Characterization of the Fas Death Receptor Antagonist in the Nervous System, Lifeguard (LFG)

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A mi familia

CONTENTS

CONTENTS

ABBREVIATIONS **1**

ABSTRACT **6**

1. INTRODUCTION **12**

1.1. Apoptosis, the background 13

1.2. Mechanisms of apoptosis 14

1.2.1. Extrinsic pathway 14
1.2.2. Intrinsic pathway 15

1.3. Apoptosis Machinery 16

1.3.1. Caspases 17
1.3.2. Inhibitors of Apoptosis Proteins (IAPs) 19
1.3.3. Bcl-2 family proteins 22

1.3.3.1. Structural and functional considerations of Bcl-2 family proteins 23
1.3.3.2. Bcl-2 family in the mitochondria 26
1.3.3.3. Bcl-2 family in the ER 28

1.3.4. Death Receptors 32

1.3.4.1. TNFR1 Signaling 34
1.3.4.2. CD95 signaling 36
1.3.4.3. Involvement of calcium in CD95 signaling 40
1.3.4.4. Non-apoptotic functions of CD95 42

1.3.5. Death Receptor Antagonists 43

1.3.5.1. FLIP 44
1.3.5.2. FAIM 47
1.3.5.3. LFG 48

1.4. Ubiquitination regulation of apoptosis 51

2. OBJECTIVES **57**

3. MATHERIALS AND METHODS **59**

3.1. Cell Lines 60

3.1.1. HEK293 60
3.1.2. HEK293T 60
3.1.3. HeLa 60

3.1.4. SK-N-AS 61
3.1.5. Mice cortical neurons 61

3.2. Cell transfection 61

3.2.1. Cationic liposoluble reagents 61
3.2.2. Calcium phosphate method 62
3.2.3. Lentiviral transduction 62

3.3. Cell death assays 63

3.3.1. Hoechst 33258 bis-benzimide staining 63
3.3.2. Caspase-3 activity assay 64

3.4. Protein extraction 64

3.5. Western Blot 65

3.6. Coinmunoprecipitation 66

3.7. Inmunofluorescence 67

3.7.1. Colocalization analysis 68

3.8. Maldi-Toff MS analysis 69

3.9. Molecular Cloning 70

3.9.1. Plasmid extraction from bacterial cultures and purification 71
3.9.2. Transformation of DNA plasmids into competent bacteria 71
3.9.3. DNA analysis in agarose gels 72
3.9.4. Cloning in mammal expression vectors 72

3.9.4.1. PCR amplification 72
3.9.4.2. Digestion by restriction enzymes and generation of blunt ends 72
3.9.4.3. Ligation 73

4. RESULTS 75

4.1. CHAPTER I. LFG inhibits Fas ligand-mediated endoplasmic reticulum-calcium release and prevents type II apoptosis in a Bcl-xL-dependent manner 76

4.3.1. LFG localizes mainly at the ER and Golgi membranes 76
4.3.2. LFG is localized along the endocytic pathway 78
4.3.3. LFG interacts with Bcl-Xl 80
4.3.4. LFG protects type II cells, but not type I, from Fas-induced apoptosis 83
4.3.5. Requirement of endogenous Bcl-xL for LFG to exert its anti-apoptotic effects 85

4.3.6. LFG overexpression downregulates calcium release from the ER after Fc-FasL stimulation 87

4.2. CHAPTER II. LFG ubiquitination 89

4.2.1. LFG is ubiquitinated 89
4.2.2. LFG ubiquitination doesn't induce its degradation 93
4.2.3. LFG ubiquitination is not modulated after DR stimulation or induction of ER stress 95
4.2.4. LFG ubiquitination can be located at N-terminal region lysins 97

5. DISCUSSION 101

6. CONCLUSIONS 114

7. REFERENCES 117

8. ANNEX 135

8.1. Transgenic mice generation 135

ABBREVIATIONS

ABBREVIATIONS

ActD	Actinomycin D
AIP	AH Receptor-Interacting Protein
ALPS	Autoimmune Lymphoproliferative Syndrome
ANT	Adenine Nucleotide Translocase
APAF-1	Apoptosis Protease Activation Factor 1
ATF6	Activating Transcription Factor 6
BAP31	B-Cell Receptor-Associated Protein 31
BAR	Bifunctional Apoptosis Regulator
Bcl	B-cell lymphoma
BI-1	Bax Inhibitor 1
BiP	Binding Immunoglobulin Protein
BIR	Baculovirus IAP Repeat
BSA	Bovine Serum Albumin
CARD	Caspase Recruitmen Domain
CED	Cell Death Abnormal
CNS	Central Nervous System
COX	Cyclooxygenase
DED	Death Effector Domain
DIABLO	Direct IAP Binding with Low PI
DISC	Death Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DNA	Desoxiribonucleic acid
dNTP	Deoxyribonucleotide
DR	Death Receptor
Drp1	Dynamin Related Protein 1
DRONC	Drosophila Melanogaster NEDD2-like Caspase
DTT	Dithiothreitol
ECL	Electrogenerated Chemiluminescence
EDAR	Ectodysplasin A Receptor
EDTA	Ethylenediaminetetraacetic Acid
EEA	Early Endosome Antigen
ER	Endoplasmic Reticulum

