffer) using an insulin syringe
29.	Use one of the following methods for protein elution
	Elution using a reducing agent and applying heat (recommended) Heating will cause recovery of some NeutrAvidin Protein monomer (15K) in the eluate.
	1. Add 50µL of 2x Laemmli buffer to the dry NeutrAvidin Agarose beads
	2. Incubate for 5min at 95°C. Vigorously shake samples every 2.5min

	Elution using a reducing agent
	1. Add 50µL of 2x Laemmli buffer to the dry NeutrAvidin Agarose beads
	2. Incubate for 1h at RT while vigorously shaking constantly or every 10min
30.	Centrifuge samples using a tabletop centrifuge
31.	Separate sample from NeutrAvidin Agarose beads by using an insulin syringe
32.	Use samples for assessing protein expression by Western blot **Depending on the expression of the protein and the quality of the antibody, 15-25µL of sample should suffice for loading**

TBS	
Reagent	**Concentration**
Tris	20mM
NaCl	150mM
Tween-20	0.1% (v/v)

3.9 DISC immunoprecipitation

The multi-protein Death-Induced Signaling Complex (DISC) is able to form upon DR activation. Analysis of the proteins and protein levels that constitute DISC formation gives insight in the regulation of DISC formation and DR-activated signaling pathways. The analysis of Fas DISC formation is performed by lysing the cells under gentle non-denaturing conditions and co-immunoprecipitating (co-IP) Fas-recruited proteins upon FasL-induced Fas activation. For the purpose, an Fc- or epitope-tagged FasL is used for treatment and after lysis the DISC is immunoprecipitated by pulling down FasL.

For Fas DISC analysis, cells were seeded on 10mm cell cultures plates, and used according to the following protocol;

Fas DISC analysis	
Treatment	
Step	
1.	Treat cells for 30min with 2.5µg/ml Fc:hFasL
2.	Place cells on ice
3.	Harvest cells by scraping
4.	Centrifuge for 3min at 4°C and 500g
5.	Wash cells with 500µL ice-cold PBS
Lysis	
6.	Lyse cells for 30min in 1ml ice-cold IP lysis buffer + protease inhibitors
7.	Remove insoluble fractions by centrifugation for 30min at 4°C and 16 000g
8.	Quantify protein concentration
9.	Adjust protein concentrations to obtain equal concentrations in all samples **Prepare protein sample in Laemmli buffer to be used as input/cell lysate**
Immunoprecipitation	
10.	Per sample, 3x wash 50µL protein G-Sepharose slurry with 500µL ice-cold IP lysis buffer + protease inhibitors
11.	Spin down beads using a tabletop centrifuge
12.	Remove IP lysis buffer using a insulin syringe
13.	Add 1ml cell lysates to the beads
14.	Incubate for 4-16h on an end-over-end rotator at 4°C
15.	Spin down beads using a tabletop centrifuge
16.	Aspirate supernatant **Store supernatant to be used as flow-through if needed.**
17.	Wash protein G-Sepharose beads 5x with 500µl ice-cold IP lysis buffer
Protein elution	
18.	Remove all IP lysis buffer using an insulin syringe
19.	Add 50µl elution buffer to the beads
20.	Incubate for 5min at RT, shake every 2min
21.	Collect eluate using an insulin syringe
22.	Add 10µl neutralization buffer
24.	Use samples for assessing protein expression by Western blot **For Fas DISC analysis, loading of the complete sample is recommended**

IP lysis buffer	
Reagent	**Concentration**
Tris-HCl pH7.4	20mM
NaCl	150mM
EDTA	2mM
EGTA	1mM
Glycerol	10% (v/v)
Igepal CA-630 (NP-40)	1% (v/v)
Added before use	
EDTA-free Complete protease inhibitor mixture	1x

Elution buffer	
Reagent	**Concentration**
Citrate pH2.2	100mM

Neutralization buffer	
Reagent	**Concentration**
Tris-HCl, pH8.5	1M

3.10 Statistical analysis

All the experiments were repeated at least three times. Values are expressed as mean ± SD. Values from qRT-PCR experiments are expressed as mean ± SEM. Statistical significance was determined by one-way or two-way ANOVA using GraphPad Prism v5 (GraphPad Software, La Jolla, CA, USA).

Results

"Inappropriate behavior makes me laugh."

Will Ferrell
American actor, comedian, producer, and writer (Born in 1967)

4. Results

4.1 Co-treatment with TNFα and FasL induces apoptosis in SK-N-AS cells

To assess whether TNFα and/or FasL induce cell death in neuroblastoma (NB) cells, the caspase-8 positve cell line SK-N-AS was treated with soluble recombinant TNFα (soluble TNFα induces TNFR1 but not TNFR2 activation [246, 247]), recombinant Fc-tagged FasL (which resembles mFasL [477]), or a combination of both. By using Hoechst staining, apoptotic cell death was assessed during a time-course of 24h (Figure 4.1AB). Our findings indicated that TNFα did not induce significant cell death. Fas stimulation on the other hand was capable of inducing around 20% cell death as of 4h of treatment. When SK-N-AS celles were treated with a combination of both TNFα and FasL, a significant increase in apoptosis could be observed after 8h of treatment when compared to signle treatments with FasL or TNFα. The apoptosis induced by double treatment continued to increase untill 24h post-treatment, when it reached near complete cell death (~80%). These data indicate that the combination of TNFα and FasL is a potent inducer of apoptosis in the caspase-8 positive SK-N-AS NB cell line.

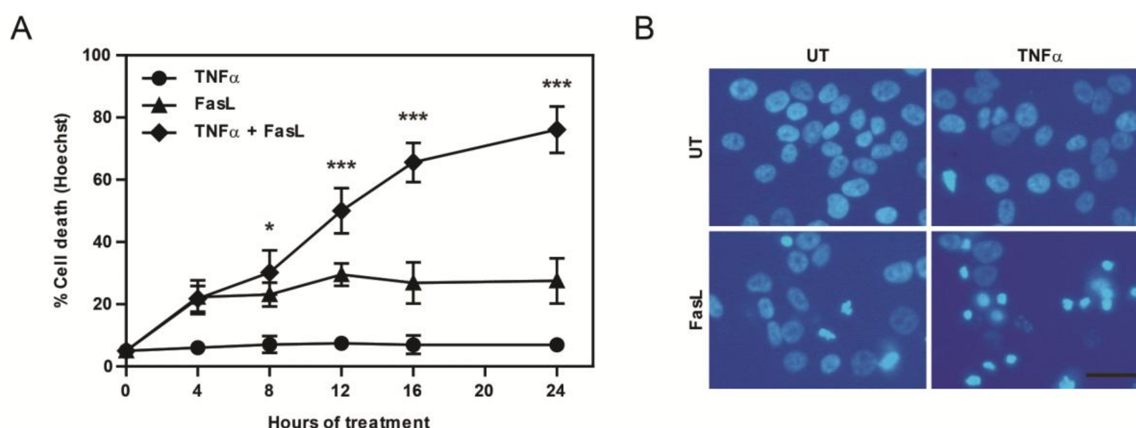


Figure 4.1: Fas and TNFα/FasL treatment induce apoptosis in SK-N-AS cells. A. SK-N-AS cells were treated for the indicated times with 100ng/ml Fc:hFasL, 100ng/ml TNFα, or a combination of both. Cell death was assessed by Hoechst staining. **B.** Representative images of nuclear Hoechst staining after 24h of treatment or no treatment (UT) for determining the levels of apoptosis as seen in **(A)**. Scale bar, 20µm. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

DR stimulation can induce apoptosis through two different apoptotic pathways, the type I and type II pathways [207]. In the type I pathway, DR stimulation induces direct caspase-8/10-mediated caspase-3 activation. In the type II pathway, DR activation induces the caspase-8/10-mediated cleavage of BID, resulting in tBID. In turn, tBID activates the intrinsic/mitochondrial apoptotic pathway by inducing MOMP [33]. Since the latter pathway passes through the mitochondria, this apoptotic pathway can be inhibited through overexpression of anti-apoptotic BCL-2 family members.

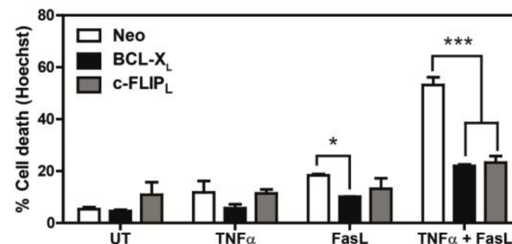


Figure 4.2: SK-N-AS cells are type II apoptotic cells. SK-N-AS cells were infected for 72h with the indicated plasmids. Thereafter, cells were treated for 24h with 100ng/ml Fc:hFasL, 100ng/ml TNFα, or a combination of both. Cell death was assessed by Hoechst staining. *p ≤ 0.05; ***p ≤ 0.001.

To determine which type of apoptosis SK-N-AS cells display upon treatment with FasL or a combination of TNFα and FasL, cells were infected by lentivirus with a plasmid containing the *BCL-X$_L$* gene. Here, BCL-X$_L$ overexpression induced an inhibition of FasL- and TNFα/FasL-induced cell death (Figure 4.2), indicating that SK-N-AS cells induce type II apoptosis when stimulated with FasL or co-stimulated with TNFα/FasL. Moreover, co-stimulation with TNFα/FasL did not induce a shift in the type of apoptosis, since both co-stimulation and single FasL-treatment induced type II apoptotic pathway activation. Overexpression of the anti-apoptotic caspase-8 homologue c-FLIP$_L$ inhibited the apoptosis induced by the TNFα/FasL-treatment as well (Figure 4.2). These data confirm that the apoptotic cell death is induced through activation of a caspase-8/10-dependent type II apoptotic pathway.

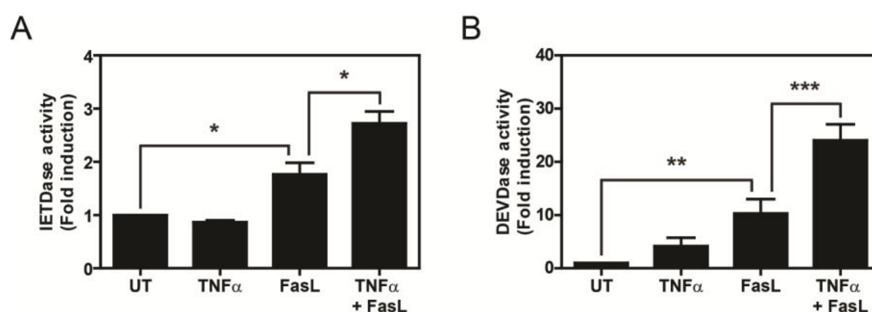


Figure 4.3: FasL and TNFα/FasL treatment induce initiator and effector caspase activation. SK-N-AS cells were left untreated (UT) or treated for 8h with 100ng/ml TNFα, 100ng/ml Fc:hFasL or a combination of both. Incubation with specific caspase substrates indicates caspase-8 activity, Z-IETD-Afc **(A)**, or caspase 3/7 activity, Ac-DEVD-Afc **(B)**. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Next, we assessed the cytokine-mediated induction of caspase activation, by using caspase-specific substrates in combination with a caspase activity assay. Treatment with TNFα did not induce caspase-8 activation, whereas for Fas stimulation a significant increase in IETDase activity was observed, indicating the activation of caspase-8 (Figure 4.3A). Double TNFα/FasL treatment further increased the IETDase activity when compared to single FasL-treatment. Moreover, the FasL- and TNFα/FasL-induced caspase-8 activation translated into effector caspase activation, as observed during caspase-3/7 activity assays using the caspase-3/7 specific Ac-DEVD-Afc substrate (Figure 4.3B). Consistent with the IETDase activity assay, where an increase in caspase-8 activity was observed, co-stimulation with TNFα and FasL also induced an increase in caspase-3/7 activation, when compared to single FasL or TNFα treatment. This increase in caspase-3/7 activity is presumably responsible for the increase in apoptosis observed in the Hoechst staining assay (Figure 4.1).

Further confirmation of the implication of caspase-8 and caspase-3/7 in the FasL- and TNFα/FasL-induced apoptosis in SK-N-AS cells was obtained through inhibition of these caspases. Inhibition of caspase 8 by using the caspase-8 specific Z-IETD-FMK substrate fully abrogated the FasL- and TNFα/FasL-induced apoptosis (Figure 4.4). Coherently, similar results were obtained when inhibiting all caspases using the pan-caspase inhibitor Q-VD-OPH.

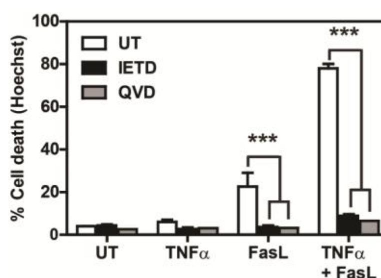


Figure 4.4: FasL- and TNFα/FasL-induced apoptotic cell death is blocked by caspase inhibitors. Hoechst staining assay with SK-N-AS cells treated or not (UT) for 24h with 100ng/ml TNFα, 100ng/ml Fc:hFasL, or a combination of both. Caspases were inhibited, using a caspase-8 inhibitor (50µM IETD) or a pan-caspase inhibitor (10µM QVD). ***$p \leq 0.001$.

4.2 TNFα primes SK-N-AS cells for FasL-induced cell death by upregulating Fas

Simultaneous treatment with TNFα and FasL did not shed light on whether the increase in apoptosis was induced through synergic DR signaling or DR-mediated sensitization of apoptosis induced by the other DR. Therefore, we continued the characterization of the TNFα/FasL-induced increase in apoptosis by using sequential treatment. While using a fluorophore-based cell viability assay, pre-treatment with FasL did not induce sensitization for TNFα-induced cell death (Figure 4.5). However, pre-treatment with TNFα and subsequent treatment with FasL produced a similar cell-death response as observed in the double treatment (Figure 4.1). Here, the TNFα pre-treatment reduced the cell viability to ~20% after FasL treatment. These data indicate that TNFα sensitizes for FasL-induced apoptosis.

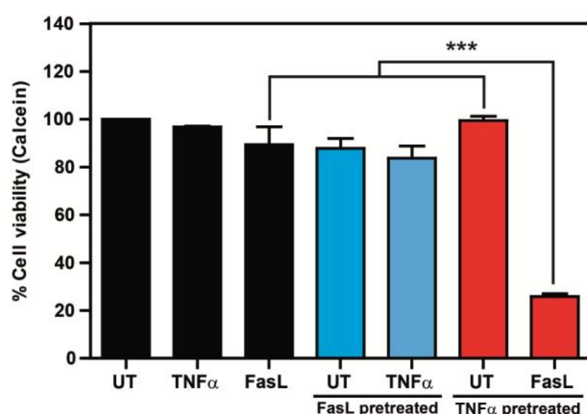


Figure 4.5: TNFα primes for FasL-induced cell death. SK-N-AS cells were left untreated or were pre-treated for 24h with 100ng/ml TNFα or 100ng/ml Fc:hFasL. Thereafter, cells were washed and left untreated (UT) or treated for another 24h with 100ng/ml TNFα or 100ng/ml Fc:hFasL. Cell viability was assessed by calcein AM staining. ***p ≤ 0.001

TNFα is known to regulate the expression of various pro- and anti-apoptotic genes [478, 479]. To determine whether TNFα-induced regulation of gene expression is implicated in the TNFα-induced sensitization to FasL-induced cell death, we performed qRT-PCR experiments evaluating various DISC and anti-apoptotic proteins. Interestingly, when analyzing the mRNA levels of the DISC proteins, Fas, caspase-8, FADD, and RIP1, an increase in Fas mRNA levels was observed as of 30min of treatment, whereas the other analyzed proteins displayed similar mRNA levels or a decrease in levels over a time course of 24h (Figure 4.6A).

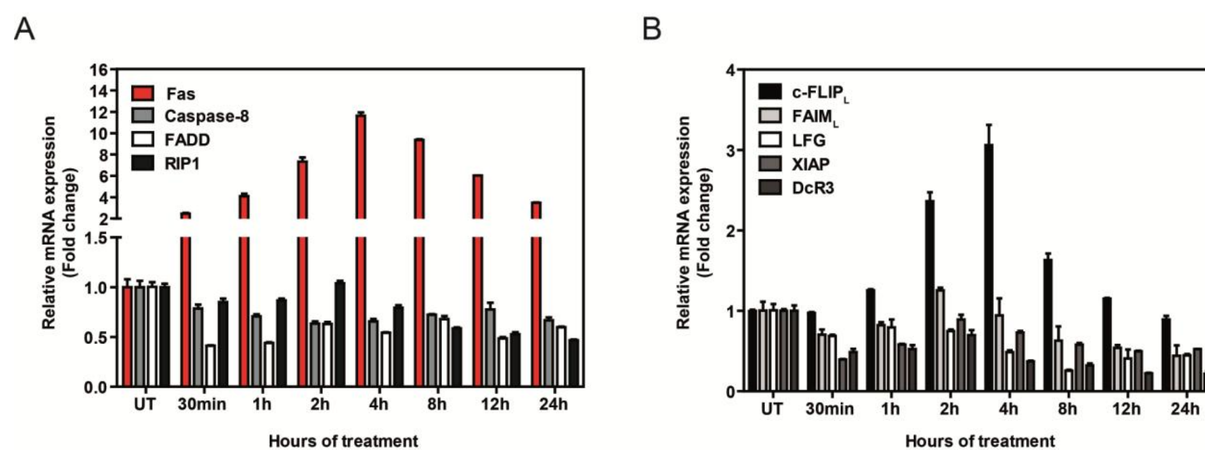


Figure 4.6: TNFα induces Fas mRNA expression. A-B. SK-N-AS cells were treated with 100ng/ml TNFα for the indicated times after which mRNA was extracted. Reverse transcriptase was used to convert mRNA into cDNA and mRNA levels of the indicated genes were assessed by qRT-PCR. mRNA expression levels were normalized using 18S.

When analyzing anti-apoptotic proteins that are able to inhibit FasL-induced apoptosis, $FAIM_L$, LFG, XIAP, and DcR3, decrease in all mRNA levels was observed (Figure 4.6B). The $FLIP_L$ mRNA levels however, showed an increase after 2h of treatment that returned to base-line levels 12h post-treatment. Although the decrease in mRNA levels of anti-apoptotic proteins could explain the TNFα-induced sensitization to FasL-induced apoptosis, we hypothesized that the increase in Fas mRNA levels had a larger contribution to the TNFα-induced sensitization for FasL-induced apoptosis. Although c-$FLIP_L$ might have an inhibitory effect, the TNFα-induced increase in its mRNA levels does not appear to inhibit FasL-induced cell death, possibly because the increase in Fas levels are higher and therefore dictate the observed sensitization. Nevertheless, first we had to ascertain that the increase in Fas mRNA levels translate to protein levels. Therefore we performed a time-course TNFα treatment and assessed the Fas protein levels by Western Blot. Here, after 8h of TNFα treatment an increase in Fas protein levels was observed, which continued to increase up till 24h post-treatment (Figure 4.7). These data are consistent with the increase in Fas mRNA levels and with the increase in apoptosis observed in the Hoechst staining assay at 8h of TNFα/FasL treatment (Figure 4.1 and 4.6).

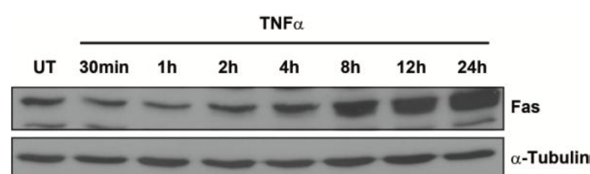


Figure 4.7: TNFα induces Fas protein expression. SK-N-AS cells treated with 100ng/ml TNFα for the indicated times and Fas expression was assessed by Western blot. α-Tubulin was used as loading control.

Analysis of various proteins involved in Fas-activated apoptotic pathways indicated that the TNFα-induced reduction in FADD and RIP1 mRNA levels translated in a reduction in protein expression levels (Figure 4.8). As for the qRT-PCR experiment, TNFα treatment induced an increase in c-$FLIP_L$ protein levels, and other anti-apoptotic proteins, such as cIAP2 and BCL-2. However, induction of these anti-apoptotic proteins did not inhibit FasL induced apoptosis, since TNFα pre-treatment increased the FasL-induced caspase-8, caspase-3, and c-$FLIP_L$ cleavage, comparing TNFα-pretreated cells to the non-pretreated condition. Protein levels of the caspase inhibitor XIAP, and the anti-apoptotic BCL-2 family member BCL-X_L, and the pro-apoptotic BCL-2 family members BAK and BAX were unaffected by TNFα treatment.

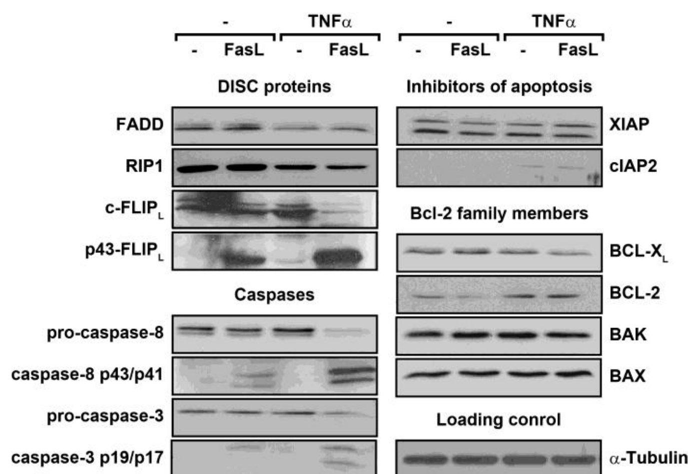


Figure 4.8: Analysis of TNFα-mediated regulation of Fas-activated apoptotic pathway proteins. Prior to 30min treatment with 100ng/ml Fc:hFasL, SK-N-AS cells were treated for 16h with 100ng/ml TNFα. Analysis of protein expression levels was assessed by cell lysis and subsequent Western blot.

4.3 TNFα-induced Fas enhances cell surface Fas exposure and DISC formation

To contribute to an increase in apoptosis, the newly synthesized Fas protein has to become exposed to the cell surface. To ascertain whether this is the case, we performed a cell surface biotinylation assay to characterize the cellular distribution of Fas after TNFα treatment. Again, an increase in total Fas protein levels was observed after 8h of TNFα treatment (Figure 4.9A). Moreover, SK-N-As cells showed an increase in cell surface Fas levels 4h after TNFα treatment, which continued to increase for another 12h after which it stabilized until at least 24h post-TNFα treatment. To confirm whether the newly cell surface exposed Fas is incorporated into a FasL-induced DISC, we performed a DISC immunoprecipitation assay before and after TNFα pre-treatment. Indeed, we were able to co-immunoprecipitate FADD and caspase-8 together with FasL in the condition where we pre-treated with TNFα (Figure 4.9B). Interestingly, in the absence of TNFα pre-treatment, we were only able to immunoprecipitate low levels of high molecular weight Fas (~150 kDa) and no immunoprecipitation of FADD and caspase-8 could be detected. Nevertheless, analysis of input cell lysates showed caspase-8 cleavage after FasL treatment in both the pre-treatment and no-pretreated conditions. Consistent with the previous findings, an increase in FasL-induced caspase-8 cleavage was observed when we pre-treated with TNFα. Moreover, the level of FasL-induced caspase-8 cleavage correlated with the level of FasL-induced caspase-3 cleavage, observing an increase in FasL-induced caspase-3 cleavage when pre-treated with TNFα. Hereby, we were able to demonstrate that TNFα-induced Fas expression translates into an increase in cell surface exposure of Fas. This TNFα-induced increase in cell surface Fas exposure enhanced FasL-induced DISC formation, which led to an increased activation of the extrinsic apoptotic pathway.

