Activation of Survival Pathways Triggered by Death Receptors in the Nervous System

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PhD Thesis

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A mis padres

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ABBREVIATIONS

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AD	Alzheimer's Disease
AIF	Apoptosis Inducing Factor
ALS	Amyotrophic Lateral Sclerosis
APAF-1	Apoptosis Protease Activation Factor 1
APP	Amyloid Precursor Protein
ASK	Apoptosis Signal-Regulated Kinase-1
ATCC	American Tissue Culture Collection
ATP	Adenosine Tri-Phosphate
BACE	β-Secretase
BAG1	Bcl-2 Associated Anthogene
BAR	Bifunctional Apoptosis Regulator
BDNF	Brain Derived Neurotrophic Factor
BH3	BIR Homology domain 3
BIR	Baculovirus IAP Repeat
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
CHX	Cycloheximide
CMV	Cytomegalovirus
CNS	Central Nervous System
CRD	Cysteine Rich Domain
DcR	Decoy Receptors
DD	Death Domain
DED	Death Effector Domain
DIABLO	Direct IAP Binding with Low PI
DISC	Death Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle's Medium

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DMSO	Dymethilsulfoxide
DR	Death Receptors
DTT	1,4- Dithiotreitol
DUB	Deubiquitinase Domain
EAE	Experimental Autoimmune Encephalomyielitis
EDTA	Ethylene Diamine Tetra Acetic Acid
ERK	Extracellular Regulated Kinase
FADD	Fas Associated Death Domain
FAIM	Fas Apoptosis Inhibitory Protein
FAP1	Fas Associated Phosphatase
FBS	Fetal Bovine Serum
FLICE	FADD-like interleukin-1-beta-converting enzyme
FLIP	FLICE-Inhibitory Protein
Fn14	Fibroblast Growth Factor Inducible 14
FrS2	Fibroblast growth factor receptor substrate 2
GBM	Glioblastoma
GEF	Guanine Nucleotide Exchange Factor
GRB2	Protein G-related alpha2M-binding 2
GSK3β	Glycogen Synthase kinase-3β
HBS	Hepes Buffered Saline
HBSS	Hank's Balanced Salt Solution
IAP	Inhibitor of Apoptosis Proteins
ICE	Interleukin 1β-converting enzyme
IkB	Inhibitor of kB
IKK	I-kB-Kinase
JNK	Jun N-terminal kinase

LRKK2	Leucine Rich Repeat Kinase 2
LUBAC	Linear Ubiquitin Chain Assembly Complex
MAPK	Mitogen-Activated Protein Kinase
MEK	MAP/ERK Kinase
MMP	Matrix Metalloproteinase
MOMP	Mitochondria Outer Membrane Permeabilization
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	Methythiazolyldiphenyl-tetrazolium bromide
NEMO	NF-kappa-B Essential Modifier
NF-kB	Nuclear Factor Kappa B
NGF	Nerve Growth Factor
NIK	NF-kB-inducing kinase
PARP	Poly-ADP ribose polymerase
PBS	Phosphate Buffered Saline
PEA15	Phosphoprotein Enriched in Astrocytes 15 kDa
PFA	Paraformaldehyde
PI3K	Phosphatidyl-inositol-3 kinase
PKA	Protein Kinase A
PS	Phosphatidylserine
PUMA	P53 Upregulated Modulator of Apoptosis
PVDF	Polyvinylidene difluoride
RBD	Raf Binding Domain
RING	Really Interesting New Gene
RIP	Receptor Interacting Protein
SDS	Sodium Dodecyl Sulphate
SMAC	Second Mitochondria-derived Activator of Caspases
SOD1	Superoxide Dismutase-1
SODD	Silencer of Death Domain

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SOS	Son of Sevenless
Src	Rous Sarcoma oncogene
SUMO-1	Small Ubiquitin-related Modifier-1
TACE	TNF-α Converting Enzyme
TNFR	Tumor Necrosis Factor Receptor
TNFα	Tumor Necrosis Factor Alpha
TRADD	TNF Receptor Associated Death Domain
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
TRK	Tropomyosin-Related Kinase
TWEAK	TNF-Related Weak Inducer of Apoptosis
UBA	Ubiquitin Associated
XIAP	X-Linked Inhibitor of Apoptosis

ABSTRACT

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Activation of the Tumor Necrosis Factor Receptor 1 (TNFR1) by its ligand TNFα induces cell death through recruitment of a Death Inducing Signaling Complex (DISC) containing caspase-8 and the adaptor proteins FADD, TRADD, TRAF2 and RIP. This complex causes the cleavage and activation of caspase-8 that leads to apoptosis. However, TNFR1 can also trigger activation of signaling pathways related to cell survival and differentiation, such as the transcription factor NF-kB or MAPK/ERK. In fact, in most cell types it has been shown that survival signaling by TNFα needs to be abrogated in order to induce apoptotic cell death. This has been classically achieved by co-treatment with protein synthesis inhibitors such as Actinomycin D or Cycloheximide, or by direct inhibition of the NF-kB pathway through chemical inhibitors or overexpression of a non-degradable form of IkBα. A previous report in our lab revealed that Actinomycin D sensitizes PC12 cells and cortical neurons to TNFα-induced apoptosis through down-regulation of the antiapoptotic protein Bcl-X_L. However, the results showed that this effect was independent of NF-kB or other antiapoptotic proteins such as FLIP-L or IAPs. This thesis analyses the activation of survival pathways by TNFα. In particular, I have established a link between the activation of NF-kB and the MAPK/ERK pathway. I have used PC12 cells, a well-known model of neuronal cells. Inhibition of the NF-kB pathway in these cells induced a downregulation of the antiapoptotic FLIP-L. My results indicate that TNFα-induced activation of the MAPK/ERK pathway depends on FLIP-L. Furthermore, this signaling cascade does not involve the classical activation of the upstream effector Ras. However, the MAPKKK Raf-1 is necessary to induce ERK1/2 phosphorylation, thus suggesting that FLIP-L subtitutes Ras in this function. The results of this thesis also demonstrate that the MAPK/ERK pathway affords protection from TNFα-induced apoptosis, since abrogation of this signaling cascade renders PC12 sensitive to cell death induced by TNFα in a manner similar to sensitization achieved by NF-kB inhibition. Characterization of the apoptotic pathway shows the implication of JNK, which is necessary to induce cell death when NF-kB is blocked and when MAPK/ERK is inhibited. Moreover, JNK induces the upregulation of Bim, a BH3-only protein classically linked tot the induction of apoptosis.

Altogether, this thesis establishes the relevance of the MAPK/ERK pathway as a negative regulator of TNFα-induced apoptosis. Importantly, the results obtained reveal that NF-kB is crucial for the induction of ERK1/2 phosphorylation by TNFα, and that this pathways are linked through the antiapoptotic protein FLIP-L.

RESUMEN

La activación del Receptor del Factor de Necrosis Tumoral de tipo 1 (TNFR1) por parte de su ligando específico, TNFα, induce la muerte celular mediante la formación de un complejo de señalización inductor de muerte (DISC) que contiene caspasa-8 junto con las proteína adaptadoras FADD, TRADD, TRAF2, y RIP. Este complejo provoca la activación de caspasa-8 que lleva a la apoptosis. Sin embargo, TNFR1 también puede iniciar la activació de vías de señalización relacionadas con la superviviencia celular y la diferenciación, tales como el factor de transcripción NF-kB o la vía MAPK/ERK. De hecho, en la mayoría de tipos celulares se ha demostrado que la señalización de supervivencia iniciada por TNFα debe ser bloqueada para inducir la muerte celular apoptótica. Esto se ha conseguido clásicamente mediante el co-tratamento con inhibidores de la síntesis proteíca como Actinomicina D o Cicloheximida, o mediante la inhibición directa de la vía de NF-kB a través de inhibidores químicos o la sobre-expresión de una forma no degradable de IkBα. Un trabajo previo de nuestro própio laboratorio reveló que la Actinomicina D sensibiliza las células PC12 y las neuronas corticales a la apoptosis inducida por TNFα a través de una disminución en la expression de la proteína antiapoptótica Bcl-X_L. No obstante, les resultados demostraron que este efecto era independiente de NF-kB o de otras proteínas antiapoptóticas como FLIP-L o las IAPs. En esta tesis se analiza la activación de vías de supervivencia por parte de TNFα. En particular, he establecido una relación entre la activació de NF-kB y la vía de MAPK/ERK. He utilizado la línea celular PC12, un modelo bien establecido de células neuronales. La inhibición de la vía de NF-kB en estas células indujo la reducción de la expression de FLIP-L. Mis resultados indican que la activación de la vía MAPK/ERK depende de FLIP-L. Además, esta cascada de señalización no implica la clásica activación de Ras, la proteina efectora que actúa por encima en la vía. Sin embargo, la MAPKKK Raf-1 es necesaria para inducir la fosforilación de ERK1/2, lo que sugiere que FLIP-L podria substituir a Ras en su función. Los resultados de esta tesis también demuestran que la vía MAPK/ERK protege a las células de la apoptosis inducida por TNFα, dado que la inhibición de esta señalización sensibiliza las células PC12 a la muerte celular inducida por TNFα de forma similar a la sensibilización conseguida mediante la inhibición de NF-kB. La caracterización de esta señalización apoptótica muestra la implicación de JNK, que es necesaria para inducer la muerte celular cuando NF-kB está bloqueado y cuando se inhibe MAPK/ERK. Es más, JNK induce un aumento en la expresión de Bim, una proteina BH3-only relacionada clásicamente con la apotosis.

En resumen, esta tesis establece la relevancia de la vía MAPK/ERK como regulador negativo de la apoptosis inducida por TNFα. Es importante destacar que los resultados obtenidos revelan que NF-kB es crucial para la inducción de la fosforilación de ERK1/2 por TNFα, y que estas vías de señalización estan relacionadas a través de la proteína antiapoptótica FLIP-L.

RESUM

L'activació del Receptor del Factor de Necrosi Tumoral de tipus 1 (TNFR1) per part del seu lligand específic, TNFα, indueix la mort cel·lular mitjançant la formació d'un complex de senyalització inductor de mort (DISC) que conté la caspasa-8 juntament amb les proteïnes adaptadores FADD, TRADD, TRAF2, I RIP. Aquest complex provoca l'activació de caspasa-8 que porta a l'apoptosi. No obstant això, TNFR1 també pot induir l'activació de vies de señalització relacionades mab la supervivència i la diferenciació cel·lular, tals com el factor de transcripció NF-kB o la vía MAPK/ERK. De fet, en la majoria de tipus cel·lulars s'ha demostrat que la senyalització de la supervivència iniciada per TNFα ha de ser bloquejada per tal d'induir la mort cel·lular apoptòtica. Això s'ha aconseguit clàssicament mitjançant el co-tractament amb inhibidors de la síntesi proteica com l'Actinomicina D o la Cicloheximida, o a través de la inhibició directa de la via NF-kB amb inhibidors químics o la sobreexpresió d'una forma no degradable d'IkBα. Un treball previ del nostre propi laboratory va revelar que l'Actinomicina D sensibilitza les cèl·lules PC12 i les neurones corticals a l'apoptosi induïda per TNFα a través d'una reducció en l'expessió de la proteïna antiapoptòtica Bcl-X_L. No obstant això, els resultats demostraven que aquest efecte era independent de NF-kB o d'altres proteïnes antiapoptòtiques com FLIP-L o les IAPs. En aquesta tesi s'analitza l'activació de vies de supervivència per part de TNFα. En particular, he establert una relació entre l'activació de NF-kB i la via de MAPK/ERK. He utilitzat la línia cel·lular PC12, un model ben establert de les cèl·lues neuronals. La inhibició de la via de NF-kB en aquestes cèl·lules va induir la reducción en l'expressió de FLIP-L. Els meus resultats indiquen que l'activació de la via MAPK/ERK depèn de FLIP-L. A més, aquesta cascada de señalització no implica l'activació clàssica de Ras, la proteïna efectora que actua per sobre en la via. Tanmateix, la MAPKKK Raf-1 és necessària per tal d'induir la fosforilació d'ERK1/2, suggerint que FLIP-L podria substituir Ras en la seva funció. Els resultats d'aquesta tesi també demostren que la via MAPK/ERK protegeix les cèl·lules de l'apoptosi induïda per TNFα, donat que la inhibició d'aquesta señalització sensibilitza les cèl·lules PC12 a la mort cel·lular induïda per TNFα, i ho fa de manera similar a la sensibilització aconseguida mitjançant la inhibició de NF-kB. La caracterització d'aquesta señalització apoptòtica mostra la implicació de JNK, que és necessària per induir la mort cel·lular quan NF-kB es troba bloquejat i quan s'inhibeix MAPK/ERK. És més, JNK inudeix un increment en l'expressió de Bim, una proteïna BH3-only relacionada clàssicament amb l'apoptosi.

En resum, aquesta tesi estableix la rellevància de la via MAPK/ERK com a regulador negatiu de l'apoptosi induïda per TNFα. És important destacar que els resultats ontinguts revelen que NF-kB és crucial per a la inducció de la fosforilació d'ERK1/2 per TNFα, i que aquestes vies de senyalització estan relacionades a través de la proteïna antiapoptòtica FLIP-L.

1.INTRODUCTION

1. Introduction

1.1 Apoptosis, Historical Background and Relevance

Apoptosis is an essential physiological process with a critical role in controlling the number of cells during development and throughout the lifetime of an organism by removal of cells at the appropriate time (Zimmermann et al., 2001). However, apoptosis is also involved in many pathological conditions such as acute neurological injuries, neurodegenerative diseases, cardiovascular diseases, immunological diseases, acquired immunodeficiency syndrome (AIDS), and cancer.

The term apoptosis was first coined by Kerr, Wyllie and Currie in 1972, to define the morphological features of a type of cell death observed in hepatocytes but consistent in other tissues and cell types, and that differed from those observed in necrotic cell death (Kerr et al., 1972). These observations suggested an underlying conserved genetic program controlling the removal of certain cells at specific time-points as an essential requirement for the correct development of the organism (Hengartner, 2000).

For a long time, apoptotic cell death has been defined by morphological changes in the cell after exposure to an apoptotic stimulus. The main morphological features characterizing apoptosis are cell shrinkage, chromatin condensation, nuclear fragmentation, formation of irregular bulges in the plasma membrane called blebbing, loss of adhesion and rounding in adherent cells, and the formation of the apoptotic bodies, without structural alterations in the organelles (Fig. 1). These apoptotic bodies are later engulfed by macrophages through a process called phagocytosis (Arends and Wyllie, 1991; Cohen et al., 1992; 1994).

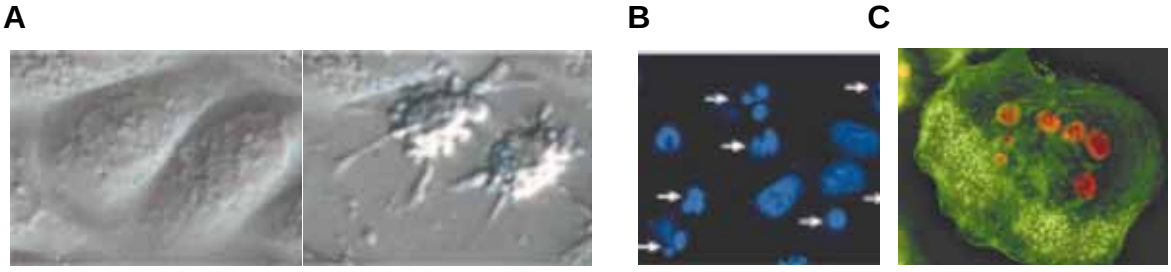


Figure 1. Hallmarks of apoptotic cell death. **A,** After an apoptotic stimulus, cells (left panel) shrink, the plasma membrane form irregular bulges called blebs, cells lose adhesion to the matrice and detach (right panel). **B,** Hoechst 33258 staining of apoptotic cells show fragmented nuclei and condensed chromatin. **C,** Apoptotic bodies stained with 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester (orange) are engulfed by macrophages (green). (Fadok et al., 2000)

The morphological characteristics of apoptosis are the consequence of biochemical events that take place during the process of apoptosis and that lead to the dismantling and elimination of the cell. The main biochemical markers of apoptosis are:

- Internucleosomal cleavage of DNA. An agarose gel analysis of the DNA of cells undergoing apoptosis shows a characteristic “ladder” pattern of fragmented DNA, with an interval between fragments of about 140 nucleotides, approximately the size of the DNA wrapped up around the nucleosome octamer (Cohen et al., 1994).

- Phosphatidylserine (PS) externalization. PS is a phospholipid that is located in the inner leaflet of the cell membrane bilayer of living cells. During apoptosis, PS flips out and is found in the external part of the plasmic membrane. It can be specifically detected by staining with Annexin V, a fluorescent molecule that attaches to PS and cannot penetrate the cell membrane, therefore will only stain cells undergoing apoptosis. Physiologically, PS externalization allows apoptotic cells to be recognized by macrophages and therefore phagocytosed and eliminated from the organism (Fadok et al., 1992; Vermes et al., 1995).

- Detection of proteolytic cleavage of intracellular substrates. Proteases are the main effectors of apoptosis. Among these, the aspartate-specific cysteine proteases (caspases) are the major components controlling cell death (Martin and Green, 1995). There are different caspases with specific roles at each step of the apoptotic process, which will be discussed below.

1.2 Molecular Components of Apoptosis

Apoptosis has been shown to be a strictly regulated process, controlled by a specific set of proteins. Before describing in detail the exact biochemical mechanisms that lead to the programmed death of the cell, it is important to begin describing its main molecular effectors.

The true molecular nature of apoptosis was first described in a model of the nematode *Caenorhabditis elegans.* This organism is relatively simple, presenting 1090 cells as an embryo. The uniqueness of this model is that during development every single cell lineage can be traced, therefore, the fate of each cell can be determined. During the development of *C. elegans* 131 of the cells undergo an apoptotic process, leaving the adult with 959 cells (Sulston and Horvitz, 1977). A genetic screening allowed the identification of genes responsible for the regulation, execution, and resolution of apoptosis. *Ced-3, ced-4 and egl-1* are required for cell death, while *ced-9* plays a role in the inhibition of apoptosis (Ellis and Horvitz, 1986).

CED-3 is an aspartate-specific cysteine protease, homologous to caspases found in mammals (Yuan et al., 1993), of which there are 14 discovered so far. CED-4 is homologous to mammalian Apoptosis Protease-Activator Factor-1 (Apaf-1) (Zou et al., 1997), and it has a role as an adaptor protein needed for CED-3 function. CED-4 is, in turn, regulated by the product of the *ced-9* gene, which acts as a suppressor of cell death. CED-9 is homologous to the antiapoptotic members of the mammalian Bcl-2 family proteins (Hengartner and Horvitz, 1994; Xue and Horvitz, 1997). The function of EGL-1 is to remove the inhibition that CED-9 exerts on CED-4, thus allowing for the apoptotic cell death to occur. EGL-1 homologues in mammals are the BH3-only pro-apoptotic members of the Bcl-2 family, (Conradt and Horvitz, 1998; Nehme and Conradt, 2009). This basic molecular pathway is evolutionary conserved, with few alterations, adding some improvements for a more efficient control of the process. All of its components are conserved in other organisms, such as *Drosophila melanogaster* (Fig.2) (Fuchs and Steller, 2011). Their homologues found in mammals will be analyzed with more details in the following paragraphs.

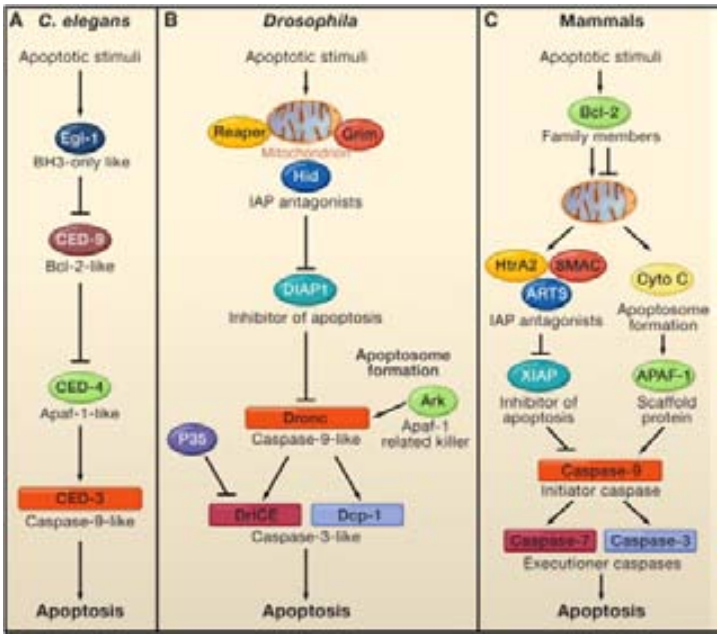


Figure 2. Apoptotic machinery in *C. elegans* and homologues in *D. melanogaster* and mammals. Egl-1 removes inhibition of Ced-4 by Ced-9. This in turn activates Ced-3, which induces ireversible changes that lead to cell death. Source: (Fuchs et al., 2011)

1.2.1 Caspases

The central components of the apoptotic response are a conserved family of cysteine proteases with a predilection for breaking bonds at aspartate residues called caspases (Cysteine-Aspartate Proteases) (Shi, 2002). Their main function is to irreversibly commit a cell to die. Although the first caspase was identified in humans (Interleukin-1β-converting enzyme, ICE, or caspase-1) (Cerretti et al., 1992; Thornberry et al., 1992), their importance in the apoptotic process was first described for the homologous gene *ced-3*, in the nematode model *C. elegans*. Since then, 14 distinct caspases have been identified in mammals, of which 11 are found in humans (Riedl and Shi, 2004). All caspases share a number of common features (Earnshaw et al., 1999). These include:

- All caspases are synthesized as catalytically inactive zymogens. They become active after being cleaved by another protease or by auto-activation.
- Caspases possess substrate cleavage specificity after an aspartate residue, unique among mammalian proteases.
- Caspases can regulate their own activity through positive or negative feedback or through amplification loops and also by their interaction with specific inhibitors.

Apoptotic caspases are generally divided into two classes: initiator caspases, which include caspases -2, -8, -9, and -10 in mammals and have homologous forms in Drosophila and other organisms; and executioner caspases, including caspase-3, and -7 in mammals and also with homologous forms in other organisms. Ced-3 is the only apoptotic caspase in *C. elegans* and exerts functions of both initiator and executioner caspases (Fig. 3) (Shi, 2002).

Besides the general characteristics, caspases show structural features that provide them with high specificity and strict regulation (Fuentes-Prior and Salvesen, 2004). Inactive zymogens are composed of an N-terminal prodomain with a size between 3 and 24 kDa, a large subunit in the middle of the enzyme, of around 17 to 20 kDa (p20), and a small subunit in the C-terminal, with a size between 10 to 13 kDa (Walker et al., 1994). Initiator caspases possess long prodomains containing one of two characteristic protein-protein interaction motifs: the death effector domain (DED) (Caspase-8 and -10) (Valmiki and Ramos, 2009) and the caspase activation and recruitment domain (CARD) (Caspase-2, and -9) (Chou et al., 1998; Bouchier-Hayes and Martin, 2002) . These domains provide the basis for the interaction of initiator caspases with upstream adaptor molecules. As for executioner caspases, which perform downstream execution steps of apoptosis by cleaving multiple substrates, they are processed and activated by upstream initiator caspases. This subgroup is characterized by the presence of a short prodomain.

The prodomain of both initiator and effector caspases contains the active site cysteine within a conserved QACXG motif. An aspartate cleavage site separates the prodomain from the large subunit (p20) and an interdomain containing one or two aspartate cleavage sites separates the large and small (p10) subunits. The presence of Asp residues is consistent with the ability of caspases to autoactivate or be activated by other caspases, as a part of an amplification cascade (Nicholson, 1999) (Zimmermann et al., 2001).

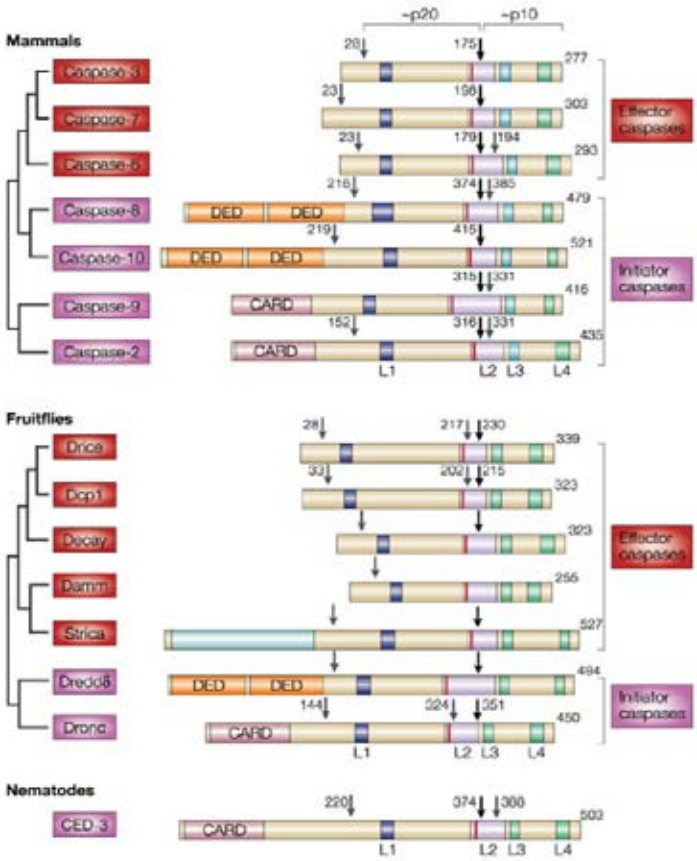


Figure 3. Apoptotic caspases in mammals, fruitflies (*D. melanogaster)* and nematodes (*C. elegans)*. Effector/executioner and initiator caspases are shown in red and purple. Position of the first activating cleavage, between the large (p20) ans small (p10) subunits, is marked by a black arrow. L1-L4 indicate the surface loops that shape the catalytic groove. Grey arrows show other cleavage sites thought to modulate caspase activity. The cysteine catalytic residue is indicated by a red line at the beginning of L2. Source: (Riedl et al., 2004).

1.2.2 Caspase Activation

It was thought initially that all caspases needed to undergo proteolysis in order to become active. In the last years it has become clear that this is a minor mechanism, belonging mainly to effector caspases (Fuentes-Prior and Salvesen, 2004).

The main activation mechanism for initiator caspases is dimerization. To facilitate dimerization initiator caspases are recruited to oligomeric platforms formed after an apoptotic stimulus. Adaptor molecules bind specifically to initiator caspases through their DED or CARD domains present in the prodomains of the catalytically inactive form. Binding of initiator caspases to adaptor proteins in activation platforms

forces a local increase in the concentration of these caspases, generating activity by proximity-induced dimerization (Riedl and Salvesen, 2007; Pop and Salvesen, 2009).

Effector caspases are found as inactive dimers that need to be cleaved in order to become activated. Dimerization occurs shortly after synthesis and the zymogen is kept inactive by a short linker separating the large from the small subunits (Fig. 4). Proteolytic cleavage favors rearrangement of the caspase structure, allowing formation of the catalytic site (Thornberry, 1998).

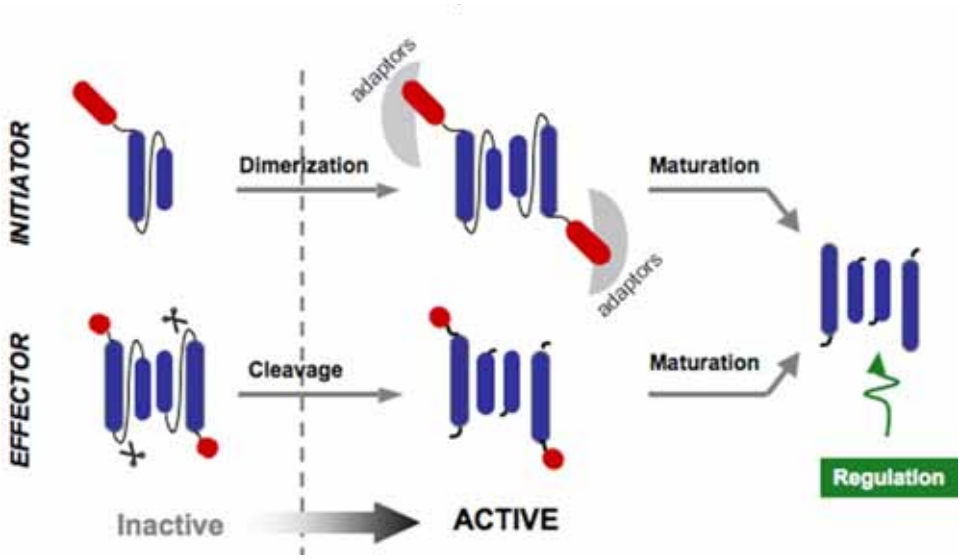


Figure 4. Activation mechanisms of caspases. Initiators are monomers that are activated by prodomain-mediated dimerization. Executioners are dimers that are activated by cleavage of intersubunit linkers. Following activation, additional proteolytic events lead to maturation of caspases to more stable forms that are prone to regulation. (Pop, Salvesen, 2009)

1.2.3 Substrate Specificity of Caspases

Caspases recognize tetrapeptide specific sequences within the target protein. They require an Asp residue in the first position (P_1), but the aminoacid found in P_4 determines a classification into three groups for all of the 14 caspases found in mammals (Thornberry et al., 1997) :

- Group I includes caspases-1, -4, -5, and -13 and has more affinity for hydrophobic residues. The optimal sequence is $W^{P4}E^{P3}H^{P2}D^{P1}$.

- Group II, caspases-2, -3, -7 and -14; show maximal activity when P_4 is occupied by an Aspartate. The preferred sequence is $D^{P4}E^{P3}X^{P2}D^{P1}$, where X may be V, T, or H.
- Group III, caspases-6, -8, -9, and -10. May have various aminoacids in P_4, but branched chain aminoacids are preferred. The optimal sequence is $(V,L)^{P4}E^{P3}H^{P2}D^{P1}$.

Interestingly, processing sites between the large and small subunits of all initiator caspases conform to their own substrate preferences, consistent with their ability to auto-activate. Effector caspases, on the other hand have processing sites that fit the preferences of caspases for the third group, which is consistent with being downstream activation targets of initiator caspases.

As it has been stated, caspases are the main effectors of the apoptotic process, and the changes they provoke within the dying cell are irreversible (Hengartner, 2000; Riedl and Shi, 2004). Therefore it is important to have a strict regulation of the apoptotic pathway through different checkpoints that assure a controlled development of apoptosis.

1.2.4 Inhibitor of Apoptosis Proteins (IAPs)

IAPs are antiapoptotic regulators that block cell death in response to diverse stimuli such as death receptors, growth factor withdrawal, radiation, viral infection, and genotoxic damage. They were first identified in baculovirus as a system to allow viral propagation by inhibiting an apoptotic response in host cells (Crook et al., 1993; Harvey et al., 1997; Uren et al., 1998). Numerous cellular homologues have been identified since, in a range of species from *Drosophila* to vertebrates. IAPs were thought to function primarily by regulating caspases (Deveraux et al., 1997, Roy et al., 1997). However, IAPs regulate many other cell processes, such as ubiquitin-dependent signaling events regulating the Nuclear Factor kB (NF-kB) transcription factor, which drives the expression of genes important for inflammation, immunity, cell migration, and cell survival (Varfolomeev et al., 2008; Mahoney et al., 2008; Leulier et al., 2006; Bertrand et al., 2009; Gesellchen et al., 2005; Paquette et al., 2010). Alterations of IAPs are frequently found in many types of human cancer and are associated with chemoresistance, disease progression, and poor prognosis (Hunter et al., 2007; LaCasse et al., 2008).

The defining feature of IAP proteins is the presence of the Baculovirus IAP Repeat (BIR) domain, an approximately 70 aminoacid zinc binding fold that mediates protein-protein interactions and is essential for the antiapoptotic potential of this family of proteins. IAPs contain between one and three of these domains (Birnbaum et al., 1994; Hinds et al., 1999; Silke and Vaux, 2001). There are eight human IAPs described so far: Neuronal IAP (NIAP), X-linked Inhibitor of Apoptosis Protein (XIAP), cellular IAP1 (cIAP1), cellular IAP2 (cIAP2), Testis-specific IAP (TsIAP), BIR-containing Ubiquitin Conjugating Enzyme (BRUCE), Survivin, and Livin (Roy et al., 1995; Ambrosini et al., 1997; Deveraux et al., 1998). Of these, XIAP, cIAP1, and cIAP2 are the most relevant in death receptor signaling and will be the ones treated in this introduction. XIAP, cIAP1, and cIAP2 each contain three BIR domains in their N-terminal portion. They also harbor other domains, such as a C-terminal Really Interesting New Gene (RING) domain, which provides them with ubiquitin E3 ligase activity (Yang, 2000). Moreover, they carry a Ub-associated domain (UBA), which allows them to interact with ubiquitylated proteins. cIAP1 and cIAP2 also have a caspase-recruitment CARD domain (Fig. 5) (Gyrd-Hansen et al., 2008). Although the relevance of the CARD domain has remained elusive, a recent work describes that the CARD domain in IAPs acts as a regulatory mechanism to prevent E3 activity by blocking RING dimerization, E2 binding, and E2 activation and is required to suppress cell proliferation and migration (Blankenship et al., 2009; Lopez et al., 2011; reviewed in Darding and Meier, 2012).

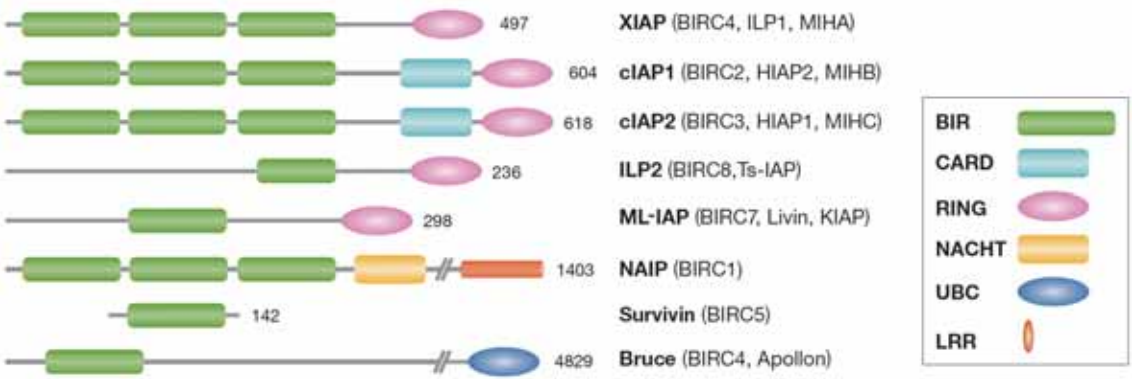


Figure 5. Human Inhibitor of Apoptosis Proteins. BIR domains provide interaction with proteins such as TRAF2, caspases, and IAP antagonists. The UBC domain binds to ubiquitin chains. CARD domain has the function of shutting down the E3 ligase activity of cIAPs. RING domains provide IAPs with E3 ligase activity It also functions as a dimerization interface and docking site for E2s. Adapted from (Eckelman et al., 2006).

Although XIAP, cIAP1, and cIAP2 are all able to bind caspases, XIAP is the only IAP that has been shown to directly inhibit them. XIAP can directly bind and inhibit caspases-3, -7, and -9 (Silke et al., 2001; Scott et al., 2005; Eckelman and Salvesen, 2006). Overexpression of XIAP efficiently inhibits caspase activation and apoptosis stimulated by the intrinsic or the extrinsic apoptotic pathways (Deveraux et al., 1997; Trapp et al., 2003; Wilkinson et al., 2004). Residues in the BIR domain 1 and 2 of XIAP bind to the active pocket of caspase-3 and -7, occluding substrate entry and resulting in its catalytic activity inhibition (Huang et al., 2001; Riedl et al., 2001; Silke et al., 2001; Suzuki et al., 2001), whereas BIR3 has been shown to bind caspase-9. The BIR3 domain of XIAP binds to the homodimerization surface of caspase-9, interfering dimerization necessary for its activation (Srinivasula et al., 2001; Shiozaki et al., 2003). However, BIR domains in cIAP1 and 2 are unable to inhibit caspases due to critical substitutions of residues that are essential for caspase inhibition in XIAP. Ubiquitylation has been implicated in inhibition of caspase mediated by cIAPs. Mutations that disrupt or eliminate the RING domain of DIAP1, the cIAP homologue in *drosophila* behave as loss-of-function alleles and die as embryos due to massive cell death (Wilson et al., 2002; Bader and Steller, 2009). Mammalian cIAP1 and cIAP2 have been reported to ubiquitylate caspase-3 and caspase-7, targeting them for monoubiquitylation or polyubiquitylation. Although the function of monoubiquitylation is not clear, polyubiquitylation targets caspases for degradation and non-degradative inactivation (Huang et al., 2000; Choi et al., 2009).

The recent development of pharmacological inhibitors of cIAPs, termed Smac Mimetics (SM) has helped to understand the role of cIAPs in normal physiology and in cancer (Petersen et al., 2010; Dynek and Vucic, 2010). SM mimic the N-terminal, IAP binding motif of the Second Mitochondria-derived Activator of Caspases (Smac, also known as DIABLO), a mitochondrial protein released during the intrinsic pathway of apoptosis and related to inhibition of IAPs (Vaux and Silke, 2003). These compounds bind selectively to the BIR2 and BIR3 domains, and exert their most prominent effect on cIAP1 and cIAP2. Binding of SM causes a conformational change allowing RING dimerization and E2 activation. This results in autoubiquitylation and proteasomal degradation of cIAP1 and 2 (Feltham et al., 2011; Darding and Meier, 2012). Inhibitors of IAPs sensitize cells to apoptosis induced by TNFα, overcoming resistance observed in most cell types and suggesting novel uses of these compounds in cancer therapies.

Besides their ability to inhibit caspases, probably the most important contribution of IAPs to cell survival and tumorigenesis is through the regulation of

ubiquitin-dependent activation of NF-kB and innate immune responses (Bauler et al., 2008; Bertrand et al., 2008; Gyrd-Hansen et al., 2008b; Krieg et al., 2009). Transcription factors of the NF-κB and Rel family are activated in response to receptor stimulation and various intracellular stressors, including DNA damage. IAPs positively regulate NF-kB signaling through non-degradative ubiquitylation of components within the pathway, which allow the formation of signaling platforms leading to activation of NF-kB (Bianchi and Meier, 2009). Interestingly, NF-kB reportedly induces expression of cIAP1, cIAP2, and XIAP, thereby promoting its activation in a positive feedback loop (Jin et al., 2009). The NF-kB pathway, as well as the role of IAP proteins in its regulation will be explained in more detail in the section corresponding to NF-kB.

1.2.5 Bcl-2 Family Proteins

The founder member of the Bcl-2 (B-cell lymphoma 2) family of proteins was first identified at the (t14;18) chromosome translocation breakpoint in B-cell follicular lymphomas (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). This translocation causes a relocation of the immunoglobulin heavy chain gene, joining it to a gene called *bcl-2*. Interestingly, instead of promoting cell proliferation, like other known oncogenes of that time, expression of Bcl-2 was able to block apoptotic cell death (Vaux et al., 1988). Thus, *bcl-2* was identified as the first member of a new category of oncogenes, the regulators of cell death.

Nowadays, Bcl-2 gives name to an entire family of proteins, with 19 members identified so far. Bcl-2 family members are important sensors that receive signals about intra- and extracellular conditions transmitted through signal transduction pathways to regulate downstream cell death effectors. Bcl-2 proteins are classified into two major groups: anti-apoptotic members and pro-apoptotic members (Cory et al., 2003). All members of this family possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1-BH4) (Adams, 1998; Gross et al., 1999). They are divided into three groups, according to their structure and function within the apoptotic-signaling pathway (Fig. 6).

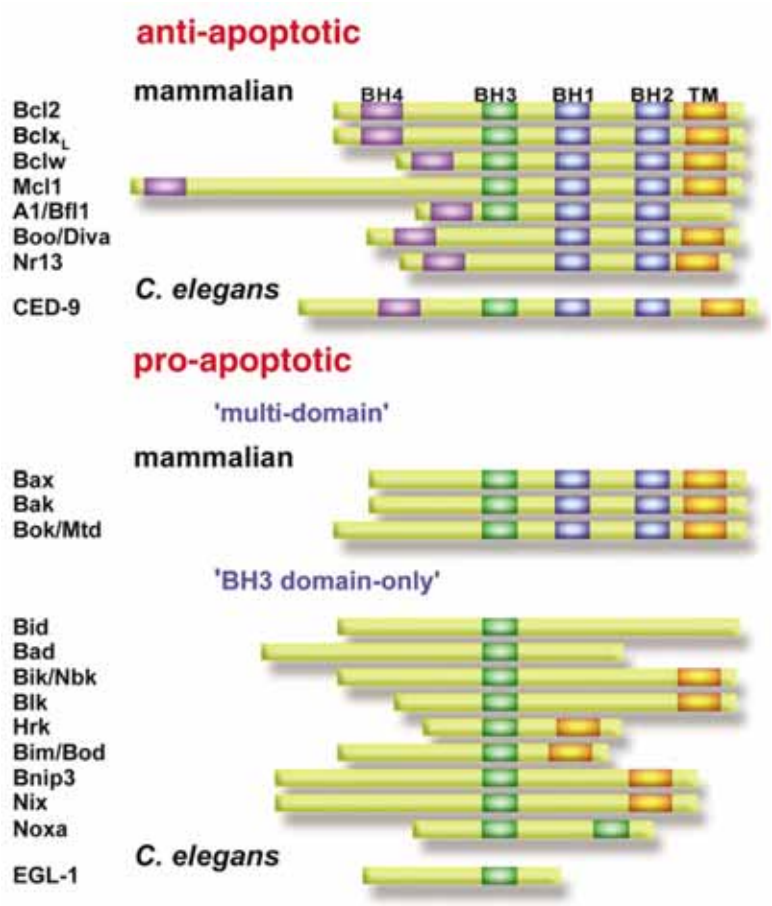


Figure 6. Summary of anti- and pro-apoptotic Bcl-2 family proteins. Bcl-2 homology (BH) domains are denoted by colored boxes. Some members also possess a carboxy-terminal hydrophobic transmembrane (TM) domain. In general, anti-apoptotic Bcl-2 family members display sequence conservation of all four BH domains. Pro-apoptotic members can be assigned to two subsets based on sequence conservation: the more fully conserved 'multi-domain' members and a divergent subset of BH3-domain only members. Adapted from (Ranger et al., 2001).