ERK Extracellular-Regulated Kinase
ES Embryonic Stem
FADD Fas Associated Death Domain
FAIM Fas Apoptotic Inhibitory Molecule
FBS Fetal Bovine Serum
FLIP FLICE-Inhibitory Protein
GFP Green Fluorescent Protein
GRP78 Glucose-Regulated Protein 78
HBS Hank Buffered Saline Buffer
HECT Homologous to E6-AP Carboxyl Terminus
HEK Human Embryonic Kidney
HIV Human Immunodeficiency Virus
HRP Horsedarish Peroxidase Enzyme
IAP Inhibitor of Apoptosis Protein
IBM IAP Binding Motif
IKK I-κB-Kinase
IP Inmunoprecipitation
IP_3 Inositol Triphosphate
IP_3R Inositol Triphosphate Receptor
IRES Internal Ribosome Entry Site
IRE1α Inositol Requiring Enzyme 1
IκB Inhibitor of Kappa B
JNK c-Jun N-terminal Kinase
LFG Lifeguard
LUBAC Linear Ubiquitin Chain Assembly Complex
LZ Leucine Zipper
MAPK Mitogen-Activated Protein Kinase
Mcl-1 Myeloid Cell Leukemia 1
MEF Mouse Embryonic Fibroblast
MVB Multivesicular Bodies
MW Molecular Weight
NF-κB Nuclear Factor Kappa B
NGF Nerve Growth Factor
NMR Nuclear Magnetic Ressonance

OGD	Oxigen-Glucose Deprivation
OMM	Outer Mitochondria Membrane
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PLCγ	Phospholipase C γ
PP2A	Protein Phosphatase 2 A
PVDF	Polyvinylidene Fluoride
RING	Really Interesting New Gene
RIP	Receptor Interacting Protein
RNA	Ribonucleic Acid
rTtA	Recombinant Tetracycline Controlled Transcription Factor
Scr	Scrambled
SDS	Sodium Dodecyl Sulfate
SERCA	Sarcoplasmic/Endoplasmic Reticulum Calcium-ATPase
SMAC	Second Mitochondria-Derived Activator of Caspases
SV40	Simian Vacuolating Virus
TAE	Tris-Acetate-EDTA
TBS	Tris-Buffered Saline
TCR	T-Cell Receptor
TGN	Trans-Golgi Network
TM	Transmembrane
TMBIM	Trans-Membrane Bax Inhibitor Motif
TNF	Tumour Necrosis Factor
TRADD	TNF Receptor Associated Death Domain
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand Receptor
TrK	Tyrosine Kinase
UBA	Ubiquitin Associated Domain
UPR	Unfolded Protein Response
UTR	Untranslated Region
VDAC	Voltage Dependent Anion Channel
WB	Western Blot
XIAP	X-linked Inhibitor of Apoptosis

ABSTRACT

ABSTRACT

Activation of the Death Receptor Fas, also called APO-1 or CD95, leads to the formation of the Death Inducing Signaling Complex (DISC). This protein complex comprises FADD and caspase-8, among other proteins, and it causes the cleavage and activation of caspase-8, that ultimately leads to apoptosis. There are two differentiated pathways in the Fas induced apoptosis. In type I cells, high levels of DISC are formed upon Fas activation, and caspase-8 levels are sufficient to directly cleave the effector caspase-3, which will trigger apoptosis. In type II cells, DISC formation levels are lower and caspase-3 is not directly cleaved by caspase-8. Instead, caspase-8 cleaves the BH3-only protein Bid, generating its truncated form tBid, which translocates to the mitochondria and induce its permeabilization. This will result in release of apoptogenic factors from the mitochondria to the cytosol, such as cytochrome c, which will activate caspase-3 through the apoptosome. Thus, type II cells need a signal amplification step through the mitochondria. Death Receptor Antagonists are proteins that are able to modulate Death Receptor activity. Among them, Lifeguard (LFG), also called NMP35 or FAIM2, is a Fas antagonist highly expressed in the nervous system. This protein has been characterized as a Fas-induced apoptosis inhibitor, and it has been shown that localizes at post-synaptic sites and dendrites. Moreover, it has been reported to interact directly with Fas receptor in the lipid rafts and inhibit caspase-8 and caspase-3 activity upon Fas activation. However, its mechanism of action has remained elusive. In this work, we try to shed some light on this problem. First, previous results in our lab have shown that LFG interacts with several proteins from the ubiquitin system. We confirmed that LFG is ubiquitinated, and we also show that this ubiquitination does not induce its degradation. In addition, we present data that suggest that LFG ubiquitination is done in a non-canonycal way. On the other hand, we make an extensive study to elucidate LFG subcellular localization. We demonstrate that LFG localizes to ER and Golgi membranes, and to a lesser extent, to endosomes. Since LFG is member of the TMBIM family proteins, that are able to modulate Bcl-2 family activity, we investigated its relationship with them, and found that it interacts with Bcl-xL and Bcl-2, through its *C*-terminal region. Moreover, we prove that LFG protects only type II cells from Fas-