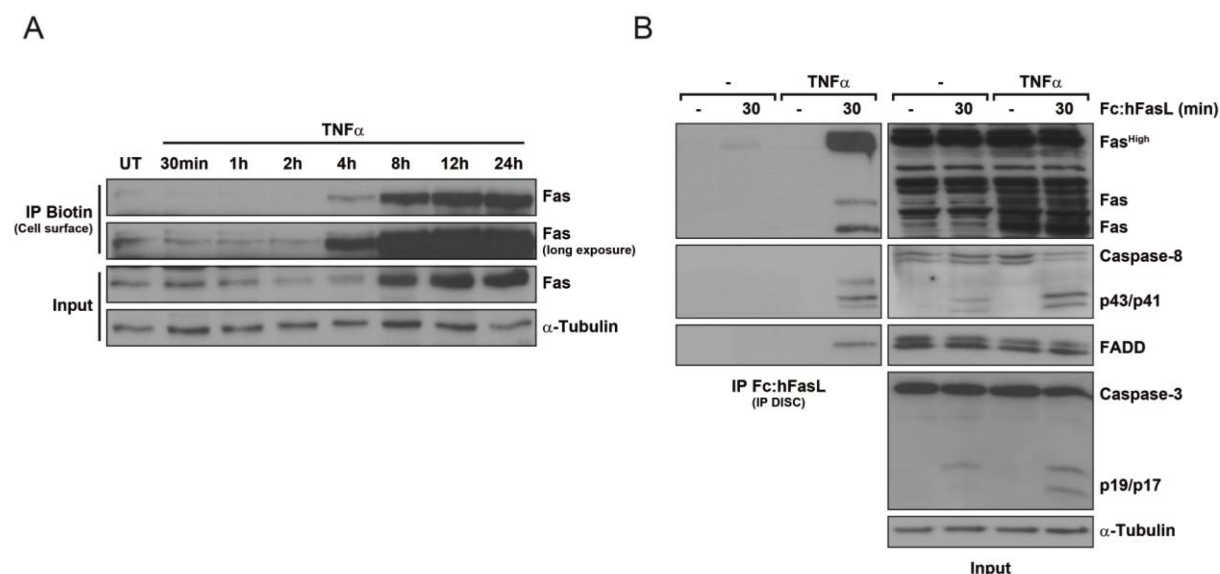


Figure 4.9: TNFα-induced Fas expression results in cell surface Fas exposure and enhances FasL-induced DISC formation. A. SK-N-AS cells were treated for the indicated times with 100ng/ml TNFα. Cell surface and total levels of Fas protein were assessed by using cell surface biotinylation assay and subsequent Western blot. **B**. DISC (Death inducing signaling complex) formation was assessed in SK-N-AS cells, pre-treated (TNFα 100ng/ml) or not (-), through stimulation with 2.5μg/ml Fc:hFasL. The DISC proteins were immunoprecipitated by pulling down Fc:hFasL with protein G-Sepharose and were thereafter analyzed by Western blot.

4.4 TNFα induces NF-κB-mediated transcriptional regulation of *FAS*

TNFR1 is known to activate various signaling pathways following TNFα stimulation, such as the caspase-dependent apoptotic pathway, MAPK pathways, and the NF-κB pathway [78, 262, 480, 481]. To determine whether one of these pathways is involved in the TNFα-induced expression of Fas, we proceeded to analyze Fas expression after TNFα treatment while specifically inhibiting the indicated pathways. Inhibition of the caspase-dependent apoptotic pathway, using the pan-caspase inhibitor Q-VD-OPH, did not induce inhibition of TNFα-induced Fas expression as assessed at the mRNA and protein level, using qRT-PCR and Western blot assays respectively (Figure 4.10AB). Nor did caspase inhibition reduce the TNFα-mediated cell surface exposure of Fas. TNFα-induced mRNA levels of the TNFα-regulated genes, *c-FLIP* [118] and *BCL-2* [482], were neither affected when using Q-VD-OPH.

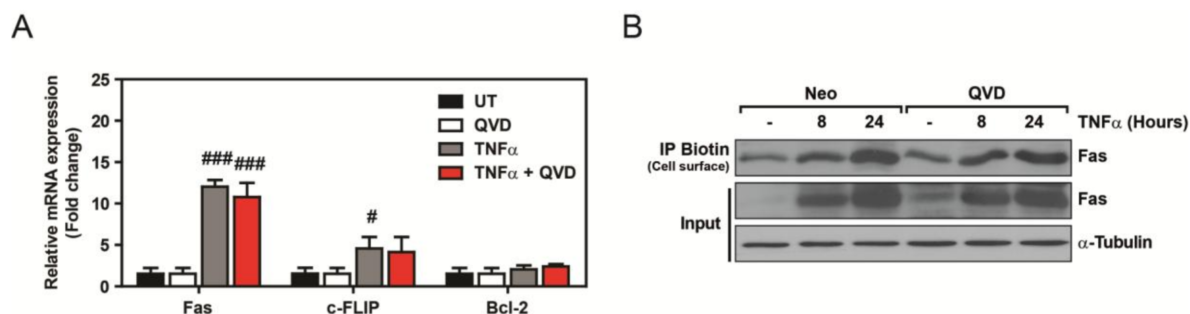


Figure 4.10: Caspase inhibition does not modulate TNFα-induced Fas expression. A. SK-N-AS cells treated or not for 1h with 10µM QVD prior to 4h treatment with 100ng/ml TNFα. mRNA levels were assessed by qRT-PCR using specific Taqman probes. mRNA expression levels were normalized using 18S. **B.** SK-N-AS cells were pre-treated for 1h with 10µM QVD and, at the indicated times, cell surface proteins were biotinylated, isolated, and analyzed by Western blot. # $p \leq 0.05$; ### $p \leq 0.001$ for comparisons between TNFα and the corresponding UT control.

We thereafter assessed the role of known TNFR1-activated MAPK pathways by treating with TNFα in combination with specific MAPK pathway inhibitors; PD98059 (ERK1/2), LY294002 (PI3K), SP600125 (JNK). Inhibition of these MAPK pathways did not affect the TNFα-induced expression of Fas (Figure 4.11). However, increases in TNFα-induced cell surface exposure of Fas were observed when using PD98059 or SP600125 in combination with TNFα, suggesting that inhibition of ERK1/2 and JNK pathway activation affects Fas trafficking to the cell surface.

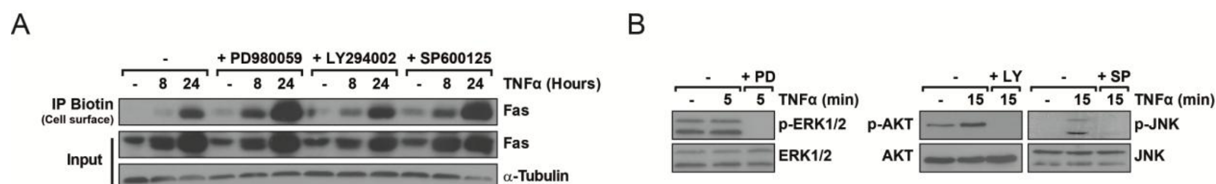


Figure 4.11: TNFα-induced Fas expression and is not modulated by MAPK pathway inhibition. A. SK-N-AS cells were pre-treated for 1h with 25µM PD98059, 20µM LY294002 or 20µM SP600125. Next, cells were treated for the indicated times with 100ng/ml TNFα and cell surface and total Fas levels were analyzed using a cell surface biotinylation assay in combination with Western blot. **B.** Phospho-protein levels assessed by Western blot to confirm efficacy of PD98059 (PD), LY294002 (LY), and SP600125 (SP). SK-N-AS cells were pre-treated for 1h with the indicated MAPK inhibitors and thereafter treated with 100ng/ml TNFα for the indicated times. For the PD experiment, prior to stimulation, cells were deprived of serum for 24h. All conditions were pre-incubated with 10µM of the caspase inhibitor QVD to avoid cell death-related effects.

We proceeded to analyze the role of NF-κB in TNFα-induced Fas expression. For the purpose, we over-expressed a mutated form of IκBα, called Super Repressor (SR). SR carries S32A/S36A mutations and can therefore not be phosphorylated by the IKK complex, thus impeding its subsequent K48 ubiquitination and degradation [483]. As a consequence, SR sequesters the NF-κB complex in the cytosol and thereby inhibits TNFα-induced NF-κB-mediated gene transcription. When overexpressing SR, TNFα-induced Fas expression was fully abrogated as observed at the mRNA and protein levels (Figure 4.12AB). The expression of known TNFα-induced NF-κB target genes, *c-FLIP* [118] and *BCL-2* [482], were inhibited as well. Moreover, overexpression of SR resulted in lack of TNFα-induced cell surface exposure of Fas.

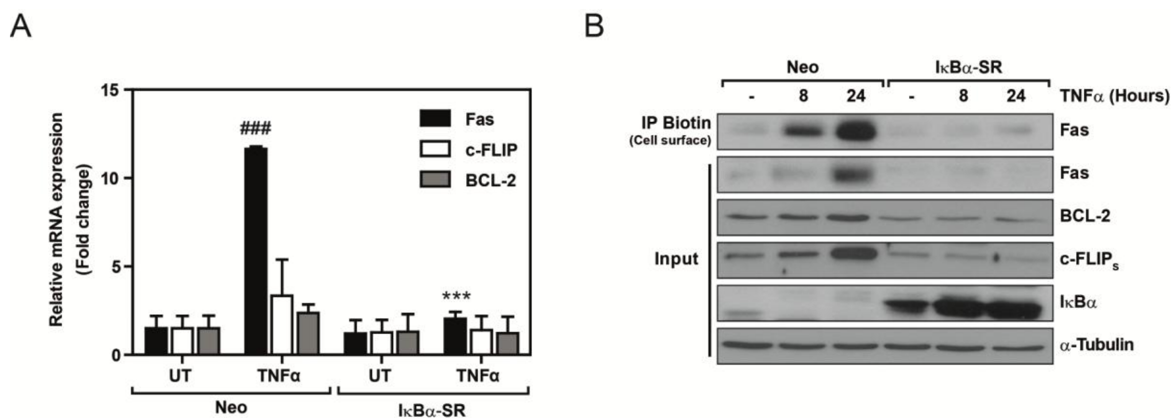


Figure 4.12: SR overexpression inhibits TNFα-induced Fas expression and cell surface exposure. A. Control (Neo) and SuperRepressor/SR-IκBα-infected SK-N-AS cells were left untreated (UT) or treated with 100ng/ml TNFα for 4h. Thereafter, mRNA levels were assessed by qRT-PCR using Taqman probes. **B.** Control (Neo) and SuperRepressor/SR-IκBα-infected SK-N-AS cells were left untreated (−) or treated with 100 ng/ml TNFα for 8h and 24h. Cell surface and total protein levels were assessed by cell surface biotinylation and analyzed by Western blot. All conditions were pre-incubated with 10µM of the caspase inhibitor QVD to avoid cell death-related effects. *** $p \leq 0.001$ for comparisons between Neo and SR. ### $p \leq 0.001$ for comparisons between TNFα and UT.

We were able to confirm these results when we inhibited the NF-κB pathway using an irreversible inhibitor of IKKα, BAY 11-7082. Again, inhibition of the NF-κB pathway induced a full abrogation of TNFα-induced up-regulation of Fas mRNA and protein levels (Figure 4.13A-C). In addition, the known TNFα-induced NF-κB target genes *c-FLIP* [118] and *BCL-2* [482] were used as a control. As observed for SR overexpression, the inhibition of the NF-κB pathway by BAY 11-7082 treatment blocked the TNFα-induced cell surface exposure of Fas. These data indicate that the TNFα-induced expression is mediated by NF-κB pathway activation. Moreover, the NF-κB-mediated expression of Fas induces the increase in Fas exposure to the cell surface.

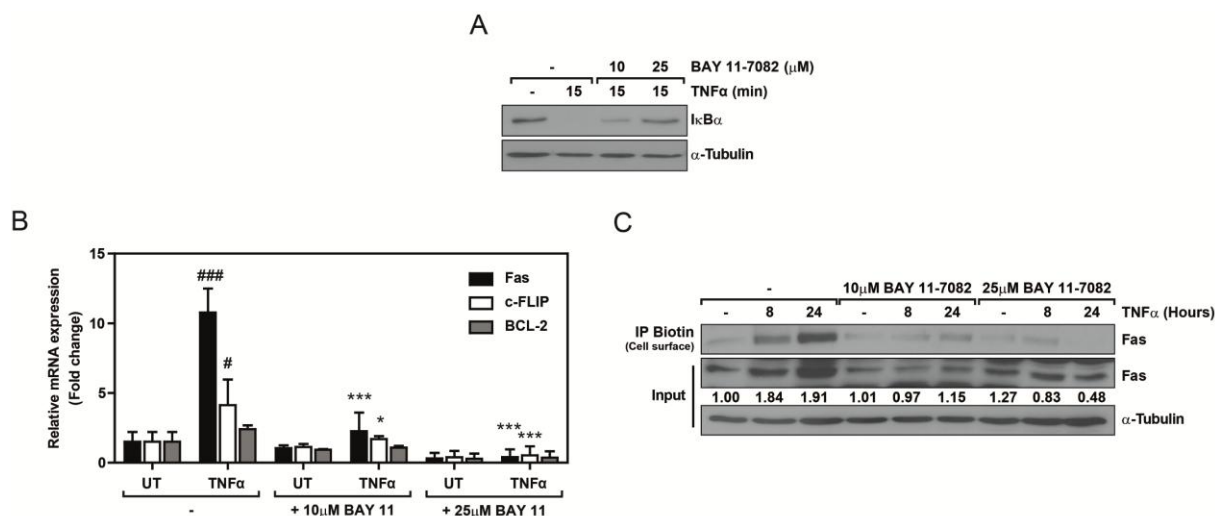


Figure 4.13: IKKα inhibition abrogates TNFα-induced expression of Fas protein and cell surface exposure. A. IκBα levels assessed by Western blot to confirm efficacy of BAY 11–7082. SK-N-AS cells were treated for 1h with the indicated BAY 11–7082 concentrations prior to treatment with 100ng/ml TNFα for 15min. **B.** Prior 100ng/ml TNFα treatment for 4h, SK-N-AS cells were left untreated or were treated for 1h with 10µM or 25µM of the IKKα inhibitor BAY 11–7082. qRT-PCR was used to analyze the mRNA levels of the indicated NF-κB target genes. **C.** SK-N-AS cells were treated for 1h with the indicated concentrations of BAY 11–7082 and subsequently treated for 8h or 24h with 100ng/ml TNFα. Thereafter, cell surface and total Fas protein levels were analyzed by cell surface biotinylation assay and Western blot. Below, quantification of total Fas expression normalized vs. matching α-Tubulin controls. All conditions were pre-incubated with 10µM of the caspase inhibitor QVD to avoid cell death-related effects. * $p \leq 0.05$; *** $p \leq 0.001$ for comparisons between (-) and BAY 11. # $p \leq 0.05$; ### $p \leq 0.001$ for comparisons between TNFα and UT.

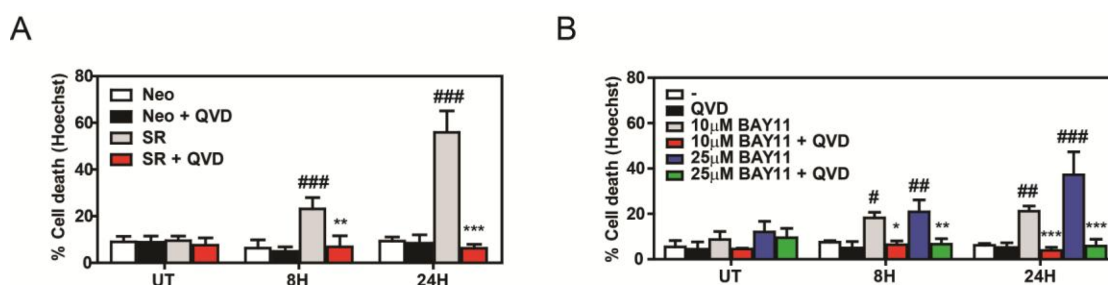


Figure 4.14: NF-κB inhibition provokes TNFα-induced apoptosis. A. Prior 100ng/ml TNFα treatment for the indicated times, SuperRepressor/SR-IκBα-infected SK-N-AS cells were pre-treated or not for 1h with 10µM QVD. TNFα-induced apoptosis was assessed by Hoechst staining. **B.** SK-N-AS cell were pre-treated for 1h with 10µM QVD with or without BAY 11–7082. TNFα (100ng/ml)-induced apoptosis was assessed by Hoechst staining after the indicated times of treatment. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ for comparisons between QVD and their corresponding control. # $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$ for comparisons between treated and UT.

Inhibition of the NF-κB pathway upon TNFα treatment is known to induce apoptosis by blocking the TNFR1-induced expression of anti-apoptotic proteins [115, 116]. When inhibiting the NF-κB pathway in the SK-N-AS NB cell line, by SR overexpression or BAY 11-7082 treatment, TNFα-induced apoptosis (Figure 4.14AB), hereby confirming previous studies. Moreover, TNFα treatment in combination with the pan-caspase inhibitor Q-VD-OPH inhibited the TNFα-induced apoptosis when the NF-κB pathway was blocked, indicating that the apoptosis was caspase dependent.

We further characterized the TNFα-induced *FAS* gene transcription and protein expression using DNA transcription (Actinomycin D) and mRNA translation (Cycloheximide) inhibitors. Pre-treatment with actinomycin D was able to inhibit TNFα-induced upregulation of Fas mRNA (Figure 4.15A). However, cycloheximide pre-treatment did not induce inhibition of Fas mRNA induction when we treated with TNFα. Nevertheless, both inhibitors were able to block the TNFα-induced expression of Fas protein and cell surface exposure (Figure 4.15B). Hereby, we confirmed that TNFα induced Fas expression through direct NF-κB-mediated transcription of the *FAS* gene, thus excluding the possibility that TNFα induces the expression of proteins that regulate the observed increase in Fas expression.

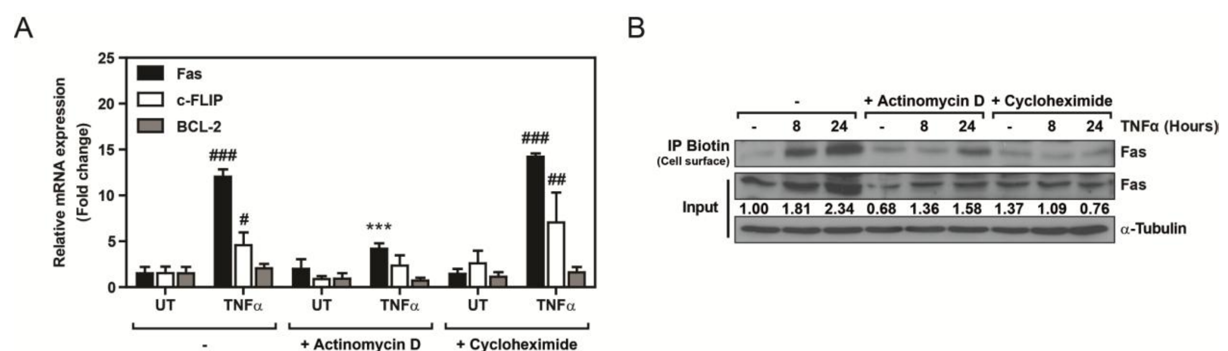


Figure 4.15: TNFα increases Fas expression through transcription and translation. A. SK-N-AS cells were left untreated (-) or were treated for 1h with 20nM actinomycin D or 1μg/ml cycloheximide, prior to 4h TNFα (100ng/ml) treatment. mRNA levels of the indicated genes were analyzed by qRT-PCR. **B.** Total and cell surface Fas levels were assessed by a cell surface biotinylation assay and Western blot. SK-N-AS cells were pre-treated for 1h with 20nM actinomycin D or 1μg/ml cycloheximide and subsequently treated with 100ng/ml TNFα for the indicated times. Below, quantification of total Fas expression normalized vs. matching α-Tubulin controls. All conditions were pre-incubated with 10μM of the caspase inhibitor QVD to avoid cell death-related effects. *** p ≤ 0.001 for comparisons between (-) and Actinomycin D/Cycloheximide. # p ≤ 0.05; ## p ≤ 0.01; ### p ≤ 0.001 for comparisons between treated and UT.

4.5 TNFα primes NB cells for cisplatin- and etoposide-induced activation of caspase-8 and apoptosis

In the clinic, NB patients are treated with chemotherapy that commonly contains the DNA-crosslinking agent cisplatin and the DNA synthesis-inhibiting agent etoposide. Both chemotherapeutic agents have been shown to induce cell death partially through activation of the Fas/FasL system by inducing FasL expression [458, 484]. Since we showed that TNFα induced the upregulation of Fas expression, we addressed if TNFα pre-treatment enhances the cytotoxic effects of these two drugs. First, we confirmed that cisplatin and etoposide induce FasL in NB, by performing qRT-PCR experiments and analyzing FasL mRNA levels at different time points. For both chemotherapeutic agents an increase in FasL mRNA was observed after 24h of treatment (Figure 4.16A).

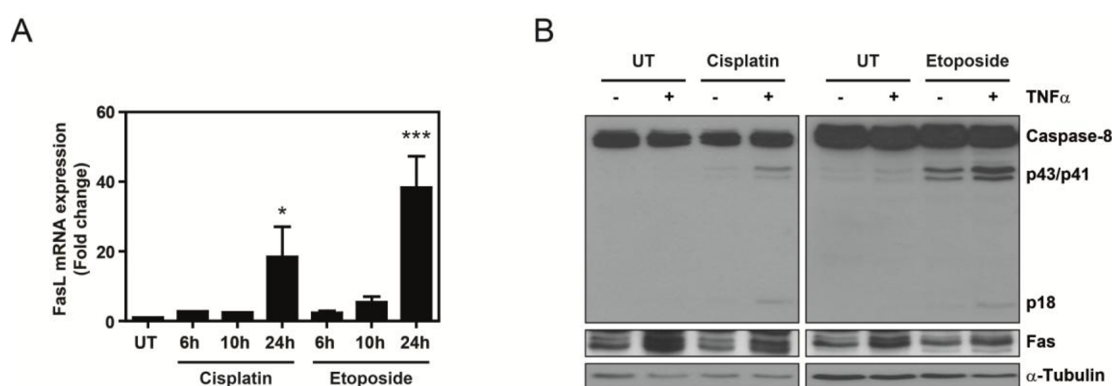


Figure 4.16: Cisplatin and etoposide induce FasL mRNA expression and caspase-8 cleavage, which is enhanced by TNFα pre-treatment. A. Cisplatin- (30µM) and etoposide- (30µM) induced changes in FasL mRNA levels were analyzed by qRT-PCR after the indicated times of treatment, using SK-N-AS cells. mRNA levels were normalized using 18S mRNA. **B.** Western blot was used to analyze caspase-8 cleavage in SK-N-AS cells pre-treated with 100ng/ml TNFα for 24 h and subsequently treated for 24h with cisplatin (30µM) and etoposide (30µM). * $p \leq 0.05$; *** $p \leq 0.001$ for comparisons between treated and UT.

The induction of FasL by treating with cisplatin or etoposide correlated with caspase-8 cleavage, which indicates caspase-8 activation (Figure 4.16B). TNFα pre-treatment induced Fas expression in SK-N-AS cells and, as we previously proposed, enhanced cisplatin- and etoposide-induced caspase-8 activation.

We thereafter analyzed whether these finding could be translated to other NB cell lines. Therefore, we performed a dose response assay in a subset of 4 NB cell lines (SK-N-AS, SK-N-SH, SH-SY5Y, and LAI-5S) and assessed cell viability by a MTT assay (Figure 4.17). Here, we determined the concentrations necessary for the induction of 50% cell death, and used these concentrations for the treatment of non- and TNFα-pre-treated NB cells. Surprisingly, when performing a Hoechst staining assay, only TNFα-induced sensitization for cisplatin- and etoposide-induced apoptosis was observed in the SK-N-AS and SK-N-SH cell lines (Figure 4.18). In contrast, no TNFα-induced changes in apoptosis were observed for the SH-SY5Y and LAI-5S cell lines. These data indicate that within the analyzed subset of NB cell lines, we can discriminate between TNFα-responsive (SK-N-AS and SK-N-SH) and TNFα-unresponsive cell lines (SH-SY5Y and LAI-5S).

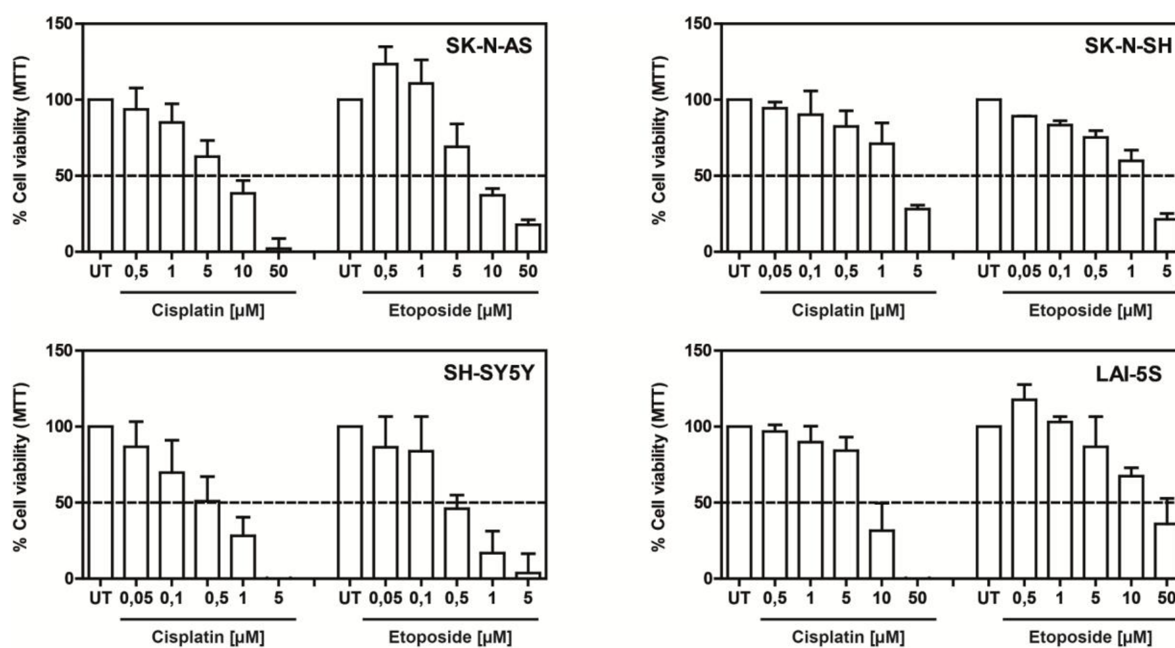


Figure 4.17: Determination of effective cisplatin and etoposide cell death-inducing doses. The indicated NB cell lines were treated for 48h with the indicated concentrations of cisplatin and etoposide. Cell viability was assessed by MTT assay.