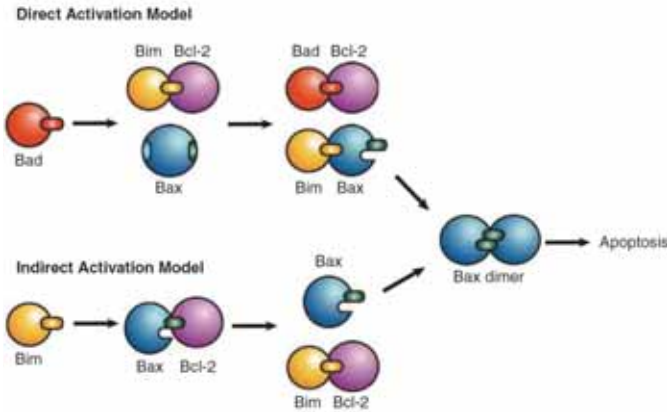
Multidomain proteins compose the first group; they possess all four BH domains and have anti apoptotic activity (Hengartner, 2000). Antiapoptotic members include Bcl-2, Bcl-x_L, A1/Bfl-1, Bcl-w, Mcl-1, Boo/Diva, Nr13 and the homologous form Ced-9 in *C. elegans* (Petros et al., 2001; Hinds et al., 2003, Muchmore et al., 1996). Proapoptotic members of the Bcl-2 family are grouped into two subfamilies, the multidomain members (Bax, Bak, Bok/Mtd) and the BH3-only proteins (Bid, Bad, Bik/Nbk, Blk, Hrk, Bim/Bod, Bnip3, Nix, Noxa, PUMA, and the *C. Elegans* homolog EGL-1). Bax and Bak possess BH1, BH2, and BH3 domains. In contrast, the rest of the proapoptotic proteins have only homology with the BH3 domain (Suzuki et al., 2000), reviewed in (Chipuk et al., 2010).

The main role of Bcl-2 family proteins is to regulate the induction of mitochondrial outer membrane permeabilization (MOMP) that takes place during the intrinsic pathway of apoptosis (Yang, 1997, Nguyen et al., 1993; Wolter et al., 1997; Gross et al., 1998; 1999). The dynamics of this regulation are still controversial, since the interplay between these family members remains elusive despite comprehensive research conducted in the past few years. Two main models have been developed to explain the interactions between different Bcl-2 family members leading to permeabilization of the mitochondrial membrane. Each of these models has been supported by two main groups of researchers, one coming from the USA, represented mainly by the group of Stanley Korsmeyer and the other from Australia, headed by DL Vaux and A. Strasser and his collaborators. Both models agree in the fact that proapoptotic multidomain members of the Bcl-2 family protein Bax and Bak are the final effectors of MOMP, since cells deficient in *bax* and *bak*, but not cells lacking only one of the two, are resistant to induction of MOMP (Wei, 2001). In its inactive conformation, Bax is localized to the cytosol and, upon apoptotic stimulation it translocates to the mitochondrial membrane by a mechanism not clearly understood (Nechushtan et al., 1999). Bak, on the other hand, is readily located to the mitochondrial membrane even in its inactive conformation. Upon activation Bax and Bak change conformation, insert into the outer membrane of the mitochondria, oligomerize, and induce its permeabilization (reviewed in (Chipuk et al., 2010)). However, the two models significantly differ as of the way in which Bax and Bak become activated.

The “direct-activation model”, postulated by Korsmeyer and his collaborators, suggests that a set of BH3-only proteins, named “activators”, directly engage Bax and Bak, inducing conformational changes which render them active and thus capable of inducing permeabilization at the mitochondrial membrane (Letai et al., 2002). According to this model, Bcl-2 subfamilies regulate MOMP in a hierarchical manner (Kim et al., 2006). Antiapoptotic Bcl-2 proteins Bcl-2, Bcl-X_L and Mcl-1 sequester “activator” BH3-only proteins Bid, Bim, and PUMA in stable complexes in the cytosol. After an apoptotic stimulus, a different subset of BH3-only proteins, including BAD, NOXA, BMF, BIK/BLK, and HRK/DP5 displace Bcl-2 antiapoptotic proteins, thus releasing Bid, Bim and PUMA to activate and induce oligomerization of Bax and Bak at the mitochondrial membrane, which leads to its permeabilization. Research has revealed that the basal conformation of Bax renders it as an inactive monomer in the cytosol by interaction of its α1 helix that keeps its α9 helix in the dimerization pocket. Activator BH3 only proteins expose the α1 helix, resulting in disengagement of the α9

helix and thus mitochondrial insertion. Activator BH3-only proteins remain associated to the BH1 domain of Bax to drive homo-oligomerization (Kim et al., 2006). Further supporting this model, Kim et al. have demonstrated that Bid, Bim, and PUMA are essential for the activation of Bax and Bak-dependent cell death, since genetic deletion of *bid, bim,* and *puma* prevented homo-oligomerization of Bax and Bak. Moreover, *bid, bim* and *puma* triple-knockout mice show the same developmental defects associated to *bax* and *bak* double-knockout, such as persistent digital webs and imperforate vaginas (Kim et al., 2009; Ren et al., 2010).

On the other hand, the "indirect activation" or "neutralization model", supported by Strasser and his collaborators, stipulates that Bax and Bak activation is a spontaneous event and, therefore, neutralization of the full repertoire of antiapoptotic Bcl-2 proteins by BH3-only proteins is sufficient to induce Bak and Bax oligomerization. As opposed to the results obtained by supporters of the "direct activation model", they have shown that Bak is sequestered by Mcl-1 and Bcl-X_L. BH3-only proteins such as Noxa and Bad can engage antiapoptotic proteins and displace Bak which can now induce cell death (Willis et al., 2005). Moreover, they have demonstrated that even in the absence of Bid and Bim and reduced expression of PUMA, Bax and Bak can induce apoptosis without engaging to BH3-only proteins (Willis et al., 2007). Strasser et al. generated a Bid knock out mouse in order to study the relevance of this protein in apoptosis induced by DNA damage and replicative stress. Results showed that in various cell types from the knock out mice cells undergo apoptosis in a manner indistinguishable from the wild-type mice, therefore concluding that Bid is dispensable at least in this model of cell-death induction (Kaufmann et al., 2007) (Fig. 7).



Since none of these

Figure 7. Bcl-2 family proteins can activate Bax and Bak through two different models. **Direct activation model** suggests a direct interaction of BH3-only proteins with Bax and Back. In the **indirect activation model,** the role BH3-only proteins is blocking Bcl-2 antiapoptotic proteins interaction with Bax and Bak. Adapted from (Giam et al., 2008)

models can fully explain all the results obtained, a third model has been proposed which integrates the first two. The “embedding together” model proposes that prosurvival proteins promote survival both by inhibiting BH3-only proteins, as in the direct model, as well as Bax/Bak, as in the indirect model. According to this model, membrane permeabilization only takes place when Bax/Bak insert multiple sequences into the mitochondrial outer membrane. Bax undergoes a conformational change upon membrane activation and a second change triggered by truncated Bid or other activators. This activated integral form of Bax then recruits more cytosolic Bax in the process of homo-oligomerization. Pro-survival proteins, such as Bcl-xL, are proposed to perform two functions: sequester the activators and also neutralize membrane-bound Bax to prevent them from homo-oligomerizing, functioning as a dominant-negative Bax molecule (Leber et al., 2007; 2010).

1.3 Signaling Pathways of Apoptosis

Two main pathways have been described for the induction of apoptotic cell death in mammals. The **intrinsic** pathway is triggered by signals within the cell that converge on the mitochondria and the formation of a death-inducing complex called the apoptosome. The **extrinsic** pathway, on the other hand, is triggered by stimulus from outside the cell and the signal is transmitted by the so-called Death Receptors. However, these two signaling pathways share some features, such as activation of effector caspases, and some events have been identified which show a crosstalk between both apoptotic mechanisms (Fig. 8)

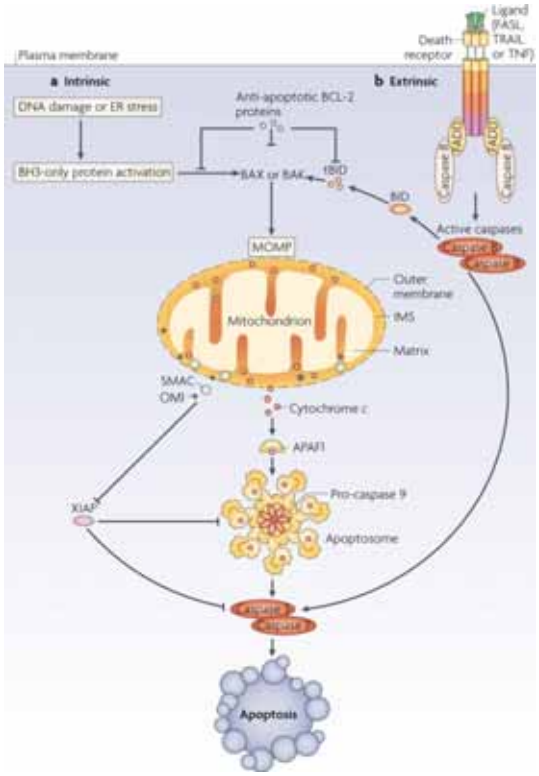


Figure 8. General view of the extrinsic and intrinsic pathways of apoptosis. **A,** Intrinsic pathway is triggered by stimulus generated within the cell, which lead to mitochondria permeabilization, formation of the apoptosome and activation of caspase-3. **B,** The extrinsic pathway is triggered by stimulus outside the cell, causing activation of caspase-8. Active caspase-8 may activate caspase-3 directly or through cleavage of protein Bid into tBid. tBid triggers permeabilization of the mitochondria in a crosstalk mechanism with components of the intrinsic pathway. (Tait et al., 2010)

1.3.1 Intrinsic or Mitochondrial Pathway of Apoptosis

In the beginning of the molecular dissection of apoptosis, mitochondria were not recognized as having any significant role in the process. It was not until the early 1990's that Monaghan and Krajewsky (Monaghan et al., 1992; Krajewski et al., 1993) described that the subcellular localization of the Bcl-2 protein was around the mitochondria. After this, investigators began to consider that the mitochondria could have a prominent role in the cell besides respiration.

Today we know that the mitochondria contain a variety of factors that are important to determine the fate of a cell undergoing apoptosis. The key event in the intrinsic pathway of apoptosis is the permeabilization of the outer membrane of the mitochondria (MOMP), which allows certain proapoptotic proteins to be released into the cytosol where they exert their function. The major molecules to regulate events leading to MOMP are the proteins of the Bcl-2 family (see previous section) (Reviewed in (Danial and Korsmeyer, 2004).

After permeabilization of the outer mitochondrial membrane, a series of proteins from the inter-membrane space are released into the cytosol. One of these proteins is the electron transporter cytochrome c (Liu et al., 1996a; Kluck et al., 1997). Besides its role in oxidative phosphorylation, once released from the mitochondria, cytochrome c binds to APAF-1 (Apoptotic Protease Activation Factor-1). Interaction with cytochrome c allows a conformational change in APAF-1, mediated by ATP, which will favor its oligomerization to form the so-called apoptosome (Hu et al., 1998; Pan et al., 1998a; Tsujimoto, 1998). Once formed, the apoptosome serves as a platform for the recruitment of inactive procaspase-9, which becomes active by a mechanism of proximity oligomerization (Li et al., 1997; Boatright et al., 2003). Once activated, initiator caspase-9 is able to activate effector caspases, like caspase-3 and -7. It has been demonstrated that through a positive feedback mechanism caspase-3 can cleave and therefore activate at least four other caspases (caspases -2, -6, -8, -10), thus acting as a signal amplifier of the apoptotic signaling process (Slee et al., 1999). Activated effector caspases are then able to cleave various substrates that are vital for the normal functioning of the cell, thus inducing its death.

During the process of MOMP other proteins that have importance in different steps of the apoptotic process are released into the cytosol. Among these are the Second Mitochondria-Derived activator of Caspases (Smac/DIABLO), High-

Temperature-Requirement Protein A2 (OMI/HTRA2), Apoptosis Inducing Factor (AIF), and Endonuclease G (EndoG).

Smac/DIABLO is a proapoptotic mitochondrial protein released into the cytosol upon activation of the intrinsic apoptotic pathway (Du et al., 2000; Verhagen et al., 2000), revised in (Wang, 2001). Smac removes the IAP-mediated inhibition of caspases. It functions as a dimer, binding to both the BIR2 and BIR3 domains of XIAP but not the BIR1. Monomeric Smac can bind strongly to BIR3, but not to BIR2. Because the linker sequence immediately preceding BIR2 is involved in binding and inhibiting caspase-3, Smac monomers cannot relieve the IAP-mediated caspase-3 inhibition. The N-terminal mitochondria-targeting sequence of Smac is proteolytically removed upon import into the cytosol. These exposed N-terminal residues play an indispensable role in Smac function. The N terminus (Ala-Thr-Pro-Phe) of the small subunit of caspase-9 was found to contain an IAP-binding tetrapeptide motif (Srinivasula et al., 2001). This sequence is indeed primarily responsible for the interactions between the processed caspase-9 and XIAP. In the absence of proteolytic processing, procaspase-9 is unable to interact with IAPs. Proteolytic processing of procaspase-9 at Asp315 leads to the exposure of the internal tetrapeptide motif AVPI, which recruits IAPs to inhibit caspase-9. Mature Smac binds IAPs, using the same N-terminal tetrapeptide, AVPI. Thus, a conserved IAP-binding motif in caspase-9 and Smac mediates opposing effects on caspase activity (Srinivasula et al., 2001; Shi, 2002).

HTRA2/Omi is expressed as a 49 kDa proenzyme targeted to the mitochondrial intermembrane space (Faccio et al., 2000). The transmembrane anchor behind the N-terminal Mitochondrial Localization Sequence (MLS) attaches the precursor protein into the mitochondrial inner membrane, where it undergoes proteolytic maturation. The fully processed protein lacks the 133 aminoacids encoding the MLS, exposing the N-terminal IAP binding motif (AVPS). HTRA2 can be released from the mitochondria after an apoptotic stimulus. Once in the cytosol it binds to cIAP1/2 and XIAP through its processed N-terminus removing their inhibitory activity towards caspases (Hegde, 2001). Binding of HTRA2/Omi to IAPs also results in the activation of its proteolytic activity, leading to caspase-independent cell death (Verhagen et al., 2002), reviewed in (Garrido and Kroemer, 2004). Transgenic *Mnd2* (Motor neuron degeneration 2) mice are homozygous for a naturally occurring mutation in HTRA22 (Ser276Cys). This mutation greatly reduces HTRA2 catalytic activity and mice carrying it display a Parkinsonian phenotype, involving loss of neurons in the striatum of the basal ganglia (Jones et al., 2003). This result was later confirmed by the targeted deletion in mice of

the gene encoding HTRA2. These animals, or cells derived from them, show loss of a population of neurons in the striatum that results in a parkinsonian phenotype, leading to death of the Mouse around 30 days alter birth (Martins et al, 2004). Before the loss of neurons became evident, HTRA2$^{-/-}$ mice displayed lack of coordination, decreased mobility, and tremor typical of Parkinson's Disease phenotype. Moreover, Strauss et al., identified two single nucleotide mutations, G399S and A141S in the *Htra2/omi* gene in German Parkinson's Disease patients and not present in healthy controls (Strauss et al., 2005). These mutations resulted in defective activation of the protease activity of the protein related to mitocondrial disfunction associated with altered mitocondrial morphology.

AIF is a flavoprotein found in the mitochondrial intermembrane space and has a critica role as a mitochondrial oxyreductase (Susin et al., 1999; reviewed in Delavallé et al., 2011). AIF is synthesized as a 67 kDa precursor. Upon being imported to the mitochondria it is processed into a mature form of 62 kDa by proteolytic cleavage. On this configuration, AIF is an inner membrane-anchored protein with an N-terminus part is exposed to the mitochondrial matrix and the C-terminal portion is exposed to the mitochondrial intermembrane space (Otera et al., 2005). Relase of AIF from the mictochondria is regulated by two elements: two families of cysteine proteases, calpains and cathepsins (Polster, 2005), and the activation of proapoptotic proteins of the Bcl-2 family, such as Bax or Bid (Landshamer, 2008; Tobaben, 2010). Bax facilitates the mitochondrial outer membrane permeabilization required for AIF release (Bidére N, 2003; Moubarak et al., 2007). Once in the cytosol, AIF translocates to the nucleus where it participates in marginalization of chromatin, although it does not participate in its fragmentation. The major feature of AIF is that it is able of inducing apoptosis even when caspase activation is blocked (Yuste et al., 2005). More recently, it has been demonstrated that AIF mediates non-apoptotic, necrotic cell death after DNA damage (Artus et al., 2010). AIF has shown various implications both in neuroprotection and induction of neuronal cell death. Microinjection of AIF-neutralizing antibodies decreased BAX-mediated neuronal apoptosis when caspases are inhibited (Cregan et al., 2002). AIF translocates to the nucleus after excitotoxicity, and *in vivo* ischemia (Joza et al., 2001). On the other hand, anotehr study using Harlequin *(Hq)* mice, a model taht has <80% reduced AIF expresión, the "Harlequin" (*Hq*) mice, suggests that AIF may also protect against oxidative stress (Klein et al., 2002).

EndoG is one of the most abundant nuclease in eukaryotic cells. It is encoded in the nucleus and imported to the mitochondrial intermembrane space (Tiranti et al.,

1995; Chipuk and Green, 2005). EndoG is released from the mitochondira during apoptosis and it translocates to the nucleus where it induces DNA degradation (Li et al., 2001). The relevance of EndoG in cell death has been a matter of discussion, since generation of null mice for this protein revealed that it is dispensable for embryogenesis and apoptosis, and mice did not show any phenotype associated to deletion of this protein (David et al., 2006; Irvine et al., 2004). In addition, EndoG has been dscribed to play a role in cell proliferation (Huang KJ, 2006). Recently, it has been demonstrated that EndoG plays a key role in cardiac hypertrophy ; McDermott-Roe et al., 2011).

1.3.2 Extrinsic Pathway of Apoptosis

Higher metazoans have evolved an additional signaling mechanism that actively directs cells to die by apoptosis called extrinsic pathway of apoptosis (Ashkenazi and Dixit, 1999). This pathway has been extensively studied in the immune system, although it has shown to have great importance also in the nervous system, during development and in pathological situations. Activation of the extrinsic pathway of apoptosis is controlled by the so-called Death Receptors (DR). Death receptors are cell surface receptors which transmit the apoptotic signal after binding to a specific ligand.

The main feature of the extrinsic apoptotic pathway is that it can lead the cell to apoptosis without the participation of the mitochondria or the mitochondrial proteins that are of key relevance in the intrinsic (Scaffidi et al., 1998) pathway. Binding of a Death Ligand to its specific receptor triggers a signaling cascade with the participation of multiple adaptor proteins, leading to the activation of the initiator caspase-8, which, in turn activates the effector caspase-3. Activated caspase-3 is able of cleaving, and thus making active, other molecules of the same protein, leading to an amplification of the signal and implying the apoptotic death of the cell (Ashkenazi, 1998).

Even though in some cellular systems the extrinsic and intrinsic pathways can be independent, in most cell types there is a tight coordination and interaction between them. The most recognized link between both apoptotic mechanisms is the BH3-only protein Bid. Bid is a proapoptotic protein located normally to the cytosol, but that can translocate to the surface of intracellular membranes (Li et al., 1998; Khosravi-Far and Esposti, 2004). Bid can be cleaved by active caspase-8 into a fragment called truncated Bid (tBid), this fragment can bind to the mitochondria and provoke MOMP,

thus releasing cytochrome c and initiating the intrinsic pathway of apoptosis (Wang et al., 1996; Luo et al., 1998; Gross et al., 1999)

1.4 Death Receptors

Death Receptors represent a subgroup of the Tumor Necrosis Factor (TNF) superfamily, which comprises around 27 members and is related to apoptotic, differentiation and proliferation events (Locksley et al., 2001). DRs are type I transmembrane proteins (their N-terminal domain is extracellular while the C-terminal domain is intracellular) (Tartaglia et al., 1993; Curtin and Cotter, 2003). This subgroup is defined by similar cysteine-rich extracellular domains, one single transmembrane domain, and an intracellular "Death Domain" (DD) of around 80 aa through which adaptor proteins can be recruited to the signaling complex (Wilson et al., 2009).

Until now, 7 DRs have been identified, all belonging to the TNF superfamily (Fig. 9). These are TNFRSF1A (also known as TNFR1, DR1, CD120a, p55 and p60), Fas (also known as CD95, DR2, APO-1), TNFRSF25 (also known as DR3, APO-3, LARD, TRAMP, and WSLT), TNF-Related Apoptosis Inducing Factor Receptor (TNFRSF10A, also known as TRAILR1, DR4 or APO-2), TNFRSF10B (also known as TRAILR2, DR5, KILLER, ad TRICK2), TNFRSF21 (DR6), and the receptor of the Nerve Growth Factor $p75^{NTR}$ (Chinnaiyan et al., 1996; Marsters et al., 1996; Bodmer et al., 1997; Pan, 1997; Schneider et al., 1997; Screaton et al., 1997; Wu et al., 1997; Pan et al., 1998b). All these receptors contain a DD and once activated by their specific ligands are able to induce the formation of signaling complexes that can lead to apoptotic cell death or to signaling pathways related to gene activation such as NF-kB or JNK.

In addition to Death Receptors, three decoy receptors have been identified and termed DcR1, DcR2 and DcR3 (Marsters et al., 1997; Simonet et al., 1997; Pitti et al., 1998). These receptors share the cysteine-rich extracellular domain, but they lack a proper intracellular death domain and thus are not able to recruit adaptor proteins and induce a signaling cascade. The main function of decoy receptors is to modulate the sensitivity to death receptors *in vivo* (Ashkenazi and Dixit, 1999).

The ligands that activate these receptors, with the exception of NGF, are structurally related and belong to the TNF superfamily. TNFα and lymphotoxin α bind to TNFR1, CD95-ligand (or FasLG) binds to Fas, TWEAK (TNFSF15, APO3L) binds to DR3 (Chicheportiche et al., 1997; Marsters et al., 1998) and TRAIL (TNFSF10, APO2L) (Wiley et al., 1995; Pitti et al., 1996) binds to DR4 or DR5. Although unknown for a long time, DR6 has been recently described to bind APP as its specific ligand, showing a role in axon pruning and neuron death (Ashkenazi and Dixit, 1999; Nikolaev et al., 2009).

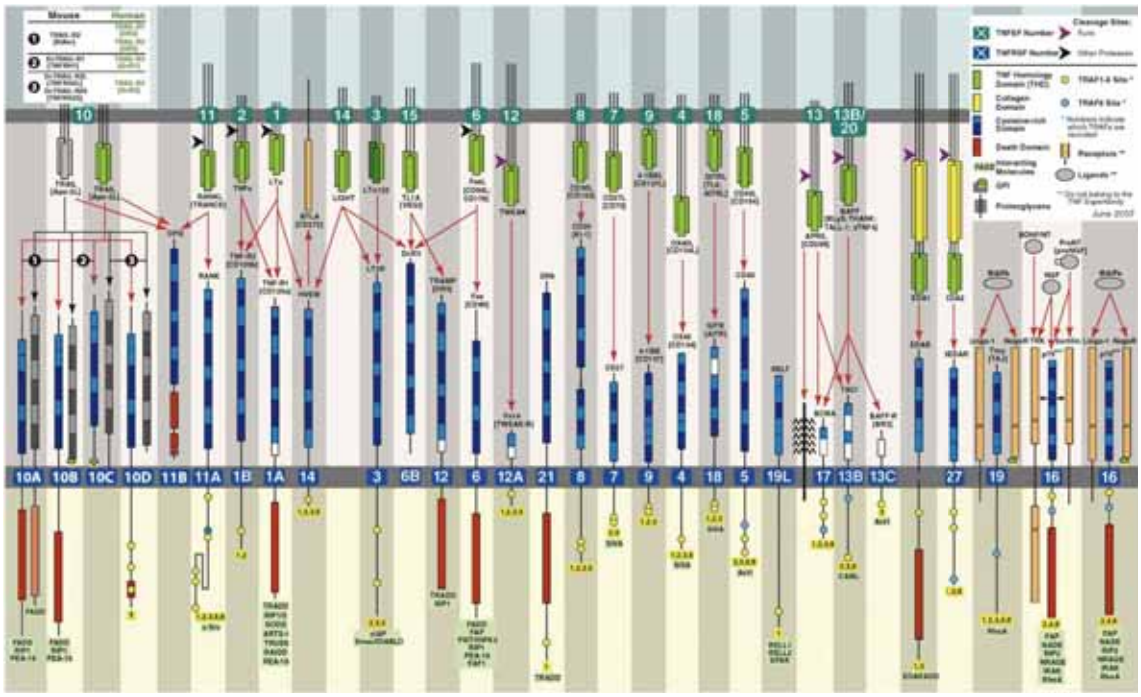


Figure 9. Members of the TNF superfamily and their ligands. Red boxes represent Death Domains (DD) characteristic of Death Receptors belonging to this family. (Tansey et al., 2009).

1.4.1 Death Receptor Signaling

The structural differences found in the extracellular cysteine-rich domains of DRs determine their affinity for different ligands. In a similar way, differences present in the intracellular death domain of these receptors will determine the recruitment of adaptor proteins leading to the formation of signaling complexes with different fates (Locksley et al., 2001) According to this, two subclasses of death receptors can be defined: we can talk about FADD (Fas Associated Death Domain) recruiting DRs and TRADD (TNF Receptor Associated Death Domain) recruiting DRs (Wilson et al., 2009) (Fig. 10). Fas and TRAIL receptors (DR4, DR5) belong to the first group. On the one hand, recruitment of FADD implies the formation of the DISC (Death Inducing Signaling Complex), which leads directly to caspase-8 activation and apoptosis of the cell (Peter and Krammer, 2003). However, it has been demonstrated that in some situations

TRAIL can also bind TRADD (Varfolomeev et al., 2005; Walczak, 2011). On the other hand, regarding TNFR1 and DR3 signalling, recruitment of TRADD is the first event in the formation of an adaptor protein complex that can lead to either cell death or survival (Chinnaiyan et al., 1996; Schneider et al., 1996; Ermolaeva et al., 2008; Pobenzinskaya et al., 2008; Marsters et al., 1996).

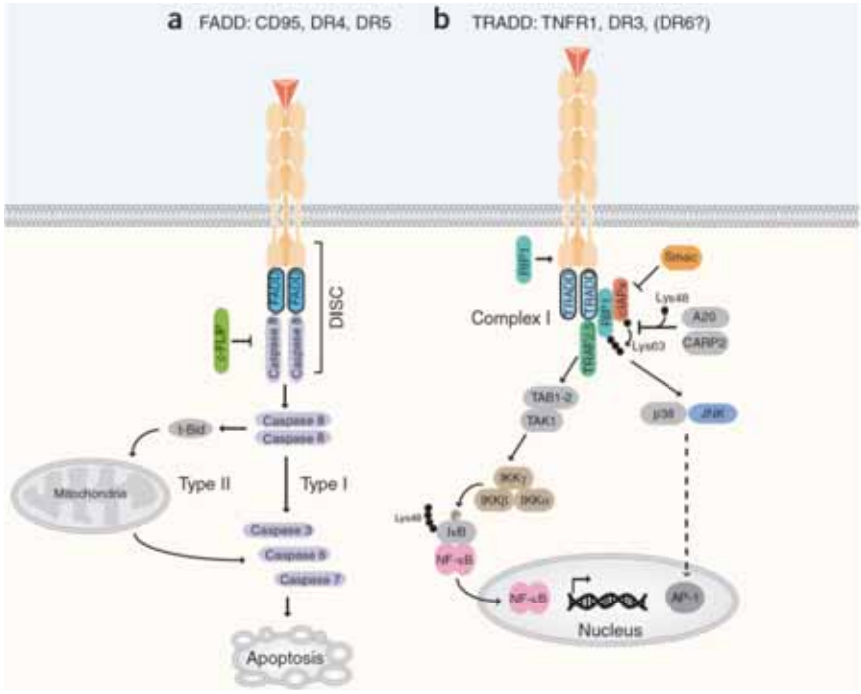


Figure 10. Signaling complexes formed alter triggering of death receptors. **A,** FADD recruiting DR induce the formation of the DISC and signal mainly apoptosis. **B,** TRADD recruiting DR induce the formation of various signaling platform that may lead to activation of pathways related to cell survival, differentiation, proliferation or apoptosis. (Wilson et al., 2009).

The best characterized death receptors are Fas (CD95) and TNFR1. The former is a FADD-recruiting DR and therefore its main signaling pathway is apoptosis. The latter TNFR1 is an example of TRADD-recruiting DR and it can recruit different signaling complexes, which will determine different outputs towards apoptotic cell death or survival, proliferation, and differentiation.

In the next sections Fas and TNFR1 signaling will be further explained as examples of death receptor signaling. Since the work in this thesis is focused on TNFR1 signaling, the regulation of this death receptor will be treated in more detail.

1.5 Fas/CD95 signaling

The membrane-bound receptor Fas (APO-1, CD95), was described for the first time as the target of monoclonal antibodies triggering apoptotic cell death in certain human tumor-derived cell lines (Yonehara et al., 1989; Itoh et al., 1991). It was described as a cell surface molecule expressed in human lymphocytes, which would trigger cell death after being activated by specific antibodies. Shortly after, its natural ligand, CD95L or FasL, was described (Itoh et al., 1991). Fas presents the classical structure of a death receptor, with three cysteine-rich domains in the extracellular fraction, joined by disulfide bonds and the typical intracellular death domain, which allows for the formation of a signaling complex leading to apoptotic cell death. FasL is produced as a membrane bound molecule (mFasL) that can be released into a soluble form (sFasL). However, it has been demonstrated that only mFasL is able to trigger the apoptotic signaling, while the soluble form triggers other cascades related to autoimmunity and tumorigenesis through the induction of non-apoptotic pathways like NF-kB (Reilly et al., 2009).

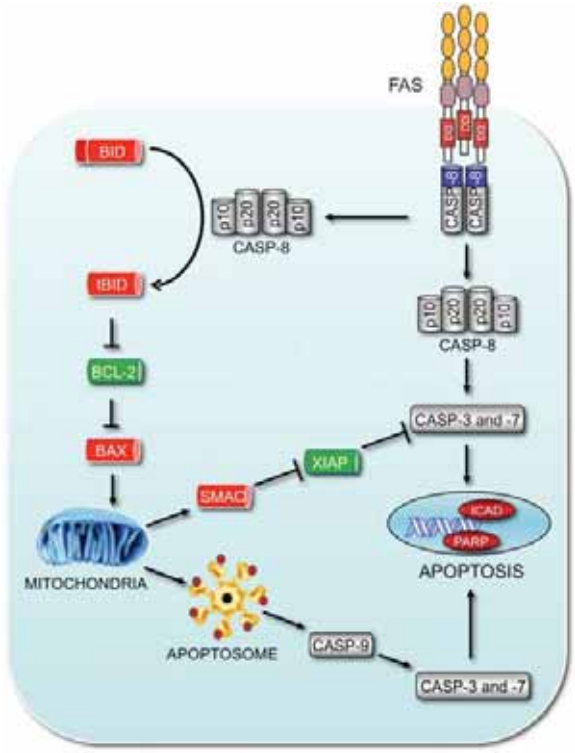


Figure 11. Fas-mediated induction of apoptosis. Ligation of FasL to Fas induces cleavage of caspase-8. This can directly cleave effector caspase-3 or induce the intrinsic pathway through cleavage of BH3-only proteín Bid. (Kaufmann et al., 2012)

Binding of FasL or agonistic antibodies to Fas leads to the formation of the DISC signaling complex at the membrane (Fig. 11). The DISC consists of oligomerized receptor molecules, the adaptor molecule FADD, procaspase-8, procaspase-10, and cFLIP. FADD interacts with Fas through its DD and the death effector domain (DED) of FADD interacts with DED domains in procaspases-8 and -10 and the DED in cFLIP. As a result of DISC formation, procaspase-8 is cleaved and thus activated, leading to apoptosis (Muzio et al., 1996; Scaffidi et al., 1999a; Siegel, 2000; Sprick et al., 2002) In the apoptotic pathway mediated by Fas two types of cells have been identified (Scaffidi et al., 1998) Type I cells are characterized by high levels of Fas oligomerization, DISC formation, and high concentration of procaspase-8, leading to direct activation of

downstream effector caspases-3 and -7. In type II cells there are lower levels of DISC formation and therefore activation of procaspase-8 is also lower. Signaling in this type of cells requires an amplification loop. This loop involves cleavage of the BH3-only protein Bid to generate truncated-Bid (tBid) and the subsequent release from the mitochondria of proapoptotic factors such as cytochrome c and Smac/DIABLO. Cytochrome C release will allow for the formation of the apoptosome as a platform for procaspase-9 activation, leading to cleavage and activation of effector caspases. Therefore, Bid cleavage into tBid, mediated by caspase-8, represents a crosstalk between the extrinsic and intrinsic pathways of apoptosis. Hepatocytes are a good example of type II cells, since tBid is necessary for the induction of apoptosis mediated by Fas. An example of type I cells are thymocytes, which do not require this amplification loop in order to undergo Fas-induced apoptosis (Yin et al., 1999; Kaufmann et al., 2007). A recent report by Jost et al. (2009) showed that levels of the antiapoptotic protein XIAP act as a switch between type I and type II cells (Jost et al., 2009). In a model of mouse hepatocytes and pancreatic β-cells (both type II cells), the loss of XIAP function, either by gene targeting or by using a Smac mimetic drug, rendered cells independent of Bid for Fas-induced apoptosis, thus turning type II cells into type I.

1.5.1 Non-Apoptotic Roles of Fas Signaling

Although Fas has been classically related to induction of apoptotic cell death, there is a growing body of evidence linking Fas signaling to non-apoptotic outcomes, including induction of proliferation in T cells and fibroblasts, hepatocyte regeneration, chemokine production, dendritic cell regulation, and neurite outgrowth.

Research has shown that physiological FasL, either its membrane-bound form or its soluble form, does not efficiently kill cells that are readily killed by commercial forms of FasL. This suggests that, in vivo, Fas signaling may have a different role besides apoptosis induction (Algeciras-Schimnich et al., 2003). One of the mediators of this kind of signaling triggered by Fas may be the transcription factor NF-kB. Fas-mediated activation of NF-kB seems to be dependent on the formation of a complex containing FADD, RIP1, and procaspase-8 (Kreuz, 2004). In this case, enzymatic activity of caspase-8 was dispensable for NF-kB activation, and suggested a role for caspase-8 as a scaffold protein in non-apoptotic signaling triggered by Fas. In the immune system it has been shown that T cell proliferation induced by suboptimal anti-CD3 stimulation is enhanced when Fas is triggered, and deletion of Fas in T cells causes lymphopenia in mice (Alderson et al., 1993; Hao, 2004). Moreover, injections of

Fas specific antibodies into mice lead to fulminant liver failure. However, Fas engagement has been shown to accelerate liver regeneration after partial hepatectomy, and liver regeneration kinetics were delayed in mutant mice with decreased surface expression of Fas (*lpr* mice) (Desbarats and Newell, 2000). The MAPK/ERK (Mitogen-Activated Protein Kinase/Extracellular signal Regulated Kinase) pathway, classically related to cell proliferation and differentiation, has also been linked to Fas signaling. The antiapoptotic protein FLIP has been shown to play a key role in Fas-induced ERK activation through recruiting of Raf-1, an upstream component of the MAPK/ERK pathway, to the DISC in thymocytes (Kataoka et al., 2000). Fas is also highly expressed in the nervous system. It is expressed in neurons and many CNS-derived tumor cells are sensitive to Fas-mediated apoptosis *in vitro* (Cheema et al., 1999; Matsushita et al., 2000; Shinohara et al., 2000; Becher et al., 1998). In 2003 Desbarats and her collaborators described that, *in vitro,* Fas is able to induce neurite outgrowth and it does so by activating ERK and p35 (Desbarats et al., 2003). Also, the MAPK pathway is activated by Fas in neural progenitor cells (Tamm et al., 2004) and neuronal branching in CNS neurons is stimulated by CD95 during development both in vitro and in vivo (Zuliani et al., 2005).

In glioblastoma, Fas-mediated signaling also has a relevant role in the invasion of brain tissue by isolated tumoral cells. Martin-Villalba and her collaborators have shown that, although the apoptotic signaling is blocked in glioblastoma cells, Fas is highly expressed in these cells at the site of tumor/host interaction, the invasion front. The authors detected the association of a Src family member, protein Yes, and PI3K to Fas. Activated PI3K leads to inhibition of glycogen synthase kinase 3-β (GSK3β) and induction of matrix metalloproteinases (MMPs) (Fig 12). Moreover, in intracranial GBM, neutralization of CD95 activity reduced the number of cells invading the contralateral hemisphere (Kleber et al., 2008).

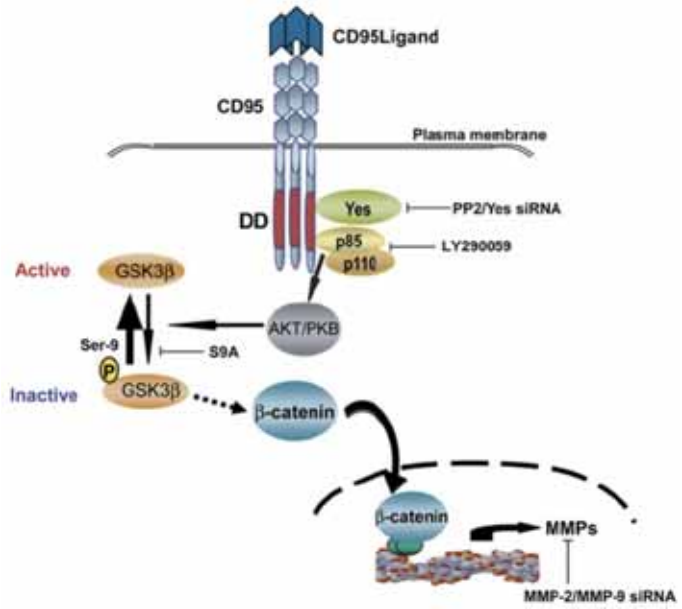


Figure 12. Model for Fas induced signaling of glioblastoma invasion. FasL induces recruitment of the Src family member Yes and the p58 subunit of PI3K to the receptor thereby activating AKT. Activated AKT phosphorylates and inactivates GSK3β, allowing β-catenin translocation into the nucleus, where it induces transcription of MMPs. This signaling pathway could be blocked by siRNA against Yes, or MMP-2 and -9, the PI3K-specific inhibitor (LY290059), or by lentiviral infection with a dominant-active mutant of GSK3β (S9A). (Kleber et al., 2008).

1.6 TNFR1 Signaling

TNFα is a cytokine that exerts multiple functions in inflammation, immunity, control of cell proliferation, differentiation, and apoptosis. TNFα is produced as a type II transmembrane protein arranged in homotrimers (Tang et al., 1996). From this form it is released from the membrane after proteolytic cleavage by the metalloprotease TNF Alpha Converting Enzyme (TACE) (Black et al., 1997). Both the membrane attached (mTNFα) and the soluble forms (sTNFα) are able to bind to the receptor and trigger a response (reviewed in (Wajant et al., 2003)).

TNFα achieves its functions through interaction with TNFR1 (also known as p55TNFR, p60, CD120a, TNFRSF1a) or TNFR2 (also known as p75TNFR, p80, CD120b, TNFRSF1b). TNFR1 is constitutively expressed in most tissues, while TNFR2 is restricted to the immune system (Banner et al., 1993; Naismith and Sprang, 1998). TNFR2 does not contain an intracellular death domain therefore it is unable to recruit the signaling platform necessary for the induction of apoptosis and is mainly

responsible for survival signaling through the transcription factor Nuclear Factor kB (NF-kB) in some tissues. TNFR1 is responsible for the apoptotic signaling induced by TNFα and can also exert prosurvival, proliferation, and differentiation signaling. (Tartaglia et al., 1993; Yang et al., 2002) (Fig. 13). In fact, in most cell types, survival signaling induced by TNFα needs to be abrogated in order to induce apoptosis. This can be achieved by co-treating cells with TNFα and an inhibitor of protein synthesis such as cycloheximide or Actinomycin D. A recent work in our own lab described a role for Bcl-X_L as a key regulator of apoptosis induced by TNFα (Gozzelino et al., 2008). The authors demonstrate that Actinomycin D induces the spcific downregulation of Bcl-X_L, rendering PC12 cells and mice cortical neurons sensitive to TNFα-induced apoptosis. Downregulation of Bcl-X_L with a specifc siRNA also sensitized cells to TNFα-induced cell death in a way similar to TNFα plus Actinomycin D treatment. However, researchers also show that in this cell models Bcl-X_L expression is independent of NF-kB, and other antiapoptotic mediators such as FLIP or IAPs are not relevant for this mechanism. Therefore, the exact mechanism by which NF-kB is able to inhibit the apoptotic pathway initiated by TNFα is still a matter of discussion.

Interaction of TNFα with TNFR1 causes the receptor to trimerize on the cell surface (Smith et al., 1994; Locksley et al., 2001). Upon TNFR1 triggering, the adaptor protein TRADD (TNF Receptor Associated Death Domain) interacts with the death domain of trimerized TNFR1. TRADD promotes the assembly of at least two complexes that initiate opposite signaling pathways: Complex I mediates activation of inflammatory and anti-apoptotic proteins, and complex II mediates activation of apoptotic cell death (Micheau and Tschopp, 2003; Wertz and Dixit, 2009). TRADD organizes the recruitment of the remaining components of complex I: RIP-1, cIAP1, cIAP2, TRAF2 and TRAF5. Proinflammatory and prosurvival pathways emanating from complex I regulate the induction of apoptosis, mainly through NF-kB regulation. This signaling pathway and its regulation through TNFR1 will be discussed in more detail below.

After complex I is formed TNFR1 complex is internalized via clathrin-dependent endocytosis, NF-κB signalling is terminated by the recruitment of the ubiquitin E3 ligases caspase-associated ring protein-2 (CARP2) and A20, leading to degradation of RIP1 (Schütze et al., 1999; Schneider-Brachert et al., 2004). TRADD then recruits FAS-associated death domain protein (FADD) and to TNFR1 at the internalized receptosomes. TRADD and RIP1 dissociate from the receptor and begin the assembly

of complex II in the cytosol. The death effector domain (DED) of FADD, in turn, recruits initiator caspases -8 and -10 to begin the apoptotic signaling cascade.

In some cell types it has been demonstrated that in order to induce apoptosis through TNFR1 activation, NF-kB activity needs to be inhibited by using a protein synthesis inhibitor such as Cycloheximide (CHX) or Actinomycin D (ActD) (Martin et al., 1990; Beg and Baltimore, 1996; Van Antwerp et al., 1996). It has been demonstrated that co-treatment of cells with TNFα plus cycloheximide can induce the formation of two other death-inducing complexes. In the first or complex 2A, TRADD binds to the death domain of FADD, inducing recruitment of caspase-8 and inducing apoptosis. Complex 2B is formed after treatment with TNFα, CHX, and inhibitors of IAP proteins. In this case, it is the non-ubiquitylated form of RIP1 that binds to the death domain of FADD, inducing the recruitment of caspase-8 and therefore apoptosis (O'Donnell et al., 2007; Wang et al., 2008).

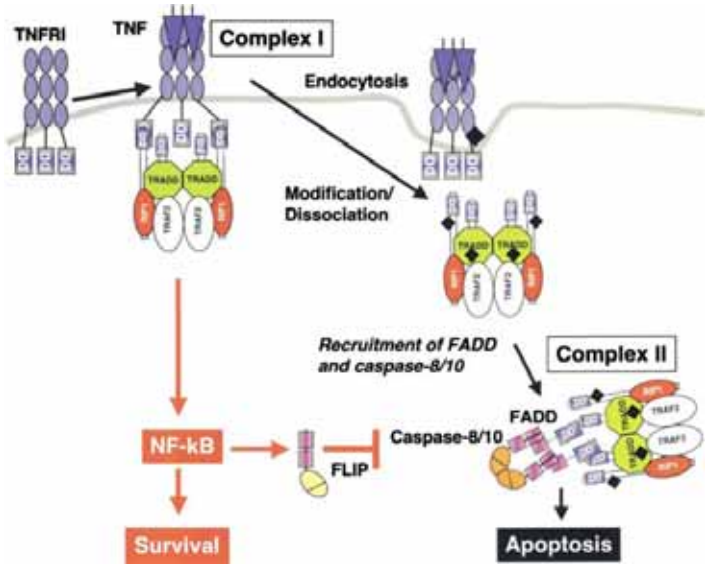


Figure 13. Apoptosis mediated by TNFR1 requires the formation of two sequential complexes. Complex I, attached to the membrane, triggers NF-kB activation pathway. Complex II is cytosolic and depends on the recruitment of FADD to signal apoptosis. (Micheau et al., 2003)

1.7 NF-kB Signaling

1.7.1 The NF-kB Transcription Factor

The NF-kB family of transcription factors consists of five members: p50, p52, p65 (RelA), c-Rel and Rel B, sharing an N-terminal Rel Homology Domain (RHD) responsible for DNA binding and homo- and hetero-dimerization. Different NF-kB dimers regulate the transcription of different genes. Transcription dimers are kept inactive in the cytoplasm by masking of their nuclear translocation sequence through association with one of three IkB (Inhibitor of Kappa B) proteins: IkBα (NFKBIA), IkBβ (NFKBIB), or IkBε (NFKBIE) (Fig. 14).

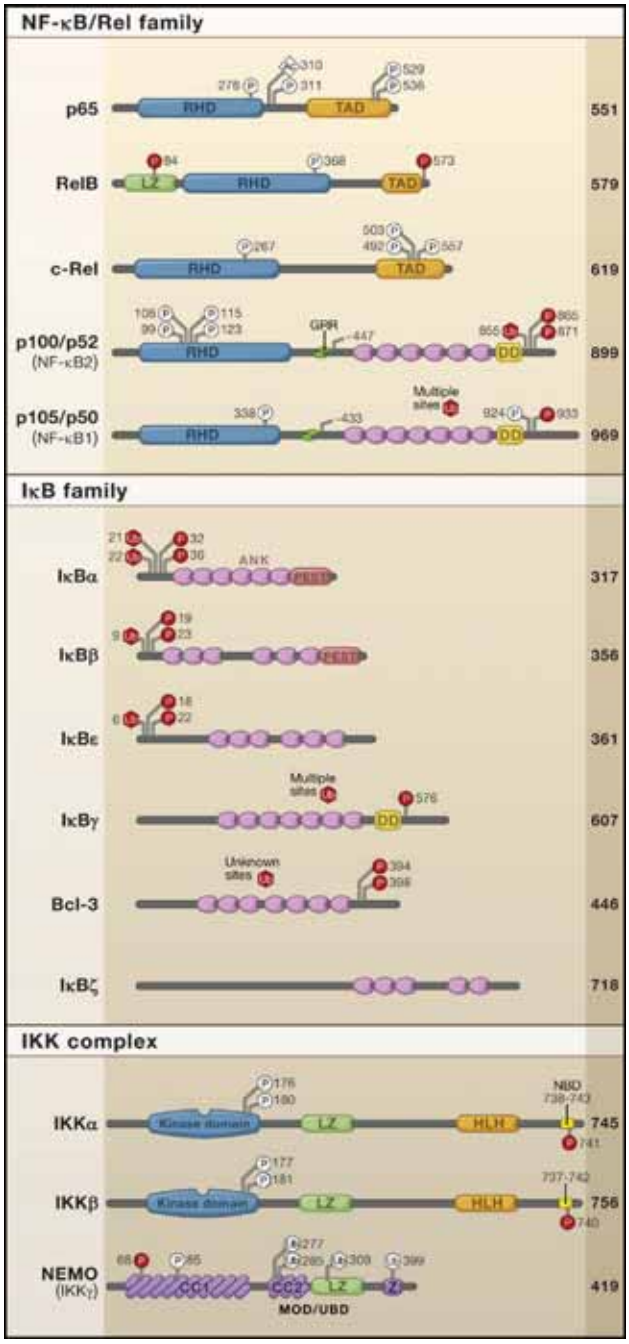


Figure 14. NF-kB, IkB and IKK complex families of proteins. The number of aminoacids of each members is indicated to the right. Posttranslational modifications are indicated with P, U, or Ac for phosphorylation, ubiquitination, or acetylation. Inhibitory events and phosphorylation and ubiq-uitination sites on p100, p105, and IκB proteins that mediate proteasomal degradation are indicated with red Ps and Us. RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper domain; GRR, glycine-rich region; HLH, helix-loop-helix domain; Z, zinc finger domain; CC1/2, coiled-coil domains; NBD, NEMO-binding domain; MOD/ UBD, minimal oligomerization domain and ubiquitin-binding domain; and DD, death domain (Hayden and Gosh, 2008).

In addition, there is a second inhibition mechanism achieved by intramolecular binding of an inhibition domain. Proteins p50 and p52 are synthesized as precursor proteins of 105 and 100 kDa respectively, and they each carry an inhibitory IkB-like domain in their C-terminal fractions (Scheidereit, 2006; Hayden and Ghosh, 2008; Wajant and Scheurich, 2011). These two inhibitory mechanisms correspond to two different activation pathways for NF-kB, the classical or canonical pathway and the alternative or non-canonical.

The classical pathway is triggered by activation of TRAF adaptor proteins. These, in turn, stimulate the IkB kinase complex (IKK). The IKK complex is formed by two homologous kinase subunits IKKα/IKK1 (CHUK) and IKKβ/IKK2 (IKBKB) and the regulatory subunit NEMO (NF-kB Essential Modulator)/IKKγ (IKBKG) (Häcker and Karin, 2006; Wertz and Dixit, 2009; Wajant and Scheurich, 2011). The activated IKK complex phosphorylates IkB proteins, triggering their proteasomal degradation. As a consequence, NF-kB dimers are released from their inhibition and can translocate to the nucleus to exert their function as transcription factors.

The alternative or non-canonical pathway of Nf-kB activation is kept inactive by continual ubiquitylation and degradtion of the NF-kB inducing Kinase (NIK) by cIAP-1 and cIAP2 as part of a cIAP1, cIAP2, TRAF2, and TRAF3 complex (Varfolomeev et al., 2007). Binding of TWEAK or CD40 disrupts this complex by promoting ubiquitylation and degradation of its components, allowing NIK accumulation (Vallabhapurapu et al., 2008; Zarnegar et al., 2008), revised in (Vucic et al., 2011). NIK then phosphorylates IKKα. IKKα phosphorylates the NF-kB transcription factor p100, triggering its parcial degradation to generate the transcriptionally active p52, which translocates to the nucleus as a homodimer or an heterodimer with RelB (Dejardin, 2006)20

1.7.2 TNFα-induced activation of NF-kB

TNFα triggers activation of NF-kB through the canonical pathway of activation (Fig. 15). Ligand-induced reorganization of TNFR1 complexes allows for the recruitment of adaptor protein TRADD and the serine-threonine kinase RIP1 (Chan, 2007). It is still a matter of discussion the way in which TRADD and RIP1 interact, since they both possess a death domain, but it is generally accepted that interaction with TNFR1 can be independent and TRADD and RIP1 can also interact strongly with each other (Devin et al., 2000). This could be relevant in the formation of the cytosolic complex formed after TNFR1 stimulation and that leads to apoptotic signaling (Wajant

and Scheurig., 2011) After binding to TNFR-1, TRADD further recruits TRAF2 through a TRAF-binding domain. TRAF2 consists of a RING (Really Interesting New Gene) domain, followed by five zinc fingers and a C-terminal TRAF domain, which mediates binding to TRADD (Hsu et al., 1995; 1996; Wajant et al., 2001). TRAF2 forms complexes with cellular Inhibitor of Apoptosis Proteins (cIAPs) 1 and 2. Up to this point, there is no evidence that protein modification, such as phosphorylation or ubiquitylation, plays a role in the formation of the signaling complex that leads to NF-kB activation. cIAP1 and cIAP2 both present a RING domain, which provides them with Ubiquitin E3 ligase activity (Zheng et al., 2010). cIAPs modify RIP1, TRAF2 and themselves with K63-linked ubiquitin chains, which have been shown to promote the assembly of signaling complexes {Bertrand:2008gg}. This creates a docking site for the Linear Ubiquitin chain Assembly complex (LUBAC, composed of HOIP/HOIL/SHARPIN). The kinase complexes TAK1/TAB2/TAB3 and IKK (Composed of NEMO, IKK1 and IKK2) are also recruited to this complex. LUBAC then modifies NEMO and RIP1 with M1-linked ubiquitin chains (linear ubiquitn linkage), stabilizing the complex and inducing a conformational change in IKK, which is thought to facilitate its activation (Haas et al., 2009; Tokunaga et al., 2009; Ikeda et al., 2011) . Activation of IKK, as it was explained above, phosphorylates IkBα, targeting this protein for proteasomal degradation and releasing NF-kB dimers that can now translocate to the nucleus.

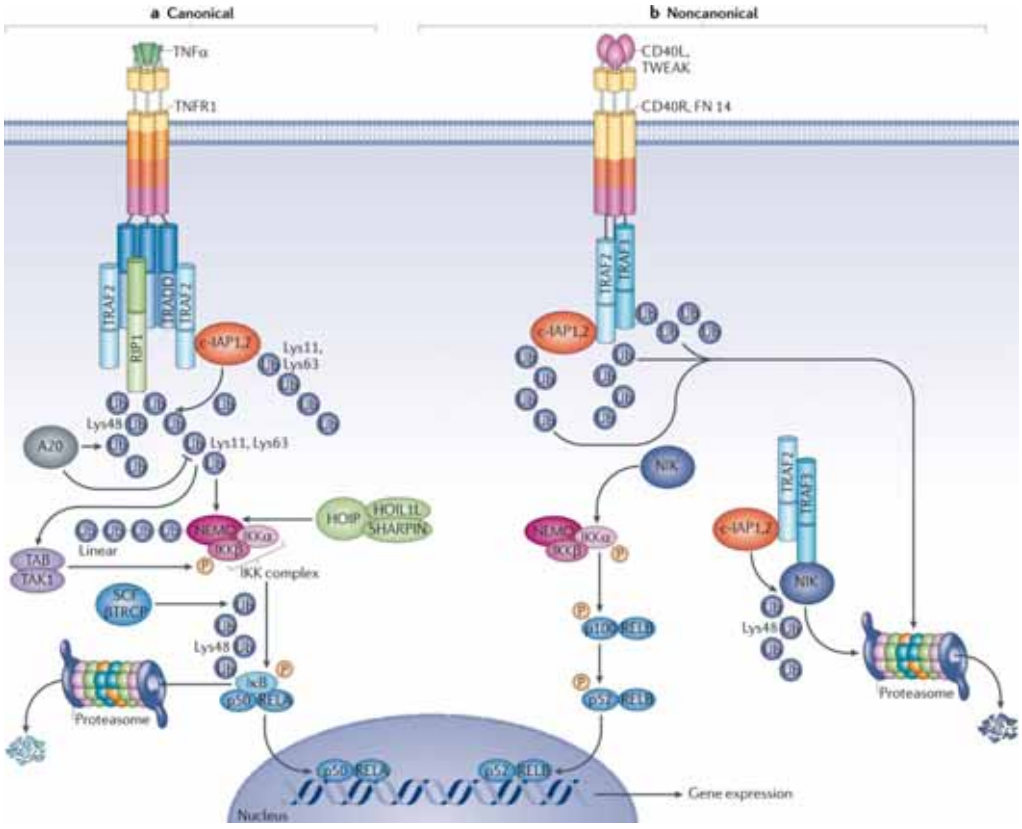


Figure 15. NF-kB activation pathways. NF-kB is activated via canonical or non-canonical pathways. **A,** TNFα activates the canonical pathway of NF-kB, dependent on IAP-mediates non-degradative ubiquitylation of RIP and themselves, forming a platform for the activation of LUBAC. **B,** The non-canonical pathway of NF-kB is activated by TWEAK or CD40. Negative regulation by c-IAP1- and c-IAP2-mediated NF-κB-inducing kinase (NIK) polyubiquitylation is abrogated by the recruitment and subsequent degradation of c-IAPs, TNFR-associated factor 2 (TRAF2) and TRAF3 at the receptor complexes, which liberates NIK and allows the activation of signalling. (Vucic et al., 2011)

NF-kB promotes cell survival through the transcription of specific genes such as cFLIP, IAPs, A20, and the antiapoptotic members of the Bcl-2 family members, which confer protection against apoptotic stimuli (Tian, 2005).

1.7.3 NF-kB in the Nervous System

NF-kB has diverse functions in the nervous system, depending on the cellular context. All DNA-binding subunits of NF-kB have been detected within the CNS, and in the adult rodent brain the major DNA-binding complexes are p50/p65 (Bakalkin GYa et al., 1993; Kaltschmidt et al., 1993; Schmidt-Ullrich et al., 1996; Meffert et al., 2003). In contrast, in the developing nervous system complexes consisting of cRel/p65, p50/p65, and p50 homodimers were reported. Transgenic reporter mouse models showed that constitutive NF-kB activity in several rodent brain regions such as the cerebral cortex, hippocampus, amygdala, olfactory lobes, cerebellum, and hypothalamus (Fig. 16) (Bhakar et al., 2002). A role for NF-kB mediated neuroprotection has also been established. Inhibition of NF-kB activation potentiates amyloid-β-dependent apoptosis in neurons (Kaltschmidt and Uherek, 1999). Ischemia induced in p502/2 mice showed a clear neuroprotective role for NF-kB in the hippocampus and striatum, in which degenerating neurons were detected 4d after 1 h of ischemia. Degenerating neurons did not show NF-kB-dependent reporter gene expression, and in addition $p50^{-/-}$ mice experienced a 2.4-fold increase in postoperative death in comparison to controls (Duckworth et al., 2006).

Figure 16. β-galactosidase expresión in embryonic transgenic reporter mice. X-gal staining of an E13 transgenic mouse shows high basal NF-kB activity in the telencephalon and along the roof plate of the midbrain. Facial staining is visible within the primordia of the vibrissae (5 parallel rows) and in the prominent tactile hair follicles. Adapted from (Bhakar et al, 2002)

1.8 JNK Signaling

JNK (c-Jun N-Terminal Kinase) 1, 2 and 3 form a sub-group of the MAPK (Mitogen Activated Protein Kinases) superfamily activated by cell stressors such as UV radiation and proinflammatory cytokines such as TNFα and interleukin-1 (Varfolomeev and Ashkenazi, 2004). JNK 1 and 2 are ubiquitously expressed, while JNK3 is neuron specific. JNK phosphorylates specific subunits, namely c-Jun, JunB, JunD and ATF-2 of the AP-1 transcription factor, turning on genes that control diverse cellular functions including proliferation, differentiation, and apoptosis. JNKs are activated by the MAP Kinase Kinases (MAPKK) MKK4 and MKK7. Several upstream MAP Kinase Kinase Kinases (MAPKKK) can activate this pathway. Among these, Apoptosis Signal regulating-1 (ASK1) and MEKK1 were shown to be activated by TNFα (Ichijo, 1999). Investigations of the link between JNK activation and TNFα-induced apoptosis have led to controversial results, with reports indicating an anti-apoptotic role of TNFα-mediated JNK activation (Liu et al., 1996b; Lee et al., 1997; Reinhard et al., 1997) and others showing that the activation of JNK by TNFα induces apoptotic cell death (Cuvillier et al., 1996; Verheij et al., 1996; Ichijo et al., 1997). The exact role of JNK on apoptosis is still a matter of debate. Its effects appear to be depending on species, type of cell, and nature and duration of the stimulus.

The involvement of JNK in apoptosis induced by TNFα may depend on NF-kB activation. TNFα activates JNK transiently, however, in cells where protein synthesis has been inhibited or NF-kB activation has been directly blocked, JNK activation is maintained (Javelaud and Besançon, 2001; Lin, 2002; Maeda et al., 2003). Moreover, it has been demonstrated that some genes under the control of NF-kB, such as *gadd45β*, *A20,* or *XIAP* are able to inhibit JNK activation (De Smaele et al., 2001; Bubici et al., 2004). A study in 2003 showed that, upon NF-kB blockade, TNFα could induce apoptosis through the JNK-dependent cleavage of the BH3-only protein Bid, generating a unique product named jBid, distinct from the already described caspase-8 cleavage product tBid. jBid, in turn, would translocate to the mitochondria and promote the preferential release of the proapoptotic protein Smac/DIABLO, but not cytochrome c. This would relieve inhibition of caspases by cIAPs and thus induce apoptosis (Deng et al., 2003). However, until now it has been impossible to identify a protease responsible for the cleavage of jBid and results from Deng et al. have not been reproduced. Another work from 2006 unveiled the role of JNK in the phosphorylation of the E3 ligase ITCH. According to the authors, phosphorylated ITCH induces ubiquitylation of the long form of cellular FLICE Inhibitory Protein (FLIP-L), a known

inhibitor of caspase-8, inducing its proteasomal degradation. They concluded that JNK antagonizes NF-kB activation during TNFα signaling (Chang et al., 2006).

Besides acting as a direct or indirect transcriptional modifier of molecules implicated in the death signaling pathway, JNK may modulate apoptosis through AP-1 dependent gene transcription. JNK has been shown to positively regulate the mRNA levels of the proapoptotic, BH3-only protein Bim through c-Jun, even in neuronal models (Whitfield et al., 2001; Putcha et al., 2003; Kaufmann et al., 2009; Sidler et al., 2011).

1.9 MAPK/ERK Signaling

ERK1/2 are ubiquitously expressed hydrophilic non-receptor proteins that participate in the Ras-Raf-MEK-ERK signal transduction cascade, which is sometimes denoted as the mitogen-activated protein kinase (MAPK) cascade (Reviewed in Roskosky, 2012). ERK signaling pathway is activated in response to several different stimuli such as cytokines, growth factors, or oxidative stress, and is involved in regulation of cell proliferation, differentiation and survival. ERK signaling is crucial for neurotrophin-induced neuronal differentiation. The signaling cascade initiated by neurotrophins such as the Neuron Growth Factor (NGF) or the Brain-Derived Neurotrophic Factor (BDNF), among others, is triggered by the binding of a neurotrophin to its specific tyrosine-kinase (Trk) receptor (Chao, 2003). Ligation to the receptor triggers its autophosphorylation, favoring the recruitment of adaptor proteins. These allow for the activation of a GTPase protein. Ras-GTPase is the main effector in neurotrophin-mediated activation, but signaling through Rap and Rac is also relevant (York et al., 1998; Arévalo and Wu, 2006). Ras-GTPase promotes the phosphorylation and activation of the first member in the MAPK signaling cascade (Marais et al., 1995). This first MAPK, termed MAPKKK (A-Raf, B-Raf, C-Raf/Raf-1) is a Ser/Thr kinase that phosphorylates the next member in the cascade, a MAPKK (MEK1/2), which has a dual specificity for Ser/Thr and Tyr in the consensus sequence T-E-Y. This will activate the last Ser/Thr MAPK (ERK1/2). Finally, the phosphorylated MAPK is responsible for phosphorylation of downstream targets and regulation of gene expression (Fig. 17).

1.9.1 Ras Activation

Ras proteins function as molecular switches as an inactive Ras-GDP is converted into active Ras-GTP in a process mediated by a guanine nucleotide exchange factor (GEF), also known as Sos1/2 (Son of sevenless) (Gupta et al., 2011). The conversion of Ras-GDP to Ras-GTP is promoted by the activation of tyrosine-kinase receptors, such as the Insulin-like Growt Factor Receptor (IGFR), the Epidermal Growth Factor Receptor family (EGFR), or neurotrophin receptors (Steelman et al., 2011).

Ligation of a specific ligand to its tyrosine kinase receptor induces its dimerization, promoting autophosphorylation of the receptor and resulting in its activation (Lemmon and Schlessinger, 2010). Phosphorylated residues act as binding sites for proteins containing a Src Homology 2 domain (SH2), a Phosphotyrosine Binding domain (PTB), or both domains. These domains are found in the adaptor protein Shc (Margolis et al., 1999). Shc in turn recruits growth factor receptor-bound protein 2 (Grb2) and Sos1/2, leading to Ras activation. All these events take place at the plasma membrane, where the next effector of this signaling cascade, the MAPKKK Raf is translocated as a crucial step for its activation (Reviewed in Wellbrock et al., 2004).

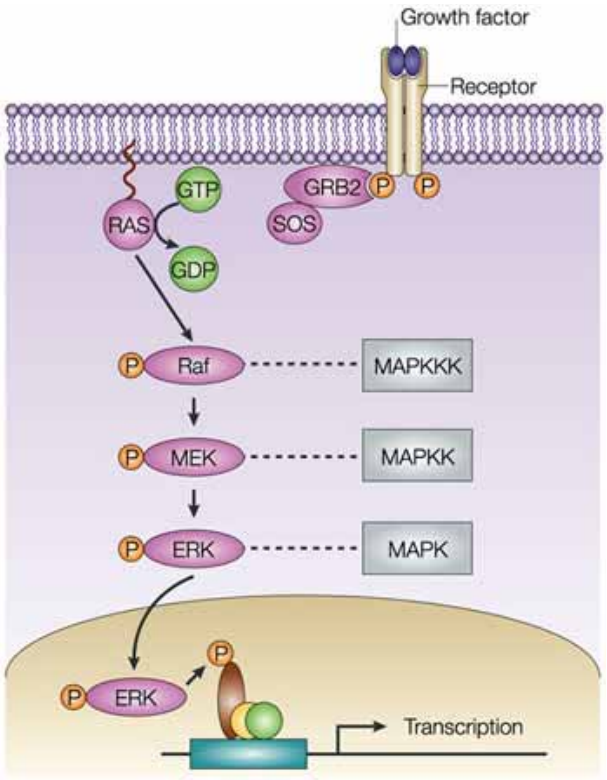


Figure 17. Activation of the MAPK/ERK pathway by growth factors. Activation of transcription factors by ERK depends on a cascade of kinase proteins, initiated by recruitment to the membrane and phosphorylation of a MAPKKK mediated by a GTPase protein. Source: (Kim et al., 2004)

1.9.2 Raf Proteins

C-Raf (Raf-1) was cloned as the cellular homologue of the *v-raf* oncogene acquired by the murine retrovirus 3611-MSV (Rapp et al., 1983). Two related genes were later found in vertebrates and termed A-Raf and B-Raf (Marais and Marshall, 1996). Raf proteins show a common architecture, with three conserved regions, two in the N-terminus (CR1 and CR2) and the third, encoding the kinase domain, in the C-terminus (C3) (Fig. 18) (Reviewed in Matallanas et al., 2011). All Raf isoforms have numerous phosphorylation sites, indicating that these proteins are subject to complex regulation. Some of these sites are conserved in all the isoforms, while others are not, showing that there may be common as well as independent regulatory mechanisms. Raf-1 is ubiquitously expressed, A-Raf is expressed particularly in urogenital organs, and B-Raf is basically restricted to neuronal tissues (Storm et al., 1990; Barnier et al., 1995). Genetic studies have shown that Raf proteins have non-redundant functions in development. Knockout mice for *a-raf* die 7 to 21 dies after birth due to neurological and gastrointestinal defects. Mouse embryos that are *b-raf*$^{-/-}$ or *c-raf*$^{-/-}$ die *in utero*. *b-raf*$^{-/-}$ embryos show growth retardation, and vascular and neuronal defects, and the *c-raf*$^{-/-}$ embryos apparently die of massive liver apoptosis (Pritchard et al., 1996; Wojnowski et al., 1997; 1998; Mikula et al., 2001).

In the recent years Raf proteins have been the target of intensive research due to the central role these proteins play in cancer (Matallanas et al., 2011).

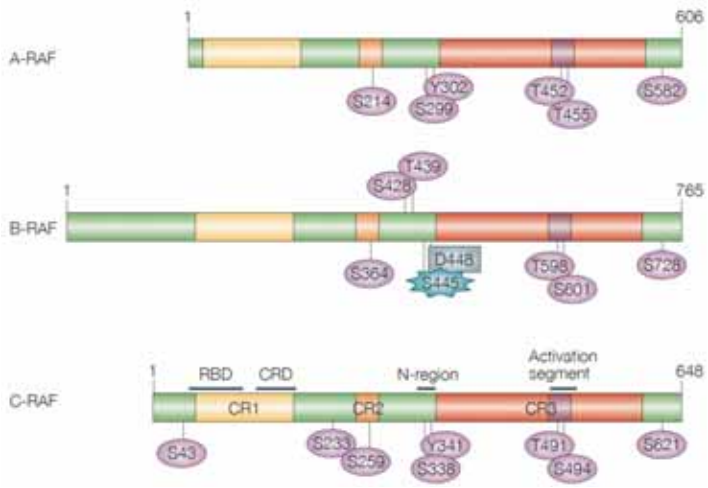


Figure 18. Structure of the RAF proteins. A-RAF, B-RAF and C-RAF, share conserved regions: CR1, CR2 and CR3. Amino acids highlighted below each isoform refer to known phosphorylation sites. The RAS-binding domain (RBD) and the cysteine-rich domain (CRD), are both required for membrane recruitment. Phosphorylation of S43, blocks C-RAF binding to RAS. CR2 contains one a 14-3-3 binding site, which encompasses S259 (the numbering refers to C-RAF). The other two 14-3-3 binding sites are centred on S233 and S621. CR3 contains the catalytic domain. The negative-charge regulatory region (N-region) is upstream of CR3 and contains residue Y341, conserved in A-RAF (Y302), but is replaced by D448 in B-RAF. S338 is conserved in all RAF proteins (S299 in A-RAF and S445 in B-RAF), it is constitutively phosphorylated in B-RAF (blue star). The catalytic domain contains the activation-segment phosphorylation sites T491 and S494, conserved in A-RAF (T452 and T455) and B-RAF (T598 and S601). (Wellbrock et al., 2004).

1.9.3 Regulation of Raf-1 Activation

Raf-1 (cRaf) is the most extensively studied of the Raf isoforms, but the precise molecular events that are required for its activation are controversial. It is clear that the activation process involves membrane recruitment, dimerization or oligomerization, binding to other proteins, conformational changes and phosphorylation (Wellbrock et al., 2004). However, crucial differences exist in this process among Raf isoforms. In its active, GTP-bound form, Ras binds to the Ras binding domain (RBD) of Raf (Wittinghofer and Nassar, 1996), but it also interacts with a cysteine-rich domain (CRD) located in CR1. Since Ras is mainly located to the inner leaflet of the plasma membrane (Hancock, 2003), this binding recruits Raf to the membrane as the initiating event in Raf activation, although this is insufficient to stimulate kinase activity. Phosphorylation plays a prominent role in the regulation of Raf-1 activation. Three sites suppress Raf-1 activity when they are phosphorylated: S43, S233, and S259. These sites seem to be targets of protein kinase A (PKA) and S259 is also phosphorylated by AKT/PKB. Of these, the most important seems to be S259, since Ras can collaborate with protein phosphatase A (PP2A) to dephosphorylate this site and activate Raf-1 (Ory et al., 2003). The five sites within or flanking the kinase domain of Raf-1 are responsible for its activation. Binding of protein 14-3-3 to the C-terminus is essential for activation mediated by phosphorylation of S621 (Yip-Schneider et al., 2000; Light et al., 2002). Another two sites that must be phosphorylated for Raf-1 activation are S338 and Y342, located at the N-terminal side of CR3 (Fabian et al., 1993; Marais et al., 1995; King et al., 1998). Src and Src family kinases have been shown to phosphorylate Y341 *in vitro* and in cell culture (Chow et al., 1995; Marais et al., 1997). Most studies agree in the requirement of S338 phosphorylation for Raf-1 activation, although the kinase or kinases that mediate this phosphorylation are not clear (Oehrl et al., 2003; Sun et al., 2000; King et al., 1998). It is well established that S388 phosphorylation stimulated by growth factor occurs at the plasma membrane in a Ras dependent manner, while another set of serine/threonine kinases termed PAKs (p21 activated Kinases) have shown to phosphorylate S338 in the cytosol in a Ras-independent manner (Chiloeches et al., 2001). Phosphorylation of T491 and S494 is also essential for Raf-1 activation. These residues are within the kinase domain, in a region named activation segment, ant their mutation blocks Raf-1 activation (Chong et al., 2001). Kinases for these two residues have not been identified and they may be subject to autophosphorylation.

A-Raf activation seems to mirror that of Raf-1. The five phosphorylation sites that are required to stimulate Raf-1 activity are conserved in A-Raf and their

mechanisms of activation are similar (Marais et al., 1997). However, B-Raf regulation is different because only four of the five phosphorylation sites are conserved and only three of these conserved sites seem to carry out similar functions in B-Raf as their counterparts in Raf-1.

1.9.4 Cellular Targets of Raf-1

MEK1/2 are the only accepted Raf-1 substrates *in vivo* and *in vitro* so far. However, several studies have indicated that Raf proteins may have other effectors. One candidate is NF-kB. Early studies implied that Raf-1 phosphorylated and induced the degradation of inhibitor of NF-κB (IκBα) (Li and Sedivy, 1993) . However, Raf-1 was later discarded as the responsible for IkBα, instead, a contaminant from the Raf-1 preparation used was responsible (Janosch et al., 1996). Therefore, although Raf-1 does seem to be able to trigger NF-kB activation (Baumann et al., 2000), the exact mechanism of this activation is not fully understood. Other studies suggest a role for Raf-1 in signaling of cell survival through binding of BAG1 (Bcl-2 associated anthogene-1), an antiapoptotic protein that binds to Bcl-2 (Wang et al., 1996a; 1996b). BAG1 is proposed to bind to Raf-1 and mediate binding of Raf-1 to the proapoptotic BH3-only Bad and inactivate it. Raf-1 can also bind to the N-terminal regulatory domain of Apoptosis-regulating kinase 1 (Ask1), an inducer of apoptosis, and suppress its proapoptotic activity (Chen et al., 2001). A recent work by Dogan et al. shows an unexpected role for XIAP and cIAPs in the regulation of Raf-1 (Dogan et al., 2008). The authors demonstrated a direct interaction between XIAP and Raf-1 both *in vitro* and *in vivo*. A reduction of XIAP through siRNA induced an increase in Raf-1 protein levels, although no significant changes in *Raf-1* gene levels were detected. Binding of XIAP promoted ubiquitylation of Raf-1. In addition, *XIAP and cIAPs* knockdown cells showed enhanced cell migration in a Raf-1 dependent manner. More recently, the same group extended this observation to a single BIR domain containing IAP family member, Melanoma IAP (M-IAP). They show that M-IAP can directly bind to Raf-1, and depletion of M-IAP leads to increased Raf-1 expression, MAPK activation, and cell migration in melanoma cells (Oberoi-Khanuja et al., 2012).

1.9.5 ERK1/2 Signaling

In the PC12 cell line, one of the best characterized models of MAPK pathway activation through NGF, transient ERK activity leads to proliferation, whereas sustained activity is related to differentiation (Marshall, 1995). Trk-mediated stimulation of Ras promotes transitory activation of ERK, but not maintained activation (Grewal et al., 1999). Termination of the transitory activation seems to be regulated by SOS phosphorylation mediated by ERK and Rsk, resulting in dissociation of the SOS/Grb2 (Kao, 2001). This activation is not sufficient to promote neurite outgrowth in PC12 cells (York et al., 1998). Maintained stimulation of ERKs depends mainly on the activity of another GTPase, Rap1, through activation of the adaptor protein Frs2 (Meakin et al., 1999; Yan, 2002). Frs2 has many binding sites for cytosolic proteins, such as adaptors Grb2 and Crk, enzymes c-Src and Shp2 and $p13^{SUC1}$ (cyclin dependent kinase substrate).

The transcriptional targets of ERKs can be divided into two main groups; early-response genes and late-response genes. Both groups differ in their action on the phenotype induced by NGF. Among the common substrates of ERK1, ERK2, and ERK5 we find the family of kinase proteins Rsk (Bonni et al., 1999). Once activated, Rsks phosphorylate and activate the cAMP response element binding protein (CREB) transcription factor. CREB regulates genes that have proven essential for the normal differentiation and maintained survival of neurons, both *in vivo* and *in vitro* (Xing et al., 1996). On the other hand ERKs also activate specific transcription factors. ERK1/2 activates Elk1, but ERK5 does not. ERK5 activates MEF2 directly but not ERK1/2 (Pearson et al., 2001). Egr-1 is the product of an early response gene activated by NGF through ERK1/2 activation (Levkovitz and Baraban, 2001). This protein activates transcription of *p35* gene, its product, p35, activates Cdk5. Egr-1 exerts its action through activation of c-Jun, instead of direct binding to DNA and it has shown to be involved in NGF-induced neurite outgrowth (Levkovitz et al., 2001).

In neurons and PC12 cells, NGF induces the activation of NF-kB through binding to TrkA and $p75^{NTR}$ (Hamanoue et al., 1999; Foehr, 2000; Wooten, 2001; Sole, 2004). Activation of NF-kB through TrkA seems to be important for neurite outgrowth, mediated by the death receptor antagonist FAIM. $p75^{NTR}$ mediated activation of NF-kB, on the other hand, has been related to the survival response of developing sensory neurons (Hamanoue et al., 1999).

A recent work by Jonathan Ham and his collaborators describes a role for the MEK-ERK pathway in regulating the expression of the BH3-only proapoptotic protein

Bim (Hughes et al., 2011). The authors report that an inhibitor of the MEK-ERK pathway, U0126, increases *bim* mRNA levels in sympathetic neurons and in the presence of NGF. They demonstrate that this regulation is attained through the 3' UTR of *bim*. By using a specific inhibitor of MEK1/2-ERK1/2 pathway they show that this mechanism is specific of this pathway. Finally, they demonstrate that MEK1/2-ERK1/2 inhibition reduces survival of NGF-treated sympathetic neurons.

The Ras-Raf-MEK-ERK signaling cascade is dysregulated in a variety of diseases including brain injury, cancer, cardiac hypertrophy, diabetes, and inflammation. Therefore this pathway has become an attractive target for the desing of new therapies (Kim and Choi, 2010; Tidyman and Raune, 2009; Tanti and Jager, 2009; Montagut and Settleman, 2009; Chico et al., 2009; Muslim, 2008).

1.9.6 MAPK/ERK and Death Receptors

As it has been mentioned, cytokines such as TNFα are also capable of triggering MAPK/ERK signaling pathway (Wajant et al., 2003). However, reports about the ability of death receptors to activate ERK have been contradictory and have led to the hypothesis that the effects of this activation might be cell specific (Vietor et al., 1993; Jupp et al., 2001; Müller et al., 1998). Several signaling pathways have been discussed to explain the mechanism of TNFα-triggered ERK phosphorylation, although none of them have been conclusive and there are no reports concerning the importance of this pathway in neuronal models. It has been reported that in HeLa cells, TNFα as well as other death ligands may trigger ERK phosphorylation pathway in order to override the apoptotic signaling triggered by binding of these ligands to their receptors (Tran, 2001). However, a specific pathway of MAPK/ERK activation triggered by these receptors could not be elucidated. Another report from 2005 describes a role for the FADD/caspase-8/cFLIP (FLICE Inhibitory Protein) signaling pathway in the TNFα-induced activation of ERK1/2 (Lüschen et al., 2005). In this paper the authors show that FADD and cFLIP are key for TNFα-induced ERK activation in various immortalized cell lines, although caspase-8 activity is not. On the other hand this work fails to specify a function for the activation of the MAPK/ERK pathway induced by TNFα. As it has been pointed out earlier in this introduction, the MAPK/ERK signaling pathway is also activated by other death receptors such as Fas (Kataoka et al., 2000). In this case, the authors show that activation of Fas leads to recruitment of FLIP-L to the DISC complex and FLIP-L can interact with Raf-1 and therefore activate the MAPK/ERK pathway. Another more recent work from 2008 reveals that, in a model of

colon epithelium cells, TNFR1 stimulation leads to Raf-1 activation. In this model, the authors show that this activation is independent of Ras (Edelblum et al., 2008).

1.10 Death Receptor Antagonists

Antagonists of death receptors are defined as molecules that interfere in the apoptotic signaling cascade, blocking it and conferring the cells protection and survival from the death-inducing stimulus. Some of the death receptor antagonists have been shown to be relevant for the regulation of various signaling cascades triggered by the binding of a death ligand to it specific receptor. This introduction will present those death receptor antagonist that have been shown to have some relevance in the nervous system.

1.10.1 Fas Apoptosis Inhibitory Molecule (FAIM)

FAIM is a Fas antagonist initially characterized by differential display as a gene that is up-regulated in B cells resistant to Fas mediated cell death and acts as an inhibitor of Fas-induced cell death (Schneider et al., 1999). FAIM was cloned from two different DNA sources, one of them from thymocytes and the other from brain tissue. Two sequences were found, one of them 66 nucleotides longer than the other in its 5' end. The brain derived sequence contained 22 more aminoacids and thus it was named FAIM long ($FAIM_L$) (Fig. 19). During the study of the genomic organization of FAIM, two putative transcription initiation points were identified in adjacent exons, indicating that $FAIM_L$ and $FAIM_S$ are generated by alternative splicing of a single gene. Expression analysis in multiple tissues showed that $FAIM_L$ is mainly expressed in the nervous system (Zhong et al., 2001). $FAIM_S$, on the other hand, is expressed in most tissues.