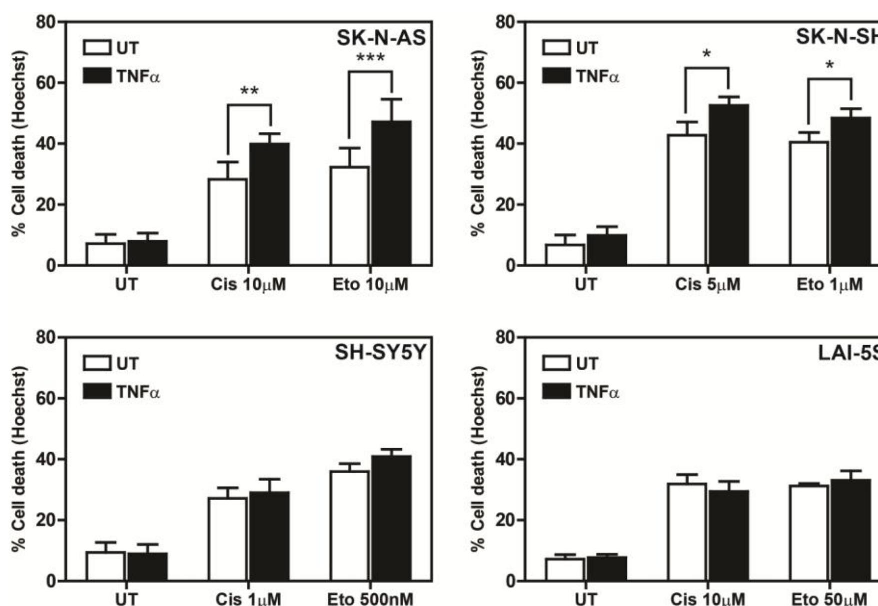


Figure 4.18: TNFα is able to sensitize NB cell lines to cisplatin- and etoposide-induced apoptosis. Prior to 48h treatment with the indicated concentrations of cisplatin (Cis) and etoposide (Eto), the indicated NB cell lines were pre-treated for 24h with 100ng/ml TNFα. Cell death was assessed by using Hoechst staining. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

4.6 NBs show heterogeneity in Fas expression and its modulation by TNFα

Heterogeneity is a hallmark of NB [349-351], which may explain why some NB cells are not primed for cisplatin- and etoposide-induced cell death when pre-treated with TNFα. Therefore we analyzed the expression of Fas and its modulation by TNFα in a set of 8 NB cell lines, 4 of which display *MYCN* amplification. First we assessed the mRNA expression levels by qRT-PCR. Here, Fas mRNA could be detected in all NB cell lines (Figure 4.19). In comparison to SK-N-AS cells, the SK-N-SH, IMR32, and Tet21N cell lines showed high levels of Fas mRNA, whereas low Fas mRNA levels were observed for the LAI-5S and SK-N-BE(2) cell lines. No correlation was observed between *MYCN* amplification state and Fas mRNA levels.

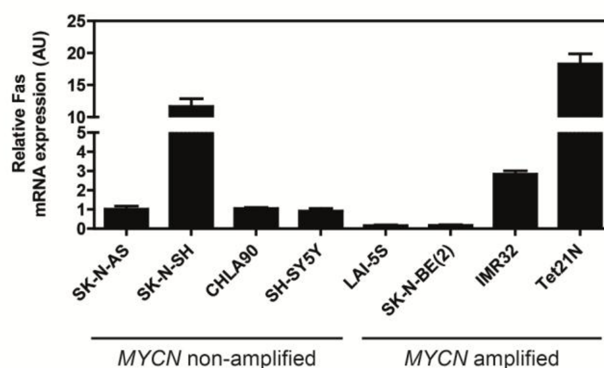


Figure 4.19: NB cell lines show heterogeneous Fas mRNA expression levels. Fas mRNA levels in NB cell lines were characterized by qRT-PCR. mRNA levels were normalized using 18S mRNA.

These observations were consistent with data obtained from Western blot experiments. Again, SK-N-SH, IMR32 and Tet21N cells showed high expression of Fas protein, whereas Fas could not be detected in LAI-5S and SK-N-BE(2) cells (Figure 4.20). Stimulation of the NB cell lines with TNFα increased the expression of Fas mRNA and protein levels in 4 (SK-N-AS, SK-N-SH, CHLA90, and Tet21N) out of the 8 NB cell lines used for the characterization (Figure 4.20 and 4.21). For the other 4 NB cell lines (SH-SY5Y, LAI-5S, SK-N-BE(2), and IMR32) no observable changes in Fas protein and mRNA levels could be detected. *MYCN* amplification has been proposed to correlate with caspase-8 expression. However, we were not able to detect a correlation since 75% of the *MYCN* amplified and 75% of the *MYCN* non-amplified NB cell lines displayed caspase-8 expression. Nevertheless, we determined that TNFα only induced Fas expression in NB cell lines that express both Fas and caspase-8, however with no correlation to *MYCN* amplification state.

We continued to analyze the TNFα-induced Fas expression by cytometry, thereby characterizing the cell surface expression of Fas and it modulation by TNFα. In the cell lines where we could detect Fas expression by qRT-PCR and Western blot (Figure 4.20 and 4.21), we were also able to detect cell surface Fas levels by flow cytometry (Figure 4.22). Low or no cell surface Fas levels were observed for the LAI-5S and SK-N-BE(2) cell lines. Moreover, we confirmed that the TNFα-induced Fas expression in the SK-N-AS, SK-N-SH, CHLA90, and Tet21N cell lines increased their cell surface Fas exposure. No observable changes were detected for the 4 TNFα-unresponsive cell lines.

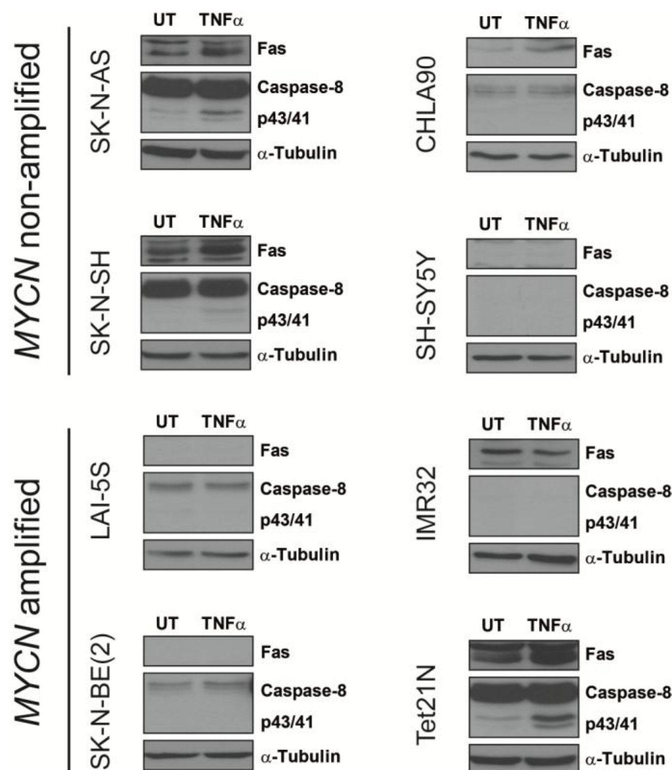


Figure 4.20: NBs show heterogeneous Fas expression in response to TNFα treatment. NB cells were left untreated (UT) or were treated for 24h with 100ng/ml. Protein expression levels were analyzed by Western blot.

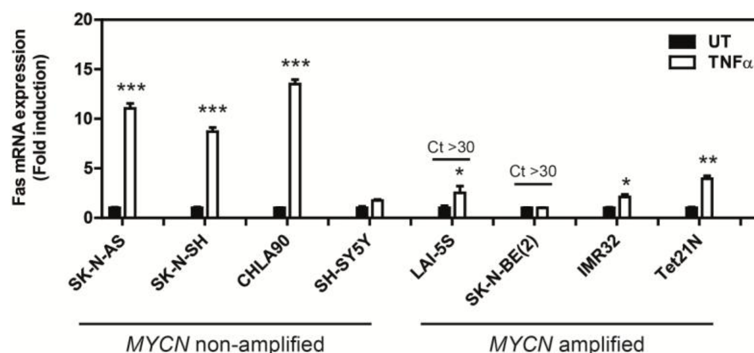


Figure 4.21: TNFα is able to induce Fas mRNA upregulation in NBs. For the indicated NB cell lines, modulation of Fas mRNA levels induced by 4h treatment with 100ng/ml TNFα was characterized by qRT-PCR. mRNA levels were normalized using 18S mRNA. *$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$ for comparison between untreated (UT) and TNFα. **Ct values higher than 30 are considered unreliable.**

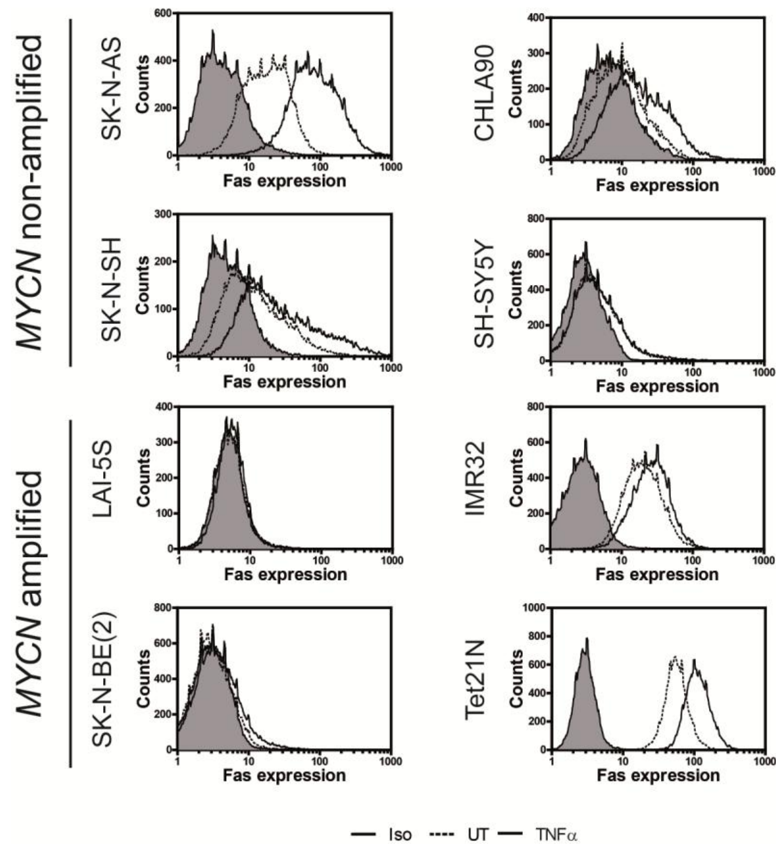


Figure 4.22: TNFα is able to increase cell surface Fas exposure in NBs. NB cell lines were left untreated (UT) or were treated for 24h with 100ng/ml TNFα. Cell surface Fas levels were analyzed by cytometry using a PE-conjugated Fas or isotype antibody.

4.7 NB cell lines show functional NF-κB activation

The failure to induce Fas in the TNFα-unresponsive cell lines could be explained by lack of NF-κB activation or functionality. To ascertain whether TNFα is able to activate the TNFR1 pathways we assessed the expression of TNFR1 by qRT-PCR. TNFR1 mRNA levels were detected in all analyzed NB cell lines (Figure 4.23), and in addition these cell lines showed TNFα-induced IκBα degradation (Figure 4.24). Hereby, we confirmed that TNFR1 is present and that TNFα-induced TNFR1 activation mediates NF-κB activation in all NB cell lines analyzed during the characterization.

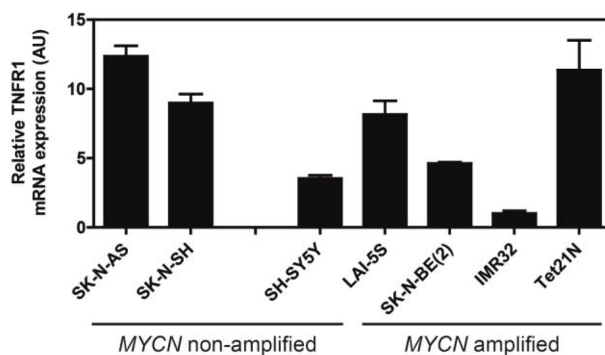


Figure 4.23: TNFR1 mRNA is detected in all NB cell lines. TNFR1 mRNA levels from the indicated NB cell lines were characterized by qRT-PCR. mRNA levels were normalized using 18S mRNA.

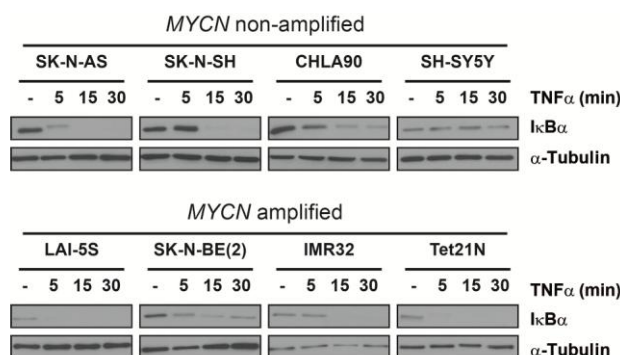


Figure 4.24: NF-κB is activated in all NB cell lines. The indicated NB cells were left untreated (−) or were treated with 100ng/ml TNFα for the indicated times. IκBα degradation was assessed by Western blot.

We next determined whether TNFα-induced activated NF-κB is functional in these NB cell lines. Therefore, we assessed the TNFα-induced expression of known NF-κB target genes, *cIAP2* [485] and *BCL-2* [482], by qRT-PCR and Western blot. Analysis of the cIAP2 and BCL-2 mRNA levels indicated that TNFα-induced NF-κB-mediated transcription (Figure 4.25AB). We confirmed induction of cIAP2 mRNA in all the NB cell lines, whereas BCL-2 induction was only observed in some. Moreover, induction of mRNA induced the upregulation of cIAP2 and BCL-2 protein levels. Hereby, we were able to confirm TNFα-induced NF-κB activation in all the analyzed NB cell lines and in addition confirmed that the activated NF-κB is functional.

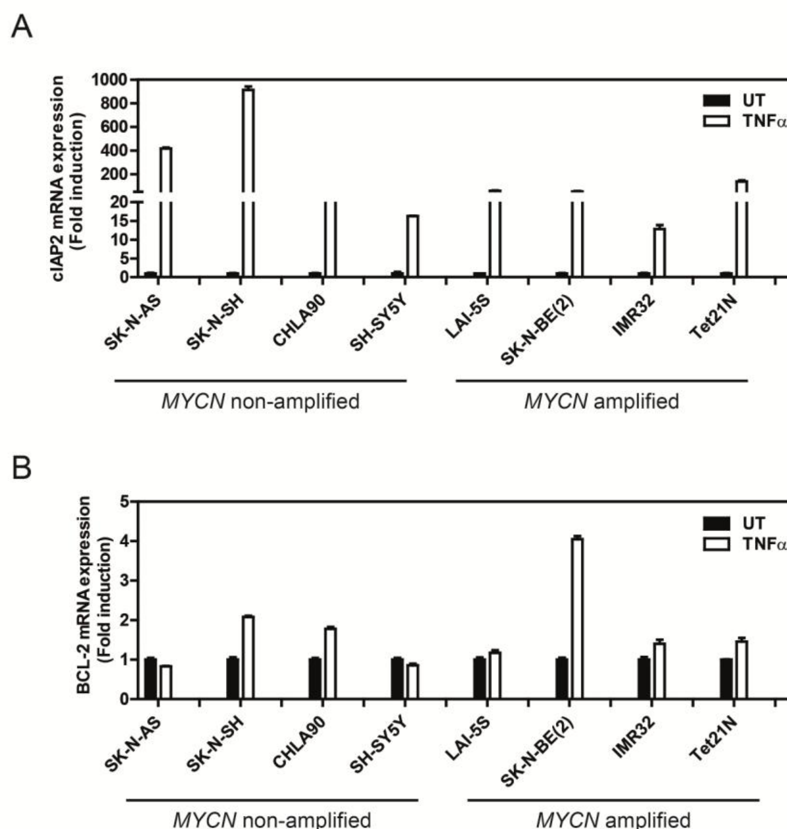


Figure 4.25: TNFα induces cIAP2 mRNA upregulation in all and BCL-2 mRNA upregulation in some analyzed NBs. The indicated NB cell lines were left untreated (UT) or were treated for 4h with 100ng/ml TNFα. cIAP2 mRNA levels were analyzed by qRT-PCR and normalized using 18S mRNA. **(Representative graphs)**

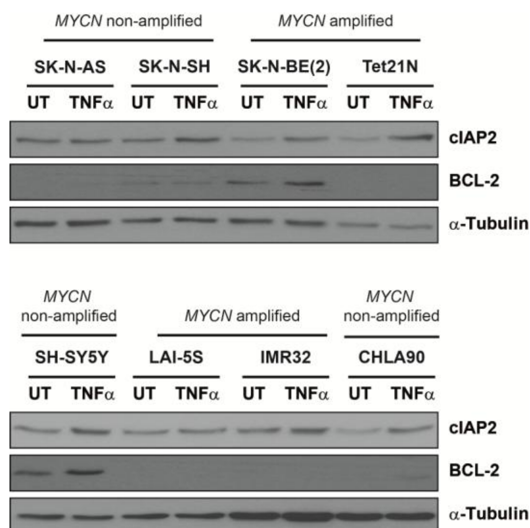


Figure 4.26: TNFα treatment of NBs induces expression of NF-κB target genes. NB cell lines were left untreated (UT) or were treated for 24h with 100ng/ml TNFα. Expression of the NF-κB-target genes *cIAP2* and *BCL-2* was analyzed by Western blot.

4.8 Fas induction by TNFα explains the priming for cisplatin- and etoposide-induced apoptosis

We previously showed in the SK-N-AS cell line that TNFα-induced expression of Fas primes for FasL-induced apoptosis. After confirming the TNFα-induced expression of Fas in the other NB cell lines, we were interested in confirming TNFα-induced priming for FasL-induced apoptosis in these cell lines as well. While performing a Hoechst staining assay, a significant increase in FasL-induced apoptosis was observed in all NB cell lines that were previously confirmed to display TNFα-induced Fas expression (Figure 4.27). Moreover, the increase in apoptosis was consistent with an increase in effector caspase activity, as assessed by a caspase activity assay using a specific caspase-3 and -7 fluorophoric substrate (Figure 4.28). Cell lines that were previously shown to lack TNFα-induced Fas expression could not be sensitized to FasL-induced apoptosis and neither displayed an increase in effector caspase activation.

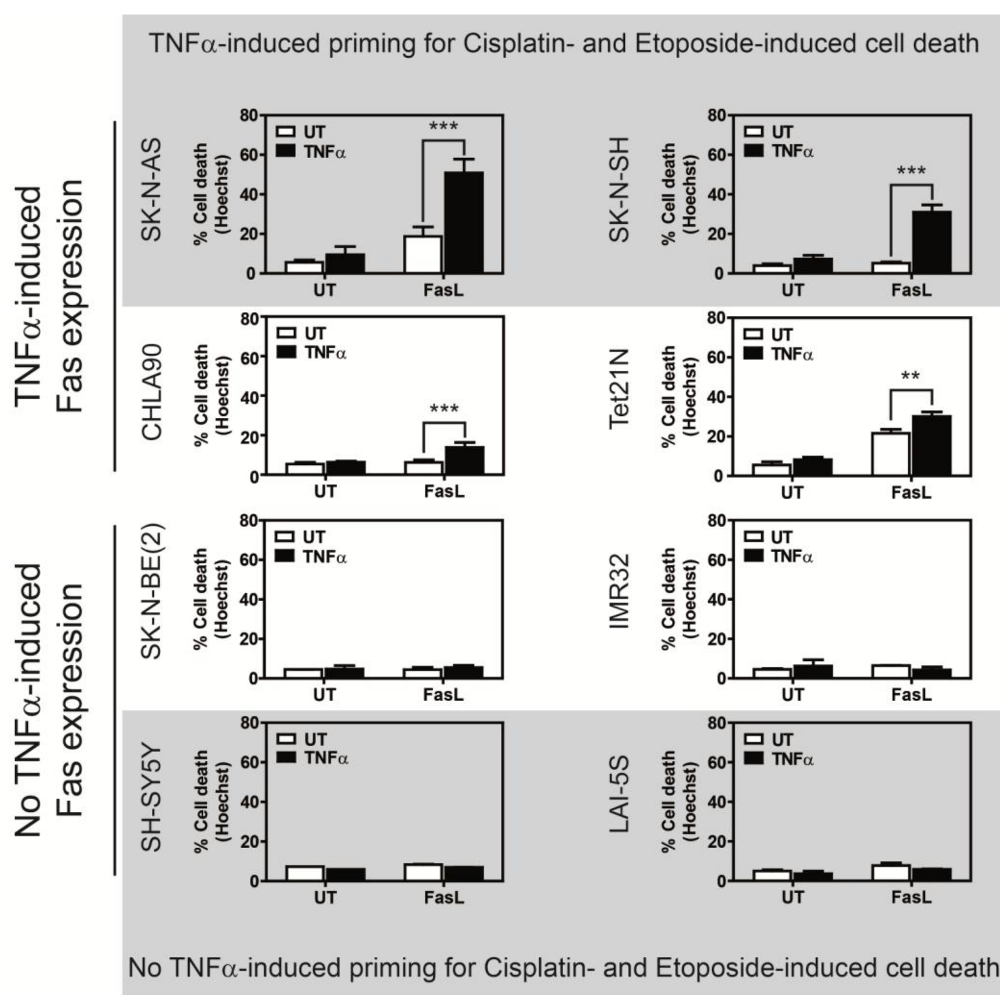


Figure 4.27: TNFα is able to sensitize NBs for FasL-induced cell death. The indicated NB cell lines were pre-treated or not (UT) for 24h with 100ng/ml TNFα. Thereafter, the cell were left untreated (UT) or treated for another 24h with 100ng/ml Fc:hFasL, or 1ng/ml Fc:hFasL for Tet21N cells. Cell death was assessed by Hoechst staining **p ≤ 0.01; ***p ≤ 0.001.

We concluded that cell lines that showed caspase-8 and Fas expression display FasL-induced apoptosis and effector caspase activation, whereas cell lines that only express one of the proteins do not show FasL-induced activation of the DR-dependent extrinsic apoptotic pathway. Moreover, we observed a correlation between Fas and caspase-8 expression levels and the FasL-induced apoptotic response. CHLA90 cells, with low levels of

Fas and caspase-8 expression, exhibit little FasL-induced apoptosis and effector caspase activation. On the other hand, Tet21N cell, with high expression levels of Fas and caspase-8, displayed a high level of apoptosis and DEVDase activity. In these cells, FasL treatment with 100ng/ml induced near complete cell death (data not shown). Therefore, Tet21N cells were treated with lower levels of FasL (1ng/ml) for the TNFα-induced sensitization to FasL-induced apoptosis to become apparent. The cell lines SK-N-AS and SK-N-SH, with intermediate expression levels of Fas and caspase-8, showed intermediate levels of apoptosis and caspase-3/7 activation, when stimulated with FasL.

In conclusion, the cell lines (SK-N-AS and SK-N-SH) that were shown to be primed by TNFα for cisplatin- and etoposide-induced cell death were cell lines that belong to the group of NB cell lines that could be sensitized for FasL-induced apoptosis by TNFα pre-treatment. On the other hand, the SH-SY5Y and LAI-5S NB cell lines could not be primed by TNFα for FasL- nor cisplatin and etoposide-induced apoptosis. Moreover, *MYCN* amplification state did not explain the TNFα-induced priming for FasL-, cisplatin, or etoposide-induced apoptotic response. Hereby, we were able to conclude that cell lines where TNFα induced sensitization to FasL-induced apoptosis could also be primed for cisplatin- and etoposide-induced cell death.