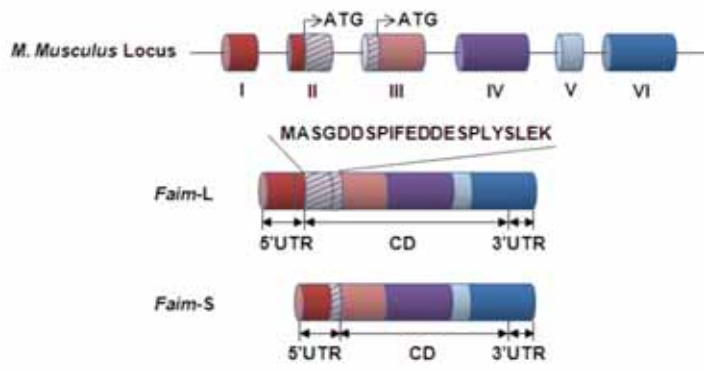


Figure 19. Genetic organization and splice variants of the FAIM gene. Source: Atlas of Genetics and Cytogenetics in Oncology and Haematology. Adapted from (Zhong et al., 2001).

Results from our own group have unveiled interesting features for both $FAIM_L$ and $FAIM_S$. Solé et al showed that overexpression of $FAIM_S$ increases NGF-induced neurite outgrowth in a neuronal model and that downregulation of $FAIM_S$ dramatically reduces neuritogenesis mediated by NGF in PC12 cells and Superior Cervical Ganglia (SCG) cells (Sole, 2004). Moreover, this work showed that neurite outgrowth promoted by $FAIM_S$ is dependent on ERK phosphorylation, and NF-kB activation, both pathways classically known to be activated by NGF (Davies, 2000; Foehr, 2000).

Another work by Segura et al. unveiled a role for $FAIM_L$ in the PC12 neuronal model and in mouse cortical neurons. This paper showed that $FAIM_L$ is specifically expressed in neurons during development. Endogenous $FAIM_L$ protects neurons from death induced by Fas or TNFR1, as it was demonstrated by RNAi experiments. Moreover, they demonstrated that $FAIM_L$ can bind to Fas and prevent caspase-8 activation induced by FasL (Segura et al., 2007).

1.10.2 Lifeguard

Lifeguard (LFG, also known as FAIM-2) was cloned in 1999 as a molecule capable of protecting cells from apoptosis induced by CD95/Fas and not other death receptors (Somia et al., 1999). Somia and his collaborators generated a cDNA library in a retroviral vector from the cell line MRC-5- This cell line is characterized by presenting resistance to cell death induced by Fas and, like other cell lines, it becomes sensitive when co-treated with a protein synthesis inhibitor such as cycloheximide, suggesting the presence of proteins which are necessary for resistance to FasL. They used the cDNA library to infect HeLa cells, a cell line sensitive to FasL treatment and they selected clones resistant to treatment with CH11, a Fas-agonistic antibody. They identified a 35kDa protein which they named Lifeguard. When comparing sequence homology it was found that Lifeguard was the human homolog for the rat protein NMP35 (Neural Membrane Protein 35), a protein highly expressed in the nervous system (Schweitzer et al., 1998), suggesting a role for Lifeguard in neurons. Structurally, Lifeguard is composed by an N-terminal end of around 106 aa, followed by seven transmembrane passes. Protection from death induced by FasL is due to a direct interaction between Lifeguard and the Fas receptor, although this interaction is not able to alter the DISC formation. It has also been described as a death receptor antagonist for Fas in the nervous system, and its regulation seems to be under the control of the PI3K survival pathway (Beier, 2005).

Others demonstrated that Lifeguard is also responsible for protection from Fas-induced cell death in mouse cortical neurons and cerebellar granular neurons. (Fernández et al., 2007). In that paper, the authors used the cell line SH-SY5Y as a model of human neuroblastoma. SH-SY5Y cells are no sensitive to Fas-induced apoptosis because they do not express caspase-8. However, treatment with Interferon-γ (IFNγ) induces caspase-8 expression and renders cells sensitive to Fas-induced cell death (Fulda and Debatin, 2002). Researchers demonstrated that LFG coul afford protection from apoptotic cell death-induction by Fas. They also suggested that interaction between Fas and LFG takes place at the lipid rafts, since overexpressed Lifeguard was only found in lipid rafts, and Fas translocated to these microdomains of the membrane after FasL stimulation. Moreover, specific LFG knockdown restored FasL toxicity to the levels observed in non-transfected SH-SY5Y cells, without affecting translocation of Fas to the lipid rafts. These results were reproduced in mouse cortical neurons in which they overexpressed Lifeguard by means of a lentiviral system. Finally, they showed that in cerebellar granule neurons (CGN), downregulation of the antiapoptotic protein FLIP did not lead to further increase of sensitivity to FasL-induced cell death. Instead, their results suggest that, in differentiated cerebellar granular neurons, protection from FasL-induced cell death could be attributed exclusively to LFG.

More recently, the group of Besirli et al. showed that Lifeguard could prevent apoptotic cell death in a model of detachment-induced photoreceptor cell death. Interestingly they described a mechanism by which Lifeguard activation was dependent on MAPK/ERK pathway (Besirli et al., 2012). They used a model of immortalized photoreceptors, 661W cells. When 661W cells were treated with a Fas-agonistic antibody, the JNK and ERK pathways became active and cells died. When the ERK activation pathway was blocked using a specific inhibitor, cell death was increased and expression levels of Lifeguard were reduced. Seemingly, using an RNAi specific for Lifeguard sensitized cell to Fas-induced cell death.

1.11.3 FLICE Inhibitory Protein (FLIP)

FLIP was originally identified as the product of viral genes (vFLIPS) during the search in the genome for proteins containing DED domains, which could interact with caspases. vFLIPS contains two DED and belong to the family of DED containing proteins together with FADD, procaspase-8, procaspase-10, and phosphoprotein enriched in astrocytes 15kDa (PEA15) among others. vFLIPS were first identified in γ-herpesvirus and molluscipoxvirus. (Bertin et al., 1997; Hu et al., 1997; Thome et al.,

1997; Searles et al., 1999). Subsequently, a mammalian homolog for vFLIP was characterized and named cFLIP (Irmler et al., 1997) (also known as CASPER, CASH, CLARP, FLAME, I-FLICE, MRIT, AND Usurpin). Various isoforms of cFLIP have also been identified, two of them expressed *in vivo:* a 26 kDa Short FLIP (FLIP-S) and a 55 kDa Long FLIP (FLIP-L) (Tschopp et al., 1998). There is also a third form of cellular FLIP isolated from the Raji cell line of lymphoblast-like cells, FLIP-R, with a molecular weight of 24 kDa (Golks, 2005).

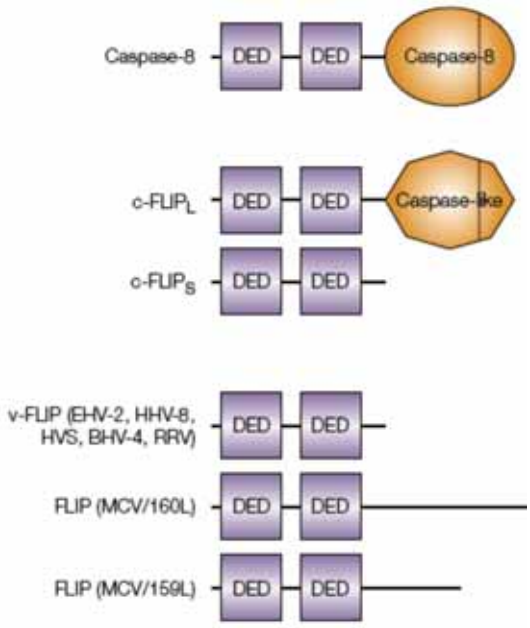


Figure 20. Molecular structure of viral and cellular FLIPs. Both herpesviral FLIP and the short form of cellular FLIP (c-FLIPS) consist essentially of two repeats of a death effector domain (DED). The long splice variant of c-FLIP (c-FLIPL) contains a carboxy- terminal inactive caspase-like domain, which confers on the molecule an overall structural homology with caspase-8 and caspase-10. (Thome and Tschopp, 2001)

The short form FLIP-S has a similar structure to vFLIP from γ-herpesvirus, except that the two DED are followed by a short c-terminal extension of around 20 aminoacids. FLIP-R was originally cloned in 2001 by Djerbi et al (Djerbi et al., 2001) and its expression has been later found in various lymphocyte cell lines and primary T-cells. FLIP-R has also the DED domain, but it lacks the carboxy terminal tail. Despite this, its features are very similar to those of FLIP-S since they both have a short half-life, it is similarly recruited to the DISC, has a comparable antiapoptotic effect, a similar expression pattern during T-cell activation, and they are both strongly induced after stimulation of T cells with CD3/CD28.

FLIP-L, on the other hand, has a longer C-terminal fraction that includes a caspase-like homology region that gives the molecule a structural similarity with caspase-8 and -10. However, this region lacks the catalytic activity found in caspases. This is due to aminoacid substitutions like the key cysteine in the motif Q-A-C-X-G of caspases-8 and -10, rather showing the motif Q-N-Y-V-V, and the histidine in the motif H-G, both crucial for catalytic activity (Irmler et al., 1997; Scaffidi et al., 1999b) (Fig. 20).

1.10.3.1 Regulation of cFLIP expression

FLIP is highly and constitutively expressed in many cell types, including cardiomyocytes, endothelial cells, keratinocytes, pancreatic β cells, dendritic cells, macrophages, CD34+ hematopoietic stem cells, and spermatocytes (Kiener et al., 1997; Rescigno et al., 2000; Bouchet et al., 2002; Kim et al., 2002; Maedler et al., 2002; Davidson, 2003; Giampietri et al., 2003; Marconi, 2004). FLIP has also been found in the nervous system. It has been demonstrated to be expressed and have an anti-apoptotic role in motoneurons (Raoul et al., 1999) and it is also highly expressed in the cortex and thalamus. FLIP expression levels are lower in the hippocampus and its expression increases after ischemic damage in neonatal brains (Graham et al., 2004).

One of the main regulators of cFLIP expression is the transcription factor NF-kB. Studies have demonstrated that cFLIP expression under the control of NF-kB results in increased resistance to FasL, TNFα or TRAIL (Kreuz et al., 2001; Micheau et al., 2001). Indeed, cFLIP has been proposed as one of the key components in apoptosis resistance to TNFα mediated by NF-kB and it has also been demonstrated that protein synthesis inhibition using cycloheximide leads to downregulation of cFLIP. cFLIP has been also shown to be ubiquitylated and subsequently degraded by the proteasome, its degradation can be rescued by the proteasome inhibitor MG132. As it has been explained earlier in this introduction, the E3 ligase ITCH becomes activated by JNK upon TNFα stimulation, specifically ubiquitylating cFLIP-L, targeting it for proteasomal degradation (Chang et al., 2006). Furthermore, ITCH-deficient mice are resistant to TNFα-induced acute liver failure, and cells from these mice do not display cFLIP-L ubiquitylation and degradation.

As for the different isoforms, cFLIP-S has a shorter half-life than cFLIP-L. It has been reported that sensitization of activated T-cells with cycloheximide slightly reduced cFLIP-L expression, while cFLIP-S expression became undetectable (Schmitz et al., 2004). This has been proposed to occur due to the structural differences in the C-terminal regions of FLIP-s and FLIP-L. Lysines 192 and 195 of cFLIP-S can get ubiquitylated and therefore target cFLIP-S for proteasomal degradation (Poukkula, 2005). However, the ubiquitin ligase involved in this process has not yet been found.

Other mechanisms of cFLIP expression regulation might include the ERK pathway through different mechanisms (Yeh et al., 1998; Wang, 2002). The PI3K/AKT

pathway has also been involved in cFLIP expression regulation at different levels (Panka, 2001; Suhara et al., 2001; Sade, 2003).

Collectively it has been shown that cFLIP has a short half-life and its turnover is tightly controlled by various mechanisms. These mechanisms provide a quick change of cFLIP levels in the cell that generate a way to provide a fast response to cellular stress.

1.10.3.2 cFLIP cellular functions

Due to its structural homology to caspase-8, FLIP interferes with activation of caspase-8 at the DISC. vFLIP and cFLIP-S are structurally very similar and therefore their mechanism of caspase-8 inhibition are also similar. The DEDs of vFLIP and cFLIP-S bind to the DED in FADD (Srinivasula et al., 1997; Thome et al., 1997), thus allowing their recruitment to the death receptor complex (complex II in the case of TNFR1). Once in the receptor they can form heterodimers with caspase-8, thus avoiding its posterior processing. Therefore vFLIP and cFLIP-S act as dominant negative forms of caspase-8, preventing the processing and release of active caspase-8 from the complex (Scaffidi et al., 1999b; Schmitz et al., 2004).

The molecular mode of action for cFLIP-L is different from vFLIP and cFLIP-S. cFLIP-L binds to caspase-8 through the DED domain and also through the caspase-like domain. Initially, it was thought that FLIP-L could act as a competitive inhibitor for the union to FADD with caspase-8, but later it was found that FLIP-L did not avoid the recruitment of caspase-8 to the DISC (Rasper et al., 1998). Once in the DISC, FLIP-L is processed into a p43 subunit, which remains attached to the DISC, and also generates a p12 subunit, which is released from the complex (Krueger, 2001). In the presence of FLIP-L, caspase-8 is also processed, generating p43 and p41 fragments, while the p10 subunit is released. According to the autocatalytic cleavage model proposed for caspase-8, there is first an autocatalytic activation and, with and adjacent caspase domain, there is transcatalytic cleavage producing the complete activation and allowing for the formation of the active heterotetramer formed by p18 and p10 subunits (Fig. 21). If the adjacent molecule is FLIP-L instead of caspase-8, the first autocatalytic cleavage takes place but, since the caspase domain in FLIP-L is not functional the final activation of caspase-8 is avoided (Irmler et al., 1997; Srinivasula et al., 1997; Rasper et al., 1998; Scaffidi et al., 1999b; Krueger, 2001).

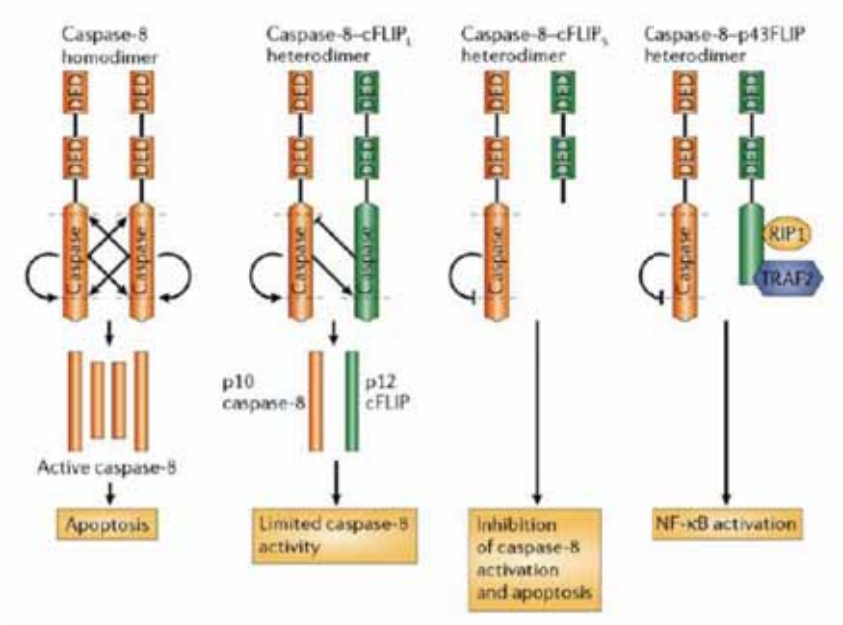


Figure 21. FLIP modulates activation of caspase-8 and NF-kB. FLIP-S inhbits caspase-8 activation by acting as a dominant negative and avoiding its activation. FLIP-L allows a partial activation of caspase-8 but it blocks further processing due to its inactive caspase-like domain. (Budd et al., 2006)

Interestingly, it has been shown that overexpression of FLIP-L can induce cell death in some models (Inohara et al., 1997; Shu et al., 1997). A recent study showed a relationship between the quantity of cFLIP-L and the amount of cell death. The work demonstrates that cFLIP-L only exerts a proapoptotic effect when expressed at moderate amounts in combination with strong Fas activation or in the presence of high amounts of cFLIP-S (Fricker et al., 2010). However, when expressed at lower levels, both cFLIP-S and cFLIP-L show a protective role from apoptosis induced by death receptors. Moreover, fibroblast cell lines derived from FLIP knockout mice are sensitive to DR-induced apoptosis (Yeh et al., 2000).

During Fas signaling, FLIP exerts its antiapoptotic function through inhibition of caspase-8 activation at the level of the DISC. In the presence of cFLIP-L, other signaling molecules are recruited such as RIP, TRAF1 and 2, and, interestingly, Raf-1. This suggests a role for FLIP-L in the regulation of signaling pathways other than apoptosis. In fact, as it has been mentioned before, cFLIP-L has a role in NF-kB and ERK signaling (Hu et al., 2000; Kataoka et al., 2000) and therefore in the regulation of

proliferation and/or differentiation of Fas stimulated cells (Fig. 22). The short form of cFLIP and the HHV-8-encoded vFLIP have also shown a certain induction of NF-kB in forced expression systems using reporter plasmids (Chaudhary et al., 1999; 2000)

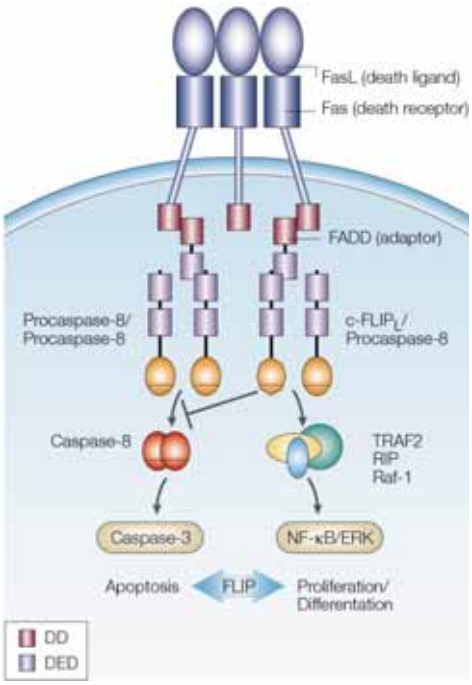


Figure 22. Regulation of death receptor-induced gene expresión by FLIP. The long form of cellular FLIP (FLIP-L) inhibits complete processing and activation of caspase-8 at the receptor level and thereby exerts an anti-apoptotic function. FLIPL mediates the recruitment of additional signalling molecules, such as TRAF1, TRAF2, RIP and Raf-1 at the death-inducing signalling complex, and might have an active role in the control of death receptor-mediated gene expression by means of the nuclear factor κB (NF-κB) and extracellular signal-regulated kinase (ERK) pathways. (Thome and Tschopp, 2001).

FLIP-induced NF-kB activation is inhibited by dominant negative versions of certain proteins implicated in NF-kB activation mediated by death receptors such as NF-kB inducing Kinase (NIK) and TRAF1 and 2. All these proteins have been shown to bind directly to FLIP in the yeast two-hybrid system (Shu et al., 1997).

In the nervous system FLIP-L has been shown to protect neurons against ischemia *in vivo* and against glucose deprivation (GD) *in vitro.* The authors of this study used mice deficient in TNFR1 and neuron-specific FLIP-L transgenic mice. Overexpression of FLIPL was sufficient to rescue TNFR1 KO neurons from GD-induced death and to enhance TNF neuroprotection in WT neurons, and neuron-specific expression of FLIPL in transgenic mice significantly reduced lesion volume

after permanent Middle Cerebral Artery Occlusion (pMCAO) (Taoufik et al., 2007). Besides its multiple roles in death receptor-induced signaling, cFLIP has been also involved in neurotrophin-mediated activation of ERK. A recent work by our own group described that FLIP-L interacts with Trk receptor and is necessary for neurite outgrowth mediated by neurotrophins (Moubarak et al., 2010). Moubarak and her collaborators showed that FLIP-L is expressed in different regions of the mouse embryonic nervous system. FLIP-L is expressed early during the neuronal development and its expression decreases at later stages. Overexpression of FLIP-L enhances neurotrophin-mediated neurite outgrowth in motoneurons, superior cervical ganglion neurons, and PC12 cells. Conversely, downregulation of FLIP-L by RNAi significantly reduces neurotrophin mediated neurite outgrowth. Interestingly, FLIP-L RNAi impairs NGF-mediated activation of ERK and NF-kB, two pathways greatly involved in the regulation of neurite outgrowth.

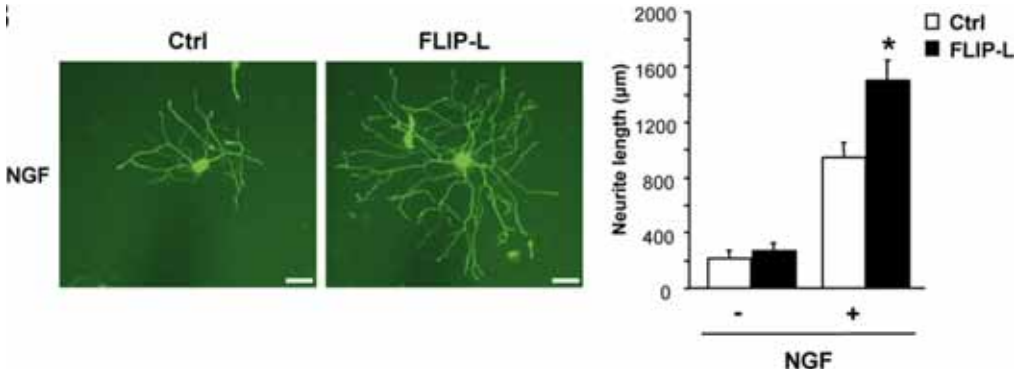


Figure 23. Ballistic transfections of cultured SCG neurons with an EGFP expression plasmid together with the control vector pcDNA3 (Ctrl) or pcDNA3–FLIP-L (FLIP-L) plasmids. Neurites were measured 24h alter transfcetion (Moubarak et al., 2010).

In addition to its roles as an antiapoptotic protein and inducer of neurite ourgrowth, FLIP-L is also relevant in another cell death process known as necroptosis or programmed necrosis. Necroptosis is a form of regulated necrosis characterized by being caspase independent and by necrotic cell death morphology (Degterev et al., 2005). This programmed necrosis can be triggered by death receptors such as Fas, TNFR1, and TRAILR1 and TRAILR2 (Reviewed in Vandenabeele et al., 2010). Treatment of some cell lines and primary cultures with caspase inhibitors unveils this death receptor triggered necrosis (Fiers et al., 1995). The most extensively characterized pathway leading to necroptosis is initiated by ligation of TNFR1. The formation of TNFR1 complex II in the presence of caspase-8 inhibitors or when caspase-8 is deleted promotes necroptosis (Vercammen et al., 1998), which depends on Rip1 and Rip3 phosphorylation (Declercq et al., 2009). In this context, Oberts et al. have found that caspase-8 inhibits death receptor-induced necroptosis without inducing

apoptosis by forming a proteolytically active complex with FLIP-L (Oberst et al., 2011). This complex is active without requiring the cleavage of caspase-8 into its active fragments, and FLIP-L seems to catalytic activity to this form of caspase-8.

1.11 Other Antagonists of Death Receptors

A20

A20, also known as TNF Apoptosis Inhibitor Protein 3 (TNFAIP3) was identified for the first time in 1990 as a gene rapidly induced by TNF stimulation in human umbilical vein endothelial cells (Opipari et al., 1990). The first indications for an A20 function were elucidated from different isolates of the breast cancer cell line MCF-7 that were either sensitive or resistant to TNFα-induced cell death. A20 was clearly upregulated in cells resistant to TNFα-induced apoptosis, and transfection of A20 into cells provided protection from TNFα-induced cell death (Opipari et al., 1992). Therefore, A20 was suggested to have a function as an apoptosis inhibitor.

Soon after, overexpression of A20 was shown to inhibit TNFα-induced activation of NF-kB, and key elements of the NF-kB activation pathway, such as TRAF2 and RIP were identified as targets of A20 (Jäätelä et al., 1996; Song et al., 1996; Cooper et al., 1996). However, a better understanding of A20 functions came from charcterization of the phenotype of A20-deficient mice (Lee et al., 2000). A20 knockout mice develop a severe and generalized inflammation in its organs and they are extremely sensitive to sub-lethal dosis of TNFα. These effects were likely due to uncontrolled activation of NF-kB, which was persistently activated in TNF-stimulated A20-deficient MEFs. This study established A20 as a negative regulador of NF-kB.

A20 was found to inhibit NF-kB through its deubiquitinase domain by hydrolyzing K63-linked polyubiquitin chains on key NF-kB signaling molecules (Wertz et al., 2004). Furthermore, one of the C-terminal Zn finger domains in A20 was found to harbor E3 ligase activity (Wertz et al., 2004). Therefore, A20 is a ubiquitin-editing enzyme with DUB and E3 ligase activity. Upon TNFα stimulation, A20 expression is induced by NF-kB, it is recruited to Rip1 and cleaves K63-linked polyubiquitin chains on Rip1. A20 was also shown to target TRAF2 to the lysosome for its degradation as another mechanism of signaling regulation. This mechanism is independent of the ubiquitn modifying activity of A20, instead, zinc finger domains seem to be key for this function (Li et al., 2009, reviewed in Harhaj and Dixit, 2012). In another recent paper,

Verhelst et al. describe that A20 inhibits LUBAC-mediated activation NF-kB through binding of linear polyubiquitin chains. The authors demonstrate that overexpression of a polypeptide corresponding to the C-terminal zinc-finger domain 7 (ZF7) of A20 is enough to inhibit TNFα-induced NF-kB activation. A20 can form a complex with NEMO and LUBAC, preventing TNFα-induced binding of NEMO to LUBAC (Verherst et al., 2012).

In another recent report, A20 was shown to mediate its inhibitory function in a complex with three other proteins; Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1, also called TXBP151 or T6BP), and two E3 ubiquitin ligases, Itch (also known as AIP3) and RING finger protein 11 (RNF11) (Shembade et al., 2009; Pranski et al., 2012a).

In the nervous system, A20 has been described to protect hippocampal neurons from cell death induced by TNFα and after ischemia-induced damage (Miao et al., 2005). Recently, the essential components of the A20 ubiquitin-editing complex were found to be present and mainly expressed in neurons. The A20 complex components are also differentially expressed throughout the human brain (Pransky et al., 2012b).

SODD (Silencer of Death Domain)

Although it does not possess a DD, SODD is a cytosolic protein that binds to TNFR1, TNFR2, and DR3, but not CD95, DR4, or DR5 and blocks activation of apoptotic signaling, NF-kB activation, or JNK by TNFR1 (Jiang, 1999). However, knockout mice for SODD have shown opposing results. SODD does not seem indispensable for the life and correct development of organs in mice. On the other hand, absence of SODD reduces the amount of TNFα needed to induce NF-kB pathway activation. These contradictory results could be explained by structural and functional redundancy with other proteins of the same family (Endres et al., 2003).

SODD has also been linked to aggressiveness of ovarian cancer. More specifically, expression of SODD in the cytoplasm marked a less aggressive ovarian cancer (Annunziata et al., 2007).

PEA-15 (Phosphoprotein Enriched in Astrocytes-1)

PEA-15 is a small protein of 15 kDa initially described as a phosphoprotein enriched in astrocytes and later found to be expressed in other tissues (Araujo et al., 1993). Soon after, PEA-15 was found to regulate apoptosis induced by Fas and

TNFR1 in MCF7 and HeLa cells. PEA-15 inhibited apoptosis induced by TNFR1 or Fas through the displacement of the FADD-caspase-8 interaction (Condorelli et al., 1999). The structure of PEA-15 contains an N-terminal DED and an irregular structure in its C-terminal. Characterization of the PEA15 null mice revealed that PEA-15 also protects astrocytes from TNFα-induced apoptosis *in vivo* (Kitsberg et al., 1999). Interestingly, PEA-15 has been involved in MAPK/ERK signaling (Formstecher et al., 2001). More precisely, the authors showed that overexpression of PEA-15 in NIH-3T3 cells impaired ERK translocation to the nucleus. To further confirm the role of PEA-15 in ERK singaling, the authors used astrocytes from PEA-15 null mice and found a greater proliferative response when compareing to control astrocytes. They linked this response to with an increased serum response element-dependent cFos transcription. By means of a yeast-two hybrid screening, researchers also demonstrated that PEA-15 bind to ERK1 and ERK2. Another report describes the importance of MAPK members in the anti-apoptotic function of PEA-15 (Condorelli et al., 2002). Condorelli and his collaborators showed that PEA-15 inhibits the signaling of JNK and p38, protecting cells form stress-induced apoptosis. Importantly, their results also show that the full anitapoptotic effect of PEA-15 requires not only inhibition of p38 and JNK, but also activation of ERK, demonstrating that PEA-15 acts as a MAPK regulatory protein. Phosphorylation of PEA-15 has been proposed as a switch determining the role of PEA-15 as a regulator of apoptosis or cell proliferation (Renganathan et al., 2005). Phosphorylation of PEA-15 at Ser-104 (phosphorylated by protein kinase C) blocks ERK binding to PEA-15 *in vitro* and *in vivo*. On the other hand, phosphorylation of Ser-116 (phosphorylated by calcium/calmodulin-dependent protein kinase II, CAMKII). was shown to promote binding to FADD.

In accordance with these results, a recent paper published by Gawecka and his collaborators identified PEA-15 and his partner ERK as potential targets for the development of new therapeutics to avoid the progression of minimal residual disease in patients with high-risk neuroblastoma. The authors tested the effects of PEA-15 overexpression on neuroblastoma cells *in vitro* and also analyzed PEA15 expression in the context of clinical and genetic features of neuroblastoma in tumor samples to determine its correlation with disease progression. Their results verified that inhibition of motility required PEA-15 interaction with its binding partners ERK and RSK2 (Gawecka et al., 2012).

SUMO-1 (Small Ubiquitin-Related Modifier-1)

SUMO-1 (also known as sentrin) was identified as a protein that could bind to the death domain in Fas, presented high homology to ubiquitin, and its overexpression could protect from death induced by Fas or TNFR1 (Okura et al., 1996). This protein is a member of the SUMO family of proteins, which functions in a way similar to ubiquitin, since it is bound to target proteins as part of a post-translational modification system (SUMOylation). SUMO-1 has been involved in a variety of cellular processes, such as nuclear transport, transcriptional regulation, apoptosis, and protein stability.

Examples of proteins that are modified by SUMO-1 are the proapoptotic protein DAXX (Jang et al., 2002), caspase-2, caspase-7 (Hayashi et al., 2006), caspase-8 (Besnault-Mascard et al., 2005), ASK-1 (Lee et al., 2005), and the NEMO/IKKγ complex (Huang et al., 2003).

BAR (Bifunctional Apoptosis Regulator)

BAR was identified in a yeast two-hybrid assay to find antagonists of Bax-induced cell death. It has a SAM (sterile alpha motif) domain that allows it to interact with antiapoptotic members of the Bcl-2 members such as Bcl-2 and Bcl-X_L. This domain is required for suppression of Bax-induced cell death, and therefore inhibits the intrinsic pathway of apoptosis. It also has a domain with high homology to the DED domain, through which it can interact with caspase-8, thus inhibiting the extrinsic pathway of apoptosis (Zhang et al., 2000). A mechanism was proposed by which BAR could sequester caspase-8 fragments in intracellular membranes, blocking the cleavage of caspase-8 substrates (Stegh, 2001). Their data suggest that in MCF-7 cells active caspase-8 is sequestered on the outer mitochondrial surface presumably by association with BAR in a way that does not allow substrates to be cleaved.

BAR has been shown to protect neurons from diverse cell death pathways (Roth et al., 2003). In that study, BAR was found to be predominantly expressed in neurons. Overexpression of BAR in different neuronal cell lines inhibited cell death induced by both intrinsic (staurosporine, serum starvation) and extrinsic (TNFα, CH11/Fas) stimulus.

Another recent report identified a role for BAR in the protection against cardiac injury. The authors generated transgenic mice expressing a modified BAR in which the RING domain was deleted to prevent autoubiquitylation. Overexpression of the

modified BAR in the hearts of mice rendered the heart more resistant to ischemia/reperfusion injury, correlating with reduced cardiomyocyte apoptosis (Chua et al., 2009).

FAP-1 (Fas Associated Phosphatase-1)

FAP-1 is a tyrosine phosphatase that can interact with the intracellular domain of Fas. It is highly expressed in those tissues that show resistance to FasL mediated cytotoxicity and it has been implicated in the inhibition of Fas induced apoptosis (Sato et al., 1995). It has been established FAP-1 blocks apoptosis induced by FasL by inhbiting Fas export from the cytoplasm to the cell surface, a necessary step to trigger the signaling cascade leading to apoptosis (Ungefroren et al., 2001; Ivanov et al., 2003). Expression of FAP-1 is regulated at the transcriptional level through NF-kB (Ivanov et al., 2006). Ivanov and his collaborators generated human melanoma cell lines with different levels of surface expression of Fas, based on the FAP-1-dependent regulation of Fas translocation. This way they established a causal connection between high basal NF-kB transcription factor activity (a hallmark in many types of metastatic tumors) and NF-kB-dependent transcriptional regulation of FAP-1 that restricts Fas protein trafficking, thereby facilitating the survival of cancer cells. In studies with astrocytomas overexpressing FAP-1 it has been shown that this protein confers high resistance to Fas-mediated cell death. FAP-1 dephosphorylates Y275 in the C-terminal of Fas that becomes phosphorylated after induction by FasL. This is the first evidence that Fas activity may be regulated by reversible phosphorylation (Foehr et al., 2005).

The importance of FAP-1 as a phosphatase of Fas was further revealed by eveidence showing that loss of FAP-1 promotes invasive growth *in vivo* (Hoover et al., 2009). The authors also descrobe the implication of FAP-1 in the MAPK signaling cascade, since functional loss of FAP-1 potentiates MAPK signaling downstreame of multiple different Ras activating oncogenes found in both Human Papilloma Virus (HPV) positive and negative epithelial cancers.

A more recent work by Winterhoff et al. described FAP-1 and Fas localization in 2D adherent cultures and after 3D differentiation of a pancreatic adenocarcinoma cell line (Winterhoof et al., 2012). Results show that in non-differentiated cells FAP-1 exhibited a nuclear and perinuclear localization. After differentiation, FAP-1 was relocated towards the actin cytoskeleton beneath the outer plasma membrane of polarised cells and no further nuclear localisation was observed. Co-localization of Fas and FAP-1 was rarely observed in undifferentiated cells, whereas differentiated cells

exhibited a strong co-localization. Also, Fas-mediated apoptotic signaling was not affected by FAP-1 in undifferentiated cells, while regulation of apoptosis by FAP-1 eas evident in differentiated adenocarcinoma cells.

FAIM3/TOSO

FAIM3 was identified as a cell surface regulator of Fas induced apoptosis in T cells. Its expression was limited to lymphoid cells it appeared limited to inhibition of apoptosis mediated by memberes of the TNF family and was capable of inhibiting T cell self-killing induced by TCR activation processes that upregulate Fas ligand (Hitoshi et al., 1998).

FAIM3 has been shown to be of key relevance in Fas-mediated resistance in Chronic Lymphocytic Leukemia (CLL). Its overexpreeion is triggered by B-cell receptor signaling and associates with progressive disease (Pallasch et al., 2008).

More recently, Toso was found to regulate death receptor induced apoptosis by facilitating the ubiquitylation of the adaptor kinase protein RIP1 in a complexe containing Toso, RIP1 and FADD. After FasL and TNFα stimulation, Toso promoted the activation of MAPK and NF-kB sigaling pathways, thus increasing survival versus apoptosis signals (Nguyen et al. 2011). The authors also analyzed the phenotype of Toso null mice, revealing that Toso is essential for TNFα-mediated liver damage.

1.12 Apoptosis in the Nervous System

1.12.1 Apoptosis during development of the Nervous System

During development of the central nervous system most cells die by a mechanism of programmed cell death (Kerr et al., 1972). Moreover, programmed cell death is a key event in most neurodegenerative diseases. Therefore apoptosis plays a central role both in the developing and pathology of the nervous system. Neurons require trophic support for survival. In the 1950's Rita Levi-Montalcini and Viktor Hamburger proposed that survival of neurons depended directly on the availability of their innervation targets, where the necessary trophic factors would be found. This was known as the neurotrophin theory (Reviewed by Oppenheim, 1989; 1991). This theory proposes that immature neurons compete for trophic factors derived from target cells and that these trophic factors are found in limited supply. Therefore, only those neurons that are able to establish correct synaptic connections obtain trophic factor and are allowed to survive. However, this theory did not provide an explanation as to

how neurons that do not obtain a trophic factor die and are eliminated. Although in the beginning it was thought that neurons died passively after trophic factor deprivation, it was soon discovered that an active process is implicated. Identification of programmed cell death genes in *Caenorhabditis elegans* and their homologues in mammals (see previous sections) offered the opportunity to test whether these components had roles in neuronal cell death. It was discovered that neuronal cell death induced by trophic factor deprivation required the participation of caspases (Gagliardini et al., 1994). This was the first evidence that trophic factor deprivation activated a program of cellular suicide in vertebrate neurons known as apoptosis (Reviewed in (Yuan and Yankner, 2000)). Apoptosis regulated by trophic factor requirement takes place mainly in postmitotic neurons that have already established both afferent and efferent synaptic connection (Oppenheim 1991; Blaschke et al., 1996).

The main model for the study of neuronal apoptosis due to trophic factor deprivation is nerve growth factor (NGF) deprivation, being sympathetic neurons the most representative neuron population dependent on NGF (Deshmukh et al., 1996; D P Martin et al., 1988). Apoptosis of sympathetic neurons after NGF deprivation requires macromolecular synthesis, Bax expression, releasing of cytochrome C to the cytosol, and caspase activity (Martin et al., 1988; Deckwerth et al., 1996; Deshmukh et al., 1996; Troy et al., 1996; Neame et al., 1998).

1.12.2 Apoptosis in Nervous System Pathology

Neuronal death underlies the symptoms of many neurological disorders including Alzheimer's, Parkinson's, and Huntington's diseases, stroke, and amyotrophic lateral sclerosis. In many diseases, aberrant regulation of apoptosis is the main abnormality. On the one hand, resistance to apoptosis is known to be responsible for many types of cancer (Wyllie, 1997). On the other hand, death of hippocampal and cortical neurons is responsible for the symptoms of Alzheimer's disease; death of neurons in the midbrain that use dopamine underlies Parkinson's disease; Huntington's disease involves death of neurons in the striatum, which controls movements; and death of lower motor neurons manifests as amyotrophic lateral sclerosis. As it has been exposed, lack of neurotrophic factor support is the best-studied signal that triggers apoptosis in neurons (Oppenheim, 1991). Overactivation of glutamate receptors in neurons can also result in apoptotic cell death by a mechanism that involves calcium influx (Glazner et al., 2000). This excitotoxicity has been involved in acute conditions such as stroke, trauma, and epileptic seizures, as well as in Alzheimer's disease and motor system disorders (Choi, 1992; Wong et al., 1998).

Formation of free radicals induced by oxidative stress can damage cellular lipids, proteins, and nucleic acids, resulting in the initiation of the apoptotic process (Sastry and Rao, 2000). Finally, environmental toxins can induce neuronal apoptosis, and some of them induce patterns of brain damage similar to Parkinson's and Huntington's diseases (Duan et al., 1999).

1.13 Death Receptors in the Nervous System

Cell death plays an important role in the shaping of the developing nervous system and in neurological disease and traumatic injury. As it has been pointed out, death receptors can trigger both apoptotic and survival or proliferation pathways. This is also true in the nervous system, where they can play roles in neural development or degeneration.

1.13.1 Death Receptors in Nervous System Development

Programmed cell death has a very prominent role in the development of the nervous system, since half of the neurons produced during neurogenesis will die before birth by this process. Death receptors and their ligands are present in the developing nervous system, and many reports show that they are able to trigger death of embryonic neurons and glia *in vitro* (Cohen and Eisenberg, 1992). However, there is little evidence of death receptor signaling in the developing nervous system *in vivo*, with the exception of $p75^{NTR}$, which can trigger cell death after binding to neurotrophins in certain cirscumstances (Deppman et al., 2008). The FasL/Fas system has been implicated in apoptosis induced by trophic factor deprivation *in vitro*, but this implication has not been demonstrated *in vivo.* Mice expressing a dominant negative of DAXX, a key protein in Fas-mediated cell death of motoneurons, do not show any alteration of spinal chord development. Moreover, the spontaneous mouse mutants *lpr and gld*, partial loss-of-function of Fas and FasL respectively, have normal numbers of cortical, hippocampal and motor neurons at birth (Cohen and Eisenberg, 1992). However, they show reduced dendritic branching of hippocampal and cortical neurons during the period at which synaptogenesis takes place. Fas seems to play a role in the induction of neurite outgrowth through the activation of the MEK1/ERK/p35 pathway (Desbarats et al., 2003) At similar stages, mice deficient in TNFα show defects in synaptic scaling and ocular dominance plasticity in the visual system (Zuliani et al., 2005; Kaneko et al., 2008). Based on our knowledge so far, death receptors may not have a role in apoptosis of the developing nervous system, but they may have functions during the developmental process instead.

1.13.2 Death Receptors in Nervous System Pathology

There is a considerable body of data concerning the role of DRs in pathologies of the nervous system. This research provides opportunities for new therapeutic strategies focused on the DR, their ligands or their signaling mediators. The next section illustrates how DRs are implicated in some pathologies of the nervous system.