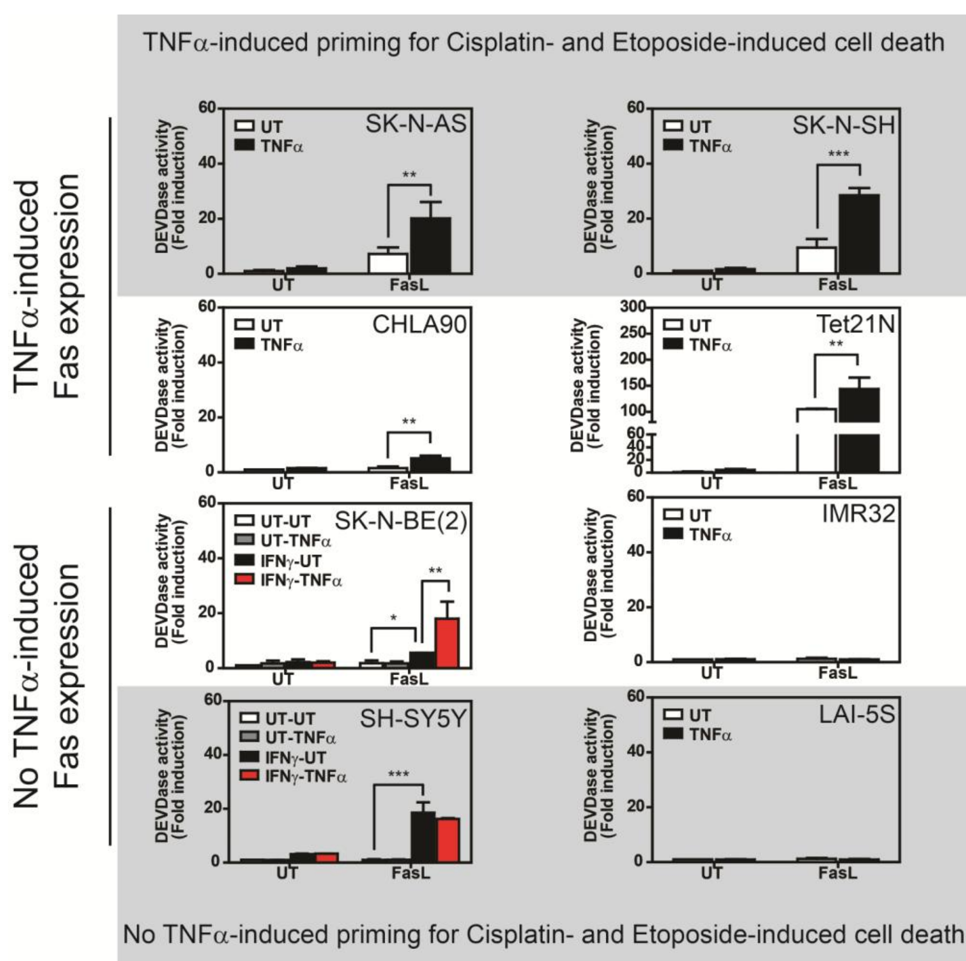


Figure 4.28: TNFα is able to prime NBs for FasL-induced effector caspase activation. NB cell lines were pre-treated or not (UT) for 24h with 100ng/ml TNFα and subsequently were left untreated (UT) or treated for 4h with 100ng/ml Fc:hFasL, or 1ng/ml Fc:hFasL for Tet21N cells. In the case of the SH-SY5Y and SK-N-BE(2) cells, before TNFα treatment the cells were treated for 24h with 100ng/ml interferon-γ (IFNγ) or not (UT). DEVDase activity was assessed using 10µM of the caspase-3/7 specific substrate, Ac-DEVD-Afc. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.9 Interferon-γ primes NB for FasL-induced cell death by inducing caspase-8 reconstitution and Fas expression

Interferon-γ (IFNγ) is known to increase Fas expression levels and is able reconstitute caspase-8 expression in NB cells, thereby restoring sensitivity for FasL-induced cell death [379, 486, 487]. Taking into account these previous studies, we assessed whether IFNγ could render NB cell lines sensitive for TNFα-induced Fas expression. Therefore, we analyzed two NB cell lines (i.e. SH-SY5Y and SK-N-BE(2)) that were previously shown to exhibit a lack of caspase-8 or Fas expression and therefore did not display FasL-induced apoptosis and TNFα-induced expression of Fas (Figure 4.20-22, 4.27 and 4.28). First, we confirmed that IFNγ induces caspase-8 and Fas expression in these cell lines (Figure 4.29B). Next, we targeted these cells with TNFα, and observed that in the SK-N-BE(2) cell line TNFα was able to induce Fas expression after IFNγ pre-treatment (Figure 4.29AB). On the other hand, subsequent TNFα treatment after targeting with IFNγ did not modulate Fas expression levels in SH-SY5Y cells. Surprisingly, IFNγ pre-treatment of SK-N-BE(2) cells also primed for TNFα-induced expression of caspase-8 (Figure 4.29B).

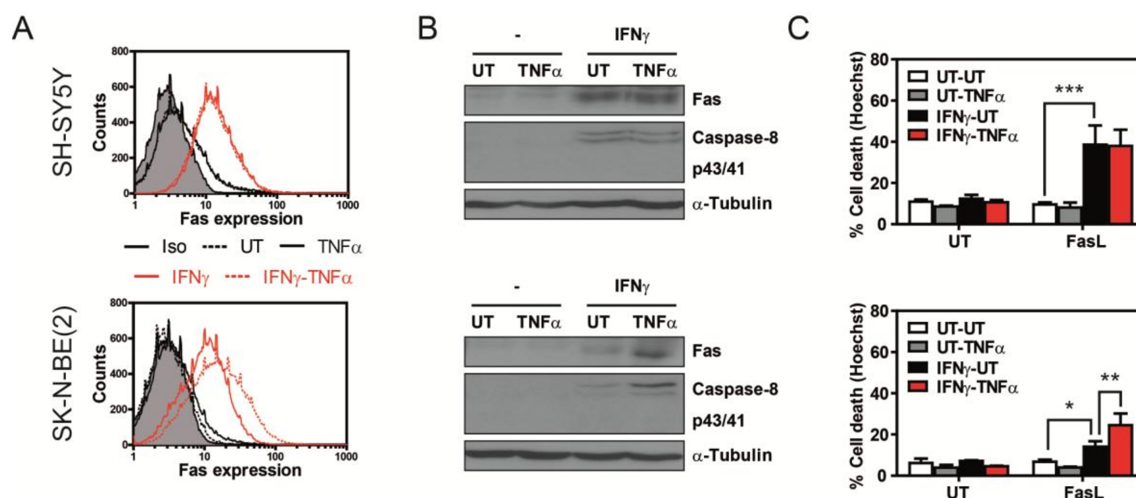


Figure 4.29: IFNγ renders caspase-8- and Fas-deficient NBs sensitive to FasL. A. SH-SY5Y and SK-N-BE(2) cells were treated with 100ng/ml interferon-γ (IFNγ) or were left untreated (UT) for 24h. Thereafter, cell were treated with TNFα or left untreated for another 24h with 100ng/ml TNFα and cell surface Fas expression was analyzed by cytometry using a PE-conjugated Fas or isotype (Iso) antibody. **B**. Cells were treated as in **(A)** and Fas and caspase-8 expression levels were analyzed by Western blot. **C**. The indicated cell lines were treated for 24h with 100ng/ml interferon-γ (IFNγ) or were left untreated (UT). Next, cells were treated for 24h with 100ng/ml TNFα or were left untreated. Cell death was assessed by Hoechst staining after 24h of 100ng/ml Fc:hFasL treatment. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

After confirming the reconstitution of casapase-8 and the expression of Fas and modulation thereof by TNFα, we assessed whether these cells were sensitized to FasL-induced apoptosis. IFNγ treatment restored sensitivity to FasL-induced apoptosis in both cell lines (Figure 4.29). Subsequent TNFα treatment only primed the SK-N-BE(2) cells for FasL-induced apoptosis, which is consistent with the previous observation that TNFα only induced Fas expression in this cell line. In addtion, for both cell lines, FasL-induced apoptosis concurred with effector caspase activation (Figure 4.28). Altogether, these data indicate that the expression of Fas in NB is regulated at various levels and that, depending on the cell line, Fas expression can be induced by IFNγ or TNFα with or without the need for IFNγ pre-treatment.

4.10 TNFα primes NB cells for NK cell-mediated cytotoxicity

Cytotoxic T Lymphocytes (CTLs) and Natural Killer (NK) cells are known to engage the Fas/FasL apoptotic pathway for the induction of cancer cell death [196-203], therefore we assessed whether TNFα-induced priming of SK-N-AS cells would sensitize for NK cell-induced cytotoxicity. NK cytotoxicity was determined by cytometry separating the SK-N-AS cell from the NKL cells. First we confirmed that FasL-induced cell death and TNFα-induced priming was detected by cytometry (Figure 4.30). Next, co-culture of SK-N-AS cells with NK cells was shown to induce a ratio-dependent increase in SK-N-AS cell death. In addition, TNFα was able to prime for NK-induced cytotoxicity. To address whether TNFα-induced priming for NK-mediated cytotoxicity was mediated by TNFα-induced Fas expression, we repeated the experiment while this time sequestering FasL by incubating with soluble Fas receptor (Fas-Fc). We concluded that TNFα primes for NK-induced cytotoxicity, however we were not able to determine that the increase in cytotoxicity was due to TNFα-induced Fas expression, since incubation with Fas-Fc did not lower NK cytotoxicity (Figure 4.31).

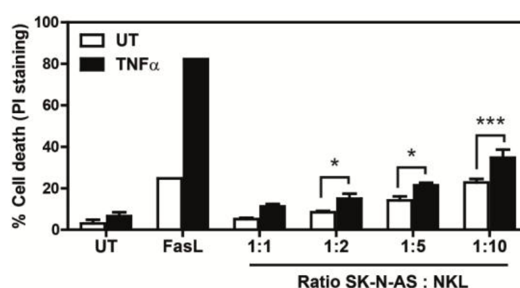


Figure 4.30: TNFα sensitizes for NK-mediated cancer cell killing. pGIPZ infected SK-N-AS cells were treated for 24h with 100ng/ml TNFα. Next, cells were co-cultured with the indicated ratios of NKL cells for 24h. Cell death of SK-N-AS cells was assessed by PI staining and gating of GFP positive cells. As a positive control, SK-N-AS cells were treated for 24h with 100ng/ml Fc:hFasL. *$p \leq 0.05$; ***$p \leq 0.001$.

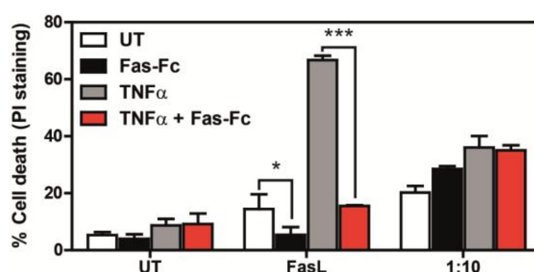


Figure 4.31: FasL sequestering does not modulate NK-induced cytotoxicity. pGIPZ infected SK-N-AS cell were left untreated or were treated for 24h with 100ng/ml TNFα. Thereafter, cell were left untreated, treated with 100ng/ml SuperFasL, or co-cultured in a 1:10 ratio with NKL cell with or without hFas-Fc (conditioned medium 2x diluted). Cell death was assessed as in figure 4.30.

Discussion

"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein
German-born theoretical physicist (1879- 1955)

5. Discussion

Neuroblastoma (NB) patients that belong to the high-risk group continue to have a poor prognosis, with 5-year survival rates below 50%. Therefore, there is an urgent need for the development of new treatment strategies or the discovery of agents that can enhance the efficacy of currently used treatments. In this thesis, we provide evidence for the targeting of the TNFR1 receptor as such a treatment that can increase the efficacy of chemotherapeutics currently used in the clinic, i.e. cisplatin and etoposide. Our findings describe that TNFα-induced TNFR1 activation is able to increase NB susceptibility to FasL-, cisplatin- and etoposide-induced cell death. Priming for these cell death-inducers is achieved through NF-κB-mediated upregulation of Fas, a therapeutic target that has received little attention for NB therapies. TNFα-induced Fas expression increases cell surface Fas exposure and enhances FasL-induced DISC formation, thereby activating the extrinsic apoptotic pathway.

Table 5.1: Neuroblastoma characteristics and their modulation by TNFα

		MYCN non-amplified [488, 489]				*MYCN* amplified [488, 489]			
		SK-N-AS	**SK-N-SH**	**CHLA90**	**SH-SY5Y**	**LAI-5S**	**SK-N-BE(2)**	**IMR32**	**Tet21N**
Expression	**Fas**	+	+	+/−	+/−	-	-	+	++
	Caspase-8	++	++	+/−	-	+/−	+/−	-	++
	p53	N [490]	F [491]	N [492]	F [491]	N [493]	N [492]	F [491]	F [491]
TNFα-induced	**Fas**	++	+	+	-	-	-	-	++
	Sensitization to etoposide/cisplatin	++	+	NA	-	-	NA	NA	NA
FasL-induced cell death	**UT**	+	+/−	-	-	-	-	-	++
	TNFα	+++	+++	+	-	-	-	-	+++

Abbreviations: F Functional, ***N*** Non-functional, ***NA*** Not available.

5.1 The heterogeneity of Fas expression in NB

5.1.1 Sensitivity of NB to FasL-induced cell death

As shown by our study, NBs are insensitive to FasL-induced cell death. Out of the 8 NB cell lines used for our study, 4 cell lines did not display FasL induced cell death before or after pre-treatment with TNFα (Table 5.1). Various mechanisms have been described which may explain the desensitization for FasL-induced cell death, from which caspase-8 silencing is the most characterized.

Caspase-8 has been described to be down-regulated in 50-70% of all NB and to desensitize for DR-induced cell death [372-374, 439-441]. This down-regulation is frequently mediated by *CASP8* promoter methylation at CpG islands [373, 439, 448, 494, 495]. Although various studies found a correlation between caspase-8 silencing and *MYCN* amplification [373, 374, 448], the link between *MYCN* amplification and caspase-8 silecning remains debated since other studies were unable to encounter a correlation between caspase-8 expression and *MYCN* amplification status [440, 494, 496]. Our data confirms the previous observations that caspase-8 is silenced in some NBs, since caspase-8 expression could not be detected in 2 out of the 8 NB cell lines used in this study, and another 3 cell lines showed low caspase-8 expression levels (Table 5.1). However, we were not able to find a correlation between *MYCN* amplification and caspase-8 expression. Nevertheless, the capase-8 silencing in the 2 NB cell lines probably mediated the unsusceptibility to FasL-induced cell death, whereas for the other 2 unsusceptible cell lines the lack of Fas expression was the probable factor. However, since we did not assess caspase-10 expression in the cell lines that lack caspae-8 expression, other mechanisms that inhibit FasL-induced cell death could be at play.

Variation in the expression of anti-apoptotic proteins could function as a mechanism for DR desensitization. Anti-apoptotic BCL-2 family members, such as BCL-2, BCL-X_L, and MCL1, are frequently found at high levels in NB samples from patients and in cell lines and correlate with poor prognosis [497-506]. Owing to their localization at the mitochondria, anti-apoptotic BCL-2 family members inhibit intrinsically- and type II extrinsically-induced apoptosis [54]. Overexpression of anti-apoptotic BCL-2 family members leads to inhibition of DR-induced cell death in type II apoptotic cells, as we were able to show for BCL-X_L overexpressing SK-N-AS cells. However, we did not characterize which type of apoptosis is displayed by the other NB cell lines used for this study. Nevertheless, NBs have been described to display type II apoptosis [507], which might explain de desensitization for FasL-induced cell death for some of the FasL-unresponsive NB cell lines used in this study.

Other anti-apoptotic proteins that have been described to frequently be expressed in NB are the DR antagonist c-$FLIP_L$ [507] and the IAP Survivin [461]. Downregulation of c-$FLIP_L$ was shown to sensitize NB for Fas-mediated apoptosis [507, 508], whereas survivin was correlated with poor prognosis and inhibited DR-induced apoptosis [461, 509].

Altogether various mechanisms for desensitization of Fas-mediated apoptosis have been described for NB. Although characterization of the anti-apoptotic protein expression is missing in our study, our data indicates that caspase-8 and Fas silencing are the most probable factors that are responsible for the unsusceptibility of NBs to FasL-induced apoptosis.

5.1.2 TNFα-induced expression of Fas

Early studies by Chan *et al.* identified the NF-κB response elements in the *FAS* promoter [510]. NF-κB activation by PMA/Ionomycin stimulation induced Fas expression in Jurkat cells which could be blocked by inhibiting IκBα degradation or NF-κB translocation. Further analysis of the *FAS* promoter confirmed these NF-κB response elements and described TNFα-induced NF-κB binding to the *FAS* promoter [511, 512]. The TNFα-induced NF-κB-mediated upregulation of Fas expression described in this thesis has been previously confirmed by various studies, using different cell type models [462, 511-520]. Likewise to our cellular model, many of these studies indicated that TNFα-induced upregulation of Fas expression could sensitize for Fas-mediated cell death. Nevertheless, to date no study has described this mechanism of TNFα-induced Fas expression and sensitization to FasL-induced cell death for NBs.

Various MAPKs (i.e. ERK1/2 [521], PI3K [522, 523], and JNK/c-Jun [523-525]), which are known to be activated by TNFR1, have been reported to regulate Fas expression. ERK1/2 and PI3K were shown to positively regulate Fas expression, whereas JNK/c-Jun has been shown to down-regulate Fas expression. In our study, inhibition of these MAPKs did not modulate basal Fas expression, nor did it modulate the TNFα-induced expression of Fas. Thereby, our data rules out the possibility for MAPK-mediated regulation of Fas expression and indicates that NF-κB is the sole inducer of Fas expression in response to TNFα. These conclusions are strengthened by the full abrogation of TNFα-induced Fas mRNA and protein up-regulation when NF-κB or *FAS* gene transcription is inhibited. Our findings show that inhibition of translation, through treatment with cycloheximide, only inhibits TNFα-induced expression of Fas protein and does not modulate Fas mRNA levels. Thereby, we are able to conclude that NF-κB directly induces *FAS* gene transcription, which concurs with previous studies [511, 512].

In NB, TNFα-induced expression of Fas primes for FasL-induced cell death, despite the TNFα-induced modulation of other regulators of extrinsically-induced apoptosis. For SK-N-AS cells, TNFα treatment was shown to down-regulate the mRNA levels of the pro-apoptotic FADD, RIP1, and caspase-8 proteins and the anti-apoptotic $FAIM_L$, LFG, XIAP, DcR3 proteins. Down-regulation of these proteins was confirmed at the protein level for FADD and RIP1, however caspase-8 and XIAP protein levels were unaffected. Protein levels of the other indicated anti-apoptotic proteins were not assessed, but the decrease in their mRNA levels might potentiate the FasL-induced apoptosis. Interestingly, TNFα treatment induced c-FLIP mRNA and protein expression in the SK-N-AS cell line and cIAP2 and BCL-2 expression in various NB cell lines. These anti-apoptotic proteins are known to inhibit DR-mediated apoptosis. However, in our experimental setting no inhibition of

FasL-induced apoptosis was observed, presumably because the increase in Fas expression is able to overcome the inhibition mediated by these anti-apoptotic proteins.

Characterization of the cell lines used in this study showed that Fas is widely expressed in NB (75% of the cell lines show Fas expression), however TNFα-induced Fas expression was only observed for 50% of these cell lines (Table 5.1). As a side note, Fas expression was difficult to detect in the CHLA90 and SH-SY5Y cell lines. IFNγ stimulation induced Fas expression in cell lines were no TNFα-induced Fas expression was observed and, in one of the two cases, IFNγ was able to prime for TNFα-induced Fas expression. Although, the characterization of the pathways involved in IFNγ-induced Fas expression and sensitization for TNFα-induced Fas remain to be elucidated, these findings indicate that in NBs the expression of Fas is silenced at the basal and the induction level.

5.1.3 Methylation of the *FAS* promoter

Various epigenetic mechanisms have been described to silence gene expression at the basal level, such as DNA methylation and histone modification [334]. Histone deacetylation has been suggested to down-regulate Fas expression in cancer [526, 527], however these studies used Histone Deacetylases (HDAC) inhibitors and did not directly detect HDAC binding to the *FAS* promoter. Nevertheless, histone deacetylation has been identified as a mechanism for Fas suppression in colonic inflammation [528] and fibroproliferative disorders [529]. On the other hand, *FAS* promoter methylation has been described for various types of cancer, including NBs [371, 512, 530-534]. These studies indicate that *FAS* promoter methylation down-regulates Fas expression. A genome-wide promoter methylation analysis on 89 primary neuroblastoma tumors by Decock *et al.* described that *FAS* promoter methylation occurs in >50% of the NBs and correlates with *MYCN* amplification [530]. A functional analysis of the promoter methylation is lacking in this study and therefore no data were obtained on Fas expression levels. Our data does not indicate a correlation between Fas expression and *MYCN* amplification status in NB cell lines (Table 5.1), despite that the promoter methylation status in these cell lines remains to be clarified. Ettou *et al.* and Santourlidis *et al.* described specific *FAS* promoter hypermethylation at the NF-κB binding sites in human carcinomas [512, 532]. This silencing mechanism could explain that Fas is basally expressed but TNFα is unable to induce Fas expression in 50% of the NB cell lines used for our study. Nevertheless, other mechanisms that regulate basal Fas expression and Fas induction could be at play.

5.1.4 Regulation of NF-κB activity

Although we confirmed that NF-κB is activated and is functional upon TNFα treatment in all the NB cell lines used for this study, we did not assess specific NF-κB dimer formation and post-translational modifications. TNFα induced the degradation of IκBα in all the NBs used for this study and the induction of Fas in SK-N-AS cells could be blocked by overexpression of a mutated form of this protein (i.e SR- IκBα). Therefore, we supposed that TNFR1 mediated the activation of the p50/p65 NF-κB heterodimer and that this NF-κB dimer was responsible for the induction of gene transcription of the NF-κB target genes (i.e. *FAS*, *cIAP2*, *BCL-2*, *and c-FLIP*). Nevertheless, IκBα has been described to bind and inhibit c-Rel/c-Rel [535-537], p50/p50 [535, 538], and p65/p65 NF-κB homodimers [539-541]. Whether TNFα induces activation of the hetero- or homo-dimers could depend on the NB's phenotype and might drive context-specific gene expression.

Differential outcome due to p65 mutations are rarely reported for solid tumors [542]. However, various post-translational modifications have been described to dictate NF-κB-induced gene expression [335]. TNFα-induced phosphorylation of p65 at S276 [543] and S536 [544, 545] has been shown to enhance the transcriptional activity of NF-κB, whereas TNFα-induced phosphorylation of S468 reduces its transcriptional activity through targeting p65 for ubiquitination and proteasomal degradation [546, 547]. In addition, Various lysines in p65 have been reported to be targeted for reversible acetylation by p300 and CREB Binding Protein (CBP) [335], which are acetyltransferases frequently observed to be mutated and over-expressed in cancer [548, 549]. Acetylation at K221 enhances NF-κB DNA binding, whereas K310 acetylation increases NF-κB transcriptional activity [550]. On the other hand, K122 and K123 acetylation inhibits NF-κB-mediated transcription by reducing p65 binding to enhancer elements [551]. Interestingly, TNFα is able to induce K314

and K315 acetylation and thereby suppresses TNFα-induced expression of specific sets of NF-κB target genes [552, 553].

Similar to phosphorylation and acetylation, NF-κB activity has been observed to be regulated by reversible p65 methylation [335]. Methylation of p65 at K310 does not affect DNA binding but renders p65 inactive [554]. However, this inhibition can be overcome by TNFα-induced phosphorylation of S311. TNFα-induced methylation of K314 and K315 has been shown to inhibit NF-κB function by inducing the ubiquitination and degradation of promoter-bound p65 [555], whereas K218 and K221 methylation increases the expression of NF-κB target genes by increasing its transcriptional activity [556]. On the other hand, K37 methylation stabilizes DNA-p65 complexes and has been shown to be an important mediator for the induction of specific NF-κB target genes [557, 558]. Lastly, ubiquitination of p65 mediates the termination of NF-κB signaling by inducing proteasomal degradation of promoter-bound p65 [559, 560]. The regulation of NF-κB activity becomes even more complex knowing that the post-translational modifications can be reversed by phosphatases, HDACs, and demethylases [335]. Altogether, the interplay between the vast amounts of p65 post-translational modifications are able to co-ordinate the outcome of TNFα-induced NF-κB activation, which might result in the differential Fas induction as observed for NBs. Analysis of these p65 post-translational modification in NB and their role in the TNFα-induced expression of Fas and other NF-κB target genes, such as *c-FLIP, cIAP2, and BCL-2*, would be an interesting study.