Cerebral Ischemia

A short ischemic event, known as ischemic preconditioning, can result in a subsequent resistance to severe ischemic damage (Ischemic Tolerance, IT) (Barone et al, 1998). Death Receptors and their ligands, especially TNFα, play an important role in this event. Liu and his collaborators demonstrated that TNFα expression is increased in the brain after an ischemic event (Liu et al., 1994). They induced focal ischemia in the brain of rats by permanent Middle Cerebral Artery Occlusion (MCAO) and monitored the levels of TNFα mRNA and protein expression. Results showed a significant increase in TNFα mRNA and protein over time, with peak expressions at 12h after MCAO. Nawasahiro et al. later established a link between TNFαand ischemic tolerance. They administered TNFα to mice 48h before MCAO and found a reduction of the infracted zone in pretreated mice when compared to control, non-pretreated mice, concomitant with a significant decrease in CD11b immunoreactivity, a marker of the inflammatory response (Nawasahiro et al., 1997). Hurtado et al., demonstrated that after after oxygen-glucose deprivation, another model of cerebral ischemia, the TNFα-converting enzyme TACE was upregulated in rat forebrain slices and in rat cortical neuron cultures. This upregulation was responsible for an increase in TNFα shedding, and in that model the authors demonstrated that TNFα inhibited apoptosis through activation of the NF-kB pathway (Hurtado et al., 2001;2002). More recently, Toll-like receptor 4 (TLR4) was also shown to mediate a neuroprotective effect by ischemic preconditioning through the up-regulation of TNFα and NF-kB activity (Pradillo et al., 2009). Lambertsen et al. have shown that microglia protect neurons against ischemia, and that they do so by synthesis of TNFα. They showed that cortical infarction and behavioral deficit were more exacerbated in TNF-knockout mice. In addition, observation of infarction size in TNFR1-KO mice compared to TNFR2-KO demonstrated that the neuroprotective effect was mediated by TNFR1 (Lambertsen et al., 2009).

An ischemic stroke results in an initial area of neuronal death, the core, where neurons die by necrosis. This area is surrounded by an area vulnerable to further

damage: the penumbra. When blood flow is reduced in the core, initial neuron death results from reduced ATP levels and protein synthesis, ionic imbalances, and glutamate mediated excitotoxicity (Kaushal and Schlichter, 2008). It is in the penumbra where apoptosis takes place and death receptors have shown to play a key role. During the stress response to ischemia, JNK kinases are activated, leading to activation of the transcription factor c-Jun, which in turn induces the expression of target genes, TNFα and FasL among them (Rosenbaum et al., 2000). Studies have shown that levels of FasL and TNFα are elevated in post-ischemic brains. The resistance of Fas-deficient mice to post-ischemic neuronal damage reinforces importance of these molecules. Martin-Villalba and her collaborators evaluated the resistance of TNFα and Fas-deficient mice to primary ischemic damage and the subsequent inflammatory reaction using a model of occlusion of the middle cerebral artery. They concluded that FasL/TNFα deficient mice were more resistant to damage caused by middle artery occlusion. The combination of protection against primary ischemic damage along with a reduced inflammatory response resulted in longer survival of TNFα/FasL-deficient animals. Moreover, the mice recovered locomotive function to an extent practically indistinguishable from that of control animals (Martin-Villalba et al., 2001).

A more recent work by Echeverry et al. showed a role for another member of the TNFR superfamily in protection from ischemic damage (Echeverry et al., 2012). According to the authors, treatment with recombinant Tumor Necrosis Factor-like Weak Inducer of Apoptosis (TWEAK) or overexpression of this molecule and its receptor, fibroblast growth factor inducible-14 (Fn14), induces hypoxic and ischemic tolerance *in vitro* and *in vivo*. The mechanism for this tolerance is through induction of TNFα expression and activation of the ERK1/2 pathway and inactivation of the proapoptotic protein Bad. Therefore, TWEAK is postulated as a new possibility for a therapeutic strategy to protect the brain from the damaged produced by ischemia.

Neurodegenerative Diseases

Neurodegenerative diseases are the result of the death of specific neuronal populations. Alzheimer's Disease (AD) for example, is characterized by the deposition of extracellular β-amyloid (Aβ) fibers (senile plaques) and intracellular deposits of Tau (fibrillary tangles) that result in extensive loss of neurons (Reviewed in Mattson, 2000). The production of Aβ is a result of the cleavage of the amyloid precursos protein (APP), the expression of which is elevated in AD (Joachim and Selkoe, 1992). In the past years, increasing evidence suggests an inflammatory component in Alzheimer's

Disease, characterized by astroglyosis, microglyosis, increased cytokine levels, and changes in acute phase proteins (Reviewed in Walsh and Selkoe, 2004). Evidence has indicated elevated levels of TNFα in the brain and plasma of AD patients, and TNFR1 has been found to be upregulated in the AD brain (Fillit et al., 1991; Li et al., 2004). A study in the triple-transgenic mouse model of AD (3xTg-AD) revealed increased levels of TNFα in the entorhinal cortex, one of the earliest affected regions in AD (Janelsins et al., 2005). Another experimental model for Alzheimer's Disease implied the intracerebroventricular injection of Aβ in mice (Reviewed in Van Dam and De Deyn, 2006). Using this model, the group of Medeiros and his collaborators established that TNFα production is one of the earliest events induced by Aβ, and revealed a crosstalk between the TNFα and inducible Nitric Oxide Synthase (iNOS) as mediators of the the Aβ-induced cognitive impairment observed in this model. Moreover, the authors also point towards the JNK or the NF-kB pathways as mediators of this crosstalk (Medeiros et al., 2007). Additionally, He et al. have described that deletion of the TNFR1 gene in AD transgenic mice inhibits β-amyloid fiber generation and prevents learning and memory deficits (He et al., 2007). According to the authors, genetic deletion of TNFR1 leads to reduced β-secretase (BACE) levels and activity. BACE is one of the molecules implicated in the processing of APP into β-amyloid. This evidence has placed TNFα as a possible target for AD therapies. A recent paper describes the use of a small molecule inhibitor for TNFα, 3,6′-dithiothalidomide, to reduce TNFα mRNA and protein expression in the brain. Reduction of TNFα improved working memory performance and the ratio of resting to reactive microglia in the hippocampus of triple transgenic mice (Gabbita et al., 2012).

Parkinson's Disease (PD) involves a loss of dopaminergic neurons in the substantia nigra pars compacta, giving rise to the characteristic symptoms of this disease: bradykinesia, tremor, rigidity, and postural instability. In this case death receptors seem to play a protective role from cell death. In PD patients, the expression of Fas and the membrane-bound form of FasL (mFas) are reduced, whereas the soluble form of FasL, sFasL, which blocks apoptotic Fas signaling, is increased.

One of the most used models of this disease is administration of 1-methyl-4-fenil-1,2,3,6-tetrahidropiridium dopaminergic toxin (MPTP), which specifically kills dopaminergic neurons and induces the symptoms of PD in mice (Mogi et al., 1996; Ferrer et al., 2000). In Fas deficient (*lpr)* mice treated with suboptimal doses of MPTP there is a dramatic loss of dopaminergic neurons, while this does not happen with wild-type mice. *In vitro*, Fas activation is able to protect cells from MPTP induced cell death

by a mechanism independent of caspase-8 (Landau, 2005). On the other hand, other experiments carried out in animal models of PD showed an increase in TNFα after MPTP administration, and mice deficient in TNFR1 and TNR2 were completely resistant to death induced by MPTP, giving rise to the possibility that TNFα is a mediator of PD neurodegeneration (Sriram, 2006).

One of the genetic causes of Parkinson's Disease is mutations in the protein Leucine Rich Repeat Kinase 2 (LRRK2). Mutations in this protein related to PD enhance its kinase activity, which has been described to cause neurite outgrowth defects *in vitro* (Parisiadou et al., 2009). Moreover, Ho and his collaborators report that LRKK2 can interact with FADD, and that LRKK2 mediated neurodegeneration *in vitro* is prevented by inhibition of FADD or caspase-8 depletion, thus suggesting a link between LRKK2 mediated cell death and the extrinsic apoptotic pathway (Ho et al., 2009). Finally, another report shows that knock out mice for LRKK2 show no hypersensitivity to MPTP, further supporting an active role for LRKK2 in the induction of apoptosis of dopaminergic neurons (Andres-Mateos et al., 2009).

Multiple Sclerosis

MS is an autoimmune pathology that affects the brain and spinal cord and is mediated by T-lymphocytes. Small areas of inflammation are formed and correlate with demyelinization caused by the specific death of oligodendrocytes. This progressive demyelinization interrupts the transmission of electric signals affecting different areas of the CNS, leading to alterations in sensation, visual problems, muscle weakness, depression, difficulties with coordination and speech, severe fatigue, cognitive impairment, problems with balance, overheating, and pain. Fas also appears to be relevant in this disease. Experimental Allergic Encephalomyelitis (EAE) is widely used as a model of MS. EAE is induced by the injection of myelin-specific peptides into mice. After 10–14 days, the animals develop a loss of muscular tone in their tails and coordinated muscular movements deteriorate (Swanborg, 1995). In this model the Fas/FasL system acts as an inducer of cell death, since the injection of anti-FasL antibodies right after the acute phase of EAE destabilizes the Fas/FasL system and results in a clinical improvement of symptoms (Wildbaum et al., 2000)

More recently, a genome wide association scan (GWAS) for multiple sclerosis susceptibility identified two new susceptibility loci in the *TNFRSF1A* gene, coding for TNFR1 (De Jager et al., 2009). Functional studies have linked one of these alleles to the expression of a novel, soluble form of TNFR1 that can block TNFα. Importantly,

TNF-blocking drugs can promote onset or exacerbation of MS, indicating that the MS-associated TNFR1 mimics the effect of TNF-blocking drugs (Gregory et al., 2012).

Amyotrophic Lateral Sclerosis (ALS)

The best evidence for a role of death receptors in a nervous system pathology comes from the study of amyotrophic lateral sclerosis (ALS). In ALS patients degeneration and death of motor neurons in the spinal cord lead to progressive and fatal muscle paralysis. The best characterized model of this disease is a transgenic mouse expressing mutant forms of SOD1 (Cu, Zn-Super-oxide dismutase 1). Mutations in this protein are known to cause ALS in a subset of patients with a familiar form of the disease. Mutant SOD1 triggers cell-autonomous neurodegeneration in motor neurons, but also drives disease progression through non-autonomous mechanisms involving microglia and astrocytes (Boillee, 2006; Di Giorgio et al., 2007; Nagai et al., 2007). TNFR, $p75^{NTR}$, and Fas/CD95 have been implicated in cell-autonomous and/or non-autonomous aspects of the disease. TNFα and TNF receptor levels have been found to be increased in SOD1 mutant mice, in parallel to the appearance of ALS symptoms and in plasma of ALS patients. However, genetic ablation of TNF showed no beneficial effect on survival or ALS symptoms in two different lines of SOD1 mutant mice (Gowing et al., 2006). Various treatments that have shown beneficial effects in SOD1 mutant mice have been related to reductions in TNFα levels. However, it does not seem likely that these treatments act through TNFα/TNFR regulation, it seems more probable that the beneficial effects are due to a reduction in inflammation secondary to neuroprotection by other mechanisms.

The strongest evidence for implication of death receptors in ALS is related to CD95/Fas. Motor neurons die *in vitro* by a Fas dependent mechanism specific for motor neurons. This pathway includes a positive feedback loop dependent on nitric oxide (NO). Moreover, motor neurons cultured from SOD1 mutant mice are hypersensitive to Fas activation (Raoul et al., 2002; Locatelli et al., 2007). Intrathecal infusion of Fas siRNAs to SOD1 mutant mice almost completely blocks pathological activation of elements in the Fas/NO pathway, protects motor neurons and extends lifespan.

2.OBJECTIVES

2. Objectives

Death receptors are known to induce a wide range of biological responses. Among them, induction of apoptotic cell death has been classically the best characterized in many cell systems and has shown to be relevant both in physiological and pathological situations. However, in the last years, the effects of death receptor activation on cell proliferation, differentiation, and survival have raised the interest of researchers. Getting to know the mechanisms by which death receptors may influence or promote the survival and proliferation of cells could open the gates to new therapeutic approaches and contribute to a better understanding of developmental processes.

The signaling pathways triggered by TNFα in the nervous system are an excellent paradigm of these processes. In the last years an increasing number of reports have been revealing the role of this cytokine as a mediator of survival in various neuronal models. However, there is a lack of data explaining the interactions among the various signaling cascades. In the present thesis I studied specifically the activation of the MAPK/ERK cascade by TNFR1 within the nervous system. More precisely I have defined the following objectives of this study:

1- Since TNFα is known to activate the MAPK/ERK pathway but the key mediators in this process have not been well defined, I wanted to characterize the activation of the MAPK/ERK pathway induced by TNFα through ligation of TNFR1.

2- Because survival induced by TNFα has been classically linked to NF-kB, I assessed if the MAPK/ERK pathway could have any interactions with the NF-kB pathway

3- Abrogation of the NF-kB survival pathway sensitizes cells to death induced by TNFα. Since the MAPK/ERK pathway has been classically linked to survival and differentiation of cells, my purpose was to assess the effects of MAPK/ERK inhibition on the treatment with TNFα and characterize the apoptotic pathway induced by this inhibition.

3.MATERIALS AND METHODS

3. Materials and Methods

3.1 Cell Culture

3.1.1 Cell Lines

PC12 cell line is derived from a Rat pheochromocytoma of the adrenal gland generated by Green and Tishcler in 1976 {Greene:1976vs}. This cell line is extensively used as a neuronal model since it responds to NGF treatment by acquiring a neuronal phenotype, releasing catecholamines, dopamine and norepinephrine. Cells were obtained from American Tissue Culture Collection (ATCC) maintained in 10 mm collagen coated culture dishes (Falcon Discovery Labware, BD Biosciences, San Agustin de Guadalix, Spain) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 6% Fetal Bovine Serum (FBS), 6% Horse Serum (HS), 10mM Hepes, 20U/ml penicillin and 20μg/ml streptomycin. PC12 cells were serum deprived for 12-16 hours prior treatment with TNFα or NGF.

HEK 293T cell line, are a good model for overexpressing and obtaining cellular and secreted or membrane proteins. They are derived from the human cell line of renal epithelium 293, transformed with the E1A adenoviral gene and over expressing the T antigen of SV40, allowing for the episomal replication of plasmids containing the promoter region SV40. In this work, HEK293T cells are used for lentiviral production. Cells were maintained in 100 mm culture dishes in DMEM medium supplemented with 10% FBS, 20U/ml penicillin and 20μg/ml streptomycin.

All cell lines were grown at 37ºC in a humidified atmosphere with 95% air and 5%CO_2.

3.2 Reagents

All the biochemicals utilized for this study were dissolved to a stock concentration according to the manufacturer data sheets. Subsequently, reagents were diluted in the cell culture medium to reach the final working concentrations. All reagents used in this study, as well as the provider and working dilutions are detailed in the following table.

Name	Description	Working Dilution	Supplier
TNFα	Cytokine, inducer of apoptosis	100ng/ml	Biotrend
NGF	Growth Factor	100ng/ml	Genentech
SP600125	JNK Inhibitor	10μM	Calbiochem
Ac-DEVD-afc	Fluorogenic caspase substrate	25μM	Calbiochem
PD98059	MEK Inhibitor	30μM	Calbiochem

Table 1. Reagents used in this study

3.3.Cell Transfection

3.3.1 Cationic Liposoluble Reagents

This method is based in the formation of cationic liposomes that engulf DNA and are able to introduce it in the target cell. Because most cell membranes have a negative net charge, anionic molecules, especially those with an elevated molecular weight, cannot be internalized. Cationic lipids aggregate polianions, such as nucleic acids, neutralizing the negative charges. The elevated efficacy of these compounds in transfection is due to the high affinity that the resulting aggregates have for cells, together with the function of lipohilic components in binding to membranes.

Lipofectamine 2000

Lipofectamine 2000 (Invitrogen) is a cationic liposoluble reagent. It has been optimized for the transfection of nucleic acids in a great variety of eukaryotic cells. This reagent was used in this work to overexpress proteins in PC12 cells. Cells were seeded in the appropriate, collagen coated plates at a confluency of 90-95% in medium without antibiotics the day before transfection. On the day of transfection the amounts of Lipofectamine 2000 and DNA were diluted separately in OptiMem I Reduced Serum Medium, keeping a proportion of approximately 1μg of DNA per 1μl Lipofectamine 2000 (Table 2). After 5 minutes of incubation DNA and Lipofectamine 2000 were combines and incubated for 20 minutes to allow lipid complex formation. After the 20 minutes incubation complexes were added to the cell culture. Culture medium was

changed 4 hours after transfection to avoid toxicity of the reagent. Correct expression of the transfected plasmids was assessed by Western Blot.

Culture vessel	Surf. area per well (cm^2)	Relative surf. area vs. 24-well	Vol. of plating medium	DNA (µg) in media vol. (µl)	Lipofectamine™ 2000 (µl) in media vol. (µl)
96-well	0.3	0.2	100 µl	0.2 µg in 25 µl	0.5 µl in 25 µl
24-well	2	1	500 µl	0.8 µg in 50 µl	2.0 µl in 50 µl
12-well	4	2	1 ml	1.6 µg in 100 µl	4.0 µl in 100 µl
35-mm	10	5	2 ml	4.0 µg in 250 µl	10 µl in 250 µl
6-well	10	5	2 ml	4.0 µg in 250 µl	10 µl in 250 µl
60-mm	20	10	5 ml	8.0 µg in 0.5 ml	20 µl in 0.5 ml
10-cm	60	30	15 ml	24 µg in 1.5 ml	60 µl in 1.5 ml

Table 2. Recommended proportions between DNA and Lipofectamine 2000 depending on the surface of the culture Vessel.

In this thesis, cells stably expressing empty pcDNA3 or SR-IkBα plasmids were also used. To obtain cells stably expressing these plasmids, they were first transfected using Lipofectamine 2000 following the above-described protocol. The day after transfection the antibiotic geneticin was added to the medium at a concentration of 0,5 µg/ml. Since these plasmids carry a gene encoding a protein that confers resistance to geneticin, all cells that had not been transfected died within a few days after antibiotic addition. Untransfected cells treated with the antibiotic were used as a control of geneticin efficiency.

Dharmafect

Dharmafect (Thermo Scientific) is a lipid-based reagent specifically designed for the transfection of small interfering RNA. We have used Dharmafect to transfect siRNA into PC12 cells. The main advantage is that it does not need a big cell confluence to reach maximum efficiency and cellular toxicity is very low. Therefore there is no need to change the medium and cells can continue growing for the time necessary for a detectable knock down of the interest protein.

3.3.2 Calcium Phosphate Transfection

This transfection method is more economical than Liposoluble Reagents and efficiency is elevated in some cell types. It is based on the formation of DNA-containing insoluble aggregates. These aggregates will precipitate over the cell monolayer,

introducing the genetic material into the cells. The elevated toxicity of this method makes it unsuitable for certain cell types, like PC12 cells. In this study, the calcium phosphate transfection method was used in the production of lentivirus in 293T cells, in which this method shows high transfection efficiency (see lentiviral production section).

For transfection by the calcium phosphate medium, four main reagents are required:

- HBS 2x (280 mM NaCl, 50 mM HEPES and Na2HPO$_4$)
- $CaCl_2$ 250mM
- Distilled Water (buffered with 2,5 mM Hepes, pH 7,3)
- DNA of interest

$CaCl_2$, distilled buffered Water and DNA are mixed in one tube in the adequate proportions. Formation of aggregates is attained by adding the DNA mix to an equal volume of the HBS 2x solution drop by drop while vortexing. The mix is incubated at room temperature prior adding to the cell monolayer. One of the most sensitive steps in this procedure is maintaining the pH of the HBS 2x between 6.95 and 7.05. Below 6.95 there is a low formation of aggregates, over 7,05 aggregates formed are too big and result in damage to cell integrity.

The main weak point of this method is its variability due to differences in the formation of the aggregates. These differences may be caused by differences in the pH of HBS 2x, concentration of the DNA used, etcetera. Variability may be reduced by controlling the pH of distilled water by adding 2,5 mM Hepes pH 7,3. Purity of DNA is another critical point in this case. It is recommended to use high purity DNA, with a minimum concentration of 1μg/μl.

3.3.3 Lentiviral Production

Retroviral vectors derived from lentivirus such as HIV are very useful tools in basic research. They can infect cells in constant division as well as terminally differentiated cells, such as neurons. Dr. Trono and his collaborators developed 3 generations of vectors for the production of lentivirus. In each generation biosafety has been improved by dividing the necessary elements for the formation of the viruses in more plasmids. In this work we used lentiviral plasmid of the second generation, with enough biosafety for *in vitro* genetic transfection. The lentivirus production protocol has

been adapted from the one described by Naldini and his collaborators in 1996. The plasmids necessary for the production of 2nd generation lentivirus are:

- pEIGW/pLVTHM: Used to overexpress or inhibit gene expression, respectively. This vector contains only the genetic material to be transferred to the target cell. The transgene cassette is flanked by cis elements necessary for encapsidation, reverse transcription, and integration in the genome. As in other retroviral vectors, we take advantage of the particularity of reverse transcription in order to obtain self-inactivating vectors derived from HIV-1, which lose the transcriptional capacity of the Long Terminal Repeats (LTR) once they have been transferred to the cell. This minimizes the risk of the appearance of new competent recombinant particles and promoter interference. In order to produce lentiviral siRNA constructs, pLVTHM was designed in such way that H1 III Pol promoter can be easily replaced by H1-siRNA cassette from pSUPER.retro.puro using EcoRI-ClaI.
- psPAX2 vector codifies for viral packaging proteins. It contains a very potent promoter (CAG), which will allow the transcription of packaging genes, among which TAT protein, DNA polymerase and reverse transcriptase are the most relevant.
- pM2G: Is the vector codifying for the virus' envelope. The viral protein comes from the vesicular stomatitis virus (VSV), allowing the infection of a great variety of tissues and cell lines.

The protocol for the generation of lentivirus is detailed below.

The first day, HEK293T cells are seeded at a density of 2,5 million cells per 100 mm culture dish. The following day plasmids are transfected in the following proportions:

- Vector pEIGW/pLVTHM 20μg
- psPAX2 13μg
- pM2G 7μg

The final 40μg are diluted in the mixture of water and $CaCl_2$ described above for the calcium phosphate transfection method. Once the precipitates are formed, they are transferred to the culture dish. Culture medium is changed 4h after transfection. Cells

are left producing virus for 2 to 3 days after transfection. After this time, the medium is removed and centrifuged at 2500 rpm for 5 minutes to eliminate cells or particles in suspension. The supernatant is filtered through a 0,45 μm filter. It is advisable to concentrate the virus, especially if components of the HEK293T cell medium may affect future experiment. To concentrate the viral particles, the supernatants are centrifuged at 50.000xg for 1,5 h. Once centrifuged, viruses are resuspended in a solution containing a carrier protein that will ensure preservation of the virus. In our protocol we use a PBS solution containing 2% BSA. This allows keeping the virus at -80ºC for a long time without apparent loss of efficiency.

Finally the approximate title of virus is calculated by infecting HEK293T cells with different virus concentrations and calculating the percentage of infection after 2 to 3 days.

3.4 Plasmid Constructions (see annex for plasmid maps)

- **pcDNA3-SR-IkBα:** Super-Repressor IκBα (SR-IκBα) cDNA was expressed under the control of a cytomegalovirus constitutive promoter in the pcDNA3 expression vector (Invitrogen). SR-IkBα codifies for the protein IkBα mutated in residues A32S and A36S, turning it into a dominant negative form of the protein.
- **pcDNA3-EYFP-hRaf-1:** The human gene of Raf-1 protein was cloned in the pcDNA3 plasmid, expressed under the control of a cytomegalovirus constitutive promoter together with yellow fluorescent protein (YFP).
- **pEXV3-MEK-CA:** The Glu217-Glu221 MAPKK1 mutant construct (MEK-CA, constitutively active MAPKK1) was kindly provided by C. E. Marshall (Institute of Cancer Research, London, United Kingdom) through A. López-Rivas (CSIC, Granada, Spain).
- **pCMV-HA-mFLIP-L:** Mouse FLIP-L cDNA was purchased from Imagenes. FLIP-L cDNA was tagged with 3xFLAG and subcloned into the pcDNA3 mammalian expression vector. HA-FLIP-L was obtained by subcloning FLIP-L from pcDNA3-FLIP-L into the vector with HA tag in 5'.

3.4.1 RNAi Oligonucleotides

For the Raf-1 knockdown experiments, two oligonucleotides were designed targeting the human sequence of Raf-1 (accession number NM_002880). Oligonucleotides were transfected into PC12 cells using Dharmafect (Thermoscientific).

Gene	Oligonucleotide Sequence (5'-3')
hRaf-1 (R1)	**(Fwd)** TCACAACTTTGCTCGGAAA **(Rv)** TTTCCGAGCAAAGTTGTGA
hRaf-1 (R2)	**(Fwd)** GGGATGAGCTTGCACGACT **(Rv)** AGTCGTGCAAGCTCATCCC

Table 3. RNAi oligonucleotides designed for Raf-1 Knockdown

3.4.2 Lentiviral Constructs

- **pEIGW-mFLIP-L:** FLIP-L-FLAG cDNA was subcloned into the pEIGW vector, giving rise to the lentiviral FLIP-L-FLAG overexpressing construct. The plasmid pEIGW was constructed by replacing the EGFP nucleotide sequence between the EF1α promoter and the woodchuck post-transcriptional regulatory element in pWPTS– EGFP with the internal ribosomal entry site (IRES)-EGFP cassette from pIRES2–EGFP (Clontech), as described by Zuliani et al. (2006).

Lentiviral FLIP-L RNAi Construct (pLVTHM-RNAi FLIP)

For RNA interference (RNAi) experiments, constructs were generated in the pSUPER.retro.puro plasmid (OligoEngine) using specific oligonucleotides for mouse and rat FLIP (sequence data available from GenBank under accession numbers NM_207653 and NM_001033864, respectively). A scrambled sequence (Scr) was also obtained (see Table 4).

Oligonucleotides were obtained from Sigma-Aldrich and cloned between BglII and HindIII sites of the pSUPER.retro.puro vector, under the control of the DNA Pol III promoter of H1. Plasmids for lentiviral production pLVTHM, pSPAX2, and pMD2G were kindly provided by Dr. Trono (University of Geneva, Geneva, Switzerland). Lentiviral constructs were achieved by digestion at the EcoRI–ClaI sites to replace H1 promoter

in the lentiviral vector pLVTHM with H1–short hairpin RNA cassette from pSUPER. The pLVTHM plasmid was subsequently used for lentiviral knockdown experiments.

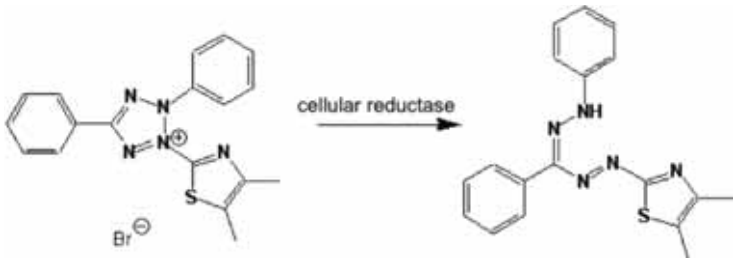
Gene	Oligonucleotide Sequence (5'-3')
FLIP-L (R1)	**(Fwd)** gatccccGAAGGAGATGATGCTCTTCttcaagagaGAAGAGCATCATCTCCTTCttttt **(Rv)** agctaaaaaGAAGGAGATGATGCTCTTCtctcttgaaGAAGAGCATCATCTCCTTCggg
Scrambled	**(Fwd)** gatccccGCATATGC-GTACGGATTAGttcaagagaCTAATCCGTACGCATATGCttttt **(Rv)** agctaaaaaGCATATGCGTACGGATTAGtctcttgaaCTAATCCGTACGCATATGCggg

Table 4. RNAi oligonucleotides designed for FLIP-L Knockdown

3.5 Cell Death Assays

3.5.1 MTT Reduction

The MTT assay is a colorimetric quantitative assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble form, formazan. Formazan forms dark blue crystals that are dissolved using organic solvents such as dimethyl sulfoxide (DMSO).



(Yellow) (Purple)

Figure . MTT structure modification after processing by a cellular reductase

Active mitochondrial dehydrogenase in living cells can induce the breaking of the ring in MTT. Therefore, this method can be used to quantify the respiratory activity in living cells in culture. The loss of metabolic activity in the mitochondria is taken as a measure of cell death, although this measure is not indicative of the type of cell death induced by the treatment.

The basic protocol used for MTT reduction assay in this thesis is as follows:

PC12 cells (25.000 cells/well, 96-well plate) were cultured in 50μl of complete medium for 24h and after this time the appropriate treatments were added. After the experiment, 50μl of MTT reactive diluted in medium without serum were added, for a final working solution of 0,5mg/ml. Plates were incubated at 37ºC for 30-45 minutes. After this time medium was removed and 50μl/well of DMSO were added. Formazan salts were allowed to dissolve for 10 minutes with gentle shaking at room temperature. Absorbance was measured at 590nm (lecture) and 620nm (reference) wavelengths. Quantification of survival was done by taking as 100% survival the values (lecture-reference) of control untreated cells.

3.5.2 Hoechst 33258 Bis-benzimide staining

Hoechst 33258 is a fluorescent dye that can penetrate through the intact cellular membrane and bind to AT (Adenosine-Thymidine) rich regions in the DNA double-strand. Once bound to DNA it is excited by ultraviolet (UV) light (350nm) and emits light in the blue spectrum (450nm), allowing for the detection of DNA without affecting cell viability. It is used as a cell death assay because it allows the visualization of condensed chromatin and fragmented nuclei typical of apoptosis.

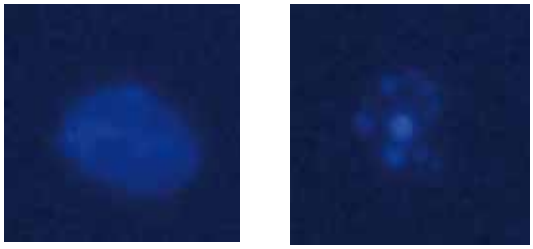


Figure . Typical aspect of viable (Left) and apoptotic (Right) nuclei of PC12 cells stained with Hoechst 33258

For the protocol used in this work, PC12 cells were transfected and treated with the corresponding treatments. After the experiment cells were fixed with 2% paraformaldehyde (PFA. After this time cells were stained with 0,05 μg/ml of Hoechst 33258 and incubated for 30 minutes at room temperature. Cells were excited with UV light. Apoptotic cell (showing type II chromatin condensation) percentage was quantified versus viable cells.

3.5.3 TUNEL Staining

Terminal Transferase dUTP End Labeling (TUNEL) is commonly used to detect fragmentation of DNA of apoptotic cells. This assay relies on the presence of nicks in the DNA that can be identified by the terminal deoxynuclotidyl transferase (TdT). This enzyme catalyzes the addition of dUTPs that are secondarily labeled with a fluorescent marker.

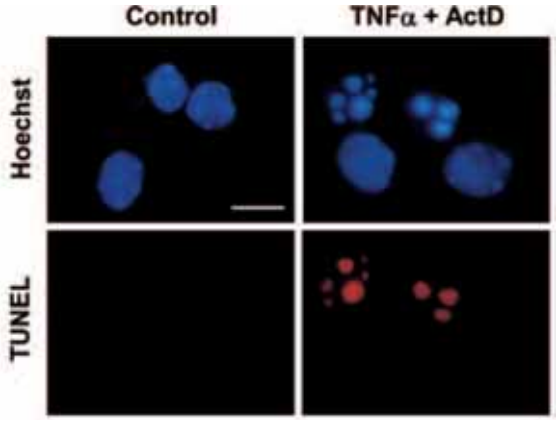


Figure . TUNEL labeling of PC12 cells treated with TNFα to induce apoptotic cell death. The top panel shows the typical staining of normal (Leith) versus condensed (right) chromatin as revealed by Hoechst 33258. The lower panel shows staining only in cells undergoing apoptosis. Source: (Segura et al., 2007)

To perform this assay, after corresponding treatment, cells were fixed in 2% PFA in PBS for 60 minutes at room temperature. Then, cells were permeabilized with 0,1% Triton X-100 0,1% sodium citrate for 20 minutes at 4ºC, and stained following the *In situ cell death detection kit* instructions (Roche, St. Cugat del Vallés, Spain). As a final step, cells were stained with Hoechst 33258, following the protocol mentioned above.

3.5.4 Caspase Activity

During the process of apoptotic cell death one of the main events that take place is activation of caspases, which can be used as a biochemical marker of the cell estate. Moreover evaluation of caspase activation allows for the definition of the temporal dynamics of apoptotic cell death.

To quantify effector caspase-like activity (DEVDase-like), after the indicated treatments cells were rinsed once with PBS and resuspended in lysis buffer containing 20 mM HEPES/NaOH ph 7.2, 10% sucrose, 150 mM NaCl, 10mM DTT, 5mM EDTA, 1% Nonidet P-40, 0.1% CHAPS and 1X EDTA-free Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 16 000 x g for 5 min. and supernatant proteins were quantified by the Bradford method. Assays were performed

in triplicate using 25µg of protein in the same specific lysis buffer supplemented with 25 µM of the fluorogenic substrate Ac-DEVD-afc. Plates were read in a fluorimeter using a 360 nm (40 nm bandwidth) excitation filter and a 530 nm (25 nm bandwidth) emission filter.

Alternatively, a method was used described by Yuste et al. {Yuste:2001cf}. By this method, an equal volume of cell lysis buffer containing the fluorogenic substrate 2x concentrated was added to the plate after treatment and the plate was read with the conditions previously described.

3.6 Protein Extraction

After each experiment, cells were rinsed once with ice-cold phosphate saline solution buffer (PBS), pH7.2, for immediate obtaining of cellular extracts. Proteins must be extracted efficiently and without degradation to ensure an accurate representation of their physiological estate in the living cell. With that purpose, different methods of protein extraction were used.

3.6.1 Lysis in denaturing conditions.

With this lysis method we obtain a total mixture of all the components in the cell. It allows for the study of protein identity, possible posttranslational modifications, and quantity because these parameters are based on the aminoacid sequence and do not require the preservation of secondary, tertiary, or quaternary structures. Extraction is vey efficient because it breaks interactions between proteins and allows the liberation of proteins bound to macromolecular structures. The composition of the lysis buffer is: 2% sodium dodecyl sulfate (SDS) and 125 nM Tris-HCl (pH 6,8) keeping a proportion of 1:50 between the volume of cell pellet and lysis buffer. Cell lysis is kept at 95ºC for 10 min to ensure fragmentation of genomic DNA and thus eliminate viscosity of the sample. Cell extracts were kept at -20ºC.

3.6.2 Lysis in non-denaturing conditions

In some cases it is important to maintain the native configuration of proteins. For that purpose non-ionic detergents are used, combined with protease and phosphatase inhibitors to avoid degradation and dephosphorylation of the sample. To study the phosphorylation of JNK, cells were resuspended in a buffer with the following composition: 20 mM Tris, pH 7.4, 140 mM NaCl, 10% glycerol, 1 M sodium

orthovanadate (Na_3VO_4), 40 mM β-glycerolphosphate, 1% NP-40, supplemented with 1X EDTA-free Complete protease inhibitor cocktail (Roche) and 1X Phosphatase Inhibitor Cocktail 3, keeping a proportion of 1:20 between cell pellet and lysis buffer Cells were incubated in ice for 20 min prior centrifugation at 16.000 xg, 4ºC for 5 min. Under these conditions, most nuclear and cytoskeleton proteins remain in the excluded pellet while the supernatant is enriched in cytosolic proteins. Supernatants were harvested and kept at -20ºC.

Protein quantification in all samples for further analysis in SDS-PAGE and Western Blot was done following the modified Lowry assay (DC protein assay; Bio-Rad, Hercules, CA, USA).

Once quantified, samples were prepared (between 15 to 40 μg of protein per condition) with Laemmli's loading buffer (2% (w/v) SDS, 10% (v/v) Glicerol, 5% (v/v), β-mercaptoethanol, 0.003% (w/v) Bromophenol (Sigma) and 62,5 mM TRIS-HCl pH 6,8). They were denatured by incubating them at 95ºC for 5 min. β-mercaptoethanol destroys disulphide bonds and SDS denatures and coats the protein to obtain isolated polypeptide chains that can be run on an SDS-polyacrylamide gel.

3.7 SDS-polyacrylamide gel electrophoresis

In order to detect and characterize complex mixtures of protein we used electrophoresis in gels with a polyacrylamide matrix in the presence of SDS (SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis). This procedure was first described by Laemmli in 1970.

Polyacrylamide gels in the presence of SDS are formed by the polymerization of acrylamide by the action of a cross-linking factor, bis-acrylamide in the presence of 0,1% SDS, an initiator and a catalizer. The relative proportion of polyacrylamide and bis-acrylamide is a solution of 40% (w/v) ultrapure acrylamide and bus-acrylamide (1,04%) in a final proportion of 37,5:1. As an initiator, 0,1% (v/v) TEMED (Sigma) is commonly used, and as a catalyzer, the ion persulfate ($S_2O_8^-$), added in the form of ammonia persulfate (APS) 0,05% (w/v) is used. The final percentage of acrylamide/bis-acrylamide determines the separation range of the gel.

For this kind of electrophoresis, two buffers are employed. This allows for the separation of relatively big volumes of sample without loosing resolution. The first buffer (125 mM Tris-HCl pH 6,8) ensures migration of all the proteins, provoking the

stacking of all the proteins loaded in the well. Separation begins when the migration front reaches the second buffer (375 mM Tris-HCl pH 8,8). The stacking gel has a bigger porus, due to a lower percentage of acrylamide/bis-acrylamide, and a lower pH than the resolving gel, which is the one that actually separates proteins.

Electrophoresis is performed in a Mini-PROTEAN cuvette (Bio-Rad, Hercules, CA), at a constant amperage (25 mA/gel) in a Tris-Glycine-SDS buffer (Tris base 25 mM, Glycine 192 mM, SDS 0,1%, pH 8,3).

3.8 Protein immunodetection by Western Blot

Immunodetection is used to identify specific antigens in the sample recognized by monoclonal or polyclonal antibodies. Once the samples have been extracted, prepared, and separated by SDS-PAGE (see previous sections), these are transferred electrophoretically to the surface of a polyvinyl difluoride (PVDF) membrane where they become accessible for immunodetection. Membranes have to be previously treated with 100% methanol and washed with MilliQ water hydrate them. Transference is done at 100 V for 90 min in transfer buffer (48 mM TRIS-HCl, 39 mM glycine, 20 % methanol).

After transference, membranes were blocked in a solution of 5% skim milk in TBS-Tween (20 mM Tris–HCl pH 7,4, 150 mM NaCl and 0,05% Tween 20), for 1 h at room temperature. Blocking of the membrane prevents unspecific binding of the detection system to the membrane. Once blocked, membranes were washed three times for 5 min with TBS-T to eliminate excess blocking solution. Primary antibody was added at the required dilution (see Table 5) and incubated overnight at 4ºC or for 1 h at room temperature.

Antibody	Antigen MW	Type	Source	Working Dilution	Supplier
Bcl-X_L	26 kDa	Polyclonal	Rabbit	1:2000	BD Transduction labs
Bid	25 kDa/ 15 kDa	Polyclonal	Goat	1:1000	R&D Systems
Bim	25 kDa	Polyclonal	Rabbit	1:4000	ProSci
FLIP-L (Dave-2)	55 kDa	Monoclonal	Rat	1:1000(WB) 5μ/mg (IP)	Alexis
IkBα (C21)	37 kDa	Polyclonal	Rabbit	1:1000	Santa Cruz
JNK	46-54 kDa	Polyclonal	Rabbit	1:1000	Cell Signaling
NF-kB p65 (C21)	65 kDa	Polyclonal	Rabbit	1:300 (IF)	Santa Cruz
PARP	116 kDa/89 kDa	Polyclonal	Rabbit	(1:1000)	Cell Signaling
phosphoERK1/2	42-44 kDa	Policlonal	Rabbit	1:2000	Cell Signaling
phosphoJNK1/2	46-54 kDa	Monoclonal	Mouse	1:1000	Cell Signaling
phosphoMEK1	71 kDa	Polyclonal	Rabbit	1:1000	Millipore
PUMA	21 kDa	Polyclonal	Goat	1:1000	Santa Cruz
Raf-1	71 kDa	Polyclonal	Rabbit	1:1000(WB) 2μg/ml (IP)	Santa Cruz
panERK	42-44 kDa	Monoclonal IgG2a	Mouse	1:5000	BD Transduction Labs
Ras	21 kDa	Monoclonal	Rabbit	1:2000	Millipore
Secondary Antibodies					
Ig Rabbit	--------	Polyclonal	Goat	1:20000	Sigma
Ig Rat	--------	Polyclonal	Rabbit	1:5000	Sigma
Ig Goat	--------	Polyclonal	Rabbit	1:10000	Sigma
Ig Mouse	--------	Polyclonal	Goat	1:10000	Sigma

Table 5. Antibodies used in this study

Before incubating with the secondary antibody membranes were washed again with TBS-T for 5 min three times to eliminate excess of primary antibody. Secondary antibody was diluted in TBS-T+5% skim milk. The secondary antibody is coupled to the horseradish peroxidase enzyme (HRP), as an inducer of detection. Incubation was done for 1 h at room temperature. After incubation, membranes were washed three

times for 10 min with TBS-T to eliminate the blocking solution and the excess secondary antibody.