5.1.5 Transcriptional repression of *FAS*

Independently and dependently on the post-translational modification, NF-κB activation can be further regulated by transcriptional repressors and enhancers. Various repressor and enhancer elements have been identified in the *FAS* promoter and suppression of Fas expression has been described for different types of cancer [561, 562]. In continuation, Lasham *et al.* characterized transcription factors that bind to these element; Y Box Binding Protein-1 (YB-1), Purine-Rich Element-Binding Protein Alpha/Beta (Purα and Purβ), and two components of the AP-1 (Activator Protein 1) complex, c-Fos and c-Jun [524]. Expression of c-Jun induced potent expression in a *FAS* promoter reporter gene assay, which was abrogated by co-expression with c-Fos. In Jurkat cells, YB-1 and Purα reduced Fas expression, whereas Purβ was able to overturn this repression.

In human melanoma cells, ASK1/MKK6 (Apoptosis Signal-Regulating Kinase 1/MAP Kinase Kinase 6)-induced activation of p38 was shown to suppress NF-κB-induced transcription of the *FAS* gene by inhibiting IκBα phosphorylation, which could be overturned by treating with the p38 inhibitor SB203580 [563]. Later, the same group described the STAT3/c-Jun mediated repression of basal Fas expression and its potentiation by inhibition of the PI3K/AKT pathway [523, 525]. Consistently, STAT3 down-regulation in a breast cancer cell line induced an increase in Fas expression [564]. Recently, unphosphorylated STAT1 was identified as a negative regulator of Fas expression in sarcoma [565]. Unphosphorylated STAT1 was shown to function directly as a transcriptional repressor but in addition is known as a transcriptional activator. Moreover, *STAT3* has been identified as a STAT1 target gene, which could drive further suppression of Fas expression [566]. Oncogenic RAS isoforms have been shown to downregulate the expression of Fas in mouse mammary epithelial and transformed NIH3T3 cells though promoter methylation [567, 568]. However, these isoforms are also known to induces c-Fos and c-Jun phosphorylation [569, 570], which might add another level to the regulation of Fas expression according to studies mentioned above.

The transcriptional repressor Yin And Yang 1 (YY1) has been described by various studies to suppress Fas expression. The group of Dr. Benjamin Bonavide documented the YY1 binding to a putative cluster at the silencer region of the *FAS* promoter [571]. Inhibition or silencing of YY1 induced Fas expression in B non-Hodgkin's lymphoma and sensitized to Fas-mediated cell death [572]. The role of YY1 in repression of Fas expression was later confirmed in a prostate cancer model [573].

The *FAS* gene is a transcriptional target of wild-type p53, which induces Fas expression during p53-mediated apoptosis [574-576]. Moreover, DNA-damaging drug-induced cell death has been shown to induce Fas

expression through p53 activation [577-579]. However, the functions of p53 are frequently inactivated in human cancers through p53 mutation/truncation [580, 581]. Mutation of p53 down-regulates apoptosis-mediating activity, but has also been shown to induce gain of function. In the case of Fas expression, mutant p53 has been described to repress the transcriptional activity of the *FAS* promoter [582]. Moreover, p53 mutation/truncation correlates with reduced Fas mRNA and Fas protein expression in nonsmall cell lung carcinoma [583] and hepatocellular carcinoma [584], respectively. These studies document the role of the tumor suppressor p53 in the induction of Fas expression and could explain the low expression levels and lack of NF-κB-mediated Fas expression observed for some of the NBs used in our study. However, our findings do not indicate a correlation between p53 functionality status and Fas expression or its regulation through TNFα treatment (Table 5.1). Nevertheless, p53 mutation might induce Fas silencing for some of the NB cell lines used in this study. Reconstitution of wild-type p53 or p53 knockdown in the NB cell lines used for our study should give us a better insight in the role of p53-mediated regulation of Fas expression.

Like p53, MYCN has been described to contain dual functionality as a transcriptional repressor and activator [585]. MYCN is able to suppress the expression of various proteins in NB, amongst which are the pro-apoptotic DR NGFR1 [586], and the anti-apoptotic DR antagonist LFG [177]. On the other hand, MYCN overexpression sensitizes for death ligand-induced apoptosis through inducing the expression of DR5 and BID [587]. MYCN-mediated regulation of Fas expression has not been previously documented. Our data does not indicate a correlation between *MYCN* amplification status and sensitivity to FasL-induced apoptosis (Table 5.1). Neither were we able to observe a correlation between *MYCN* amplification status and Fas expression or TNFα-mediated Fas induction.

5.2 The role of Fas in cancer therapy

5.2.1 Fas and chemotherapy-induced toxicity

The participation of the Fas/FasL pathway has been previously described to contribute to cell death induced by DNA-damaging agents such as cisplatin and etoposide [458, 484, 588-591]. Therefore, we studied the possibility to improve the efficacy of these drugs by inducing Fas expression through TNFα treatment. Our study confirms the previous findings by determining FasL induction and caspase-8 activation. In addition, TNFα-induced Fas expression correlated with sensitization for cisplatin- and etoposide-induced cell death (Table 5.1).

Various studies have shown that cisplatin- and etoposide-induced cell death can be inhibited by blocking the Fas/FasL pathway through treatment with antagonistic Fas and FasL-sequestering antibodies [458, 484, 588-591]. Moreover, Fas overexpression has been shown to sensitize to cisplatin-induced cell death [592, 593]. In our study, it remains to be determined whether cisplatin- or etoposide-mediated FasL induction is responsible for the observed caspase-8 activation. Nevertheless, FasL induction has been shown to be obsolete for Fas activation on some occasions. Micheau *et al.* described the cisplatin-induced activation of a Fas apoptotic pathway that is FasL-independent and mediated by FADD [594]. In their model, treatment with an antagonistic Fas antibody did not rescue from cisplatin-induced cell death. They discovered that cisplatin (and doxorubicin) treatment induced Fas-FADD binding and FADD overexpression sensitized for cisplatin-induced cell death. Similar results were obtained by Shimada *et al*. who showed that etoposide induced cell death which could not be inhibited by an antagonistic Fas antibody [595]. However, treatment with etoposide induced Fas-FADD binding and FADD-DN expression inhibited etoposide-induced cell death in prostate cancer cells. FADD-mediated induction of cell death has been confirmed for the cisplatin, etoposide, and vinblastin treatment [594]. Moreover, treatment with these agents induced Fas clustering on the cytoplasmic membrane. The cisplatin-induced recruitment of FADD to Fas has also been documented by Spierings *et al.* [589]. However, here antibody-mediated FasL sequestering inhibited cisplatin-induced cell death.

The role of Fas clustering in response to cisplatin treatment was further characterized by Lacour *et al.* [596]. In the human colon cancer cell line HT29, cisplatin treatment induced Fas clustering in lipid rafts on the cytoplasmic membrane. These lipid rafts were also positive for FADD and capsase-8. Although they observed cisplatin-induced sensitization to Fas-mediated apoptosis, the role of Fas in cisplatin-induced cell death was not assessed. Membrane-stabilizing agents that inhibit lipid raft formation have been shown to inhibit cisplatin-induced apoptosis without affecting cisplatin uptake [597]. In addition, these agents inhibited Fas aggregation. Later, the same group documented the cisplatin-induced redistribution of Fas, FADD, and procaspase-8 into membrane lipid rafts and showed that silencing of Fas inhibited cisplatin-induced cell death [598]. In addition, the lipid raft component sphingomyelin was described to be essential for cisplatin-induced cell death, caspase-8 activation, and sensitization for Fas-mediated cell death [599].

Although these studies suggest a role for Fas-sequestering in lipid rafts during cisplatin- and etoposide-induced cell death, no direct evidence is presented that indicates that lipid rafts mediate activation of the Fas apoptotic pathway. Nevertheless, data from other studies performed by the group of Dr. Faustino Mollinedo, using the chemotherapeutic edelfosine, indicate that lipid raft formation is able to induce Fas activation. The group of Dr. Faustino Mollinedo has extensively studied the mechanism of action of edelfosine-induced cell death [214, 600-602]. In these studies, sensitivity to edelfosine-induced cell death correlated with cell surface Fas expression and cell lines deficient for Fas expression could be sensitized to edelfosine-induced cell death through overexpression of Fas [214, 600, 602]. Treatment with an antagonistic Fas antibody did not rescue from edelfosine-induced cell death, indicating that edelfosine-induced cell death is FasL-independent [600, 602]. On the other hand, Fas silencing or expression of FADD-DN was sufficient to inhibit edelfosine-induced cell death [603]. Moreover, expression of a truncated form of Fas that lacks the death domain did not reconstitute sensitivity to edelfosine in Fas negative cell lines [602]. These data indicate that edelfosine

induces cell death through activation of the Fas apoptotic pathway. The role of lipid rafts in edelfosine-induced cell death were elucidated by treating with the raft disrupting agent methyl-β-cyclodextrin [601]. Treatment with this agent inhibited edelfosine-induced Fas clustering and cell death.

Altogether these data indicate that cisplatin and etoposide are able to induce cell death through the Fas apoptotic pathway. Whether this pathway is activated trough FasL binding or Fas clustering in lipid rafts remains to be determined and might be stimulus- or cell type-specific.

Additionally, there remains the possibility that the cisplatin- and etoposide-induced activation of caspase-8 is independent of Fas. In 1998, etoposide treatment of thymocytes from lpr/lpr mice, which carry the lpr mutation in the *FAS* gene that renders Fas functionally deficient, was reported to induce caspase-8 cleavage, thus in a Fas-functionally deficient manner [604]. Later, these results were confirmed in a Fas deficient Jurkat cell line and it was shown that the etoposide-induced caspase-8 activation was FADD-independent [605]. Moreover, etoposide treatment induced caspase-3 activation in caspase-8 deficient Jurkat cells. Incubation of Jurkat extracts with recombinant caspase-3 induced caspase-8 cleavage, and caspase-3 was therefore proposed as the mediator of etoposide-induced caspase-8 activation. Like etoposide-induced cell death, cisplatin has been reported to induce caspase-8 activation in a FADD-independent manner [606]. Both etoposide- and cisplatin-induced cell death and caspase-8 activation could be blocked by overexpression of anti-apoptotic BCL-2 family members, implicating the mitochondria in drug-induced caspase-8 activation [605, 606]. It was shown that caspase-9 inhibition reduced etoposide-induced caspase-8 activity, whereas cisplatin-induced caspase-8 activity was unaffected [606, 607]. Moreover, MEFs negative for the apoptosome component Apaf1 were resistant to etoposide-induced cell death and caspase-8 cleavage [608].

For both cisplatin and etoposide, inhibition of caspase-8 activity has been shown to be sufficient to reduce drug-induced cell death [605-607]. However, these findings are questioned by other studies, which indicate a more prominent role for caspase-9 and find caspase-8 inhibition insufficient to abrogate drug-induced cell death [608-611].

A new signaling platform, called the ripoptosome, has recently been described to induce apoptosis upon treatment with etoposide and possibly cisplatin [110-112]. This platform is able to assemble independently of mitochondrial and death receptor activation and relies on the RIP1-mediated recruitment of caspase-8 and FADD. Lack of c-FLIP$_L$ and cIAPs drives further caspase-8 activation and ripoptosome-induced apoptosis. Our data shows etoposide- and cisplatin-induced activation of caspase-8, which might have been induced by ripoptosome formation. Since a clear characterization of the mechanism that induces capsase-8 activation in our study is missing, we are unable to attribute the cisplatin- and etoposide-induced cell death to ripoptosome formation.

Altogether, cisplatin and etoposide are able to induce caspase-8 activation and cell death through various mechanisms. However, knowing that TNFα-induced sensitization to cisplatin and etoposide-induced cell death was only observed in NB cell lines that upregulate Fas expression upon TNFα treatment, we propose that TNFα-induced sensitization to cisplatin- and etoposide-induced cell death occurs due to upregulation of Fas. This leaves us to conclude that in these cell lines an upregulation of Fas increases cisplatin- and etoposide-induced Fas activation, resulting in enhanced caspase-8 activation and cell death. In addition, other cell death-inducing agents might benefit from TNFα-induced Fas expression since they are described to activate the Fas apoptotic pathway (Table 5.2). Whether in our model Fas was activated through FasL stimulation or Fas aggregation in lipid rafts, remains to be determined.

Table 5.2: Agents known to induce Fas-apoptotic pathway activation

Stimulus	References
DNA binding or DNA synthesis inhibiting agents	
Cisplatin	[458, 588-590, 592-594, 598]
Etoposide	[458, 591, 594, 595, 612]
Teniposide	[612]
Doxorubicin	[458, 459, 590, 594, 613, 614]
Methotrexate	[459]
Cytarabine	[459]
Camptothecin	[615]
Bleomycin	[577]
5-Fluorouracil	[616-618]
UV radiation	[612, 619-622]
γ-irradiation	[590, 623]
Microtubule damaging agents	
Vinblastin	[594]
Paclitaxel	[624, 625]
Lipid raft-inducing agents	
Edelfosine	[214, 600-603]
Perifosine	[591, 602]
Mitochondria modulating agents	
Resveratrol	[626, 627]
CD20 activating antibodies	
Rituximab	[628]
MDM2/p53 interaction inhibitors	
Nutlin-3	[629]
Other	
Curcumin	[630]
Avicin D	[631]
Plitidepsin	[632]
Ceramide	[633]

5.2.2 TNFα-induced sensitization to cisplatin and etoposide

Although we conclude that TNFα treatment primes for cisplatin- and etoposide-induced caspase-8 activation and cell death through NF-κB mediated upregulation of Fas, some studies have described other mechanism for TNFα-induced sensitization.

As of 1989, it was shown that *in vitro* treatment of a human stomach adenocarcinoma cell line with a combination of cisplatin and TNFα increased cell death compared to single treatment [634]. These results were later confirmed for various cellular models [635-639]. However, these studies only assessed synergic effects [635, 637-639] or described cisplatin-induced sensitization to TNFα-induced cell death [636]. Contrarily, Gordon *et al.* described the TNFα-induced resistance to cisplatin-induced cell death [640]. For most studies, a clear characterization of the mechanism that induces an increase in TNFα/cisplatin-induced cell death is missing. Nevertheless, Benedetti *et al.* described the mechanism for synergic TNFα/cisplatin-induced cell death in renal proximal tubular cells [638, 639]. Here, TNFα/cisplatin treatment induced cell death through inhibiting TNFα-induced NF-κB translocation to the nucleus and prolonging JNK activation. Data presented in this thesis does not provide evidence that confirms these studies, since our data indicates a role for NF-κB-induced Fas expression and increased caspase-8 activation.

Despite the lack of a clear characterization of the mechanism that increases cell death in TNFα/cisplatin treatment, some studies have assessed the use of TNFα/cisplatin in *in vivo* xenograft models. These studies show promising results for the use of TNFα/cisplatin combination therapy [641, 642], however unlike the *in vitro* models, these studies did not assess or did not detect direct effects of TNFα on cisplatin-induced cell death when treating *in vitro* tumor cell lines [642].

In 1987, combination treatment with TNFα and etoposide was shown to increase cytotoxicity *in vitro* and *in vivo* [643, 644]. After then, few studies were performed on TNFα/etoposide-induced cytotoxicity. Donaldson *et al.* confirmed the increased cell death-inducing potential of TNFα/etoposide combination treatment in a human renal cell carcinoma xenograft model [645], and Doyle *et al.* documented a TNFα/etoposide-induced increase in cell death using the H209 human lung cancer cell line [646]. Surprisingly, for other human lung cancer cell lines, TNFα was found to induce protection or had no effect compared to cell death induced by etoposide alone [646]. Like the data presented in this thesis, Schmelz *et al.* showed that TNFα pre-treatment of various malignant cell lines sensitized for etoposide-induced cell death [647]. However, unlike our findings, etoposide did not induce caspase-8 cleavage and TNFα pre-treatment did not sensitize for etoposide-induced caspase-8 activation. Cell death was proposed to be induced by mitochondrial cytochrome c release, thereby driving capase-9 activation through apoptosome formation.

Recently, while describing etoposide-induced ripoptosome formation, Tenev *et al.* described an increase in cell death when treating with a combination of TNFα/etoposide compared to single treatments [111]. They observed an increase in RIP1-caspase-8 association after treatment with TNFα/etoposide and determined that the cell death was caspase-8 dependent. The increased cytotoxicity was proposed to occur due to etoposide-induced depletion of IAPs which sensitized for TNFα-induced cell death through activation of the TNFR1-apoptotic pathway. However, only TNFα/etoposide combination treatments were used in their study and no characterization of the activation of the TNFR1 pathway was described while treating with the TNFα/etoposide combination. This leaves doubt for whether the increase in cell death was induced by TNFR1-mediated apoptotic pathway activation or whether TNFα primed for etoposide-induced cell death through DR-mediated caspase-8 activation, as proposed by our study.

5.2.3 TNFα-induced sensitization to NK co-culture

Due to the role of Fas-mediated cell death in cytotoxic T lymphocyte (CTL) and Natural Killer (NK) cell-mediated cytotoxicity [196-203], we assessed the possibility of TNFα-induced sensitization to NK-mediated cancer cell death. Indeed, TNFα pre-treatment of SK-N-AS cells sensitized for cell death induced by NK co-culture. However, blocking the interaction between Fas and FasL by sequestering FasL through treatment with soluble Fas receptor (Fas-Fc) did not result in inhibition of NK-mediated cytotoxicity, hence we were unable to attribute the increased target cell killing to TNFα-induced Fas expression on the NB cells. One might hypothesize that in our model Fas-Fc was unable to sequester FasL since NK cell have been shown to induce FasL expression and cell surface exposure upon target cell engagement and NK activation [648-651], thus concealing FasL until the moment of target cell assault. Nevertheless, TNFα might have induced other changes that increase NK-mediated cytotoxicity besides promoting Fas-mediated cell death. For example, TNFα treatment of cancer cells has been shown to induce expression of the adhesion molecule ICAM-1 (Intercellular Adhesion Molecule 1), thereby promoting NK-mediated cell adhesion and cytotoxicity [652-654]. Despite the lack of a clear characterization which explains the TNFα-induced sensitization to NK-mediated cytotoxicity, TNFα pretreatment of NB cells was found to be possibly beneficial for NB treatment. Immunotherapy is a common strategy for high-risk NB patients whose efficacy might increase from targeting TNFR1 activation on NB cells.

5.3 Targeting TNFR1 in the clinic

5.3.1 Overcome TNFα toxicity

Data presented in this thesis indicate that TNFα has the capacity to prime Fas- and caspase-8-expressing NBs for cisplatin- and etoposide-induced cell death. These findings highlight a new approach to improve clinical outcome of currently used NB treatments. However, care has to be taken when administrating TNFα, since systemic treatment can induce severe side effects [655, 656]. To overcome this toxicity, patients are currently treated through isolated limb perfusions [407-411]. This TNFα treatment strategy has been found effective to improve the efficacy of anti-cancer agents, such doxorubicin and melphalan, for the treatment of solid tumors and metastases.

NBs originate from neuronal crest cells, most frequently from the adrenal glands (35%) but also from nerve tissues in the abdomen (30-35%), chest (20%), neck (1-5%), and pelvis (2-3%) [349-352]. Since these sites cannot be treated through isolate limb perfusions, other TNFα treatment strategies have to be developed. To overcome systemic toxicity, different modes for targeted TNFα treatment have been described, such as gene therapy, TNFα-fusion proteins, and nanoparticle delivery systems.

Intratumoral injection of TNFerade™, an adenoviral based *TNFα* gene delivery system that induces TNFα expression upon chemo- or radiation-therapy, has been described to lack systemic toxicity and shows potential for the treatment of multiple tumor types [657, 658]. Moreover, its use has been combined with cisplatin treatment and showed an increase in tumor regression using a xenograft mouse model [659].

TNFα-fusion antibodies developed against tumor-associated markers possess enhanced antitumor activity and reduced systemic toxicity *in vivo* [660]. For NB patients, the tumor-associated disialoganglioside GD2 has been used as a target for immunotherapy [661]. Administration of a chimeric anti-GD2 monoclonal antibody has been shown to mediate tumor cell killing through the recruitment of immune cells [662-666]. Moreover, the anti-GD2 antibody has been proven efficient for the treatment of NB patients and is used in combination with GM-CSF (granulocyte macrophage colony stimulating factor) and IL-2 (Interleukin-2), to further increase immune system-mediated NB eradication [661, 667, 668]. Thus, the development of TNFα-fusion antibodies that target specific NB tumor-associated markers, such as GD2, is a possible TNFα delivery route that can be utilized for increasing the efficacy of chemo-therapeutics that induce Fas activation, such as cisplatin and etoposide. In addition, due to its immune system mobilizing activity, anti-GD2 antibody treatment in combination with TNFα might increase the efficacy of NB immunotherapy.

Recently, advances are made in the development of nanoparticles that reduce then systemic toxicity of TNFα and are able to release TNFα in a controllable manner. Tumor necrosis factor-alpha coated gold nanospheres (Au-TNF) show enhanced tumor permeability and retention in solid tumor *in vivo* models [669-674]. Moreover, they are less toxic compared to systemic TNFα treatment and increased drug action can be achieved through inducing TNFα release by laser-induced photothermal effects. Phase I clinical trials have shown promising results for their application in humans [675], which suggest that these particles could be used in combination with cisplatin and etoposide to increase their efficacy, according to our data.

5.3.2 The dual role of NF-κB

The activation of NF-κB-mediated upregulation of Fas expression by TNFα treatment is suggested as a new approach for the treatment of NB patients, by priming for cisplatin- and etoposide-induced cell death. However, these findings are contradictory to current cancer therapies which utilize NF-κB inhibition as a treatment strategy due to its role in promoting cancer initiation, development, and metastasis [321, 322, 676]. NF-κB is frequently found constitutively active in most types of human cancer, due various mechanisms, such as genetic alterations in NF-κB subunits and IκB proteins, abnormal activation of NF-κB pathway proteins, or autocrine and paracrine cytokine- and growth factor-induced NF-κB activation. NF-κB-mediated production of cytokines and growth factors, such as TNFα, IL-1α, IL-6, IL-8, VEGF and HGF, has been shown to induce

proliferation, tumor growth and cell survival [677-682]. In addition, NF-κB-dependent upregulation of cyclooxygenase-2, cyclin D, angiogenic factors, chemokine receptors, and adhesion molecules further promote tumor survival, chemoresistance, angiogenesis, invasion, and metastasis [321, 322, 676].

In the regulation of apoptosis, NF-κB has been shown to play a dual role. Anti-apoptotic NF-κB target genes, such as *XIAP, cIAPs, TRAFs, c-FLIP*, and anti-apoptotic BCL-2 family members are known to inhibit the intrinsic and extrinsic apoptotic pathway and in addition have been shown to drive cell survival through promoting NF-κB and MAPK pathway activation [33, 78, 83, 124, 137, 218, 248, 333]. Moreover, anti-apoptotic BCL-2 family members and c-FLIPs are described to be upregulated in cancer and give rise to chemoresistance [54, 380-389, 683, 684]. On the other hand, NF-κB has also been described to repress the expression of anti-apoptotic proteins such as cIAPS, XIAP, TRAFs, c-FLIPs, BCL-X_L, and A20, thereby sensitizing for intrinsically- and extrinsically-induced apoptosis [343-345]. Moreover, various pro-apoptotic NF-κB target genes have been identified that promote apoptosis, such as the tumor suppressors *p53* and *MYC*, the pro-apoptotic *BCL-2* family members *BAX* and *BIM*, the death ligands *FASL* and *TRAIL*, and the death receptors *FAS, DR4, DR5*, and *DR6* [333].

Our data indicates that although TNFα treatment induced expression of anti-apoptotic proteins (c-FLIP, cIAP2, and BCL-2), the NF-κB-mediated upregulation of Fas overcomes their inhibition and sensitizes for FasL-, cisplatin-, and etoposide-induced apoptosis. However, the TNFα-mediated effects on NBs that can promote cancer development and metastasis were not assessed in this study and should be taken into consideration. Nevertheless, our data shows that, in NBs, TNFα treatment is able to disturb the balance between anti- and pro-apoptotic proteins and thereby promote drug-induced apoptosis.

5.3.3 Upregulating Fas in NB

The question remains whether TNFα-mediated Fas induction is favorable for NB eradication. Our data indicates that Fas induction primes for drug-induced cell death, however we did not assess the Fas-mediated tumor-promoting effects. Increased Fas expression might drive tumorigenesis and metastasis in the absence of drug treatment, since Fas expression and activation have been related to tumor progression [100, 362-367]. According to our data, only caspase-8- and Fas-expressing NBs should be targeted for Fas upregulation, since NBs that lack expression of one or both the proteins could not be sensitized for cisplatin- and etoposide-induced cell death. Although we did not observe TNFα-induced upregulation of Fas in these latter NBs, one might hypothesize that this event could still occur at later time points or after repetitive TNFα treatment. In cases where caspase-8 is silenced, Fas upregulation could aggravate tumor aggressiveness, due to lack of activation of the extrinsic apoptotic pathway and activation of pathways that leads to survival, migration, and proliferation [100, 362-367].

Despite the characterization of the role of Fas in tumor progression, limited data is available on the induction of Fas and its effects on tumor homeostasis. Some studies have been performed with murine xenograft models that over-expressed Fas [402, 403, 405]. These studies described that Fas over-expression delays tumor progression or increases survival, indicating that induction of Fas might be beneficial for cancer treatment. On the other hand, silencing of Fas expression was also shown to induce cancer cell death [397].

Lack of Fas expression has been linked to resistance to chemotherapy-induced cell death [458, 459, 590, 592, 593, 613, 614], suggesting that Fas down-regulation is a cell death evasion mechanism. Moreover, the immune system is known to induce cancer cell death through targeting the Fas/FasL pathway [196-203]. NK and CTLs have been shown to activate the Fas apoptotic pathway but have also been described to induce Fas expression on target cells, thereby sensitizing for NK and CTL-mediated cytotoxicity [685-688].

In this light, Fas upregulation could increase the response to chemo- and immuno-therapy-induced cell death, through sensitizing for chemotherapy-induced Fas activation or priming for immune system-mediated Fas engagement. However, more detailed studies and extensive *in vivo* models should be assessed before introducing Fas upregulation as a treatment strategy for NBs to the clinic. Nevertheless, our findings break ground for a new approach to improve the clinical outcome of NB patients that are treated with currently used strategies, such as chemo- or immuno-therapy.

Conclusions

“Life is really simple, but we insist on making it complicated.”

Confucius

Chinese teacher, editor, politician, and philosopher (551 BC- 479 BC)

6. Conclusions

First: Combined treatment of TNFα and FasL increases apoptosis in SK-N-AS cells, which correlates with an increase in caspase-8 and caspase-3/7 activity.

Second: FasL and TNFα/FasL induce caspase-8-dependent type II apoptosis in SK-N-AS cells.

Third: TNFα sensitizes neuroblastomas with detectable expression of Fas and caspase-8 to FasL-induced cell death through additional induction of Fas expression.

Fourth: Fas increased by TNFα stimulation is exposed to the cell surface and is able to enhance the DISC formation after FasL treatment.

Fifth: TNFα-induced expression of Fas is mediated through activation of the NF-κB pathway.

Sixth: Inhibition of NF-κB sensitizes SK-N-AS cells to TNFα-induced apoptosis.

Seventh: Cisplatin and etoposide induce FasL and activate caspase-8.

Eighth: TNFα primes caspase-8 and Fas expressing neuroblastomas for cisplatin- and etoposide-induced cell death.

Ninth: Neuroblastomas that lack basal Fas or caspase-8 expression do not respond to FasL-induced cell death neither before nor after TNFα treatment. These neuroblastomas do not induce Fas expression after TNFα treatment, and cannot be primed for cisplatin- or etoposide-induced cell death.

Tenth: Fas and caspase-8 expression in neuroblastomas does not correlate with *MYCN* amplification status nor p53 functionality.

Eleventh: IFNγ is able to reconstitute caspase-8 and Fas expression in Fas- and/or caspase-8 deficient neuroblastomas, thereby rendering them susceptible for FasL-induced apoptosis.

Twelfth: IFNγ treatment is able to prime for TNFα-induced expression of Fas and caspase-8, thereby increasing FasL-induced apoptosis.

Thirteenth: TNFα increases the susceptibility of SK-N-AS cells for natural killer cell-induced cytotoxicity.

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"The roots of education are bitter, but the fruit is sweet."

Aristotle
Greek philosopher and scientist (384 BC-322 BC)

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Annex

"Amigos de la prensa, yo me voy. Felicidades"

Translation: "Friends of the press, I'm going. Congratulations."

Louis van Gaal

Dutch football manager and former player (Born in 1951)

8. Annex

8.1 Annex 1: Most NBs are unsusceptible to TRAIL-induced apoptosis before and after TNFα priming

Apart from Fas, NF-κB activation induced by various stimuli, amongst which is TNFα, has been described to induce TRAIL receptor (DR4 and DR5) expression [462, 689-692]. Therefore, we analyzed the susceptibility of NBs to TRAIL-induced apoptosis, before and after TNFα priming.

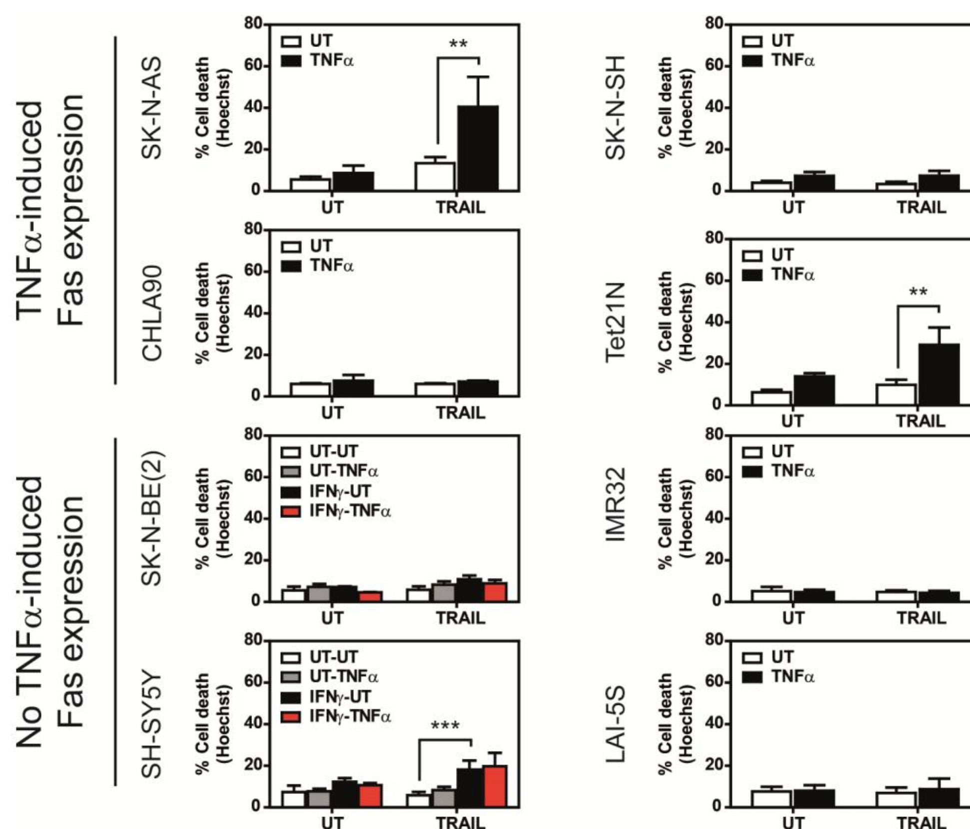

Figure 8.1: Most NBs are unsusceptible to TRAIL-induced apoptosis before and after TNFa priming. NB cell lines were pre-treated or not (UT) for 24h with 100ng/ml TNFα and subsequently were left untreated (UT) or treated for 24h with 100ng/ml TRAIL. In the case of the SH-SY5Y and SK-N-BE(2) cells, before TNFα treatment the cells were treated for 24h with 100ng/ml interferon-γ (IFNγ) or not (UT). $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

8.2 Annex 2: Publications

Galenkamp KMO, Carriba P, Urresti J, Planells-Ferrer L, Coccia E, Lopez-Soriano J, Barneda-Zahonero B, Moubarak RS, Segura MF, Comella JX: *TNF alpha sensitizes neuroblastoma cells to FasL-, cisplatin- and etoposide-induced cell death by NF-kappa B-mediated expression of Fas.* ***Molecular Cancer*** **2015**, **14:**14.

Garcia-Negredo G, Soto D, Llorente J, Morato X, **Galenkamp KMO**, Gomez-Soler M, Fernandez-Duenas V, Watanabe M, Adelman JP, Shigemoto R, et al: *Coassembly and coupling of SK2 channels and mGlu5 receptors.* ***Journal of Neuroscience*** **2014**, **34:**14793-14802.

Marques-Fernandez F, Planells-Ferrer L, Gozzelino R, **Galenkamp KMO**, Reix S, Llecha-Cano N, Lopez-Soriano J, Yuste VJ, Moubarak RS, Comella JX: *TNF alpha induces survival through the FLIP-L-dependent activation of the MAPK/ERK pathway.* ***Cell Death & Disease*** **2013**, **4**

Urresti J, Ruiz-Meana M, Coccia E, Carlos Arévalo J, Castellano J, Fernández-Sanz C, **Galenkamp KMO**, Planells-Ferrer L, Moubarak RS, Llecha-Cano N, et al: *LFG/FAIM2/TMBIM2 inhibits Fas ligand-mediated endoplasmic reticulum-calcium release mandatory for apoptosis in type II cells.* ***Manuscript under review, 2015***

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MOLECULAR CANCER

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TNFα sensitizes neuroblastoma cells to FasL-, cisplatin- and etoposide-induced cell death by NF-κB-mediated expression of Fas

Koen MO Galenkamp[1], Paulina Carriba[1], Jorge Urresti[1], Laura Planells-Ferrer[1], Elena Coccia[1], Joaquín Lopez-Soriano[1], Bruna Barneda-Zahonero[1], Rana S Moubarak[1], Miguel F Segura[2*] and Joan X Comella[1*]

Abstract

Background: Patients with high-risk neuroblastoma (NBL) tumors have a high mortality rate. Consequently, there is an urgent need for the development of new treatments for this condition. Targeting death receptor signaling has been proposed as an alternative to standard chemo- and radio-therapies in various tumors. In NBL, this therapeutic strategy has been largely disregarded, possibly because ~50-70% of all human NBLs are characterized by caspase-8 silencing. However, the expression of caspase-8 is detected in a significant group of NBL patients, and they could therefore benefit from treatments that induce cell death through death receptor activation. Given that cytokines, such as TNFα, are able to upregulate Fas expression, we sought to address the therapeutic relevance of co-treatment with TNFα and FasL in NBL.

Methods: For the purpose of the study we used a set of eight NBL cell lines. Here we explore the cell death induced by TNFα, FasL, cisplatin, and etoposide, or a combination thereof by Hoechst staining and calcein viability assay. Further assessment of the signaling pathways involved was performed by caspase activity assays and Western blot experiments. Characterization of Fas expression levels was achieved by qRT-PCR, cell surface biotinylation assays, and cytometry.

Results: We have found that TNFα is able to increase FasL-induced cell death by a mechanism that involves the NF-κB-mediated induction of the Fas receptor. Moreover, TNFα sensitized NBL cells to DNA-damaging agents (i.e. cisplatin and etoposide) that induce the expression of FasL. Priming to FasL-, cisplatin-, and etoposide-induced cell death could only be achieved in NBLs that display TNFα-induced upregulation of Fas. Further analysis denotes that the high degree of heterogeneity between NBLs is also manifested in Fas expression and modulation thereof by TNFα.

Conclusions: In summary, our findings reveal that TNFα sensitizes NBL cells to FasL-induced cell death by NF-κB-mediated upregulation of Fas and unveil a new mechanism through which TNFα enhances the efficacy of currently used NBL treatments, cisplatin and etoposide.

Keywords: Neuroblastoma, Fas (CD95/APO-1), TNFα, NF-κB, Cisplatin, Etoposide, Apoptosis

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Background

Neuroblastoma (NBL) is a solid tumor that arises from neuronal crest cells of the sympathetic nervous system. The most common form of cancer in infancy, NBL causes 15% of cancer-related deaths in children. The tumors have remarkable heterogeneity, which become evident in the clinic where patients can show spontaneous regression or rapid and fatal tumor progression. Over the years, significant advances have been made in the treatment of low- and intermediate-risk patients, thus allowing reaching high survival rates; however, the 5-year survival rate of patients in the high-risk group is still below 50% [1-3].

High-risk NBLs are treated with surgery, chemotherapy, radiotherapy, and/or the use of biological agents. Most of the therapeutic strategies used in NBL interfere with cell cycle progression and DNA synthesis or function, thereby causing DNA damage and the induction of apoptosis through the intrinsic and extrinsic apoptotic pathways [4].

The extrinsic or Death receptor (DR) pathway is activated by cell surface receptors of the tumor necrosis factor receptor (TNFR) family, which includes receptors for TNFα, FasL, and TNF-related apoptosis-inducing ligand (TRAIL) [5-7]. These receptors contain a death domain in their cytosolic tail which upon receptor activation leads to context-dependent outcomes such as apoptosis, necroptosis, or pro-survival signaling. The targeting of DR signaling has been proposed and studied for the treatment of various types of cancers [8-10]. For NBL tumors, this strategy has been largely disregarded, possibly because caspase-8 silencing occurs in 50-70% of all human NBLs [11-14]. However, a significant group of NBL patients do express caspase-8 and could benefit from treatments that induce DR activation. Given that TNFα is able to upregulate Fas expression in human cancer cell lines and sensitize them to FasL-induced cell death [15-17], we sought to investigate whether TNFα and FasL combination could be therapeutically relevant in NBL.

We found that TNFα treatment primes a subset of NBLs for FasL-induced cell death by triggering the NF-κB-mediated upregulation of Fas. Moreover, TNFα pretreatment increased cisplatin- and etoposide-induced caspase-8 cleavage and cell death in NBL cells that express both Fas and caspase-8. Our findings suggest that selected NBL patients could benefit from treatments that target TNFR1 and upregulate Fas expression.

Results

TNFα and FasL co-treatment induces cell death in SK-N-AS cells

To ascertain whether simultaneous treatment with FasL and TNFα induces cell death in NBL cells, we used the caspase-8-expressing NBL cell line SK-N-AS. TNFR1 was activated with soluble TNFα and the Fas receptor with trimeric Fc:hFasL [18-20]. Cell death assessment by Hoechst staining showed that TNFα or FasL treatment alone barely induced cell death (~5% and 20% respectively). However, the combination of the two cytokines caused an increase in cell death after 8 h of treatment, as compared to treatment with TNFα or FasL alone (Figure 1A). After 24 h, nearly all cells in the co-treatment condition were dead. Moreover, an increase in caspase-8 and caspase-3/7 activity was observed after simultaneous treatment with TNFα and FasL (Figure 1B-C). The use of the caspase-8 specific inhibitor IETD or the pan-caspase inhibitor QVD fully abrogated TNFα/FasL-induced cell death, thereby indicating that apoptosis triggered by FasL is the main mechanism of loss of viability (Figure 1D).

TNFα primes SK-N-AS cells for FasL-induced cell death by upregulating Fas

Next, we analyzed whether the phenotypic effects of the TNFα/FasL co-treatment were caused by the FasL and TNFα receptors signaling in synergy or whether one DR was sensitizing for apoptotic signaling by the other DR. Sequential treatment with TNFα or FasL followed by FasL or TNFα administration, respectively, revealed that TNFα was able to sensitize SK-N-AS cells to FasL-induced cell death (Figure 2A). On the contrary, FasL pre-treatment did not sensitize the cells to TNFα. The increase in caspase-8 activity during the co-treatment and the abrogation of cell death when using the caspase-8 inhibitor IETD suggested that TNFα induces molecular changes upstream of caspase-8 activation. Therefore, to address this point, we characterized the effects of TNFα on the expression of various components of the DISC complex (Figure 2B). Interestingly, a significant increase was observed only in Fas mRNA levels whereas minimal variations were detected for caspase-8, FADD, and RIP1 (Figure 2B). The increase in Fas mRNA levels was confirmed at the protein level by Western blot as early as 8 h after TNFα treatment (Figure 2C), which is consistent with the increase in cell death observed after simultaneous treatment with TNFα and FasL.

Newly synthesized Fas is exposed to the plasma membrane and favors DISC formation

The increase in FasL-mediated cell death could be explained by exposure of the newly synthesized Fas receptor to the cell surface. To test this notion, we used a cell surface biotinylation assay to analyze the cellular distribution of Fas after TNFα treatment. An increase in total and cell surface Fas was already observed after 4 h of TNFα treatment, peaking at 12 h, after which the expression was maintained for at least another 12 h (Figure 3A). To further confirm the functionality of the

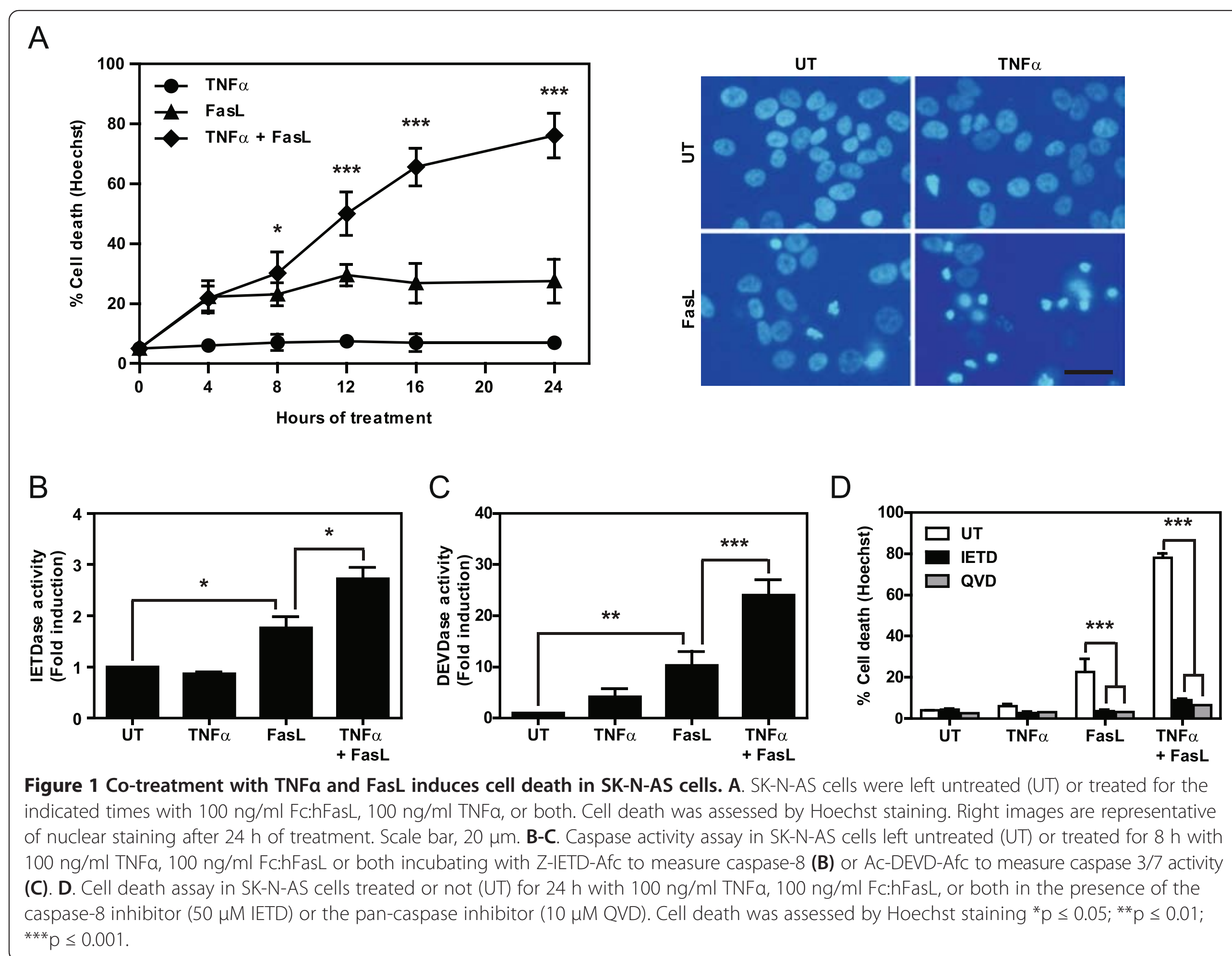

Figure 1 Co-treatment with TNFα and FasL induces cell death in SK-N-AS cells. A. SK-N-AS cells were left untreated (UT) or treated for the indicated times with 100 ng/ml Fc:hFasL, 100 ng/ml TNFα, or both. Cell death was assessed by Hoechst staining. Right images are representative of nuclear staining after 24 h of treatment. Scale bar, 20 μm. **B-C**. Caspase activity assay in SK-N-AS cells left untreated (UT) or treated for 8 h with 100 ng/ml TNFα, 100 ng/ml Fc:hFasL or both incubating with Z-IETD-Afc to measure caspase-8 **(B)** or Ac-DEVD-Afc to measure caspase 3/7 activity **(C)**. **D**. Cell death assay in SK-N-AS cells treated or not (UT) for 24 h with 100 ng/ml TNFα, 100 ng/ml Fc:hFasL, or both in the presence of the caspase-8 inhibitor (50 μM IETD) or the pan-caspase inhibitor (10 μM QVD). Cell death was assessed by Hoechst staining *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

newly synthesized Fas, a DISC formation assay was performed before and after TNFα pre-treatment. Indeed, FasL co-immunoprecipitated with FADD and caspase-8 only in cells pre-treated with TNFα (Figure 3B). Interestingly, in the absence of this pre-treatment, we were only able to immunoprecipitate low levels of high molecular weight Fas (~150 kDa) and in these conditions we did not detect caspase-8 or FADD immunoprecipitation. The analysis of input cell lysates confirmed that caspase-8 and −3 were cleaved only after FasL treatment and could be increased by TNFα pre-treatment (Figure 3B). These data demonstrate that cell surface exposure of Fas shows similar kinetics as the newly synthesized Fas, thereby suggesting a rapid translocation of newly synthesized Fas to the surface. Furthermore, the increased cell surface expression of Fas enhanced FasL-induced DISC formation, which led to the activation of the extrinsic apoptotic pathway.

TNFα-induced Fas is transcriptionally regulated by NF-κB

TNFα has been shown to induce gene expression by activating various signaling pathways, such as those of ERK1/2, PI3K, and JNK kinases and transcription factors like NF-κB [5,6,21]. Therefore, we proceeded to analyze Fas expression after TNFα treatment combined with specific inhibitors: PD98059 (ERK1/2), LY294002 (PI3K), SP600125 (JNK), BAY 11–7082 (NF-κB), or overexpressing Super Repressor (SR), a mutated form of the NF-κB inhibitor IκBα that inhibits NF-κB signaling [22,23]. While the inhibition of ERK1/2, PI3K or JNK did not block the upregulation of Fas by TNFα (Figure 4A), the inhibition of the NF-κB -pathway by overexpression of SR or treatment with BAY 11–7082 did fully abrogate the upregulation of Fas induced by TNFα at the protein level (Figure 4B). Other known NF-κB targets, such as Bcl-2 [24] and c-FLIP$_S$ [25], were used as controls. Moreover, the use of SR overexpression or treatment with BAY 11–7082 blocked the TNFα-induced upregulation of Fas mRNA or known NF-κB targets such as c-FLIP and Bcl-2 (Figure 4C). Further assessment of TNFα-induced gene transcription and mRNA translation was performed with the use of DNA transcription (Actinomycin D) and mRNA translation (Cycloheximide) inhibitors (Figure 4D-E). Both inhibitors blocked the

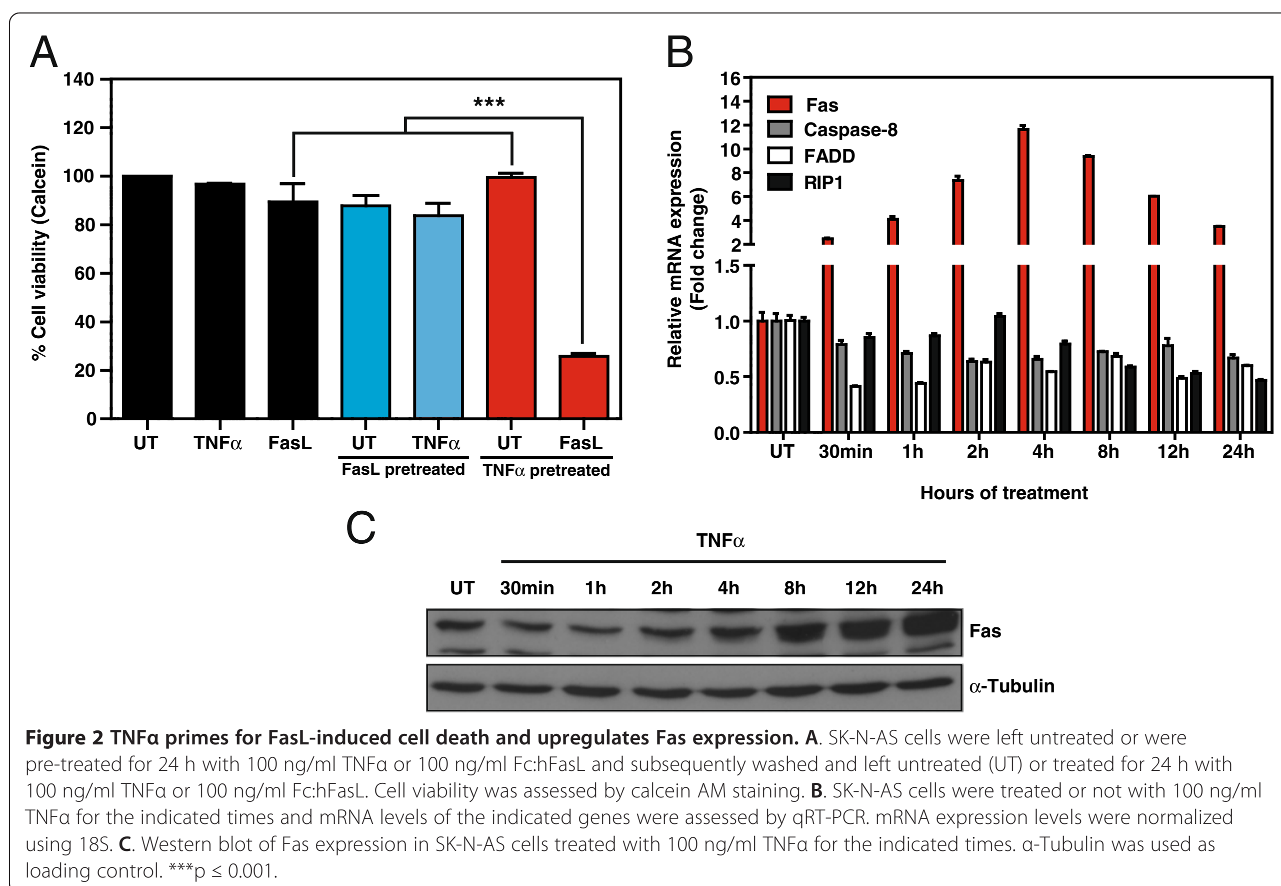

Figure 2 TNFα primes for FasL-induced cell death and upregulates Fas expression. A. SK-N-AS cells were left untreated or were pre-treated for 24 h with 100 ng/ml TNFα or 100 ng/ml Fc:hFasL and subsequently washed and left untreated (UT) or treated for 24 h with 100 ng/ml TNFα or 100 ng/ml Fc:hFasL. Cell viability was assessed by calcein AM staining. **B**. SK-N-AS cells were treated or not with 100 ng/ml TNFα for the indicated times and mRNA levels of the indicated genes were assessed by qRT-PCR. mRNA expression levels were normalized using 18S. **C**. Western blot of Fas expression in SK-N-AS cells treated with 100 ng/ml TNFα for the indicated times. α-Tubulin was used as loading control. ***p ≤ 0.001.

upregulation of Fas protein levels induced by TNFα, whereas only actinomycin D was able to inhibit TNFα-induced upregulation of Fas mRNA. Cycloheximide did not result in abrogation of Fas mRNA induction, thereby confirming the direct NF-κB -mediated transcriptional regulation of this protein (Figure 4D-E). In summary, our results reveal that the upregulation of Fas mRNA occurs rapidly after TNFα treatment through activation of the NF-κB -mediated transcription of the Fas gene. Moreover, the inhibition of Fas upregulation by the overexpression of SR, treatment with BAY 11–7082 (Figure 4B), or by blocking its synthesis with actinomycin D and cycloheximide treatment (Figure 4E) prevented the cell surface exposure of Fas.