Detection was done using the EZ-ECL (Biological Industries, Israel), or SuperSignal chemiluminescent (Amersham Pharmacia Biotech, Pierce, USA) kits. HRP bound to the secondary antibody, in the presence of peroxides, cataluzes the oxidation reaction of luminol, which produces light or chemoluminiscence.

3.9 Co-Immunoprecipitation

Immunoprecipitation is a technique by which a protein is isolated by binding it to a specific antibody, which is bound to or precipitated using a sedimentable matrix.

Co-Immunoprecipitation (Co-IP) is used to isolate a specific protein along with any other proteins forming a complex with it in order to analyze interactions between proteins. It implies the usage of an antibody that specifically binds to that particular protein, this antibody has to be coupled to a solid substrate at some point in the procedure. For co-immunoprecipitation studies in this thesis, cells were lysed in non-denaturing conditions, following the protocol described above in order to keep protein-protein interactions. 1 mg of total protein was incubated with 5 μg of the primary antibody of interest overnight at 4ºC. Immunocomplexes were collected with protein A or G coupled to sepharose beads by orbital shaking for 1 h at 4ºC and washed five times with IP lysis buffer. Beads were resuspended in 40 μl of Laemmli's loading buffer and boiled for 5 min. Samples were loaded onto an SDS-polyacrylamide gel and the presence of proteins that could be part of a complex was analyzed by Western Blot. As input controls, 25 μg of the total lysates were blotted with specific antibodies.

3.10 Immunofluorescence

For the p65 immunofluorescence assays performed in this thesis, PC12 cells were treated with TNFα for the indicated time points. After treatment medium was removed and cells were fixed with 100% methanol for 5 min at room temperature. Fixing with methanol precipitates proteins and it is suitable to detect proteins located in the nucleus and the cytoskeleton. However, cytosolic proteins may be lost. In one single step, methanol fixes and permeabilizes the cells. Next, cells were washed repeatedly with PBS, and, without needing to block with albumin or other agents to

avoid unspecific binding of the antibodies, they were incubated with a primary polyclonal antibody anti-p65 (C-20) (Santa Cruz Biotechnology, California, USA) for 1h at room temperature. After washing three times with PBS, a secondary antibody was used, conjugated to Alexa Fluor 488 (Molecular Probes) incubated for 1h at room temperature and protected from light. After washing two more times to eliminate secondary antibody, cells were stained with Hoechst 33258 for 20 min at 4ºC protected from light. Pictures were taken using an inverted microscope and the proportion of cells showing nuclear p65 was determined by direct counting.

3.11 Cellular Subfractioning

To study the translocation of Raf-1 to the cellular membrane after stimulation of PC12 cells with TNFα, a subfractioning assay was performed to separately analyze the presence of this protein in the membrane and the cytosol.

After appropriate treatment, cells were rinsed once with ice-cold PBS and scrapped from the plate in subfractioning buffer (10 mM HEPES pH 7.4, 2 mM EDTA, 1 mM Na_3VO_4), supplemented with 1X EDTA-free Complete protease inhibitor cocktail (Roche) and 1X Phosphatase Inhibitor Cocktail 3 (Sigma). Samples were sonicated 3 times for 5 seconds and cell debris were pelleted by centrifugation for 10 minutes at 800 G, 4ºC. Supernatants were harvested and centrifuged for 80 min at 100.000 xg, 4ºC. Supernatants were recovered, and labeled as cytoplasmic fraction while pellets were solubilized in subfractioning buffer supplemented with Triton-X-100 1%, and incubated at 4ºC for 30 min. This latter fraction was labeled membrane fraction. Protein concentration in both fractions was quantified by the modified Lowry method, and samples were boiled in Laemmli buffer for 5 min prior Western Blotting.

3.12 Determination of Ras Activity

In order to determine activity of Ras protein after treatment with TNFα, a Ras activation assay kit (Millipore) was used. This assay uses the Ras-binding domain (RBD) of the Ras effector kinase Raf-1. The Raf-RBD domain has been shown to bind specifically to the GTP-bound form of Ras. The fact that the Raf-RBD has a high affinity for GTP-Ras and that its binding results in a significantly reduced intrinsic and catalytic rate of hydrolysis of Ras make it an ideal tool for affinity purification of GTP-Ras from cell lysates. The Raf-RBD is in the form of a GST fusion protein, which

allows one to "pull-down" the Raf-RBD/GTP-Ras complex with glutathione affinity beads. The assay therefore provides a simple means of quantifying Ras activation in cells. The amount of activated Ras is determined by a western blot using a Ras specific antibody.

The general protocol used is as follows: After treating cells with the appropriate conditions, culture media was removed and cells were rinsed twice with ice-cold PBS. Mg^{2+} Lysis/Wash Buffer 5X (MLB, 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% NP-40, 50 mM $MgCl_2$, 5 mM EDTA and 10% glycerol) was diluted to 1X by adding 4 ml sterile, distilled water containing 10% glycerol to 1 ml of 5X MBL. Assay buffer was also supplemented with 1X Complete protease cocktail (Roche) and 1 mM sodium orthovanadate. MLB 1X was added to the rinsed plate and cells were detached and lysed by scraping with a rubber cell scrapper and lysates were transferred to a centrifuge tube on ice. Lysates were cleared of insoluble cell debris by centrifugation (5 min., 14.000xg, 4ºC). To perform the pull-down assay, 0,5 ml of cell extract for each sample (from a 90% confluent 100 mm plate) were used. 10 μg of Ras assay reagent (Raf-1 RBD agarose) were added to each sample and the reaction mixture was incubated for 45 min at 4ºC with gentle agitation. Agarose beads were pelleted by brief centrifugation (10 seconds, 14.000xg, 4ºC). Supernatant was discarded. Beads were washed three times with 0,5 ml of 1X MLB. In the last wash, buffer was eliminated using insulin syringes to avoid loss of beads. Finally, beads were resuspended in 40 μl of Laemmli reducing buffer and boiled for 5 minutes, adding 2 μl of 1M dithiothreitol (DTT) to improve release of Ras from the beads. After this time, beads were pelleted by brief centrifugation. Western blot was performed using 20 μl of supernatant and agarose bead mixture, since adding of beads does not affect electrophoresis and loading the slurry helps maintaining consistent results. 20 μg of total protein were loaded in parallel as a control of Ras expression in the samples.

3.13 Determination of Raf-1 Kinase Activity

To determine activation of Raf-1 kinase in cells treated with TNFα, a Raf-1 kinase assay kit (Millipore) was used. This assay uses inactive recombinant MEK-1 as a target for Raf-1 purified by immunoprecipitation from a cell lysate.

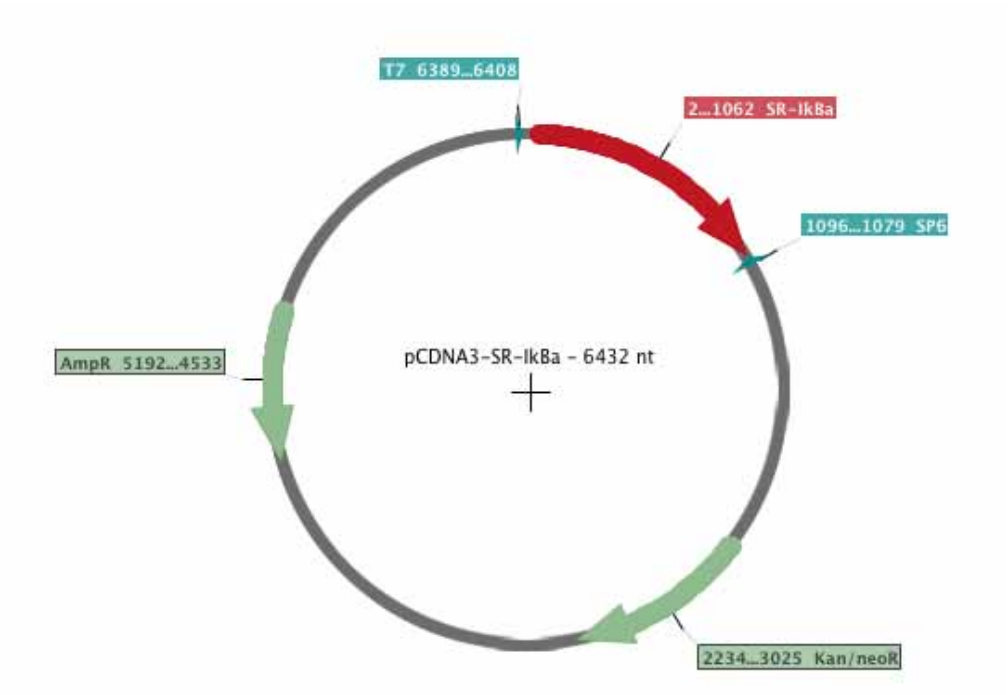
As a previous step for this assay, Raf-1 needs to be immunoprecipitated from the protein extract of PC12 cells treated. This was done by using a specific antibody against Raf-1, following the immunoprecipitation protocol specified above (section 3.8).

Once Raf-1 was purified, and before releasing it from agarose beads, these were washed once with assay buffer ADBI without DTT, to avoid early release from Raf-1. The assay mix was then added to each sample, following the manufacturer's protocol. The assay mix contains Assay Dilution Buffer I (ADBI, 20 mM MOPS, pH 7,2, 25 mM β-glycerolphosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT), Magnesium/ATP cocktail (500 μM ATP and 75 mM magnesium chloride in ADBI) and inactive MEK1. Samples were incubated for 30 minutes at 30ºC in a shaking incubator. As a positive control, assay mixed was incubated in the presence of recombinant, active Raf-1. After incubation, an equal volume of Laemmli's loading buffer was added to the samples and these were boiled for 5 minutes. SDS-PAGE was performed using 15 μl of sample. As an input control, 20 μl of total cell lysate were used. After electrophoresis, proteins were transferred to a nitrocellulose membrane and western blot was performed. The membrane was immunoblotted using a specific phosphorylated-MEK1 antibody.

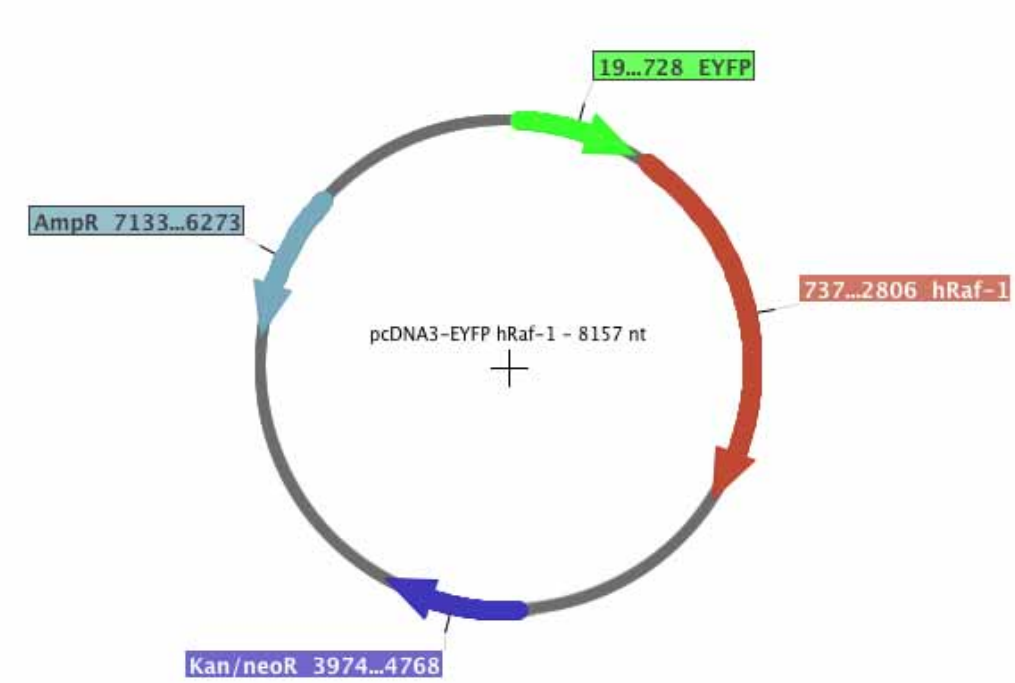
MATERIALS AND METHODS ANNEX

Plasmid Maps

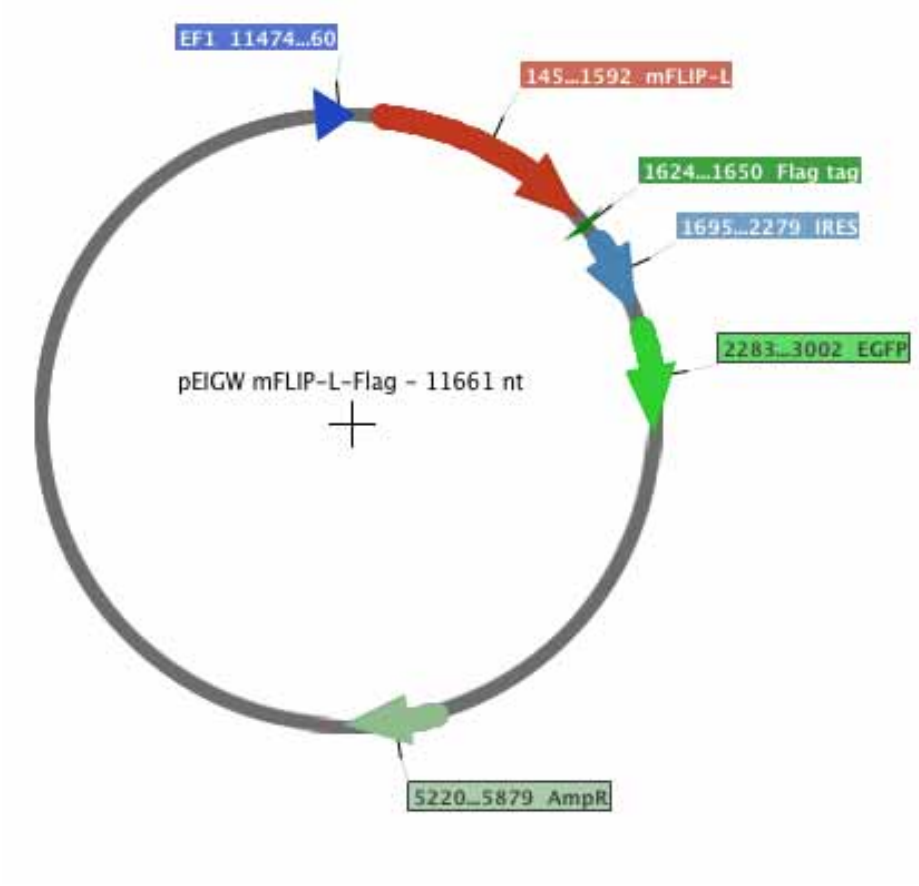
- pCDNA3-SR-IkBα



- pCDNA3-EYFP hRaf-1



- pEIGW mFLIP-L-Flag



4.RESULTS

4.RESULTS

4.1- NF-kB inhibition sensitizes PC12 cells to the apoptotic effect of TNFα.

TNFα can exert apoptotic or survival effects in most cell types. In order to induce apoptotic cell death through activation of TNFR1, survival signaling needs to be abrogated. This can be achieved by treating cells with RNA transcription or protein synthesis inhibitors, such as Actinomycin D or cycloheximide, respectively. Actinomycin D has been shown to down-regulate the expression of Bcl-X_L as a mechanism for sensitization to cell death induced by TNFα. Moreover, this is independent of the survival-related proteins under the control of the NF-kB transcription factor (Gozzelino et al., 2008).

However, in many cellular models, NF-kB has been postulated to be the main regulator of the anti-apoptotic response triggered by TNFR1 activation. Therefore, the exact mechanism by which NF-kB regulates the anti-apoptotic response to TNFα still needs to be clarified. To confirm that NF-κB inhibition sensitizes PC12 cells to apoptosis induced by TNFα, we stably transfected PC12 cells with an empty plasmid or with a non-degradable form of human IkBα, named SR-IkBα. This plasmid contains mutations in the two serine residues (S32A, S36A) that become phosphorylated after TNFR1 stimulation, targeting IkBα for proteasomal degradation. Therefore overexpression of SR-IkBα blocks the activation of NF-kB mediated by TNFα. To assess wether NF-kB inhibition sensitized PC12 cells to TNFα-induced apoptosis, cells were left untreated or treated with TNFα for different time points and executioner caspase activity was analyzed by means of a DEVDase-like activity assay, as detailed in the matherial and methods section (Fig. 1). The results for this assay show a time-dependent increase in caspase-3-like activity induced by TNFα only when PC12 cells overexpress the SR-IkBα plasmid, suggesting that TNFα is able of inducing caspase activation only when NF-kB activation is abrogated

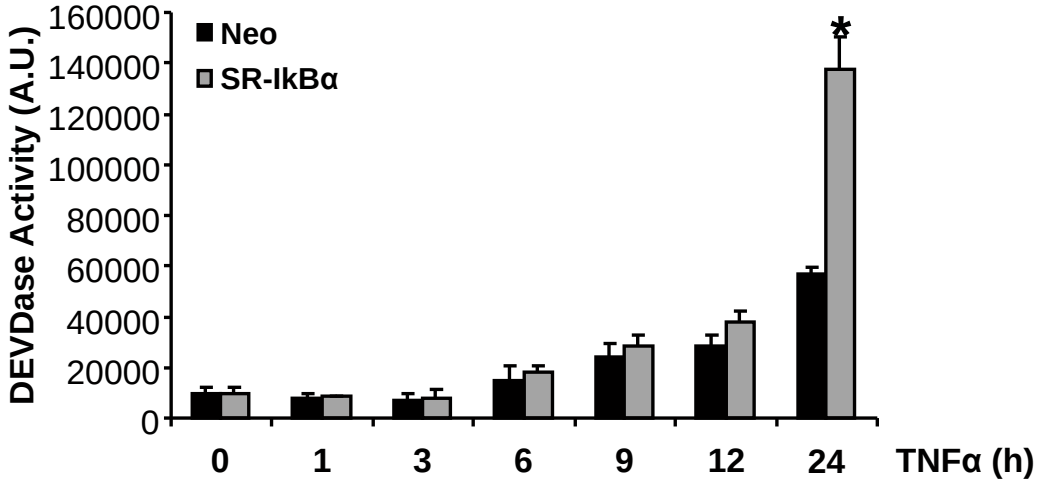


Figure 1. PC12 cells stably transfected with an empty (Neo) or with SR-IκBα plasmid were treated for the indicated time points with 100 ng/ml of TNFα and a caspase-3-like activity assay was performed using Ac-DEVD-afc fluorogenic substrate. Significant differences are indicated (* p<0.001, t test)

In order to confirm that caspase activation induced by TNFα correlated with an apoptotic type of cell death in PC12 cells, we analyzed chromatin condensation, a hallmark of apoptosis, as revealed by Hoechst 33258 staining. Since maximal caspase activation was measured at 24h of TNFα treatment, cell death was determined by direct counting of apoptotic nuclei at the same time point. Results show that PC12 cells over-expressing the SR-IκBα plasmid becomes sensitive to apoptosis induced by TNFα, as compared to cells transfected with the empty control plasmid (Neo).

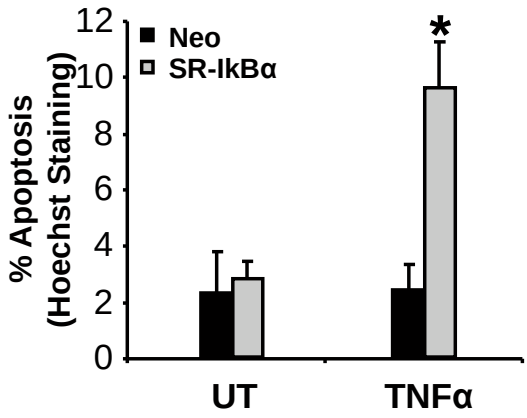


Figure 2. PC12 cells stably expressing an empty plasmid (Neo) or SR-IκBα were left untreated or treated with 100 ng/ml of TNFα for 24h. Apoptotic cell death was quantified by direct counting of condensed nuclei stained with Hoechst 33258. Significant differences are indicated (* p<0.01, t test)

Additionaly, to further confirm that cell death observed in PC12 cells overexpressing SR-IkBα was apoptotic, we carried out a Terminal Deoxynucleotydil transferase dUTP nick end-labeling (TUNEL) assay. By fluorescently labeling DNA nicks that result from the activation of the apoptotic signaling cascade, we determined that TNFα was only able to induce apoptotic cell deathin PC12 cells transfected with the super-repressor plasmid (SR-IκBα). Figure 3 shows representative images of TUNEL labeled cells and the same cells stained with Hoechst 33258, showing a correlation between condensed chromatin and the presence of fragmented DNA.

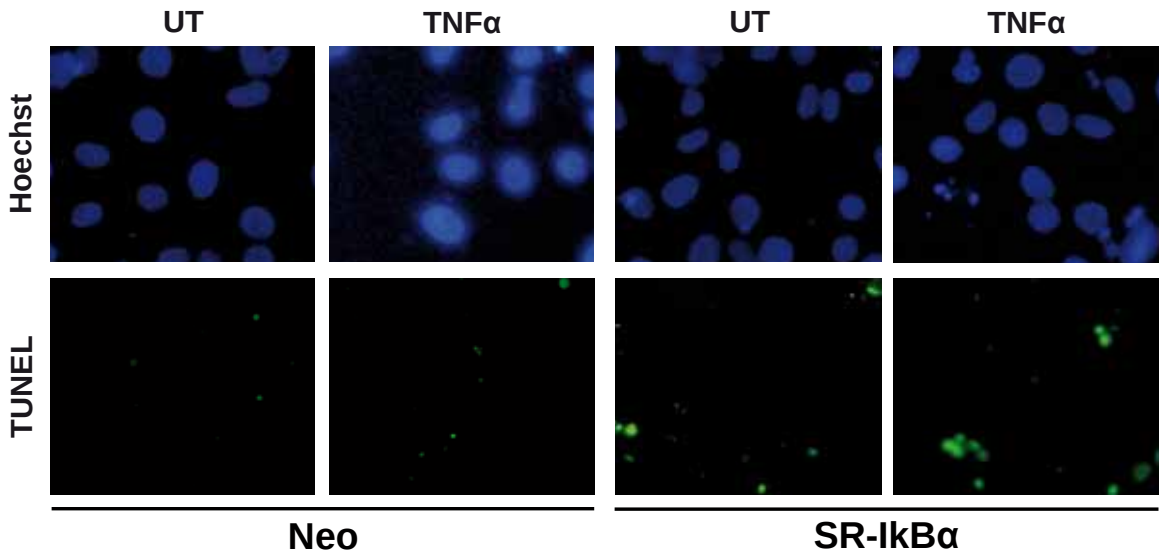


Figure 3. Cells were treated as in figure 2 and TUNEL assay was performed, with co-staining with Hoechst 33258

To control the efficient blockade of NF-kB activation we assessed the nuclear translocation of p65. TNFR1 activation results in degradation of IkBα, allowing translocation of p65 from the cytosol to the nucleus. Therefore, we overexpressed the SR-IkBα plasmid in PC12 cells and treated them with TNFα for 15 minutes before performing a p65 immunofluorescence using a specific antibody. Images in figure 4A show that, in cells transfected with an empty plasmid, TNFα induces p65 translocation from the cytosol to the nucleus, whereas overexpression of SR-IkBα abrogates this translocation. Quantification was evaluated by direct counting of nuclear versus cytosolic stained cells (Fig. 4B).

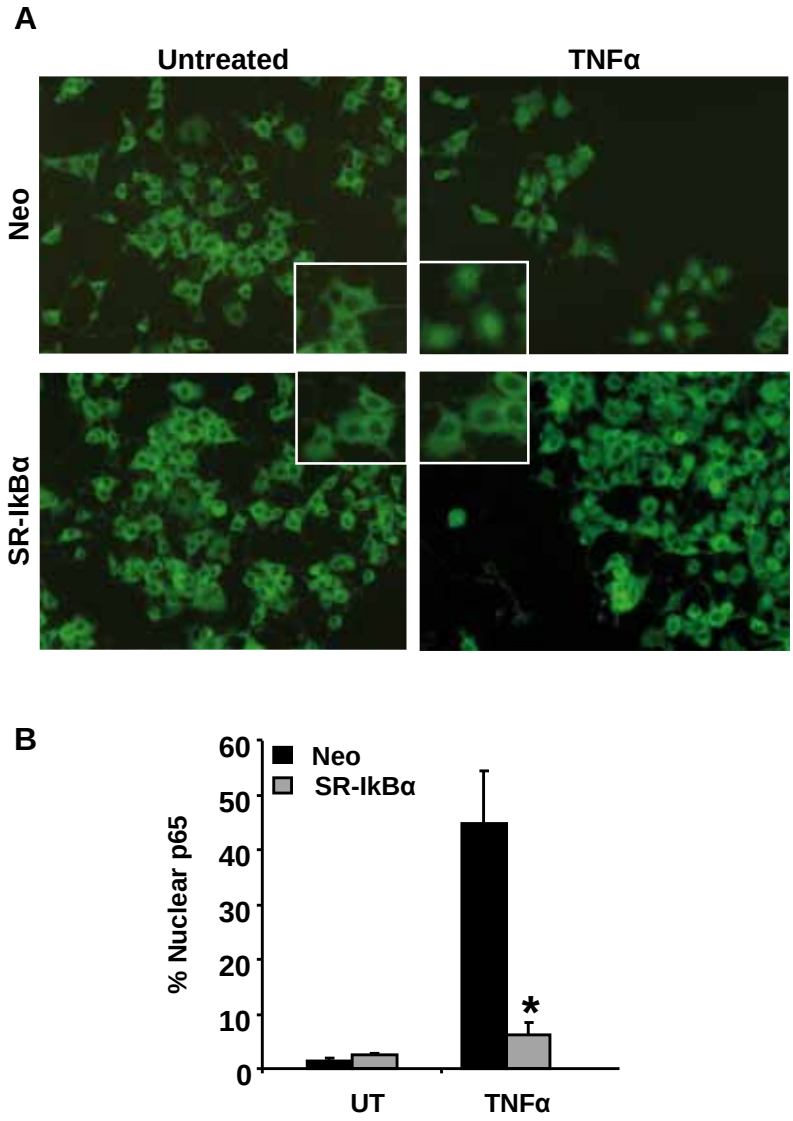


Figure 4. Cells were left untreated or treated with 100 ng/ml of TNFα for 15 minutes. Immunocytochemistry was performed to detect the nuclear translocation of the p65 subunit of NF-κB. **A,** Representative images of three independent experiments show nuclear translocation of p65. **B,** The percentage of cells displaying nuclear translocation of p65 was determined. Significant differences are indicated (* $p<0.001$, *t* test).

Overexpression of the SR-IkBα plasmid was assessed by western blot using a specific antibody against IkBα. The SR-IkBα overexpression plasmid contains the mutated human sequence of IkBα. PC12 are rat cells, and the IkBα protein they express endogenously has a lower molecular weight (35 kDa) than its human homologue (37 kDa). Therefore, detection of a band with a higher molecular weight in the western blot of cells transfected with the SR-IkBα plasmid (Fig. 5) confirms overexpression of the mutated protein.

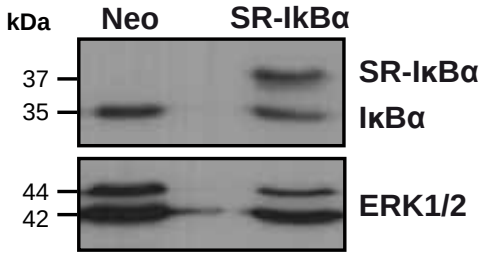


Figure 5. PC12 cells were stably transfected with an empty plasmid (Neo) or SR-IκBα. The expression of the human mutated form of IkBα after SR-IκBα plasmid transfection was validated by western blot, leading to a higher band. Equal loading was confirmed by reblotting with an anti-ERK1/2 antibody

4.2- NF-kB-induced FLIP-L expression controls MAPK/ERK activation

Although it has been previously reported that DRs can activate the MAPK/ERK pathway, the mechanism and relevance of DR-induced MAPK activation in the nervous system remain unknown (Wallach et al., 1999; Ahn, 2001; Desbarats et al., 2003; Micheau and Tschopp, 2003). Therefore, we wanted to assess the possibility of an interaction between the NF-kB and MAPK/ERK signaling pathways as a possible mediator in the inhibition of the apoptotic signaling induced by TNFα.

First, in order to characterize the activation of MAPK/ERK by TNFα in PC12 cells, we analyzed the time-course and dose-dependence of ERK1/2 activation by TNFα treatment. As shown in figure 6, TNFα induces a rapid phosphorylation of ERK1/2. Phosphorylation is maximal at 5 minutes, and it decreases with time until it is almost undetectable after 60 minutes of treatment (Fig. 6). Interestingly, the activation profile of ERK1/2 induced by TNFα is similar to the profile observed when cells are treated with Nerve Growth Factor (NGF) (Moubarak et al., 2010).

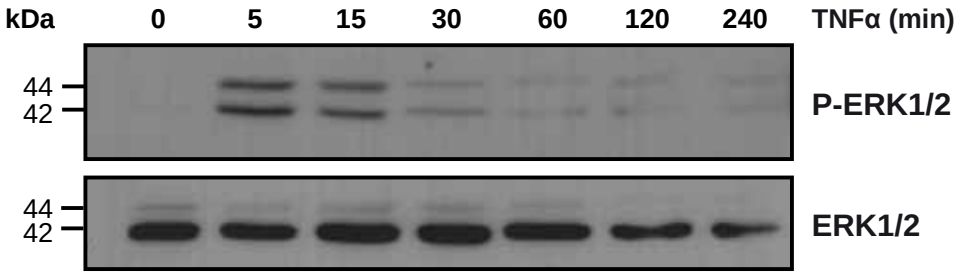


Figure 6. PC12 cells previously serum-deprived for 12h were left untreated or treated for the indicated time points with 100 ng/ml of TNFα. Total cell lysates were analyzed by Western Blot using a specific P-ERK1/2 antibody.

For the dose-dependence analysis we treated serum-deprived PC12 cells with increasing concentrations of TNFα for 5 min., since we observed maximal activation of ERK1/2 at that time point. As shown in figure 7, increasing concentrations of TNFα have the same effect on TNFα-induced MAPK/ERK activation after 5 minutes of treatment (Fig. 7). Therefore, this effect seems to be independent of the dose of TNFα used. In the subsequent f experiments we used a dose of 100 ng/ml of TNFα to induce ERK1/2 phosphorylation, since it is at that dose that we can also observe apoptosis induced by TNFα after blocking NF-kB activation.

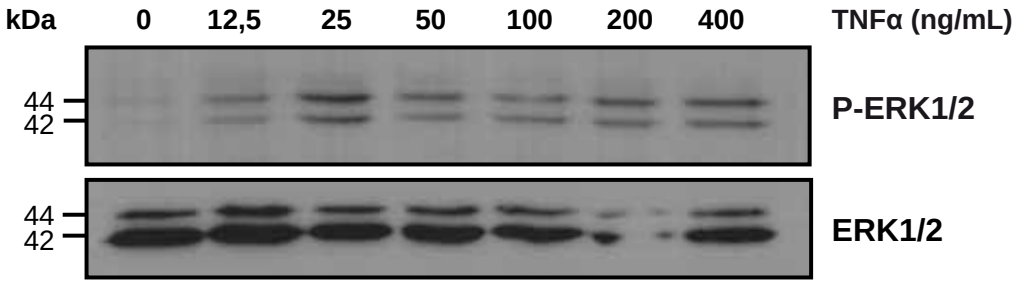


Figure 7. Serum-deprived PC12 cells were left untreated or treated with increasing concentrations of TNFα for 5 minutes and MAPK/ERK activation was assessed as in figure 6.

One of the proteins that have been proposed to mediate resistance to TNFα-induced apoptosis is the anti-apoptotic protein cFLIP-L. In addition, in many cell types, cFLIP-L has been shown to be one of the genes regulated by NF-kB (Micheau et al. 2001). Furthermore, in the immune system, FLIP-L has revealed a role in the activation of MAPK mediated by another death receptor, Fas/CD95 (Kataoka et al., 2000).

Having established that TNFα can induce MAPK/ERK activation in PC12 cells in a time-dependent manner, we explored the putative role of NF-kB in FLIP-L expression and MAPK/ERK activation. Transient transfection of PC12 cells with an empty pcDNA3 plasmid (Neo) or with the SR-IkBα plasmid shows that NF-κB regulates FLIP-L expression, since it decreases over time until it is almost undetectable 2 days after SR-IκBα transfection. We also analyzed Bcl-xL expression in these cells to confirm that it remains unchanged and therefore its expression is not controlled by NF-kB in this model (Fig. 8).

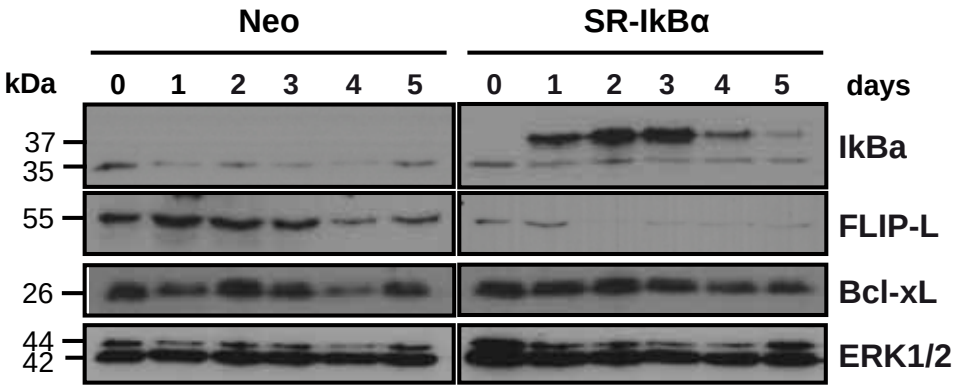


Figure 8. Expression of IκBα, FLIP-L and Bcl-xL in PC12 cells transiently transfected with an empty-plasmid (Neo) or SR-IκBα plasmid was detected by western blot at different days after transfection

We assessed the contribution of NF-kB activation to the MAPK/ERK activation induced by TNFα stimulation in PC12 cells transfected with the SR-IκBα plasmid. By contrast with empty-vector transfected cells, SR-IκBα-transfected cells do not show any phosphorylation of ERK1/2 (Fig. 9). This result indicates that TNFα-mediated activation of ERK1/2 is dependent on NF-κB activation.

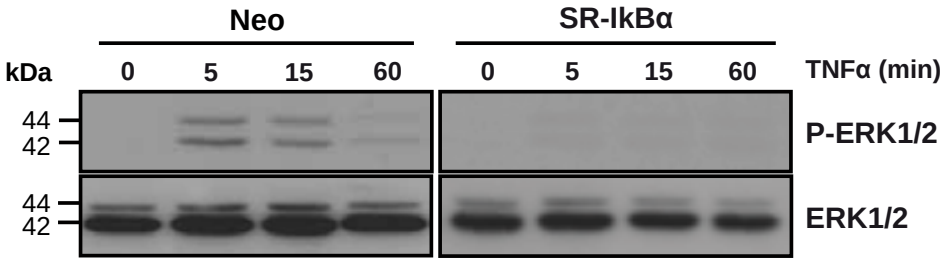


Figure 9. PC12 cells were stably transfected with an empty (Neo) or SR-IκBα plasmid, serum-deprived then left untreated or treated with 100 ng/ml of TNFα for the indicated time points. Total cell lysates were analyzed by western blot using an anti-P-ERK1/2 antibody.

Since FLIP-L expression is under the control of NF-kB and inhibition of this pathway by overexpression of SR-IkBα blocks ERK1/2 phosphorylation, we assessed whether FLIP-L overexpression had any effect on phosphorylation of ERK1/2. As a positive control we stimulated PC12 cells with NGF, a well-known inducer of the MAPK/ERK pathway that has also been shown to depend on FLIP-L to for the phosphorylation of ERK1/2 (Fig 10). Results show that overexpression of FLIP-L causes an increase in ERK1/2 phosphorylation induced by TNFα as well as by NGF.

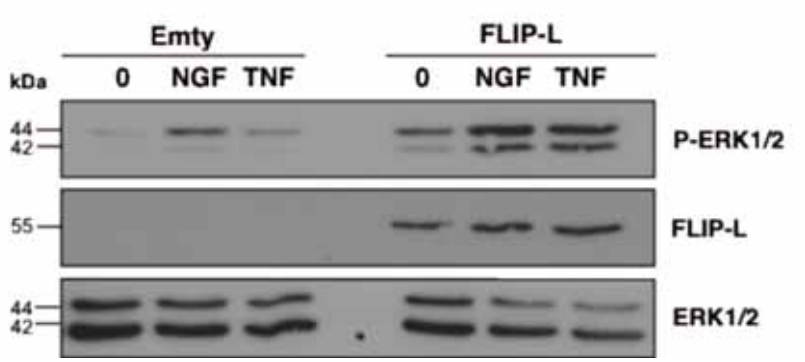


Figure 10. PC12 cells were infected with a lentivirus carrying an overexpression plasmid for FLIP-L. 48h after infection, cells were serum-deprived for 12h and then treated as indicated.

We further analyzed the relevance of FLIP-L in the TNFα-induced activation of the MAPK/ERK pathway. PC12 cells were infected with FLIP-L overexpression lentivirus and, after 48h of lentiviral infection, cells were treated with TNFα for the indicated time-points. Activation of MAPK/ERK pathway was analyzed (Fig. 11). Our results show that in cells overexpressing FLIP-L, TNFα induces a more prolonged ERK1/2 phosphorylation when compared to control cells infected with an empty plasmid. Furthermore, our findings agree with the results obtained by Kataoka et al. in a lymphocyte model stimulated with FasL. As we have shown with TNFα in PC12 cells, they demonstrated that transfection of FLIP-L induces a maintained ERK1/2 phosphorylation after FasL stimulation.

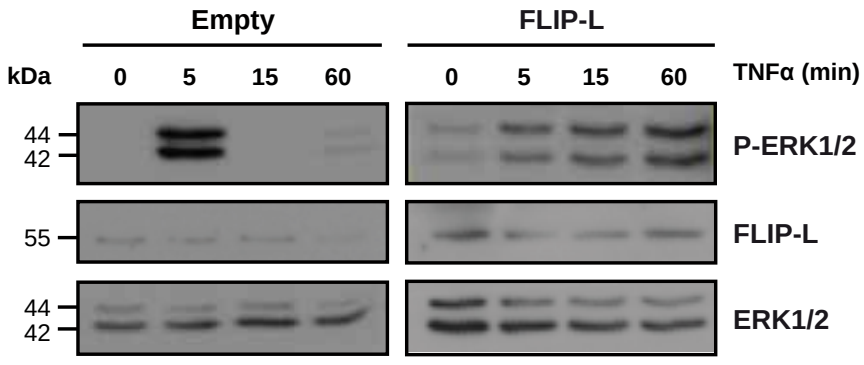


Figure 11. PC12 cells were transduced with Empty or FLIP-L overexpression lentiviruses, serum-deprived 2 days post-transduction, then left untreated or treated with 100 ng/ml of TNFα for the indicated time points. Total cell lysates were analyzed by immunoblotting using an anti-P-ERK1/2 antibody. An anti-FLIP antibody was used to control efficiency of transduction

In order to validate the relevance of FLIP-L as a mediator of ERK1/2 phosphorylation induced by TNFα, we carried out a lentiviral-based knockdown of FLIP-L (shFLIP-L). Cells were infected with lentivirus carrying a FLIP-L specific shRNA. Three days after infection, cells exhibited a strong reduction in FLIP-L expression, as assessed by western blot analysis (Fig. 12). A western blot for phosphorylated ERK1/2 revealed that FLIP-L knockdown prevents ERK1/2 phosphorylation in a way very similar to the inhibition observed in cells overexpressing SR-IkBα (see Fig. 9). Altogether, these results confirm that NF-kB-regulated FLIP-L plays a key role in MAPK/ERK activation induced by TNFα.

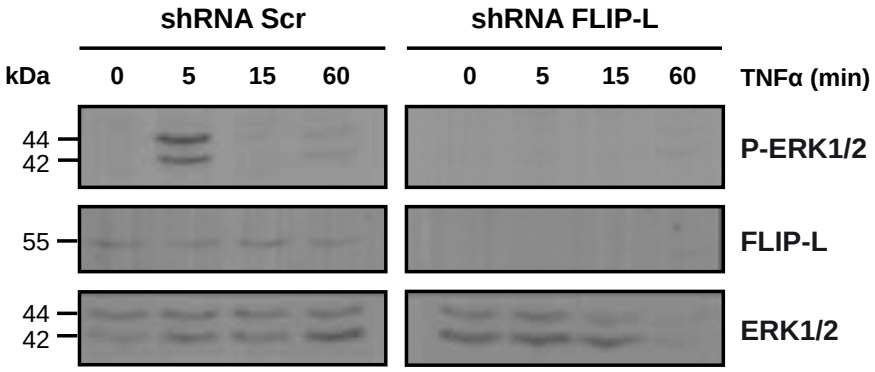


Figure 12. PC12 cells were transduced with scrambled sequence (shRNA Scr) or shRNA against FLIP-L (shRNA FLIP-L) lentiviruses, serum-deprived 3 days post-transduction, then left untreated or treated with 100 ng/ml of TNFα for the indicated time points. Total cell lysates were analyzed by immunoblotting with a specific antibody against P-ERK1/2. FLIP-L knockdown efficiency was assessed using the anti-FLIP antibody. In all panels, equal loading was confirmed by reblotting the membranes with an anti-ERK1/2 antibody.

4.3- TNFα induces FLIP-L-dependent Raf-1 activation

Although the signaling pathway that leads to MAPK/ERK activation by several stimuli, such as NGF or other growth factors, has been extensively described, there is a lack of data concerning the mediators of ERK1/2 activation mediated by death receptors.

Since we demonstrate here that FLIP-L is necessary for TNFα-induced MAPK activation, we further analyzed the upstream step that is critical for this event. ERK1/2 activation is frequently a result of Ras activation, which recruits to the membrane the MAPKKK Raf-1, leading to the activation by phosphorylation of the MAPKKs MEK1/2, which in turn phosphorylates ERK1/2 {Leevers:1992vd}. To test whether activation of ERK1/2 by TNFα induces this activation pathway in PC12 cells, a Ras activation assay kit (Millipore) was used, based on the pull-down of active Ras from cell lysates by binding to the Ras Binding Domain (RBD) of Raf-1.

We stimulated PC12 cells transfected with SR-IkBα or an empty plasmid for 5 minutes with TNFα. Treatment with NGF was used as a positive control for Ras activation. After peforming the pull-down assay, GTP bound Ras presence was analyzed by western blot. Interestingly, results show that unlike NGF treatment, TNFα treatment does not activate Ras (Fig. 12). Therefore, according to this result Ras is not the upstream mediator of TNFα-induced MAPK/ERK activation.

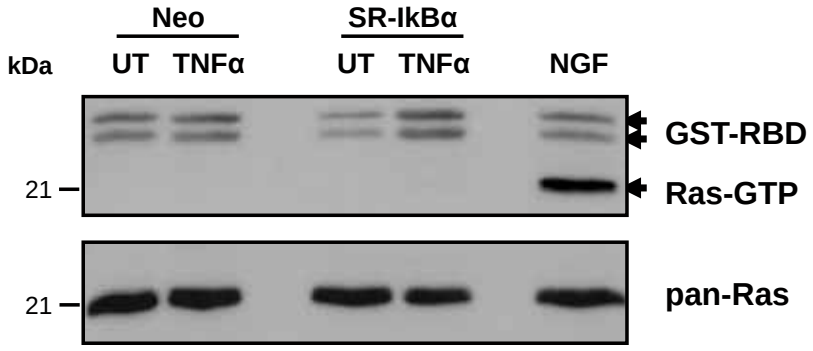


Figure 13. Serum-deprived PC12 cells were treated with 100 ng/mL of TNFα or NGF for 5 minutes, and activated Ras was pulled down using Raf-RBD conjugated agarose beads. GTP-bound Ras was detected by western blot using an anti-pan-Ras antibody.

Next, we analyzed the TNFα-mediated activation of Raf-1, classically linked to ERK1/2 phosphorylation mediated by NGF downstream of Ras. For that purpose, cells were treated as indicated in figure 14, using NGF as a positive control, and Raf-1 was immunoprecipitated from cell lysates. A kit was used based on the immunodetection of phosphorylated MEK1 as a measure of Raf-1 activation (Millipore, see materials and methods). TNFα induces activation of Raf-1, and it does so at the same level of NGF. (Fig. 14). Therefore, although the activation of Ras is not necessary for ERK1/2 phosphorylation mediated by TNFα, Raf-1 does seem to have a role in this signaling cascade.

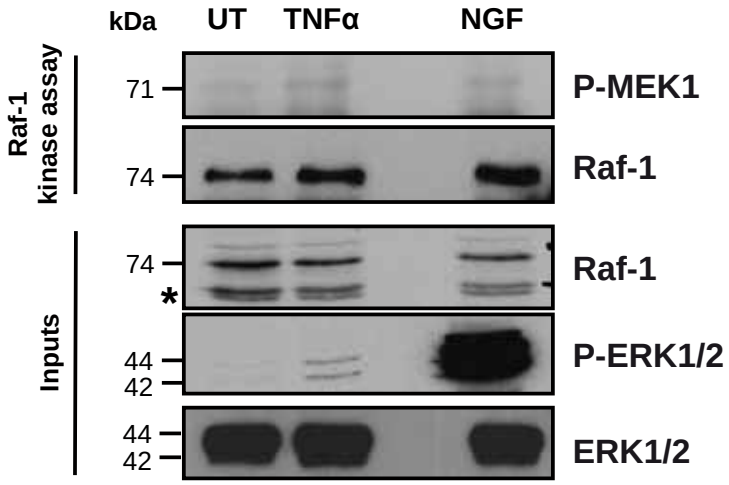


Figure 14. Endogenous Raf-1 was immunoprecipitated from PC12 cells treated with TNFα or NGF and immunoprecipitates were incubated with recombinant MEK and ATP *in vitro* in order to detect Raf-1 kinase activity. Western blot analysis was performed for Raf-1 and P-MEK1. Inputs were blotted using anti-Raf-1, anti-P-ERK1/2, and anti-ERK1/2 antibody as a loading control.

Since we showed that blockade of NF-kB completely abrogates ERK1/2 phosphorylation induced by TNFα, we wanted to assess the activation of Raf-1 in PC12 cells overexpressing SR-IkBα. As expected, when NF-kB activation is inhibited TNFα not only is unable to induce ERK1/2 phosphorylation, but it als cannot induce activation of the upstream effetor Raf-1 (Fig. 15).

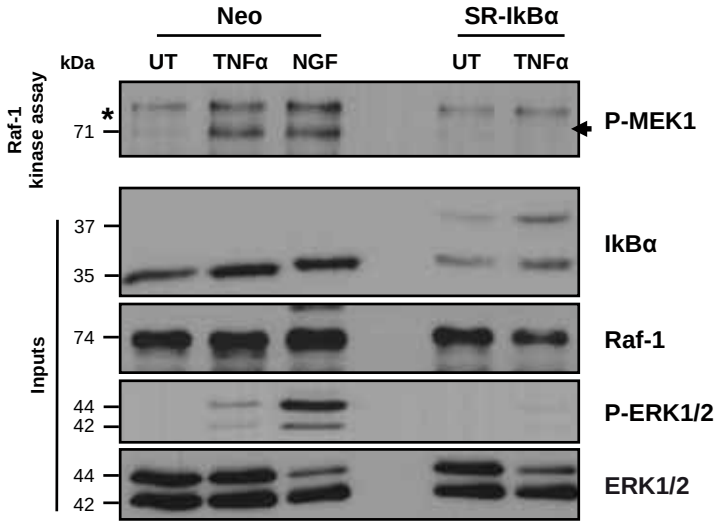


Figure 15. Raf-1 kinase activity was assessed as in figure 13**,** in PC12 cells transfected with Neo or SR-IkBα

In the same manner, FLIP-L knockdown abrogates TNFα-induced Raf-1 activation and subsequent ERK1/2 activation (Fig. 16), further supporting a direct role of FLIP-L in Raf-1 activation and subsequent ERK1/2 phosphorylation.

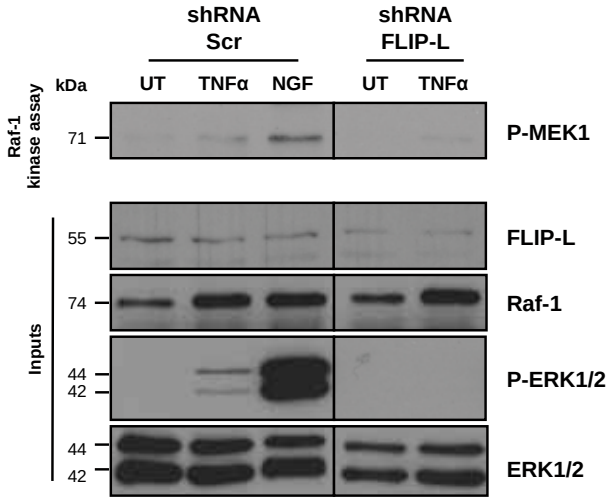


Figure 16. After 3 days of PC12 transduction with scrambled sequence (shRNA Scr) or shRNA against FLIP-L (shRNA FLIP-L) lentiviruses**,** Raf-1 activity was assessed as in Figure 13. Black lines indicate that intervening lanes have been spliced.

Additionally, we assessed Raf-1 activation by its recruitment to the membrane, since this has been shown to be a main step for Raf-1 activation in the classical Ras/Raf/MEK/ERK pathway. A subfractionation analysis revealed that Raf-1 is clearly enriched in membrane fractions in cells treated with TNFα for 15 minutes, in comparison with a treatment of 5 minutes or untreated cells. We also show that most of the phosphorylated ERK1/2 is located in the cytosol (Fig. 17).

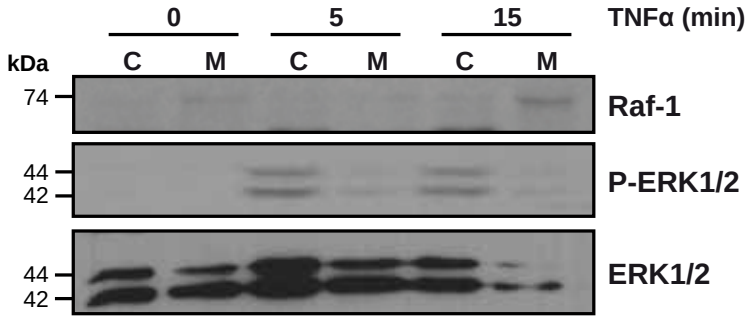


Figure 17. Serum-deprived PC12 cells were treated with TNFα for the indicated times prior harvesting and subcellular fractionation. Lysates corresponding to cytosolic (C) and membrane fractions (M) were resolved by SDS-PAGE and Raf-1 subcellular localization was assessed by western blot using an anti-Raf-1 antibody. ERK1/2 phosphorylation was also detected to control MAPK/ERK activation following TNFα stimulation.

To demonstrate that Raf-1 is necessary to induce ERK1/2 phosphorylation, we designed RNAi oligonucleotides targeting the human sequence of Raf-1, which holds great homology to the rat form of PC12 cells. To test the efficiency of the designed RNAi, we transfected PC12 cells with both oligonucleotides at different concentrations and performed western blot to assess the specific down-regulation of Raf-1 (Fig 18).

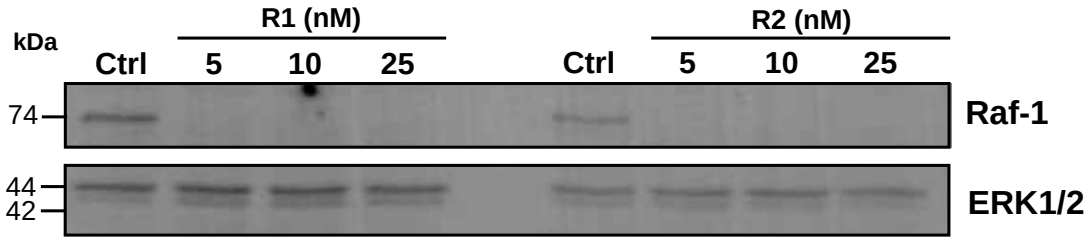


Figure 18. PC12 cells were transfected with increasing concentrations of two different RNAi against Raf-1 (R1, R2). Three days alter transfection, total cell lysates were extracted and Western Blot was performed to analyze Raf-1 levels. Membrane was also blotted for ERK1/2 as a loading control.

We demonstrate that Raf-1 activation is necessary for MAPK/ERK pathway activation, since Raf-1 knockdown significantly impairs TNFα-induced ERK1/2 phosphorylation (Fig. 19). Finally, we show that Raf-1 and FLIP-L interact by immunoprecipitation of FLIP-L in PC12 cells transfected with HA-tagged FLIP-L and/or Raf-1 (Fig. 20), as a suggestion of a structural basis for Raf-1 activation. Taken together, these results allow us to conclude that FLIP-L is able to activate Raf-1 by an alternative mechanism to the classical Ras activation.

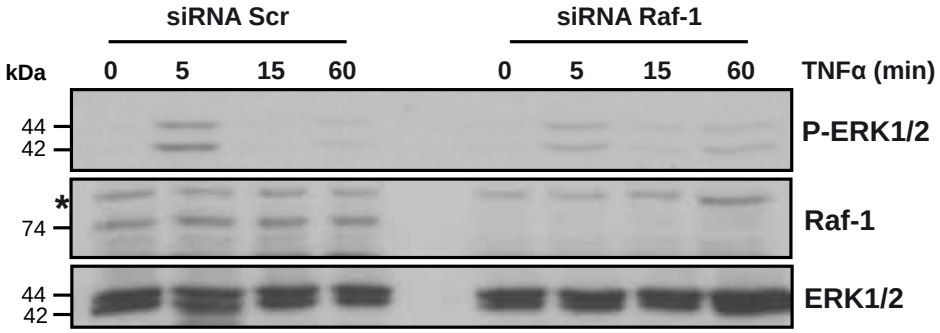


Figure 19. PC12 cells were transfected with siRNA targeting Raf-1 or a scrambled sequence. Three days after transfection, cells were treated with TNFα for the indicated time points and western blot was performed to detect P-ERK1/2, Raf-1 and total ERK1/2 as loading control.

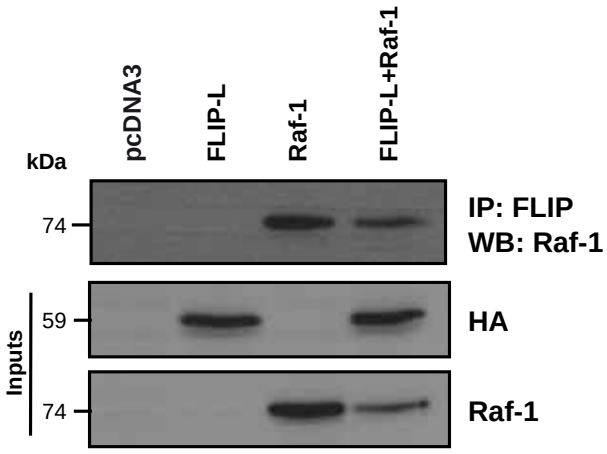


Figure 20. PC12 cells were transfected with pcDNA3-HA-FLIP-L, pcDNA3-Raf-1 or both plasmids. Cells were harvested 24h later and FLIP-L was immunoprecipitated using a specific anti-FLIP antibody prior western blot using anti-Raf-1 antibody. Transfection efficiency of both plasmids was checked in the inputs.

4.4- NF-kB and MAPK/ERK inhibition are equivalent for sensitization of PC12 cells to the apoptotic effect of TNFα.

After describing the pathway triggered by TNFα and leading to ERK1/2 phosphorylation, we wanted to invetigate the relevance of this signaling cascade as a regulator of apoptosis induced by TNFα, given that the MAPK/ERK pathway has been classically related to cell survival and proliferation.

As shown in Figure 1, when NF-kB pathway is blocked, TNFα induces apoptotic cell death. Since we have linked NF-kB activation and FLIP-L regulation to MAPK/ERK activation, we further investigated whether inhibition of MAPK/ERK is equivalent to NF-kB blockade in terms of sensitivity to TNFα-induced apoptosis. To this end, we performed a series of experiments in which we co-treated PC12 cells with TNFα and the MEK1 inhibitor PD98059. First we analyzed the effects of TNFα in presence of the PD98059 on cell survival as assessed by an MTT assay. Cells pretreated with PD98059 and co-treated with TNFα show a decrease in cell viability when compared with untreated cells or cells treated with TNFα or PD98059 alone (Figure 21).

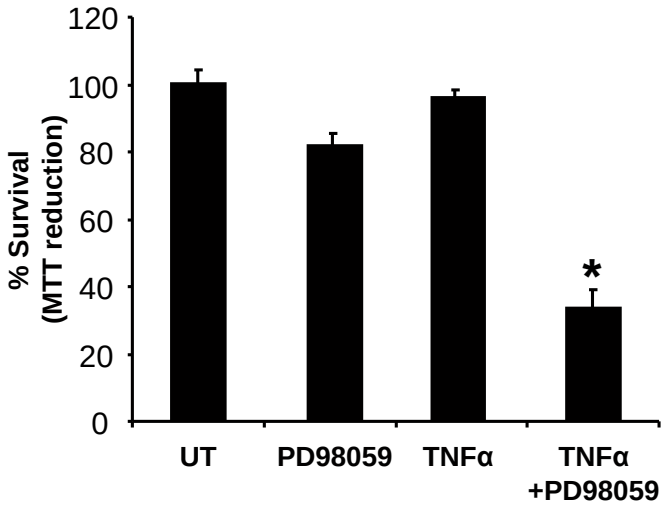


Figure 21. PC12 cells pretreated or not with 30μM of PD98059 were left untreated or treated with TNFα and/or PD98059 for 24h before MTT reduction assay was performed

Second, a DEVDase activity assay revealed that TNFα significantly induces effector caspase-like activity upon MEK1 inhibition, when compared to an untreated control or the single TNFα or PD98059 treatments (Figure 22). Finally, apoptotic cell death was evaluated by quantification of condensed nuclei stained with Hoechst 33258 (Figure 23). A higher percentage of apoptotic cell death is noticeable in cells co-treated with TNFα and PD98059, and the level of apoptotic cell death reached is similar to the one observed in cells stably transfected with SR-IκBα and treated with TNFα alone (Fig. 2). These results allow us to conclude that the inhibition of the MAPK pathway, as well as NF-kB pathway abrogation, sensitizes cells to the pro-apoptotic function of TNFα.

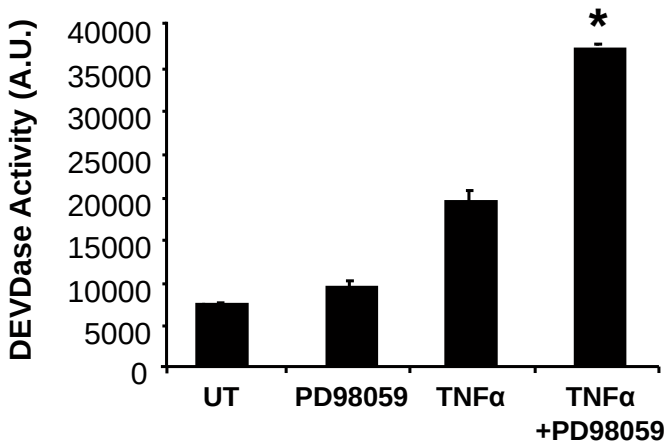


Figure 22. Cells were treated as in figure 18 before caspase-3-like activity was measured using the Ac-DEVD-afc fluorogenic substrate. Significant differences are indicated (*p<0.001, t test).

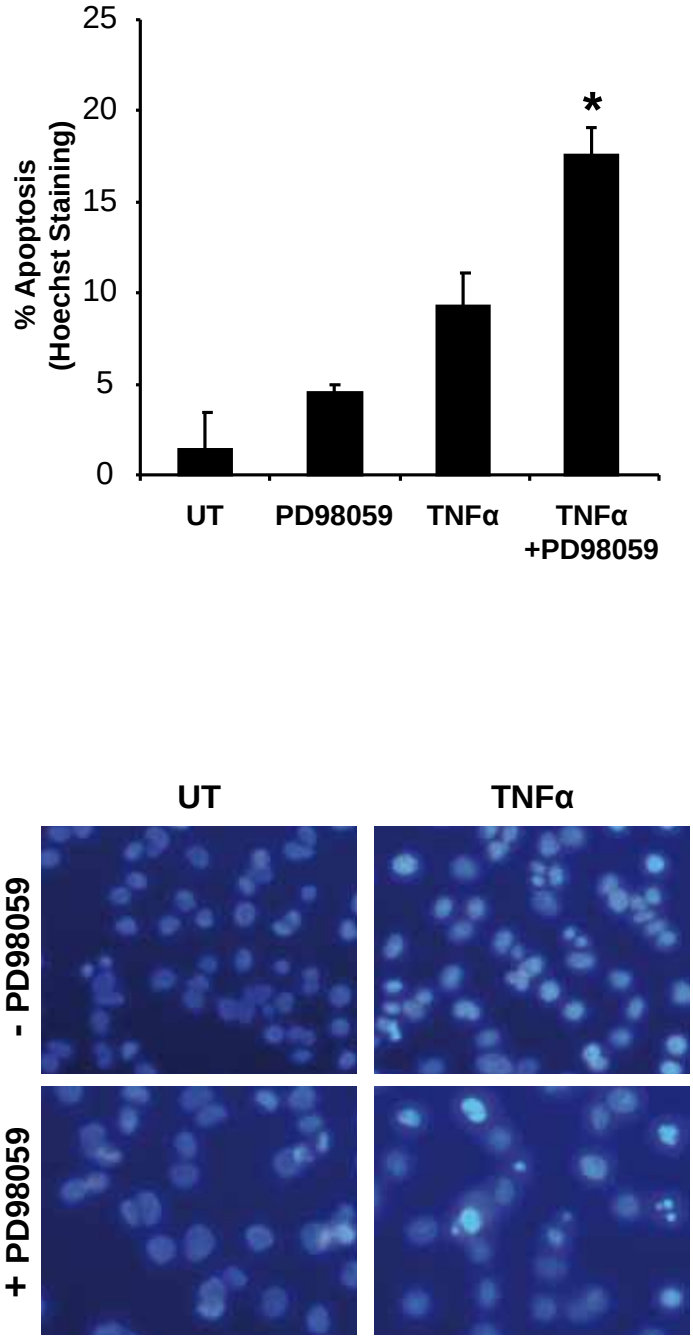


Figure 23. Apoptotic cell death was quantified by direct counting of condensed nuclei using Hoechst 33258 staining. Significant differences are indicated (*p<0.001, t test). Representative images of Hoechst 33258 stained nuclei are shown

4.5- MAPK/ERK activation is essential in the cell survival pathway elicited after TNFα treatment

To further assess the link between NF-kB and MAPK/ERK pathways, we checked whether sustained activation of the MAPK pathway in SR-IκB-expressing cells would protect them from TNFα-induced cell death. Neo- or SR-IκBα-expressing PC-12 cells were transfected with a plasmid encoding the constitutively active form of MEK (MEK-CA) or pcDNA3. Figure 24 shows a significant increase in DEVDase activity after 24h of TNFα treatment in cells expressing SR-IκBα, which is restored to control levels when MEK-CA is co-expressed with the IkBα mutant.

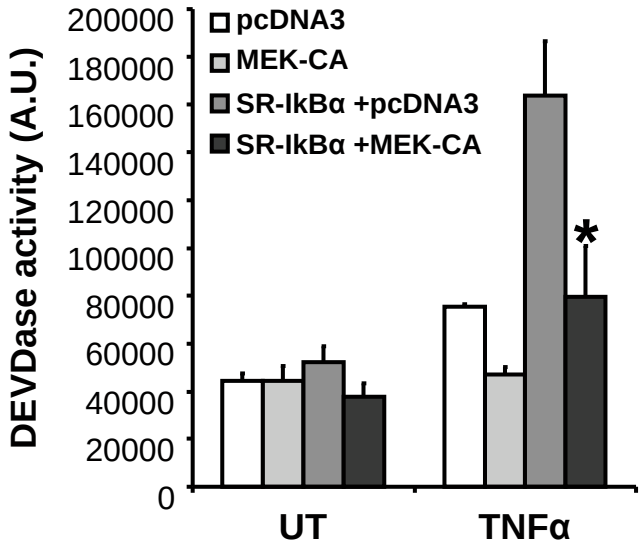


Figure 24. PC12 cells stably transfected with an empty plasmid (Neo) or SR-IκBα were transiently transfected with or without a plasmid carrying a constitutively active form of MEK (MEK-CA). 24h after transfection, cells were left untreated or treated with 100 ng/mL of TNFα for 24h. Caspase-3-like (DEVDase) activity was measured using the Ac-DEVD-afc fluorogenic substrate. Significant differences are indicated (* p<0.01, *t test)*

In the same sense, apoptotic cell death was quantified by chromatin condensation, and we show that the TNFα-induced cell death in SR-IκB-expressing cells is significantly reduced to the same level as untreated controls, when both SR-IκBα and MEK-CA are co-expressed (Fig. 25A). PC12 cells transfected with the MEK-CA show constitutive phosphorylation of ERK1/2 as compared with Neo-transfected cells (Fig. 25B).

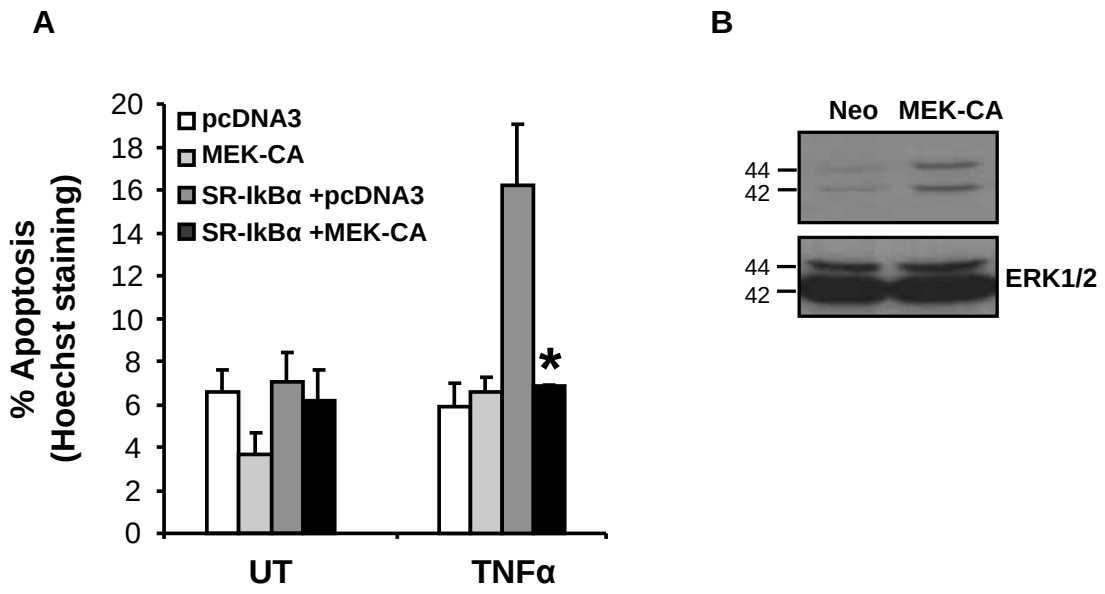


Figure 25. A, Percentage of cell death was measured by counting apoptotic nuclei after Hoechst 33258 staining. Significant differences are indicated. **B**, Cells lysates of Neo or MEK-CA-transfected cells were immunoblotted using anti-P-ERK1/2 antibody. Note that ERK1/2 is basally phosphorylated in untreated cells. Equal loading was confirmed by reblotting with an anti-ERK1/2 antibody

In addition we analyzed by immunoblot the cleavage of PARP, a well-known target of activated caspase-3 (Fig. 26). In cells overexpressing both the SR-IkB and the constitutively active form of MEK, PARP cleavage was significantly inhibited, suggesting that activation of the MAPK/ERK has an inhibitory effect on caspase-3 cleavage and therefore on apoptosis.

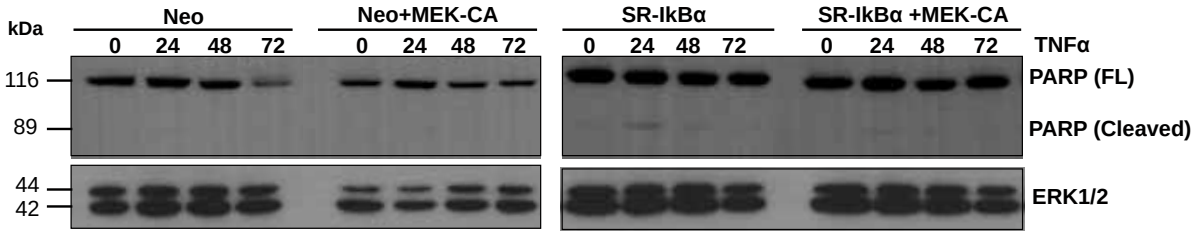
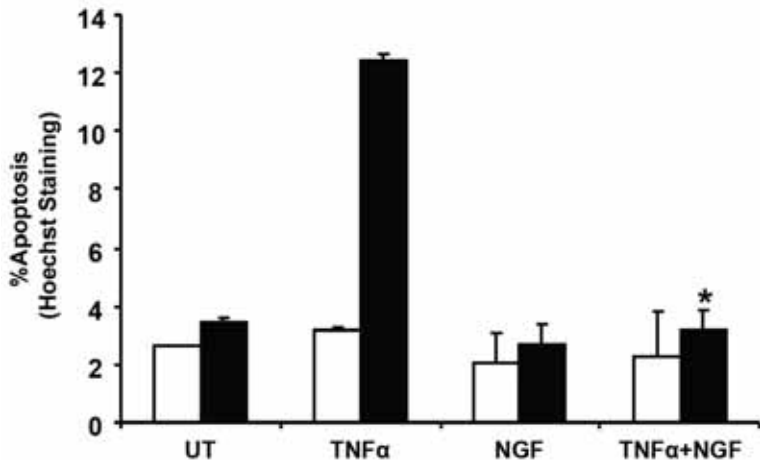


Figure 26. PC12 cells were transfected as in Fig 24. Cells were treated with 100ng/ml of TNFα for the indicated time points. Cell lysate was resolved in SDS-PAGE and PARP was detected with an antibody specific for Full-length (FL) and cleaved forms of PARP.

Alternatively, to demonstrate that the activation of MAPK/ERK is essential for neuroprotection upon TNFα treatment, we treated stably transfected Neo and SR-IkB PC12 cells with TNFα, in the presence or not of 100 ng/mL of NGF for 24 hours. NGF is a neurotrophin that activates MAPK/ERK through tyrosine kinase receptors by a classical Ras-dependent manner (Fig. 27). As evidenced in Figure 27A, TNFα treatment induces apoptosis when the NF-kB pathway is blocked, as compared with Neo control cells. Interestingly, co-treatment with NGF protects these cells from apoptosis induced by TNFα, even though the NF-kB pathway is blocked (Fig. 27A and 27B). These results support that the MAPK/ERK pathway activation is necessary and sufficient for the pro-survival effects of TNFα.

A



B

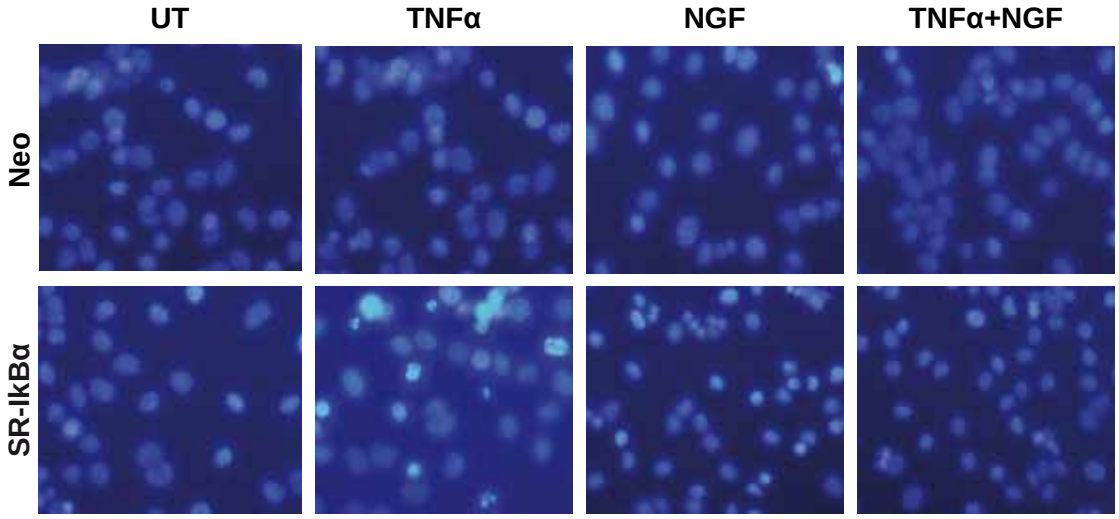


Figure 27. PC12 cells stably transfected with an empty plasmid (Neo) or SR-IκBα were treated with 100 ng/ml of TNFα and/or NGF for 24h. **A,** Apoptotic cell death was assessed by direct counting of condensed nuclei stained with Hoechst 33258. Significant difference between SR-IkBα-transfected and TNFα – or TNFα+NGF-treated cells is indicated (* $p<0.01$, *t* test). **B**, Representative pictures of Hoechst 33258-stained cells are shown

4.6- Inhibition of NF-κB or MAPK/ERK sensitizes PC12 cells to TNFα-induced apoptosis through the JNK pathway.

It has been reported that TNFα might induce apoptosis through the activation of c-Jun-N-terminal kinase (JNK) (De Smaele et al., 2001; Javelaud and Besançon, 2001; Tang et al., 2001; Wang et al., 2006). The BH3-only protein Bim has been identified as a key mediator of apoptosis acting downstream of JNK via the intrinsic pathway in several models, including neuronal cells (Whitfield et al., 2001; Putcha et al., 2003; Kaufmann et al., 2009). In order to determine if the JNK pathway is implicated in the TNFα-induced cell death when the NF-kB pathway is blocked, empty-vector or SR-IκBα-transfected PC12 cells were treated with TNFα and the activation of JNK1/2 and its downstream mediators of apoptosis was analyzed. As shown in Figure 28, SR-IκBα-expressing PC12 cells show a significant and sustained increase in phosphorylated JNK1/2 levels (P-JNK1/2) after 24 hours of TNFα treatment, as compared to their respective empty-vector control.

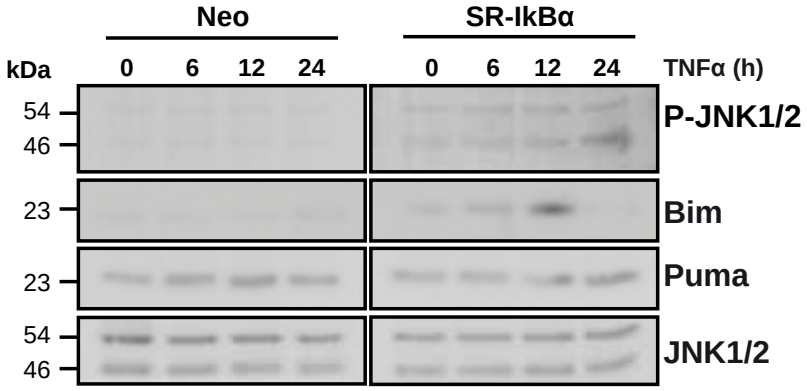


Figure 28. PC12 cells stably expressing an empty (Neo) or SR-IκBα plasmid were treated with 100 ng/ml of TNFα or TNFα plus 10 μM of the specific JNK inhibitor SP600125 for the indicated time points. Total cell lysates were analyzed using specific anti-P-JNK1/2, anti-Bim, and anti-Puma antibodies. A specific antibody against total JNK1/2 was used as a loading control.

Moreover, the observed JNK1/2 activation correlates with an increase in the expression of the pro-apoptotic BH3-only protein Bim but not PUMA (Fig. 28). The upregulation of Bim in SR-IκBα-expressing PC12 cells treated with TNFα is dependent on the activation of the JNK pathway, since the pretreatment with the JNK1/2 inhibitor SP600125 followed by TNFα treatment completely inhibited JNK1/2 phosphorylation (Fig. 29).

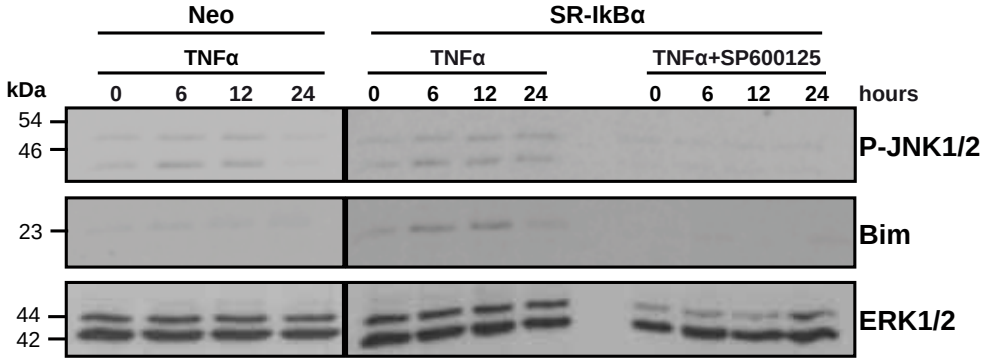


Figure 29. PC12 cells stably transfected with an empty (Neo) or SR-IκBα plasmid were treated with 100 ng/ml of TNFα or TNFα plus 10 μM of SP600125 for the indicated time points. Total cell lysates were analyzed using specific anti-P-JNK1/2, anti-Bim and anti-ERK1/2 antibodies.

In order to link the activation status of JNK1/2 to the sensitization to TNFα-induced apoptosis, stable Neo or SR-IκBα-transfected PC12 cells were treated with the JNK1/2 inhibitor SP600125, with or without TNFα. Cell death counting shows that inhibition of JNK1/2 phosphorylation rescues PC12 cells from TNFα-induced apoptosis when the NF-kB pathway is impaired (Fig. 30).

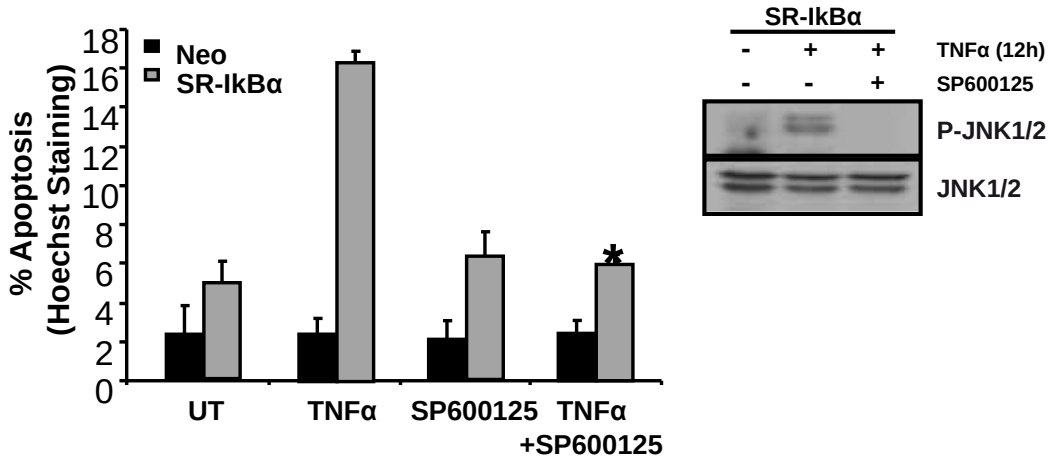


Figure 30. Cells were pretreated or not with 10 μM of SP006125 prior treatment with 100 ng/ml of TNFα and/or SP006125 for 24 hours. Apoptotic cell death was quantified by counting condensed nuclei stained with Hoechst 33258. Significant difference between SR-IkBα-transfected and TNFα or TNFα+NGF-treated cells is indicated (* p<0.01, t test). Efficiency of the inhibitor was analyzed by western blot of P-JNK1/2 in SR-IκBα-transfected cells treated with TNFα and/or SP600125 for 12h.

Similarly, we characterized the apoptotic pathway induced by TNFα in PC12 cells in which both JNK1/2 and ERK1/2 phosphorylation is inhibited. PC12 cells treated as in Figure 26 were also pretreated or not with PD98059. The inhibition of the JNK pathway is able to rescue PC12 cells from TNFα-induced apoptosis when the MAPK/ERK pathway has also been blocked (Fig. 31).

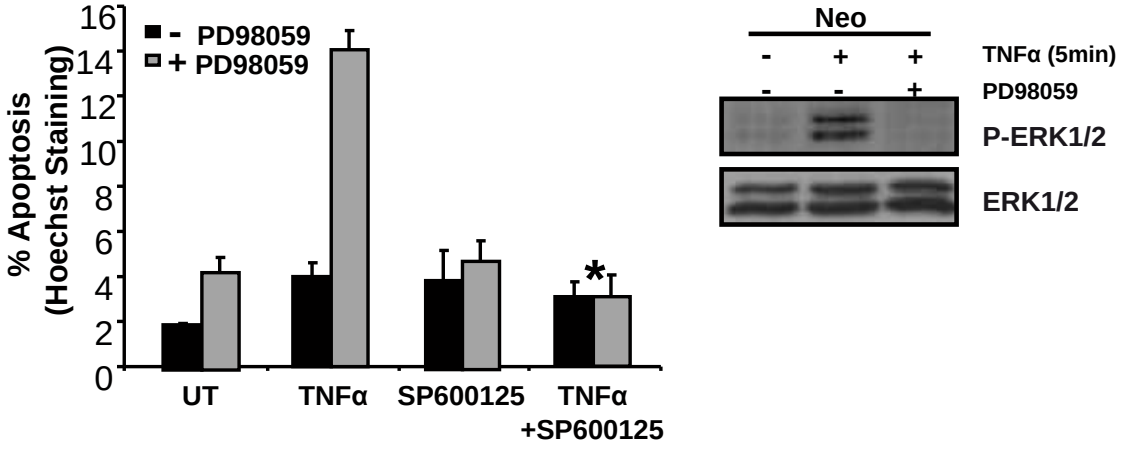


Figure 31. PC12 cells were pretreated or not with 30μM of PD98059 prior treatment with TNFα and/or SP600125 for 24 hours. Apoptotic cell death was quantified as in figure 26**.** Significant differences are indicated (*p<0.001, *t* test). Efficiency of the MEK1 inhibition was analyzed by western blot of P-ERK1/2 in cells treated with TNFα and/or PD98059 for 12h.