TNFα primes for cisplatin- and etoposide-induced activation of caspase-8 and cell death

The FasL/Fas system has been shown to participate in cell death mechanisms triggered by DNA-damaging agents currently used in NBL therapy such as cisplatin and etoposide [26,27]. Therefore, we addressed whether TNFα treatment enhances the cytotoxic effect of these two drugs. Cisplatin and etoposide have been shown to induce FasL expression in NBL cells [27]. We confirmed these observations in SK-N-AS cells, as an increase in FasL mRNA was detected after 24 h of etoposide or cisplatin treatment (Figure 5A). Furthermore, the induction of FasL by treatment with these chemotherapeutic agents concurred with the activation of caspase-8, as reflected by caspase-8 cleavage (Figure 5B). Indeed, TNFα treatment upregulated Fas expression in SK-N-AS cells and enhanced the cleavage of caspase-8 induced by cisplatin (Figure 5B left) and by etoposide (Figure 5B right). We next analyzed the functional consequences of these observations in a subset of NBL cell lines (SK-N-AS, SK-N-SH, SH-SY5Y, and LAI-5S) and assessed cell death by Hoechst staining (Figure 5C). Notably, only the SK-N-AS and SK-N-SH cell lines showed an increase in cisplatin- and etoposide-induced cell death when primed with TNFα, whereas no changes in cell death were observed for the SH-SY5Y and LAI-5S cell lines.

NBLs show heterogeneity in TNFα-induced Fas expression, thereby explaining the priming for cisplatin- and etoposide-induced cell death

NBLs are known to have a high degree of heterogeneity [1-3], which may explain why some NBL cell lines are not primed by TNFα for cisplatin- and etoposide-induced cell death. Therefore, we assessed the expression of Fas and its modulation by TNFα treatment in a

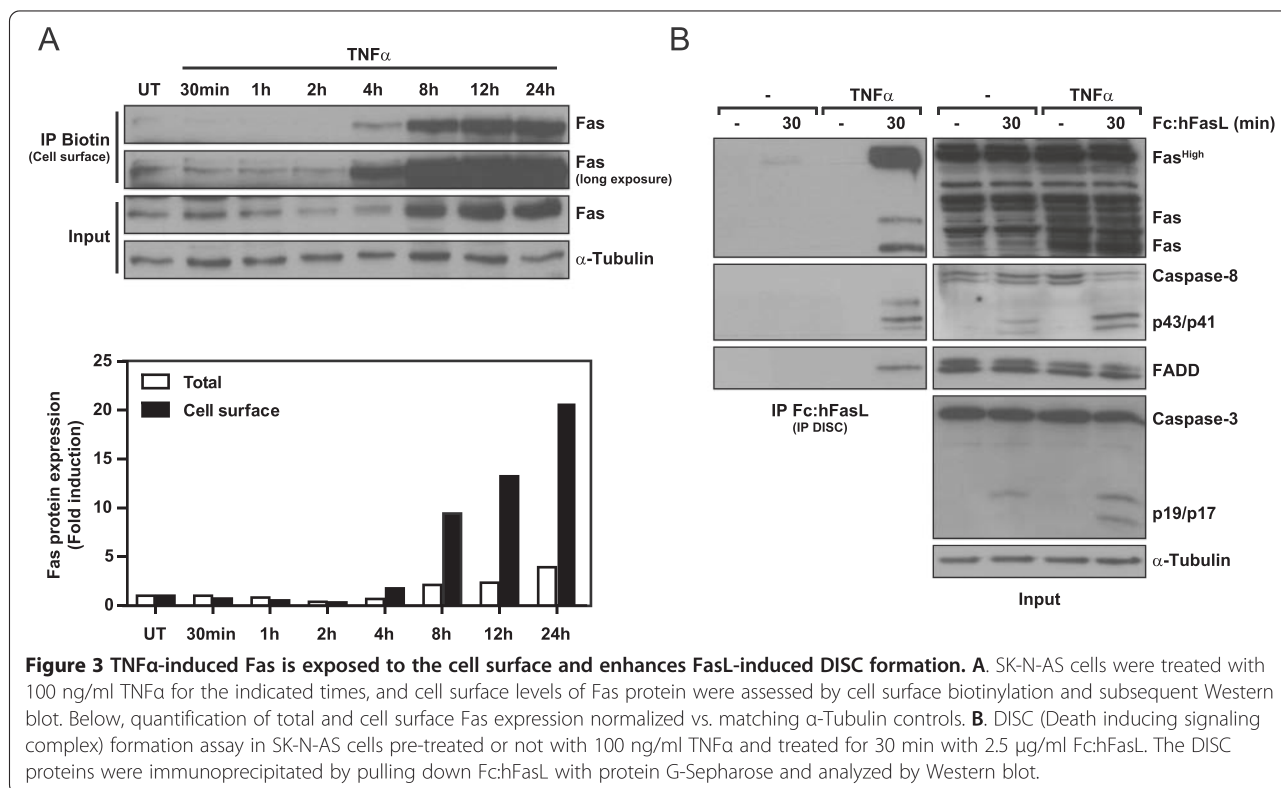

Figure 3 TNFα-induced Fas is exposed to the cell surface and enhances FasL-induced DISC formation. A. SK-N-AS cells were treated with 100 ng/ml TNFα for the indicated times, and cell surface levels of Fas protein were assessed by cell surface biotinylation and subsequent Western blot. Below, quantification of total and cell surface Fas expression normalized vs. matching α-Tubulin controls. **B**. DISC (Death inducing signaling complex) formation assay in SK-N-AS cells pre-treated or not with 100 ng/ml TNFα and treated for 30 min with 2.5 μg/ml Fc:hFasL. The DISC proteins were immunoprecipitated by pulling down Fc:hFasL with protein G-Sepharose and analyzed by Western blot.

set of eight NBL cell lines. In addition to the SK-N-AS cell line, TNFα upregulated Fas expression in the SK-N-SH, CHLA90, and Tet21N cell lines, as observed by flow cytometry (Figure 6A) and Western blot (Figure 6B). Furthermore, TNFα was also able to sensitize these cells to FasL-induced apoptosis, as determined by Hoechst staining (Figure 6C) and caspase-3/7 activity (Additional file 1: Figure S1). In contrast, the SH-SY5Y, LAI-5S, IMR32, and SK-N-BE(2) cell lines did not show changes in Fas expression (Figure 6A-B), FasL-induced cell death (Figure 6C), or caspase-3/7 activity (Additional file 1: Figure S1) after TNFα treatment. Interestingly, these cells showed TNFα-induced IκBα degradation and the upregulation of other known NF-κB -target genes, such as Bcl-2 [24] and/or cIAP2 [28] (Additional file 2: Figure S2A-B).

A correlation could be observed between the expression of both Fas and caspase-8, the induction of cell death by FasL and the sensitization thereof by TNFα. Cells that only express one of the two proteins did not show FasL-induced cell death and could not be sensitized by TNFα. However, cells that express both proteins, Fas and caspase-8, did show FasL-induced cell death and TNFα-induced sensitization. Furthermore, the levels of Fas and caspase-8 expression concur with the FasL-induced cell death response. CHLA90 cells, with low levels of Fas and caspase-8, exhibit little FasL-induced cell death when compared to Tet21N cells, which show high levels of Fas and caspase-8 expression. Due to these high levels and the corresponding cell death response, Tet21N cells had to be treated with lower levels of FasL for the TNFα-induced sensitization to become apparent, since 100 ng/ml FasL induced a near complete cell death response (data no shown).

These findings demonstrate that although NF-κB is activated and induces gene transcription in all the NBL cell lines studied here, there is a subset of NBL cell lines in which Fas expression is not upregulated in response to TNFα treatment. These observations concur with our previous observations in which we determined that these cells were primed neither for cisplatin- nor etoposide-induced cell death when targeted with TNFα.

Interferon-γ reconstitutes caspase-8, upregulates Fas expression, and primes NBL for FasL-induced cell death

Interferon-γ (IFNγ) is known to render NBL cells sensitive to FasL-induced cell death by reconstituting caspase-8 and upregulating Fas expression [29-31]. Here we studied whether NBL treatment with IFNγ promotes TNFα-induced Fas expression in cell lines that previously did not show Fas induction in response to TNFα treatment (i.e. SH-SY5Y and SK-N-BE(2)). We confirmed that IFNγ upregulates caspase-8 and Fas expression in these cell lines (Figure 7A-B). The SK-N-BE(2) cell line showed a TNFα-induced increase in caspase-8 and Fas expression after IFNγ treatment. However, in the SH-SY5Y cells, TNFα did not modulate the expression of either protein.

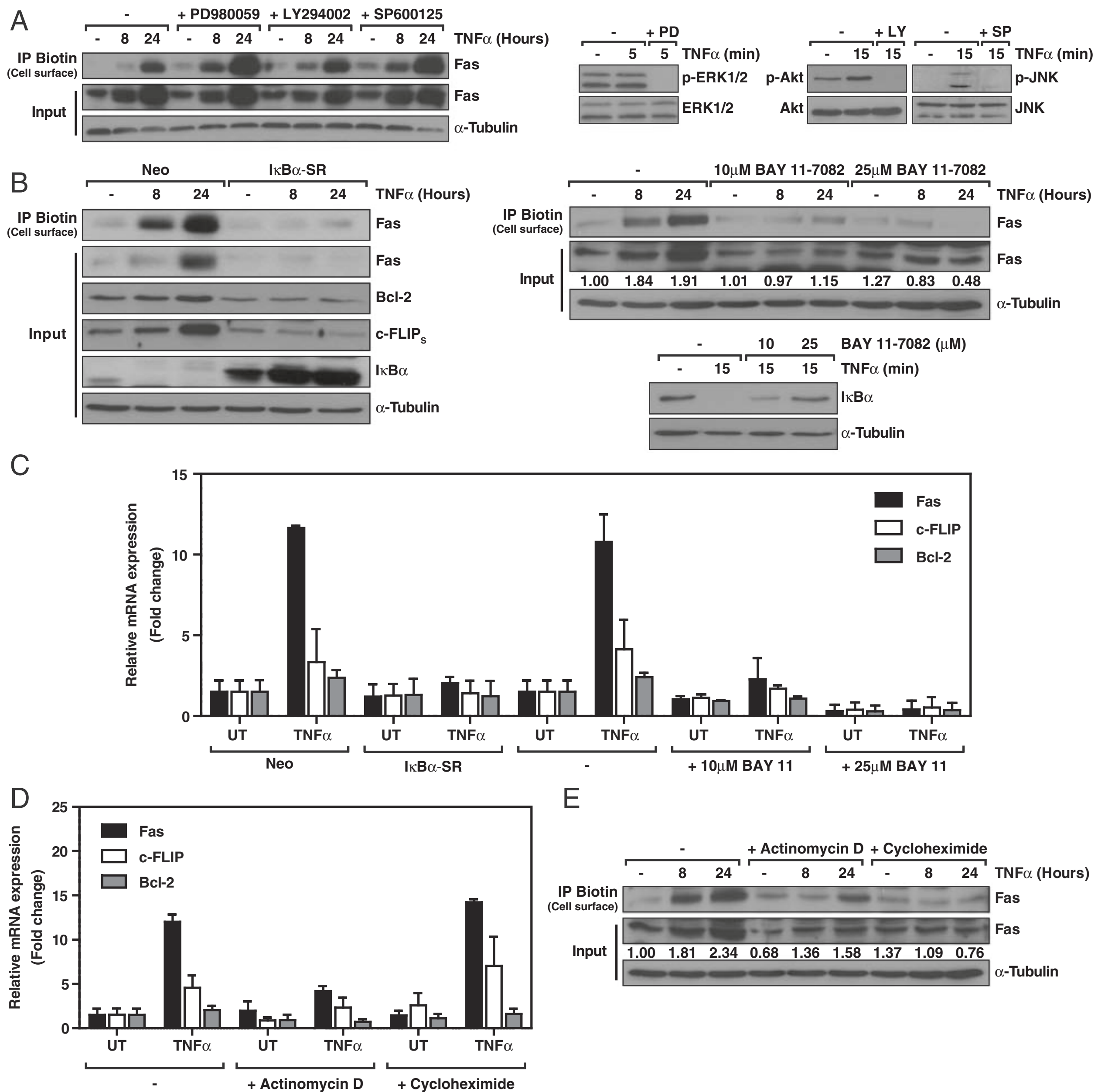

Figure 4 NF-κB mediates TNFα-induced Fas expression and exposure to the cell surface. A. Left panel, SK-N-AS cells were treated with 25 μM PD980059, 20 μM LY294002 or 20 μM SP600125 prior to treatment with 100 ng/ml TNFα. At the indicated times, cell surface proteins were biotinylated, isolated, and analyzed by Western blot. Right panel, phospho-protein levels assessed by Western blot to confirm efficacy of PD980059 (PD), LY294002 (LY), and SP600125 (SP). **B**. Left panel, control (Neo) and SuperRepressor/SR-IκBα-infected SK-N-AS cells were left untreated (–) or treated with 100 ng/ml TNFα for 8 h and 24 h. Cell surface proteins were biotinylated, isolated, and analyzed by Western blot. Right panel, analysis of cell surface and total Fas levels. SK-N-AS cells were incubated with 10 μM and 25 μM of the NF-κB inhibitor BAY 11–7082 prior treatment with 100 ng/ml TNFα. Below, quantification of total Fas expression normalized vs. matching α-Tubulin controls. IκBα degradation Western blot confirmed the efficacy of BAY 11–7082. **C**. Control (Neo) and SuperRepressor/SR-IκBα-infected or control (–) and BAY 11–7082 pre-treated SK-N-AS cells were left untreated (UT) or treated with 100 ng/ml TNFα. mRNA levels of the indicated genes were assessed after 4 h by qRT-PCR. **D**. qRT-PCR assessment of the indicated genes, 4 h after 100 ng/ml TNFα treatment of SK-N-AS cells in combination with 20 nM actinomycin D or 1 μg/ml cycloheximide. **E**. Cell surface biotinylation assay in SK-N-AS cells pre-treated with 20 nM actinomycin D or 1 μg/ml cycloheximide and treated with 100 ng/ml TNFα for the indicated times. Protein levels were analyzed by Western blot. Below, quantification of total Fas expression normalized vs. matching α-Tubulin controls. All conditions were pre-incubated with 10 μM of the caspase inhibitor QVD to avoid cell death-related effects.

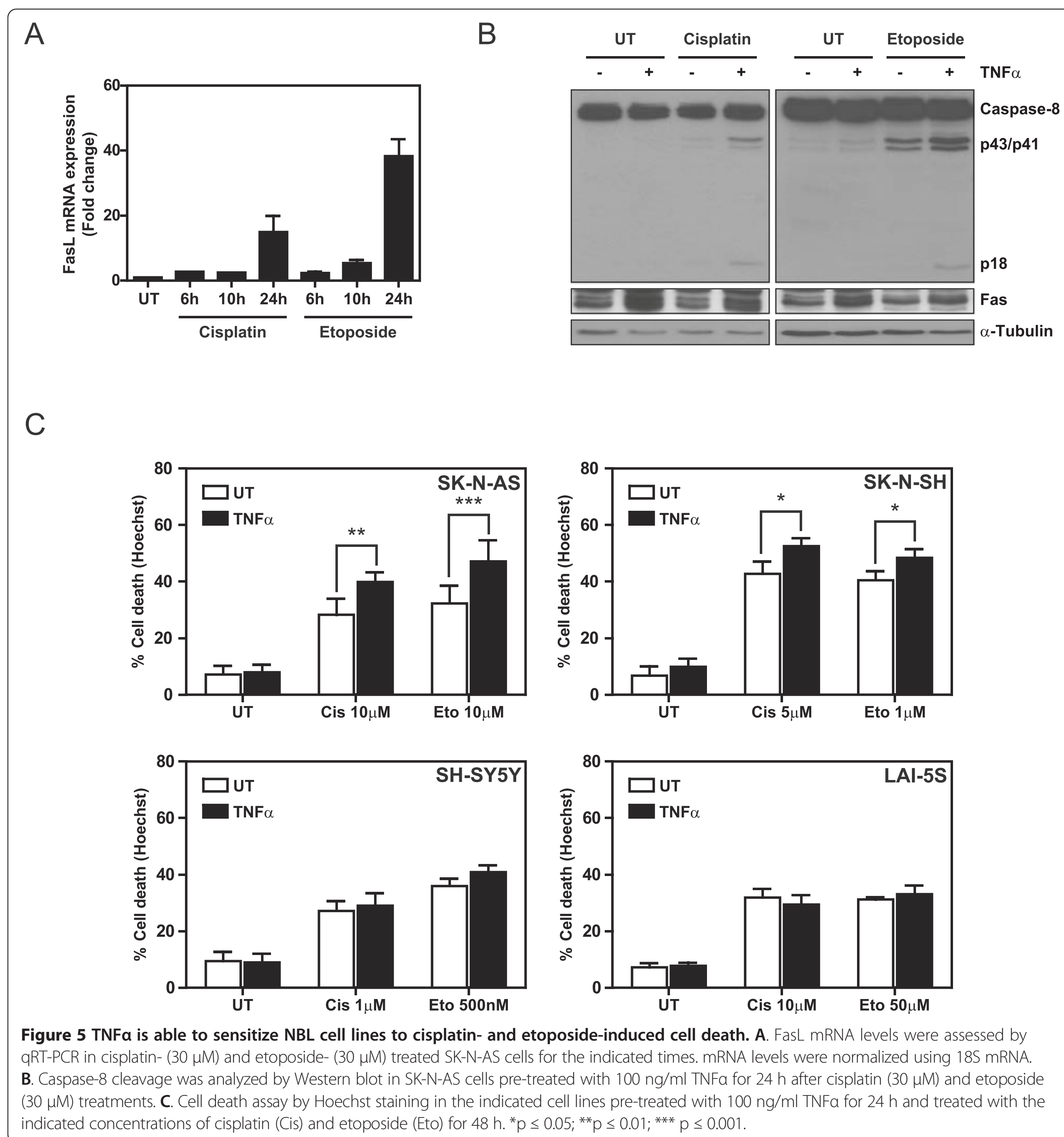

Figure 5 TNFα is able to sensitize NBL cell lines to cisplatin- and etoposide-induced cell death. A. FasL mRNA levels were assessed by qRT-PCR in cisplatin- (30 μM) and etoposide- (30 μM) treated SK-N-AS cells for the indicated times. mRNA levels were normalized using 18S mRNA. **B**. Caspase-8 cleavage was analyzed by Western blot in SK-N-AS cells pre-treated with 100 ng/ml TNFα for 24 h after cisplatin (30 μM) and etoposide (30 μM) treatments. **C**. Cell death assay by Hoechst staining in the indicated cell lines pre-treated with 100 ng/ml TNFα for 24 h and treated with the indicated concentrations of cisplatin (Cis) and etoposide (Eto) for 48 h. *$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$.

According to these observations, IFNγ sensitized SK-N-BE(2) and SH-SY5Y cells to FasL-induced cell death (Figure 7C) and caspase 3/7 activity (Additional file 1: Figure S1). For the SK-N-BE(2) cells, subsequent TNFα treatment further increased sensitization to FasL-induced cell death. In contrast, SH-SY5Y cells, which did not show a further increase in caspase-8 or Fas levels, did not show further sensitization to FasL-induced cell death after stimulation with TNFα. These data indicate that the induction of Fas expression by TNFα in NBLs cannot always be recovered by IFNγ treatment, thus pointing to different levels of Fas regulation.

Discussion

Many patients with high-risk NBL tumors continue to have a poor prognosis. Consequently, ongoing efforts are being channeled into the development of new treatments or the discovery of therapeutic agents that can increase the efficacy of current clinical regimes—cisplatin and etoposide being examples of such drugs [2]. Here

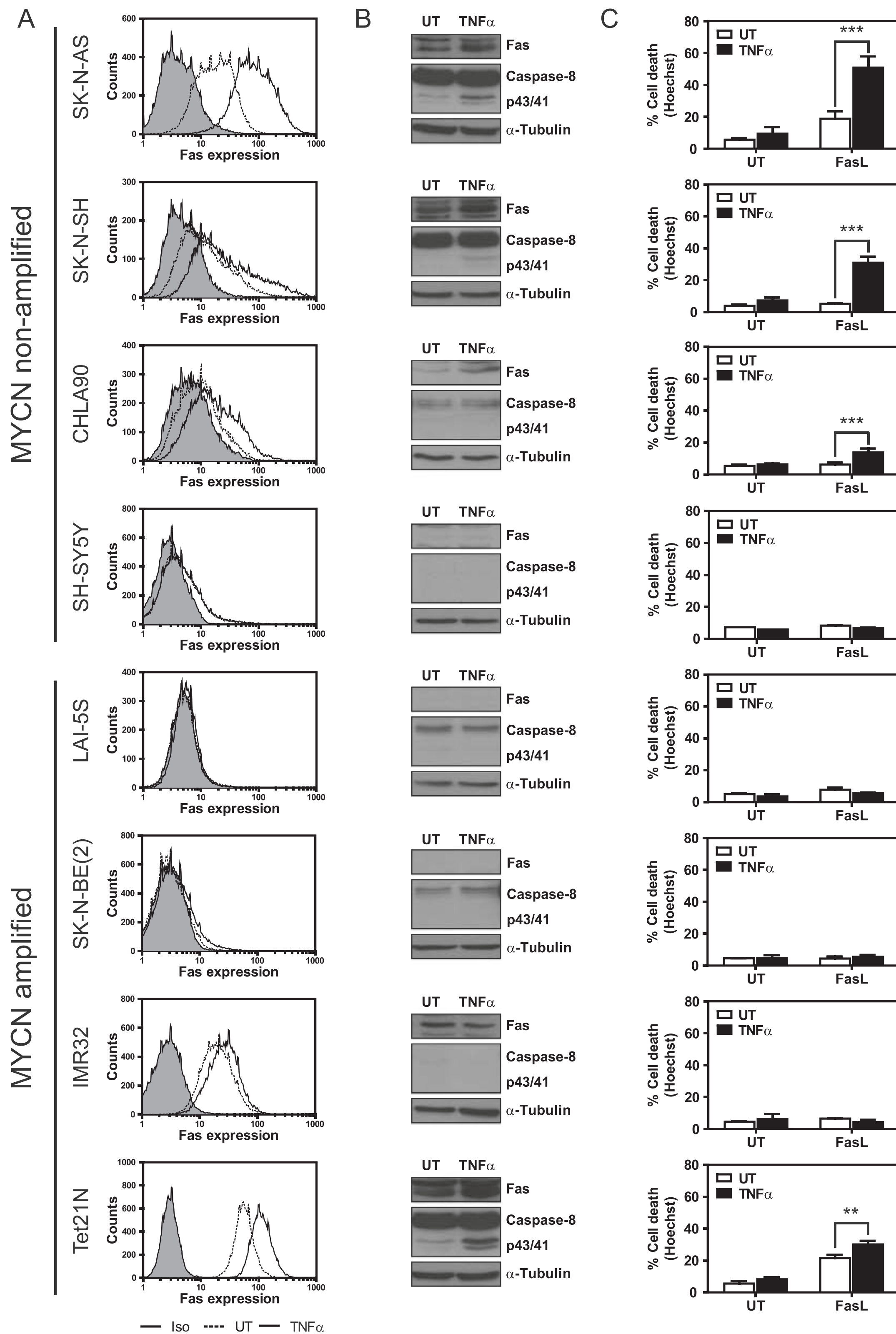

Figure 6 (See legend on next page.)