Finally, we show that the inhibition of the MAPK/ERK pathway by PD98059 leads to a sustained JNK1/2 phosphorylation and the upregulation of Bim as compared with cells treated with TNFα alone (Fig. 32), and in a similar manner to NF-kB inhibition (Fig. 29). Also, the simultaneous inhibition of the MAPK/ERK and the JNK pathway inhibits TNFα-induced Bim upregulation (Fig. 32). Altogether, our results demonstrate that TNFα induces apoptosis when either NF-kB or the MAPK/ERK pathway is inhibited, and in both cases through the upregulation of the BH3-only protein Bim which is under the control of JNK1/2 activation.

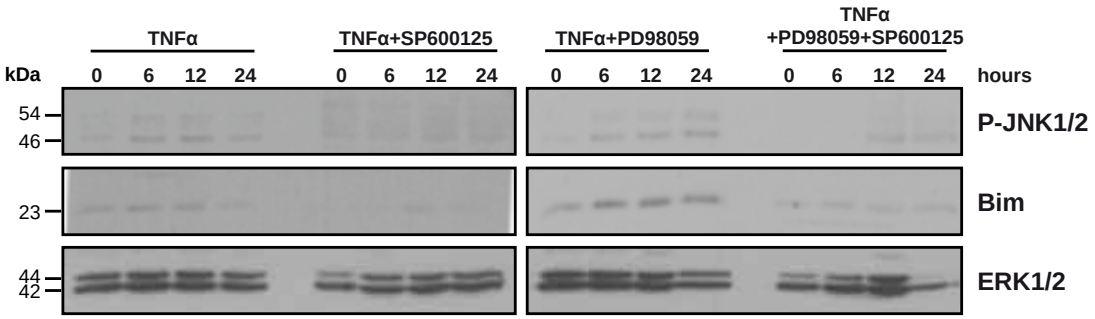


Figure 32. PC12 cells were pretreated or not with 30 μM of PD98059 and/or SP600125 prior treatment with TNFα and/or PD98059 and/or SP600125 for the indicated time points. Total cell lysates were analyzed using specific antibodies against P-JNK1/2, Bim and ERK1/2.

5.DISCUSSION

5.DISCUSSION

Since their discovery and characterization, Death Receptors have been linked to both induction of cell death and cell survival and proliferation. Initially, the relevance of DR function was thought to be confined to the immune system, where these molecules have been better characterized, proving to be crucial for apoptosis, survival, proliferation, and secretion of proinflammatory cytokines (Wilson et al., 2009). However, death receptors are highly expressed in many tissues, including the nervous system. In the nervous system, death receptors have shown to play a role both in development of the CNS and in several pathologies such as neurodegenerative diseases, ischemia, multiple sclerosis, or Amyotrophic Lateral Sclerosis (see introduction).

Of all the death receptors belonging to the TNF superfamily, CD95/Fas is one of the best described in the nervous system. Fas has shown to induce neurite outgrowth and to accelerate peripheral nerve regeneration (Desbarats et al., 2003; Pettmann and Henderson, 2003). The MAPK/ERK pathway has been implicated in this effect. Moreover, Fas deficient *lpr* mice display cognitive and sensorimotor deficits, stria vascularis cell degeneration, progressive atrophy of pyramidal neuron dendrites, and delayed neurite regeneration. On the other hand, Fas has also been related to the induction of cell death in the nervous system, further supporting the dual role of these receptors in the nervous system. More specifically, the accumulation of ROS caused by mutations in the protein SOD observed in some cases of ALS induces overexpression of Fas and FasL that leads to motoneuron degeneration (Raoul et al., 2002).

TNFR1 has also been implicated in pathways promoting either survival or cell death in the nervous system (ref). However, the signaling cascades induced through TNFR1 are less well known and are currently being the subject of intensive studies due to their potential role in many pathological, as well as physiological conditions. The present thesis is focused in the activation of the MAPK/ERK pathway by TNFα in the PC12 cell line as a model of neuronal cells. We demonstrate that NF-kB has a pivotal role in activation of the MAPK/ERK pathway through the antiapoptotic protein FLIP-L. Moreover, our results show that ERK activation is necessary for the protection of PC12 cells from apoptosis induced by TNFα. Finally, we have shown that abrogation of this pathway leads to TNFα-induced apoptosis dependent on JNK1/2 phosphorylation.

NF-kB protects PC12 cells from apoptosis induced by TNFα

As it was mentioned in the introduction, binding of TNFα to TNFR1 triggers the activation of multiple signaling pathways within the cell. Activation of these pathways may result in apoptotic cell death through the extrinsic apoptotic pathway, leading to cleavage and activation of caspase-8, but it can also promote cell survival or differentiation. The transcription factor NF-kB has a crucial role in TNFα-mediated cell survival, since it controls the transcription of many genes that have been related to the inhibition of cell death, like IAPs, cFLIP, and, in some models, Bcl-2 family proteins like Bcl-X_L. It is well established that in order to induce cell death, the survival pathway triggered by TNFα needs to be abrogated. Protein synthesis inhibitors, such as Actinomycin D or Cycloheximide have been extensively used to block survival signaling and sensitize cells to the pro-apoptotic activity of TNFα. However, neither Actinomycin D nor cycloheximide are specific inhibitors of the survival signaling mediated by NF-kB. In fact, Actinomycin D has been shown to mediate sensitization of PC12 cells and cortical neurons through the down-regulation of Bcl-X_L in a mechanism independent of NF-kB or NF-kB target genes such as cFLIP or IAPs(Gozzelino et al., 2008). Therefore, there is a lack of conclusive data regarding the mechanism by which NF-kB activation inhibits cell death triggered by TNFα.

Although the role of NF-kB as a regulator of cell survival, inflammation and immune response has been described extensively (Van Antwerp et al., 1996; Barkett and Gilmore, 1999), its function in the nervous system remains controversial. Several studies have demonstrated that NF-kB facilitates neuronal survival, while others report that neuronal death may be mediated by NF-kB. To clarify the role of NF-kB in the CNS, Bhakar and his collaborators generated transgenic mice that provide a sensitive readout for NF-kB activity in the nervous system. That study shows that NF-kB transcriptional activity is prominent in the developing and adult nervous system. The authors showed that NF-kB is necessary for neuronal survival, and that NF-kB overexpression in primary cortical neurons confers neuroprotection through the induction of antiapoptotic genes such as cIAP1 or cIAP2 (Bhakar et al., 2002). However, the stimuli that contribute to constitutive activation of NF-kB or the mechanism through which it promotes neuronal survival remain unclear.

In this study we used a non-degradable form of IkBα (SR-IkBα) to block TNFα-mediated activation of NF-kB. SR-IkBα bears mutations in two residues that are crucial for its phosphorylation by upstream kinases and subsequent degradation (S32A, S36A). Therefore, SR-IkBα overexpression acts as a dominant negative of IkBα, thus

avoiding translocation of NF-kB to the nucleus where it can exert its function. We stably expressed SR-IkBα on PC12 cells and showed that blocking of NF-kB renders these cells sensitive to apoptosis induced by TNFα, as indicated by apoptotic nuclear condensation and DEVDase activity. These results support the protective role of NF-kB in neuronal cells, as it has been pointed out in the literature. Furthermore, from these results it can be hypothesized that TNFα is a key activator of NF-kB during development of the nervous system.

TNFα triggers a MAPK/ERK signaling pathway that depends on the regulation of FLIP-L by NF-kB

The MAPK/ERK pathway has been classically related to cell survival and proliferation after activation by growth factors, such as the Nerve Growth Factor (NGF) or the Brain Derived Neurotrophic Factor (BDNF) in the nervous system. However, death receptors are also known to activate this pathway in certain conditions, although the exact activation cascade is not fully understood. In this study we show that TNFα induces a rapid ERK1/2 phosphorylation in PC12 cells in a time-dependent manner. More importantly, overexpression of the SR-IkBα plasmid in this model completely blocks activation of MAPK/ERK by TNFα. This result suggests that the NF-kB pathway has a role in the phosphorylation of ERK1/2 mediated by TNFα. In fact, this is not the only evidence linking NF-kB to MAPK/ERK. In the same model used for this study, PC12 cells, Foehr et al. found that NF-kB inducing kinase (NIK) induces cell differentiation and prevents apoptosis (Foehr, 2000) and that these effects involve activation of IKK and the MAPK pathways. NIK is a MAPKKK that potently activates NF-kB. Although the upstream activator of NIK remains unclear, some studies have linked NIK activation to the TNFR1 signaling cascade. These results support our observation of a relationship between MAPK/ERK and NF-kB pathways and further suggest that the role of NF-kB-regulated MAPK/ERK activation is related to cell survival and proliferation.

We have shown that in our model NF-kB controls the expression of the antiapoptotic protein cFLIP-L, as demonstrated by the down-regulation of this protein in cells transfected with SR-IkBα for different days. We also analyzed the expression of the Bcl-2 family protein Bcl-X_L, confirming that the expression of this protein is not altered by the inhibition of the NF-kB pathway, as proven by Gozzelino et al. One interesting work by Kataoka et al. (Kataoka et al., 2000) uses a lymphocyte cell model to show that FLIP-L mediates activation of ERK and NF-kB signaling pathways by

death receptors. In that case, the authors used another death receptor, Fas, as an inducer of MAPK activation.

We provide evidence for a role of FLIP-L in the activation of ERK1/2 initiated by TNFα, as indicated by the fact that ERK1/2 phosphorylation is maintained in time in PC12 cells where FLIP-L has been overexpressed. Moreover, through a loss of function approach, we show that FLIP-L is essential for TNFα-induced MAPK activation. Therefore, our results point to FLIP-L as the key regulator of this activation, linking the pro-survival function of NF-kB to the anti-apoptotic role of FLIP-L. Other reports have suggested before that FLIP may be important for TNFα-induced activation of the MAPK/ERK pathway. Luschen et al. provide evidence of this event in various cell lines in which overexpression of FLIP led to prolonged and enhanced ERK1/2 phosphorylation (Lüschen et al., 2005). However, the authors fail to define a role for ERK1/2 phosphorylation mediated by TNFα. FLIP has also been shown to be involved in MAPK/ERK signaling pathway in epithelial cells (Gilbert et al., 2004). Importantly, so far there is no previous evidence demonstrating a link between FLIP-L and ERK in neuronal models.

The MAPKKK Raf-1 interacts with FLIP-L, independently of Ras activation and mediates ERK1/2 phosphorylation induced by TNFα

Besides describing the key role of FLIP-L in MAPK/ERK activation induced by Fas, Kataoka and his collaborators show that FLIP-L can interact with Raf-1, the kinase upstream MAPK/ERK in the Raf-MEK-ERK pathway. This is not the only study linking FLIP-L to Raf proteins. Park et al. have shown that alternative splicing variants of FLIP contibute to TNFα-induced cell proliferation through differential pathways (Park et al., 2001). The authors show that cFLIP-L activates Raf, whereas FLIPs is involved in the activation of JNK.

As explained in the introduction, the classically described signaling cascade that leads to phosphorylation of ERK begins with the translocation of the MAPKKK Raf-1 to the membrane. This translocation depends on the GTP-bound protein Ras. Once in the membrane Raf-1 undergoes various phosphorylation and dephosphorylation events that render it active to phosphorylate MEK, which in turn phosphorylates ERK1/2. In our model, TNFα is unable to trigger Ras activation, as evidenced by an active Ras pull down assay. Despite this, a Raf-1 kinase activity assay demonstrated that TNFα induces the activation of this protein, confirmed by immunoblotting analysis of the phosphorylated downstream kinase MEK1. As in the model proposed by

Kataoka and his collaborators, in the PC12 neuronal cell model FLIP-L interacts with Raf-1. Additional work is required to elucidate the detailed mechanistics of Raf-1 interaction with FLIP-L, since there is no evidence of a direct interaction between these two proteins. Additionally, the down-regulation of FLIP-L by a specifically designed shRNA abrogates the activation of Raf-1 induced by TNFα. Therefore, according to our results, we propose a model in which the activation of the MAPK/ERK pathway mediated by TNFα does not follow the classical signaling cascade initiated by activation of Ras. Instead, FLIP-L behaves as a "Ras-like" protein, in the sense that it is necessary for the activation of Raf-1, which in turn is indispensable for TNFα-mediated ERK1/2 phosphorylation, as we have demonstrated by the knock-down of Raf-1 by a specific siRNA. Previous evidence exists of the independence of Ras in the Raf-1 activation mediated by TNFR1 in a model of colon epithelial cells in which Raf-1 seems to be important for survival of the cell through a mechanism dependent on NF-kB. However, these results do not focus on MAPK/ERK as the effector of cell survival and this has not been previously described in the nervous system (Edelblum et al., 2008).

Putting these results together, it seems clear that the classical Ras-Raf-MEK-ERK is not the signaling cascade triggered by TNFα, instead, a FLIP-L-Raf-1-MEK-ERK model seems to be responsible for the activation of the MAPK/ERK through TNFR1.

TNFα- triggered MAPK/ERK activation promotes survival through abrogation of JNK-induced apoptosis

We have shown that inhibition of the MAPK/ERK pathway sensitizes PC12 cells to apoptotic cell death induced by TNFα. As we hypothesized, cell death induced by TNFα in the presence of a MEK1 specific inhibitor (PD 98059) is very similar to that observed when cells that overexpress the SR-IkBα plasmid are treated with TNFα, confirming the link between the NF-kB and MAPK/ERK pathways. Moreover, overexpression of a constitutively active form of MEK (MEK-CA) in cells already expressing SR-IkBα rescued cells from apoptosis induced by TNFα. JNK1/2 seems to play a relevant role in the apoptotic pathway induced by TNFα when NF-kB is blocked or when the MAPK/ERK pathway is inhibited.

The c-Jun N- terminal kinases (JNK) are predominantly activated by pro-inflammatory cytokines and stress stimuli. Upon activation, these kinases translocate to

the nucleus, where they activate transcription factors by mediating their phosphorylation. Investigations of the link between activation of JNK and induction of apoptosis by TNFα have led to controversial results (Cuvillier et al., 1996; Verheij et al., 1996; Liu et al., 1996b; Ichijo et al., 1997; Lee et al., 1997; Reinhard et al., 1997). In fact, results from several studies suggest that it is the duration of JNK activation induced by TNFα that is a crucial determinant of the cell response (Chen et al., 1996; Guo et al., 1998; Sánchez-Perez et al., 1998). In normal conditions TNFα activates JNK1/2 transiently. Inhibition of the JNK1/2 activation soon after TNFα induction has been previously linked to the NF-kB pathway. More specifically, NF-kB induces the transcriptional upregulation of *gadd45β*, a member of the *Gadd45* family of inducible factors associated with cell-cycle control and DNA repair(De Smaele et al., 2001). Gadd45β downregulates TNFα-mediated phosphorylation of JNK. The initial and rapid activation of JNK has not been linked to cell death, in fact, reports show that activation of JNK1/2 is independent of the cell death pathway and could regulate proliferation and survival in some models (Lee et al., 1997; Reinhard et al., 1997). On the other hand, abrogation of the NF-kB pathway in SR-IkBα-expressing cells allows a maintained activation of JNK1/2, as demonstrated by Javelaud et al. (Javelaud and Besançon, 2001) in a model of Ewing sarcoma cells and by ourselves in PC12 cells in this work(Javelaud and Besançon, 2001) (Javelaud and Besançon, 2001) (Javelaud and Besançon, 2001). We show here that persistent activation of JNK1/2 is responsible for apoptosis induced by TNFα in PC12 cells when NF-kB pathway is blocked. This is further supported by the fact that co-treatment with a JNK1/2 specific inhibitor, SP600125, is able to prevent cell death in cells that express SR-IkBα. Interestingly, inhibition of the MAPK/ERK pathway with PD98059 shows the same effect on TNFα-mediated JNK1/2 activation as blocking of NF-kB, adding to the evidence that support a link between the MAPK/ERK and the NF-kB pathways.

Role of BH3-only protein BIM in JNK1/2 mediated apoptosis initiated by TNFα

Although JNK has been previously shown to be important for TNFα-induced apoptosis, the exact mechanism that leads to cell death has remained elusive. Several models have been proposed to explain this event. In an interesting work, Chang et al. coupled JNK activation initiated by TNFα to the turnover of FLIP-L (Chang et al., 2006). The authors show that JNK can specifically phosphorylate and activate Itch, an E3

ubiquitin ligase that can target FLIP-L for proteasomal degradation as a possible mechanism of sensitization to cell death induced by TNFα. In another study, Deng et al. suggested that after being activated by TNFα, JNK induces the processing of the BH3-only protein Bid (Deng et al., 2003). According to the authors the product of this processing, which they termed jBid, is different from the well-known product of caspase-8-mediated processing, tBid. Their results show that jBid induces the preferential release from the mitochondria of SMAC but not cytochrome c. Smac would then disrupt the cIAP-TRAF2 complex, releasing its inhibition on caspase-8 activation. This would represent another cross-talk point between the extrinsic and intrinsic pathways of apoptosis, besides the classically described caspase-8 processing of Bid. However, these results are controversial since, so far, the protease responsible for the processing of Bid into jBid has not been described and the results of Deng and his collaborators have not been reproduced.

Another mechanism that has been proposed to explain apoptosis induced by maintained JNK activation after TNFα treatment involves the BH3-only protein BIM. Whitfield et al. (Whitfield et al., 2001) studied apoptosis induced by NGF deprivation in a model of sympathetic neurons. They showed that NGF withdrawal induced an increase in BIM mRNA. Overexpression of a dominant-negative form of c-Jun, an effector protein downstream of JNK, promoted neuronal survival by reducing BIM expression. Likewise, overexpression of BIM in neurons induced cytochrome c release and apoptosis even in the presence of NGF, and neurons injected with a *Bim* antisense oligonucleotide or isolated from *Bim*$^{-/-}$ mice died more slowly after NGF withdrawal. Putcha et al. describe the relevance of JNK-mediated BIM phosphorylation in a model of trophic factor deprivation-induced apoptosis. In this case, the authors show that BIM is critical for Bax-dependent cytochrome c-release, and they provide evidence that links JNK activation to regulation of proapoptotic activity of BIM, both transcriptional and posttranscriptionally (Putcha et al., 2003).

In agree with these studies, we have observed that the maintained phosphorylation of JNK induced by TNFα in SR-IkBα-expressing PC12 cells correlates with an increase in BIM expression. Interestingly, direct inhibition of the MAPK/ERK pathway together with TNFα treatment also induces an increase in BIM expression that correlates with JNK activation. In both cases, overexpression of BIM is lost when cells are co-treated with a specific inhibitor of the JNK pathway. In line with this result, Ham et al. have shown that the MAPK/ERK pathway negatively regulates the expression of BIM in sympathetic neurons. This regulation seems to take place at the level of

transcription. They also demonstrate that inhibition of the MAPK/ERK pathway significantly reduces survival in sympathetic neurons treated with NGF (Hughes et al., 2011). These data could also provide insight into the mechanism of cell survival or cell death mediated by TNFα in PC12 cells, explaining the link between MAPK/ERK inhibition and BIM upregulation we observe.

Significance of our results in the physiological context

On the one hand, we have characterized the molecular players intervening in the apoptotic response that might be triggered upon TNFα stimulation, but only when the NF-kB and/or MAPK pathway is blocked. On the other hand, we confirm the relevance of the MAPK/ERK activation in the TNFα-induced survival decision by rescuing TNFα-induced cell death with the NGF-induced MAPK activation. Previous results in our own group show that NGF activates MAPK/ERK and NF-kB in two different and redundant pathways (Moubarak et al., 2010), since both pathways need to be blocked in order to inhibit neurite outgrowth induced by NGF.

Quite differently, this study demonstrates that TNFα treatment induces NF-kB and MAPK/ERK activation in one unique pathway since blocking either component leads to the same biological response, more specifically JNK-mediated apoptosis. We demonstrate here that the activation of MAPK/ERK is key for neuroprotection upon TNFα treatment, since apoptotic pathway triggered by NF-kB inhibition in TNFα-treated PC12 cells can be rescued by the MAPK/ERK activation induced by NGF stimulation. While the role of MAPK/ERK activation by neurotrophins as mediators of neuronal survival, differentiation and synaptic plasticity has been well characterized (Chao, 2003) there is little information about the role of MAPK/ERK activation induced by DRs in the nervous system. However some reports draw attention to the relevance that DR-induced MAPK/ERK pathway may have in various processes related to the nervous system. Desbarats *et al.* described that crosslinked Fas is able to induce neurite outgrowth in dorsal root ganglia neurons through ERK activation and p35 upregulation (Desbarats et al., 2003). The authors used the neuroblastoma cell line SH-SY5Y, which does not express caspase-8 therefore is not sensitive to Fas-induced apoptosis, and showed that Fas stimulation with activating antibodies or FasL constructs induces ERK phosphorylation. This activation is very similar to the one we observed in PC12 cells, reaching its maximum after 5 minutes of treatment. The authors also observed an ERK-dependent upregulation of p35, a protein related to neurite outgrowth. They

determined a correlation between Fas-induced ERK/p35 pathway and neurite outgrowth using the dorsal root ganglion (DRG) neurite outgrowth assay, a well-characterized model for axon regeneration from primary neurons *in vitro.* Moreover, the authors report endogenous Fas expression accelerates in vivo functional recovery after sciatic nerve crush injury in mice. Recovery was significantly slower in Fas-deficient *lpr* mice.

The group of Martin-Villalba investigated the relevance of another survival pathway linked to Fas signaling (Kleber et al., 2008). The authors link the activation of Fas by FasL to glioblastoma invasion mediated by PI3K/AKT and regulated by the Src family protein Yes.

The effects of TNFα on survival or proliferation of neurons have been less characterized, although some reports point in the same direction as our own results concerning the protective role of TNFα in the nervous system. Echeverry et al. report that The Tumor Necrosis Factor-like Weak inducer of apoptosis (TWEAK) has a neuroprotective role in the central nervous system. Sub-lethal hypoxia promotes the acquisition of tolerance to a subsequent lethal hypoxic injury. Exposure to sub-lethal hypoxia in neurons induces the expression of TWEAK and its receptor Fibroblast growth factor-inducible 14 (Fn14). The authors found that TWEAK ligation to its receptor induced a rapid increase in the expression of TNFα in neurons, and that TNFα is responsible for the neuroprotective effect of TWEAK. In addition, the authors determined that TNFα-induced activation of ERK1/2 mediated hypoxic tolerance (Echeverry et al., 2012).

Altogether, we present a mechanism through which NF-kB is able to block TNFα-induced apoptosis in a neuronal model. The pro-apoptotic role of TNFα and other DRs in neurodegenerative diseases has been extensively documented. In Alzheimer's disease, TNFα has been shown to mediate the inflammatory response in the hippocampus of a mouse model of the disease (He et al., 2007; Jimenez et al., 2008). A report by Nikolaev et al. proposes APP as a specific ligand of death receptor 6 (DR6), triggering axon pruning and neuron death via activation of caspases (Nikolaev et al., 2009).

Our results provide an answer to questions related to observations done in the nervous system in which, apart from their well-documented apoptotic role in

neurodegenerative diseases, TNFα and other DRs exert a protective role. This role seems relevant in physiological processes such as development of the nervous system (Martin-Villalba et al., 1999), and pathological conditions, such as the protection provided by TNF family molecules after an ischemic insult (Esposito and Cuzzocrea, 2011). In summary, this study uncovers a single activation pathway for MAPK/ERK, regulated by NF-kB through the transcriptional regulation of the classical anti-apoptotic protein FLIP-L, and directly involved in the regulation of neuronal death and survival.

Results obtained from this thesis are synthesized in the scheme presented in figure 1.

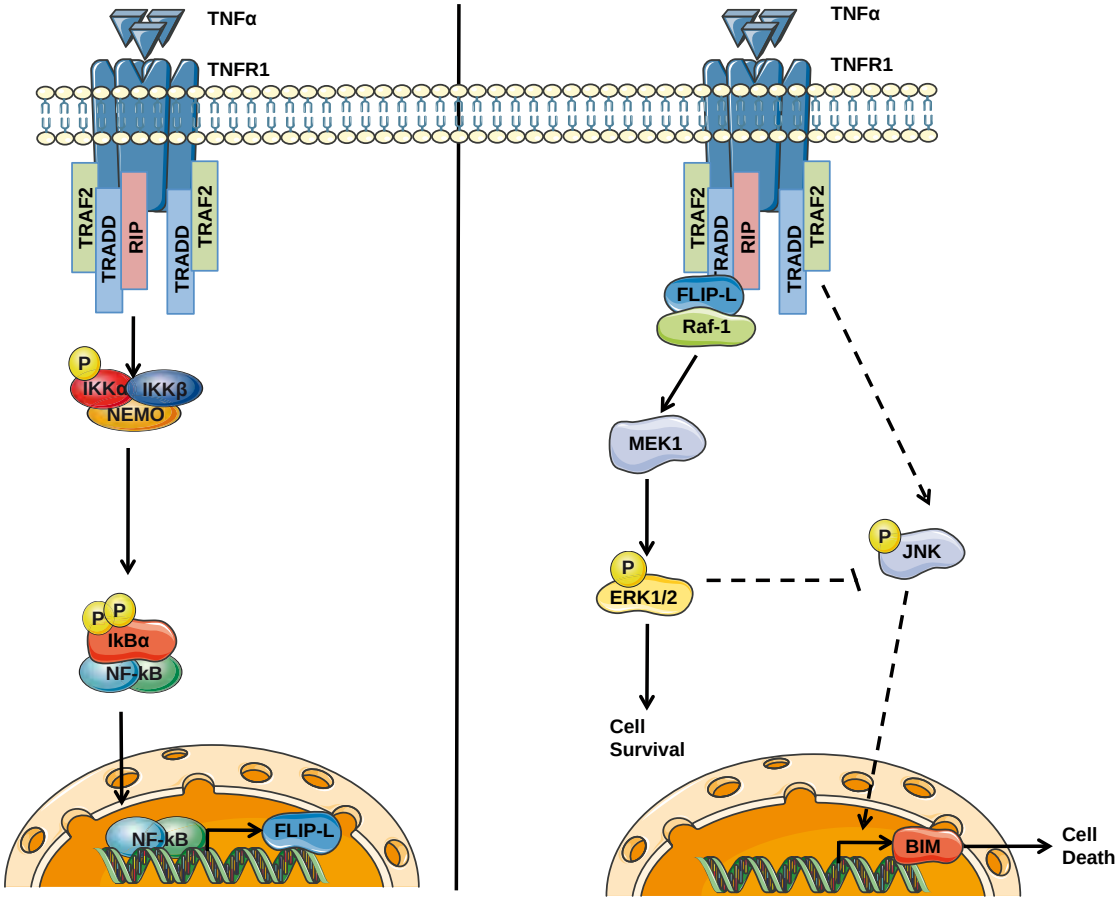


Figure 1. Model proposed to explain the activation of the MAPK/ERK mediated by stimulation of TNFR1 and theapoptotic pathway triggered when the survival signaling is blocked. Left panel: Ligation of TNFα to TNFR1 triggers activation of NF-kB through induction of IkBα phosphorylation and degradation. NF-kB translocates to the nucleus inducing transcription of FLIP-L among others. Right panel: FLIP-L is recruited to TNFR1 complex. There it binds Raf-1 and induces its activation. Activated Raf-1 phosphorylates MEK1, which in turn phosphorylates ERK1/2 inducing its activation and affording protection from apoptosis. When ERK1/2 activation is abrogated, TNFR1 complex induces phosphorylation of JNK, which becomes active and induces an increase in BIM expression, thus favoring cell death. Dotted lines indicate interactions that need to be further characterized.

6.CONCLUSIONS

Conclusions

First: TNFα treatment of PC12 cells triggers the NF-kB pathway activation. When activation of this pathway is prevented, survival signaling is blocked and PC12 cells die by apoptosis.

Second: Super-repressor of IkBα plasmid efficiently inhibits NF-kB activation, as evidenced by abrogation of TNFα-induced p65 translocation to the nucleus.

Third: TNFα induces the activation of the MAPK/ERK pathway in PC12 cells in a time and dose-dependent manner.

Fourth: NF-kB inhibition by the SR-IkBα plasmid overexpression completely abrogates activation of the MAPK/ERK pathway mediated by TNFα.

Fifth: Expression of the anti-apoptotic protein FLIP-L is down-regulated by overexpression of SR-IkBα. This indicates that the transcription factor NF-kB regulates FLIP-L expression in PC12 cells

Sixth: FLIP-L overexpression increases TNFα-induced MAPK/ERK activation, while downregulation of endogenous FLIP-L inhibits this signaling. This demonstrates that FLIP-L is a key regulator of MAPK/ERK mediated by TNFα.

Seventh: TNFα leads to the activation of MAPK/ERK without the Ras activation. However, Raf-1 becomes activated after 5 minutes of TNFα treatment.

Eighth: FLIP-L interacts with Raf-1, and this interaction is necessary for TNFα to induce ERK1/2 phosphorylation.

Ninth: Raf-1 is necessary for TNFR1-mediated activation of MAPK/ERK**.**

Tenth: Inhibition of ERK1/2 phosphorylation sensitizes PC12 cells to apoptosis induced by TNFα.

Eleventh: NGF rescues cell death induced by TNFα in PC12 cells when NF-kB activation is blocked.

Twelfth: NF-kB or MAPK/ERK inhibition lead to maintained JNK1/2 phosphorylation that induces apoptotic cell death in PC12 cells. Maintained activation of JNK1/2 correlates with an increase in the proapoptotic, BH3-only protein BIM.

7.ANNEX

7.ANNEX- Other results

Apart from the results presented in this thesis, I have also participated in another in another project in collaboration with the group of Dr. Victor Yuste at the Universitat Autònoma de Barcelona (UAB). The project consisted in defining the relevance of Bcl-2 family proteins in the sensitization to TNFα-induced cell death in HeLa cells. In this section I present the results obtained.

Down-regulation of Bcl-X_L sensitizes HeLa cells to TNFα-induced apoptosis better than down-regulation of other antiapoptotic Bcl-2 family proteins. (Fig A1)

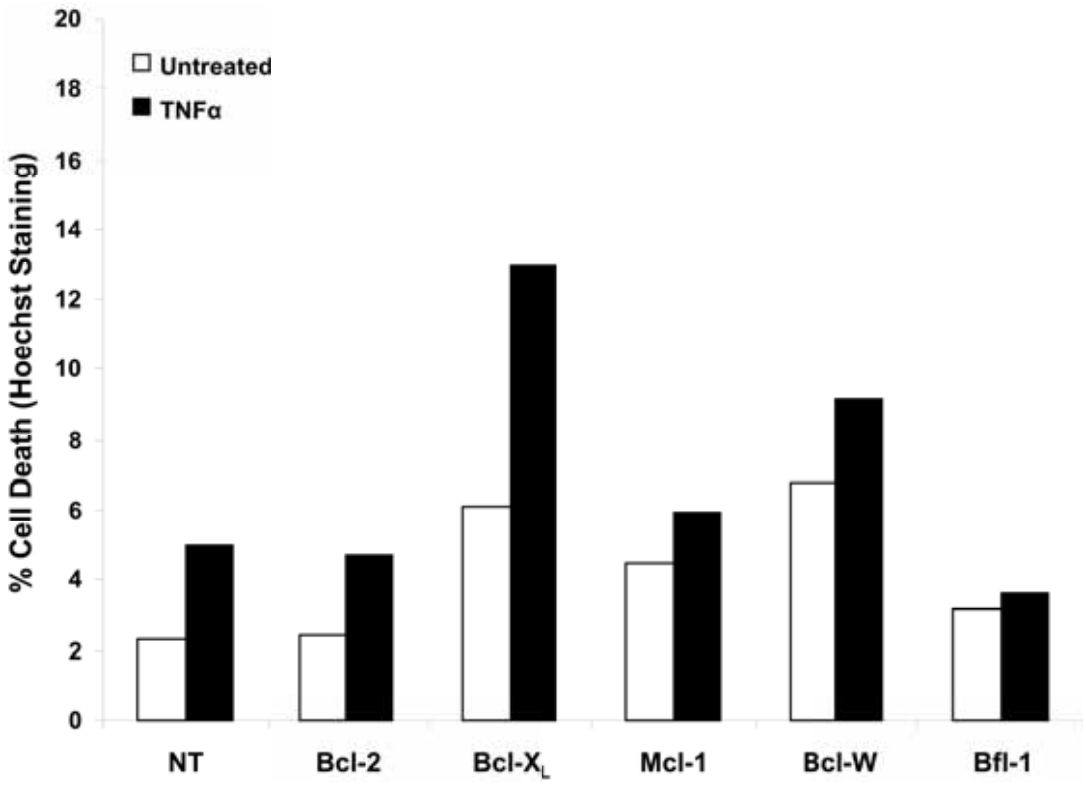


Figure A1: HeLa cells were transfected with siRNA oligonucleotides for the proteins indicated. 3 days alter transfection cells were left untreated or treated with 50ng/ml TNFα for 24h prior staining with Hoechst 33285 to identify nuclear condensation

Down-regulation of Bcl-X_L sensitizes HeLa cells to caspase-8-dependent apoptosis induced by TNFα (Fig A2 and A3).

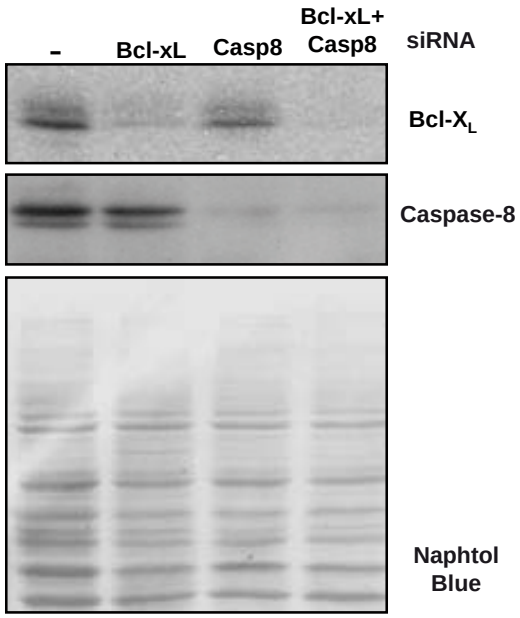


Figure A2: HeLa cells were transfected with siRNA targeted to Bcl-X_L or Caspase-8. Three days alter transfection cells were lysed and western blot was done to check efficiency of the transfection.

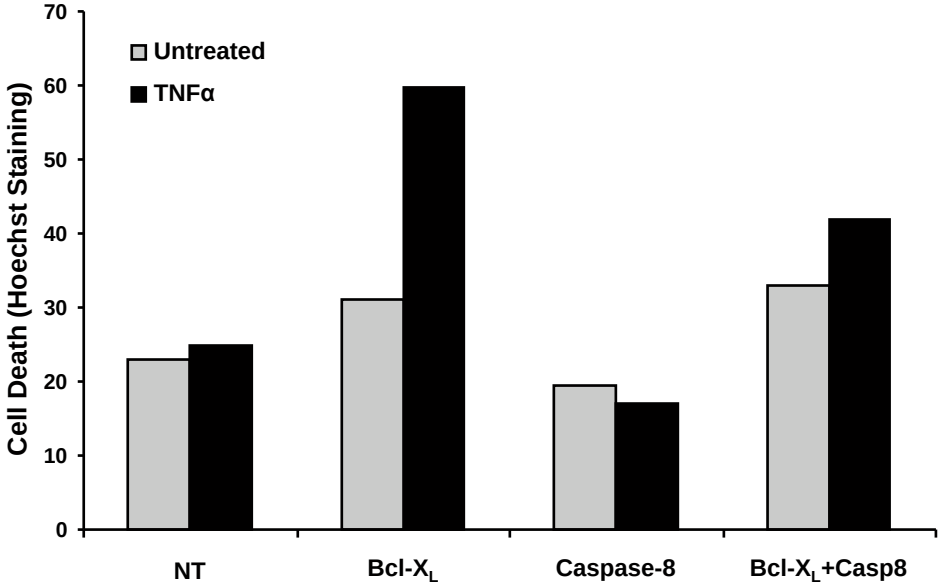


Figure A3: HeLa cells were transfected with siRNA as in Fig A2. Three days after transfection cells were left untreated or treated with 50ng/ml TNFα for 24h. Apoptotic cell death was evaluated by direct counting of condensed nuclei stained with Hoehcst 33258.

TNFα+ActD induces the release of proapoptotic proteins cytochrome C and SMAC from the mitochondria (Fig. A4)

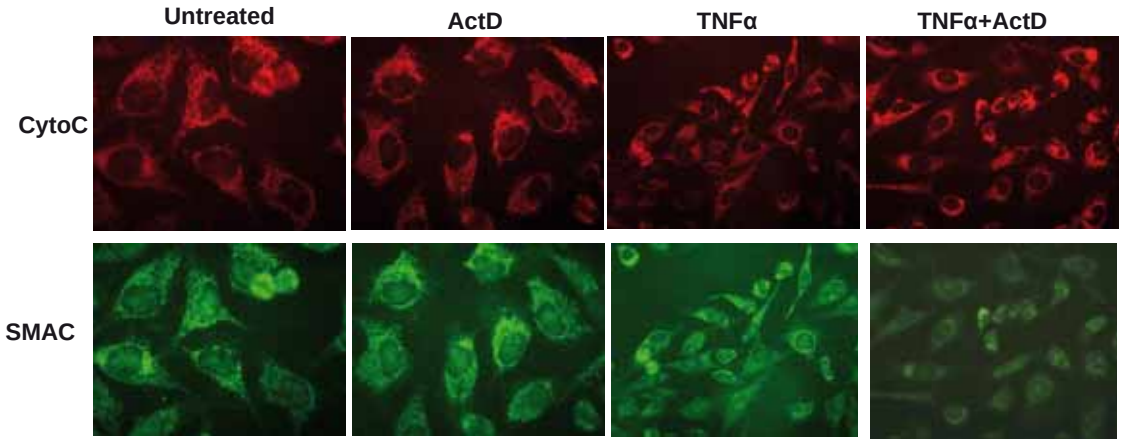


Figure A4. HeLa cells were treated with 20nM ActD, 50ng/ml TNFα, or both for 24h. Immunofluorescence against CytochromeC (Cyto C) or SMAC was performed to analyze the subcellular localization of these proteins before and after treatment. The homogeneous pattern evidences translocation of the proteins to the cytosol.

TNFα-induced cell death in HeLa cells depends partially on SMAC release from the mitochondria (Fig. A5 and A6).

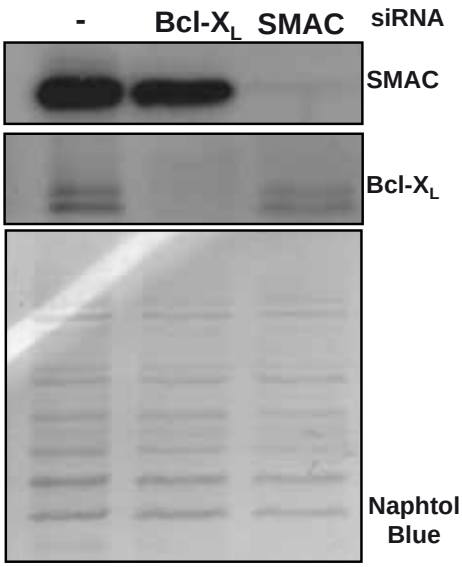


Figure A5. SMAC and Bcl-X_L downregulation by specifically designed siRNA oligos was analyzed by western blot three days alter transfection

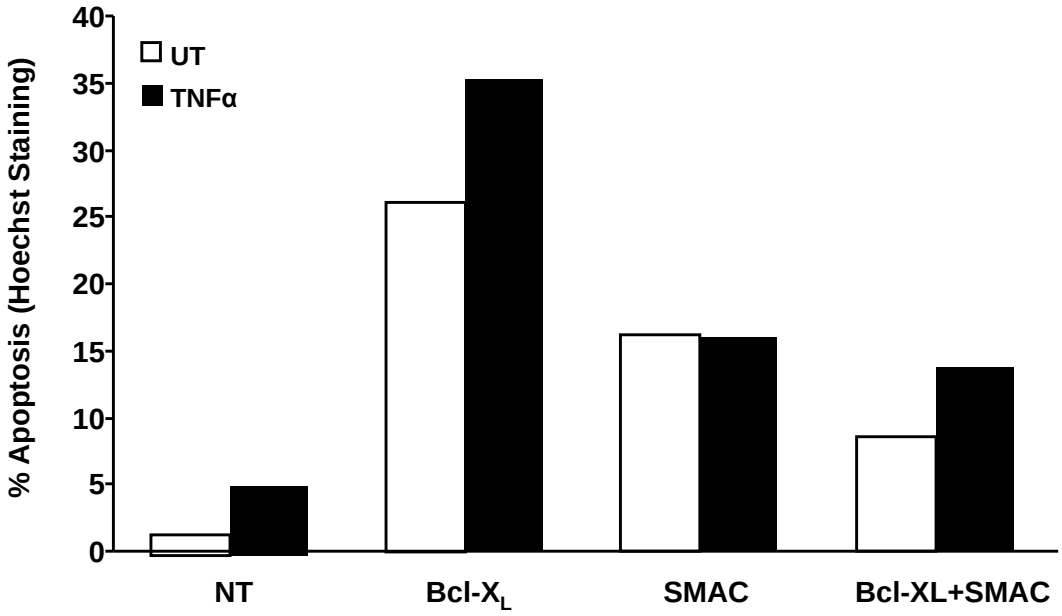


Figure A6. Apoptotic cell death was analyzed in HeLa cells after down-regulating Bcl-X_L, SMAC, or both.

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