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Figure 6 NBLs show heterogeneous Fas expression in response to TNFα treatment. A. NBL cell lines were treated with 100 ng/ml TNFα or left untreated (UT) for 24 h. Fas expression was analyzed by cytometry using a PE-conjugated Fas or isotype antibody. **B**. Cells were treated for 24 h with 100 ng/ml TNFα or were left untreated (UT). Protein expression levels were analyzed by Western blot. **C**. Cell death assay by Hoechst staining in the indicated cell lines pre-treated with 100 ng/ml TNFα for 24 h and treated for another 24 h with 100 ng/ml Fc:hFasL, or 1 ng/ml Fc:hFasL for Tet21N cells. **p ≤ 0.01; ***p ≤ 0.001.

we describe that the activation of TNFR1 increases susceptibility to FasL-, cisplatin- and etoposide-induced cell death through the NF-κB -mediated upregulation of Fas, a target that has received little attention for NBL therapies. The newly synthesized Fas is exposed to the cell surface and incorporated into the DISC complex upon ligand binding, thereby triggering the activation of caspases and inducing apoptotic cell death.

Soluble TNFα exerts its effects through the binding and activation of the ubiquitously expressed TNFR1 receptor [5-7,18,19]. Depending on the cellular context, TNFα stimulation induces apoptosis, necroptosis, or pro-survival signaling through the activation of caspases, kinases, and transcription factors such as NF-κB [5-7,21]. For NF-κB activation, TNFR1 binds the adaptor protein TRADD through interaction with its death domain. This interaction allows the recruitment of the adapter protein RIP1 and the E3 ligases TRAF2/5 and cIAP1/2, thereby inducing the ubiquitination of RIP1. This shapes the platform for recruitment and activation of the IKK complex that induces phosphorylation of the cytoplasmic NF-κB inhibitor IκBα, thereby targeting it for ubiquitination and subsequent proteasomal degradation. Degradation of IκBα mediates the release of NF-κB and allows its translocation to the nucleus where it can induce gene transcription. According to our data and data from others, *FAS* is amongst the genes that can be induced by NF-κB. Chan *et al.* and Liu *et al.* have previously identified the p65/RelA binding site in the Fas promoter and confirmed TNFα-induced NF-κB -mediated upregulation of Fas [32,33]. Here, we were able to demonstrate the NF-κB -mediated regulation in NBLs and discarded regulation of Fas expression by other pathways known to be activated by TNFR1 (i.e. ERK1/2, PI3K, and JNK).

Given the participation of the Fas/FasL system in the mechanisms of cell death caused by DNA-damaging agents such as cisplatin and etoposide [26,27], we studied the possibility of improving the efficacy of these drugs by combined treatment with TNFα. Our results showed that TNFα pre-treatment increased cisplatin- and etoposide-induced cell death in two of the four NBL cell lines studied. Similarly, Benedetti *et al.* reported that TNFα acts in synergy with cisplatin in renal proximal tubular cells, inducing an increase in cell death by prolonging JNK activation and inhibiting NF-κB translocation to the nucleus [34,35]. However, our data

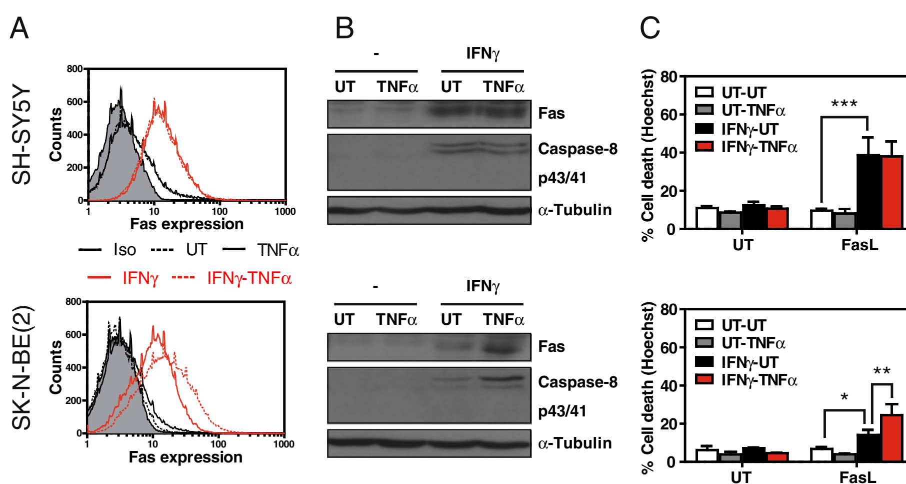

Figure 7 IFNγ renders caspase-8- and Fas-deficient NBLs sensitive to FasL. A. The indicated cell lines were treated with 100 ng/ml interferon-γ (IFNγ) or were left untreated (UT) for 24 h. Cells were then treated or not for 24 h with 100 ng/ml TNFα and Fas expression was analyzed by cytometry using a PE-conjugated Fas or an isotype antibody. **B**. Fas and caspase-8 expression analysis by Western blot in the indicated cell lines pre-treated for 24 h with 100 ng/ml interferon-γ (IFNγ) and treated with 100 ng/ml TNFα for 24 h. **C**. Cell death assay in NBL cell lines pre-treated or not (UT) with 100 ng/ml IFNγ for 24 h and thereafter treated or not with 100 ng/ml TNFα for 24 h. Next, cells were treated for an additional 24 h with 100 ng/ml Fc:hFasL. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

indicate that the TNFα-induced priming for cisplatin- and etoposide-induced cell death depends on NF-κB -mediated induction of Fas expression and caspase-8 cleavage.

Remarkably, not all the NBL cell lines studied were primed by TNFα for cisplatin- and etoposide-induced cell death. To predict the benefit of the TNFα combination therapy, we analyzed the expression of Fas and the modulation thereof by TNFα in a set of eight NBL cell lines. In four of the eight NBL cell lines, TNFα upregulated Fas expression. Furthermore, we observed that only the cell lines that showed TNFα-induced upregulation of Fas expression also displayed TNFα-induced priming to FasL-, cisplatin-, and etoposide-induced cell death. The cell lines that showed TNFα-induced priming also displayed Fas and caspase-8 expression, whereas cell lines that were not primed by TNFα showed the expression of only one of the two proteins. The response to TNFα treatment was not related to other frequent NBL alterations, such as MYCN amplification or p53 functional status (see Table 1).

The mechanism by which Fas is silenced in NBL and why some cell lines do not respond to the TNFα-induced Fas regulation remains to be clarified. In the NBL cell lines addressed, we confirmed NF-κB activation after TNFα treatment and detected the induction of other known NF-κB target genes, such as cIAP2 and Bcl-2 [24,28]. One possible mechanism to explain this lack of Fas induction is that TNFα treatment stimulates the formation of different NF-κB heterodimers or NF-κB was post-transcriptionally modified, which may drive specific gene expression [42]. An alternative mechanism to account for the incapacity of TNFα to induce Fas expression can be found at the level of epigenetic regulation of the Fas gene. Methylation of the Fas promoter has been reported in various types of tumors, including NBL [43-45]. IFNγ has been shown to restore caspase-8 and Fas expression in NBL cells [29-31,46,47] and to render them sensitive to FasL treatment. Consequently, IFNγ may also prime caspase-8- or Fas-deficient NBL cells for the TNFα combination therapy. Indeed, we confirmed that IFNγ primes these NBL cells for FasL-induced cell death. However, IFNγ treatment did not sensitize all the NBL cell lines to the TNFα-induced upregulation of Fas. These findings suggest that the expression of Fas in NBLs is regulated at various levels and that it differs between NBLs.

Recent studies have described the benefits of TNFα in combination with doxorubicin [48] or melphalan [49] for the treatment of solid tumors. Due to its low toleration in systemic treatment, various TNFα fusion proteins have been developed for localized treatment [50], some of which show promise and have entered clinical trials [49,51,52]. These findings break ground for the use of TNFα in the treatment of NBL in combination with cisplatin and etoposide.

Our results suggest that NF-κB -mediated upregulation of Fas by TNFα could be a new approach for the treatment of NBL patients. These findings are in contradiction to the current dogma in which NF-κB inhibition is seen as a strategy for cancer treatment, since NF-κB has been implicated in promoting cancer initiation, development, and metastasis [53,54]. NF-κB activation is known to promote cell survival by upregulating anti-apoptotic proteins, such as Bcl-2, c-FLIP, and cIAP2 thereby inhibiting DR-induced apoptosis [24,25,28]. However, NF-κB is also able to promote apoptosis through the induction of pro-apoptotic proteins, such as Fas [32,33], Bax [55], DR5 [56], and DR6 [57]. Our study supports the evidence that NF-κB triggers pro-apoptotic signaling in a subset of NBL cells through Fas upregulation, which tips the scale towards apoptotic cell death.

Conclusions

The results of this study contribute to our understanding of Fas expression, its regulation by TNFα in a NBL setting, and its implications in the treatment of NBL tumors. Although TNFα is mostly known for its pro-survival signaling [24,25,28], our results indicate that this cytokine has the capacity to prime caspase-8- and Fas-expressing NBLs for cisplatin- and etoposide-induced cell death. These findings pave the way for a new approach to improve clinical response to current NBL treatments.

Table 1 Neuroblastoma characteristics and their modulation by TNFα

		MYCN non-amplified [36,37]				MYCN amplified [36,37]			
		SK-N-AS	**SK-N-SH**	**CHLA90**	**SH-SY5Y**	**LAI-5S**	**SK-N-BE(2)**	**IMR32**	**Tet21N**
Expression	**Fas**	+	+	+/−	+/−	-	-	+	++
	Caspase-8	++	++	+/−	-	+/−	+/−	-	++
	p53	N [38]	F [39]	N [40]	F [39]	N [41]	N [40]	F [39]	F [39]
TNFα-induced	**Fas**	++	+	+	-	-	-	-	++
	Sensitization to etoposide/cisplatin	++	+	NA	-	-	NA	NA	NA
FasL-induced cell death	**UT**	+	+/−	-	-	-	-	-	++
	TNFα	+++	+++	+	-	-	-	-	+++

Abbreviations: F Functional, *N* Non-functional, *NA* Not available.

Methods

Reagents

Unless stated otherwise, all biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant Fc:hFasL was a generous gift of Dr. Pascal Schneider (University of Lausanne, Epalinges, Switzerland). Recombinant human TNF*α* and IFNγ were supplied by Biotrend (Köln, Germany). PD98059, SP600125, BAY 11–7082, Z-IETD-FMK, and Q-VD-OPH were purchased from Merck Millipore (Billerica, MA, USA).

Cell culture

The human NBL cell lines SK-N-AS, LAI-5S, IMR32, SK-N-BE(2), and SH-SY5Y and the renal epithelial cell line HEK293T were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% or 15% (SH-SY5Y) heat-inactivated FBS (FBSi, Thermo Fisher Scientific). The NBL cell lines SK-N-SH and CHLA90 were cultured in IMDM (Thermo Fisher Scientific) supplemented with 20% FBSi. The NBL cell line Tet21N was maintained in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% FBSi, 25 mM HEPES (Thermo Fisher Scientific), 200 μg/ml geneticin (G418), 0.5 μg/ml amphotericin B, and 10 μg/ml hygromycin B. Cell culture media was supplemented with 100U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific). Cultures were maintained at 37°C in a saturated atmosphere of 95% air and 5% CO_2. CHLA90 cells were acquired from the Children's Oncology Group Cell Line repository. SK-N-BE(2) and LAI-5S cells were from the Public Health England Culture Collections (Salisbury, UK). Tet21N cells were a kind gift from Dr. Manfred Schwab (DKFZ, Heidelberg, Germany). All other cell lines were acquired from the American Type Tissue Collection (ATCC, Manassas, VA, USA).

Hoechst staining

After the indicated treatments, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton™ X-100, and stained with 0.05 μg/ml Hoechst 33342. Cell death was assessed by counting viable and dead cells, by discriminating condensed and fragmented nuclei (apoptotic nuclear morphology type II), as described by Yuste *et al.* [58]. Quantification was performed in blind testing, and at least 500 cells were counted per condition.

Caspase activity

After the indicated treatments, cells were harvested, washed with ice-cold PBS, lysed in caspase activity buffer (20 mM HEPES-NaOH, pH7.2, 10% sucrose, 150 mM NaCl, 5 mM EDTA, 1% Igepal CA-630, 0.1% CHAPS, and 1× EDTA-free Complete protease inhibitor mixture), and insoluble fractions were removed by centrifugation. The protein concentration of the lysate was quantified using the Lowry-based DC protein assay (Biorad, Hercules, CA, USA). Next, caspase activity was assessed by incubating 10 μg protein at 37°C in caspase activity buffer supplemented with 10 mM DTT and 50 μM of the fluorogenic substrate Z-IETD-Afc for caspase-8 activity or Ac-DEVD-Afc for caspase-3/7 activity (Merck Millipore). Caspase activity was assessed in a fluorometer using excitation and emission wavelengths of 405 nm and 535 nm, respectively.

Calcein AM

After the indicated treatments, cells were incubated for 1 h at 37°C with 1 μM Calcein AM (Merck Millipore) diluted in DPBS (Thermo Fisher Scientific). Fluorescence was then assessed in a fluorometer using excitation and emission wavelengths of 485 nm and 535 nm, respectively.

qRT-PCR

After treatment, cells were harvested, washed with ice-cold PBS, and RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Next, the RNA was retrotranscribed to cDNA using the High Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific) and subjected to PCR analysis using Taqman® probes and Universal PCR Master Mix (Thermo Fisher Scientific). Taqman® probes: Fas (Hs00531110_m1), Caspase-8 (Hs01018151_m1), FADD (Hs00538709_m1), RIP1 (Hs00169407_m1), FasL (Hs00181225_m1), c-FLIP (Hs01116280_m1), Bcl-2 (Hs00608023_m1), and 18S (Hs03928990_g1).

Cell surface biotinylation

Cell surface proteins were biotinylated, isolated, and collected by using the Pierce® Cell Surface Protein Isolation Kit (Thermo Fisher Scientific), following the manufacturer's instructions, with the only exception of equalizing protein quantity and concentration before immunoprecipitation. Protein levels were determined by Western blot.

DISC immunoprecipitation

For Fas DISC analysis, cells were treated with Fc:hFasL (2.5 μg/ml) for 30 min. The cells were then washed with ice-cold PBS, harvested, and lysed in ice-cold Triton lysis buffer (NaCl 150 mM, EDTA 10 mM, Tris–HCl pH7.4 10 mM, 1% Triton™ X-100, 1x EDTA-free complete protease inhibitor cocktail (Roche, Basel, Switzerland)). After lysate clearance by centrifugation, Fc:hFasL was immunoprecipitated from the supernatant by incubation with protein G-Sepharose beads for 1 h on an orbital shaker at 4°C. Next, the beads were washed 5x with ice-cold Triton lysis buffer, and the immunocomplexes were collected with elution buffer (Citrate 0.1 M, pH2.5). The pH was adjusted by adding 1/6 neutralizing buffer (Tris HCl 1 M, pH8.5). Protein levels were determined by Western blot.

Western blot

Cells were harvested, washed with ice-cold PBS, and lysed in ice-cold Triton lysis buffer or boiling SET buffer (Tris–HCl pH7.4 10 mM, EDTA 1 mM, NaCl 150 mM, 1% SDS). Insoluble fractions were removed by centrifugation, and protein concentration of the supernatant was quantified. The cell lysates obtained (25 μg of protein) were resolved in SDS-polyacrylamide gels. Next, proteins were transferred onto PVDF Immobilon-P membranes (Merck Millipore) by electrophoresis. Membranes were blocked with 5% non-fat dry milk in 1× TBS and 0.1% Tween-20 and probed with the appropriate primary antibodies [anti-Fas (C-20), anti-FADD (S-18), anti-c-FLIP$_{S/L}$(H-202), anti-IκBα (C-21), anti-cIAP2 (H-85) (Santa Cruz, Biotechnology, Santa Cruz, CA, USA), anti-α-Tubulin (Sigma-Aldrich), anti-Bcl-2 (Dako, Agilent Technologies, Santa Clara, CA, USA), anti-Caspase-3 and anti-Caspase-8 (Cell Signaling Technologies, Beverly, MA, USA)] and the corresponding peroxidase-conjugated secondary antibodies (Sigma-Aldrich).

Plasmids

The Super-repressor IκBα (SR) cDNA was subcloned from the validated pcDNA3 expression vector [22,59] into the lentiviral pWPI expression vector. SR was expressed under the control of the constitutively active EF-1 Alpha promoter.

Lentiviral production and cell infection

Lentiviruses were produced in HEK293T cells by Lipofectamine 2000 (Thermo Fisher Scientific) co-transfection of pWPI-derived constructs, pSPAX2, and pM2G in a 3:2:1 ratio, respectively. Cells were allowed to generate lentiviruses for 48 h, after which the lentivirus-bearing medium was collected and passed through a Whatman® 45 μm filter (GE Healthcare, Little Chalfont, UK). For infection, the lentivirus-bearing medium was added to the host cells in combination with 8 μg/ml polybrene. Infection efficiency was assessed by direct counting of GFP-positive cells, and infection was repeated until an efficiency of ≥95% was reached.

Flow cytometry

After the indicated treatments, cells were detached with cell dissociation buffer (PBS, 5 mM EDTA), harvested, washed 2× with ice-cold PBS and 1× with ice-cold FACS buffer (PBS, 2% FBSi, 0.02% sodium azide), and then incubated for 30 min on ice with a PE-conjugated monoclonal antibody against Fas or its matched isotype (Becton Dickinson, Franklin Lakes, NJ, USA). Thereafter, cells were washed 2× and resuspended in ice-cold FACS buffer. Fas expression was assessed by a FACSCalibur™ flow cytometer (Becton Dickinson).

Statistical analysis

All the experiments were repeated at least three times. Values are expressed as mean ± SD. Statistical significance was determined by one-way or two-way ANOVA using GraphPad Prism v5 (GraphPad Software, La Jolla, CA, USA).

Additional files

Additional file 1: Figure S1. TNFα pre-treatment sensitizes a subset of NBLs to FasL-induced caspase-3/7 activity. The indicated cell lines were pre-treated or not for 24 h with 100 ng/ml TNFα and were left untreated (UT) or treated for 4 h with 100 ng/ml Fc:hFasL, or 1 ng/ml Fc:hFasL for Tet21N cells. Before Fc:hFasL treatment, SH-SY5Y and SK-N-BE(2) cells were treated for 24 h with 100 ng/ml interferon-γ (IFNγ) or not (UT), and an additional 24 h with 100 ng/ml TNFα or were left untreated (UT). DEVDase activity was assessed using 10 μM Ac-DEVD-Afc as substrate. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Additional file 2: Figure S2. NF-κB is functional in all NBL cell lines. **A**. Cells were left untreated (–) or were treated with 100 ng/ml TNFα for the indicated times. IκBα degradation was assessed by Western blot. **B**. Expression of the NF-κB -target genes cIAP2 and Bcl-2 were analyzed by Western blot in NBL cell lines left untreated (UT) or treated with 100 ng/ml TNFα for 24 h.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JXC, MFS, and KMOG designed the experiments. KMOG, PC, LP-F, JU, EC, JL-S, and RSM performed the laboratory work and collected the data. KMOG, JXC, and MFS analyzed and interpreted the data. KMOG, MFS, JXC, and BB-Z wrote the manuscript. The final manuscript was read and approved by all signing authors.

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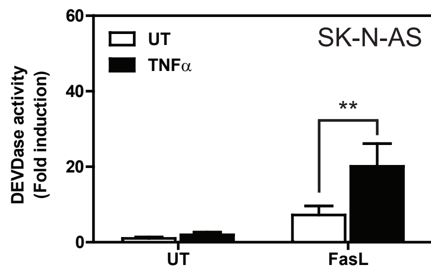

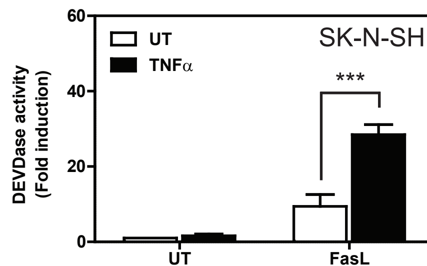

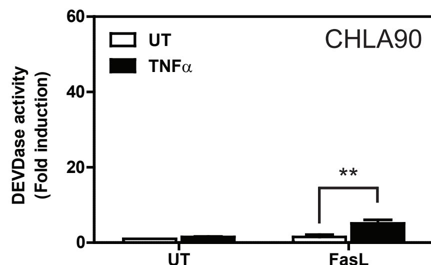

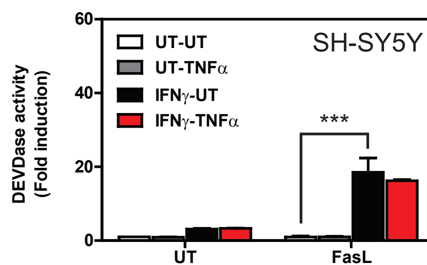

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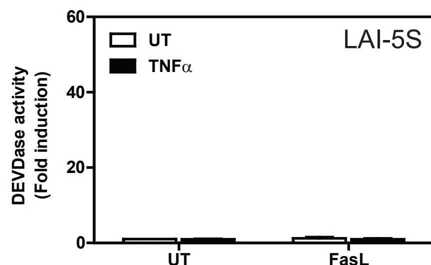
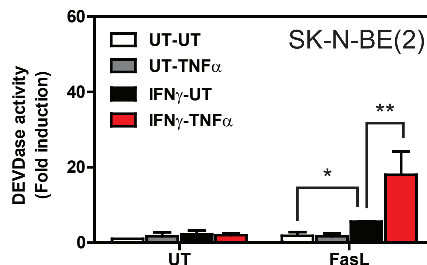
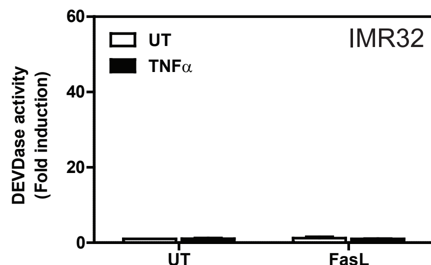
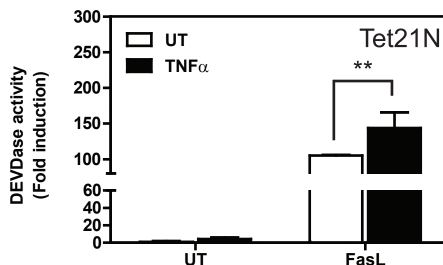
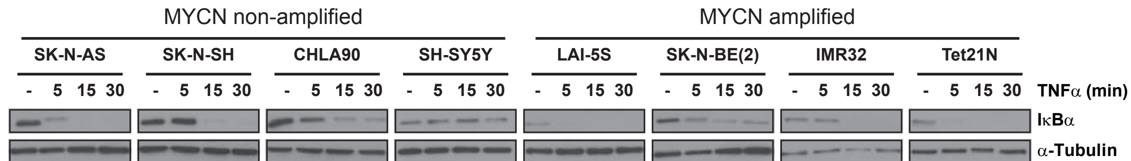


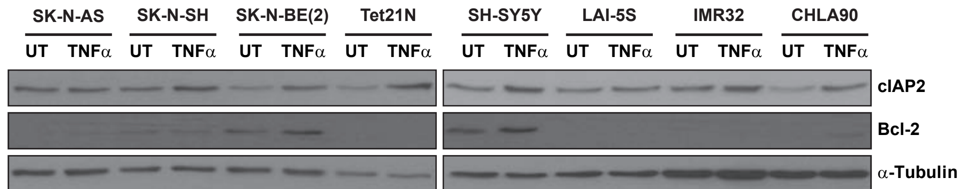






Galenkamp *et al.* Figure S1





Galenkamp *et al.* Figure S2