induced apoptosis, and this protection is dependant on Bcl-xL endogenous expression. Finally, our results reveal a hitherto undescribed step in the signaling pathway in type II cells. Calcium mobilization from the ER has been shown to be relevant in Fas apoptotic signaling. We demonstrate that LFG modulates calcium release from the ER after Fas stimulation and inhibits Fas-induced apoptosis in type II cells. On the basis of our observations, we propose that LFG protects against Fas-induced apoptosis by modulating calcium release from the ER.

RESUMEN

La activación del Receptor de Muerte Fas, también llamado APO-1 o CD95, da lugar a la formación del Death Inducing Signaling Complex (DISC). Este complejo proteico contiene FADD y caspasa-8, entre otras proteínas, y causa el corte y activación de caspasa-8, que finalmente da lugar a la apoptosis. Existen dos vías de señalización diferenciadas en la apoptosis inducida por Fas. En las células tipo I, la activación de Fas da lugar a la formación de altos niveles de DISC, y los niveles de caspasa-8 son suficientes para cortar directamente la caspasa-3 ejecutora, que desencadena la apoptosis. En las células tipo II, los niveles de formación de DISC son más bajos y caspasa-3 no es cortada directamente por caspasa-8. En vez de ello, caspasa-8 corta la proteína BH3-only Bid, dando lugar a su forma truncada tBid, que transloca a la mitocondria e induce su permeabilización. Esto conlleva la salida de factores apoptogénicos de la mitocondria al citosol, como citocromo c, que da lugar a la activación de caspasa-3 a través del apoptosoma. Así, las células tipo II necesitan un paso de amplificación de la señal a través de la mitocondria. Los Antagonistas de los Receptores de Muerte son proteínas que son capaces de modular la actividad de los Receptores de Muerte. Entre ellos, Lifeguard (LFG), también llamado NMP35 o FAIM2, es un antagonista de Fas altamente expresado en el sistema nervioso. Esta proteína ha sido caracterizada como un inhibidor de la apoptosis inducida por Fas, y se ha comprobado su localización en los sitios post-sinápticos y en las dendritas. Además, se ha comprobado que interacciona directamente con el receptor Fas en los *rafts*, e inhibe la actividad de las caspasas-8 y -3 tras la activación de Fas. Sin embargo, su mecanismo de acción aún no ha sido descrito. En este trabajo tratamos de arrojar algo de luz sobre este problema. Primero, resultados previos en nuestro laboratorio mostraron que LFG interacciona con varias proteínas del sistema ubiquitina. Confirmamos que LFG está ubiquitinado, y también mostramos que esta ubiquitinización no induce su degradación. Además, nuestros resultados sugieren que la ubiquitinización de LFG es no-canónica. Por otro lado, llevamos a cabo un extenso estudio para elucidar la localización subcelular de LFG. Demostramos que LFG localiza en las membranas del RE y del Golgi, y en menor medida, también en los endosomas. Dado que LFG es miembro de la

familia de proteínas TMBIM, que son capaces de modular la actividad de las proteínas de la familia Bcl-2, investigamos su relación con ellas, y encontramos que interacciona con Bcl-xL y Bcl-2 a través de su región *C*-terminal. Además, demostramos que LFG solamente protege a las células tipo II de la muerte inducida por Fas, y esta protección es dependiente de la expresión endógena de Bcl-xL. Para finalizar, nuestros resultados revelan un paso no descrito hasta el momento en la vía de señalización de las células tipo II. La movilización de calcio del RE se ha comprobado que es relevante en la vía de señalización apoptótica inducida por Fas. Aquí, demostramos que LFG modula la salida de calcio del RE tras la estimulación de Fas, e inhibe la apoptosis inducida por Fas en células tipo II. Basándonos en estas observaciones, proponemos que LFG protege de la apoptosis inducida por Fas mediante la modulación de la salida de calcio del RE.

INTRODUCTION

Apoptosis, a background

Cell death is an irreversible process that leads to loss of the cellular function. There are diverse types of cell death depending on the criteria used to classify them, such as morphology of the cell after death, mechanisms involved, stimulus that trigger cell death, and many others.

Apoptosis is a mode of cell death used by multicellular organisms to get rid of unwanted cells in different contexts. It is a key component in many processes such as cell turnover, embryonic development or proper function of the immune system [1, 2]. In the other way, deregulation of apoptosis is an important factor in many pathological situations, such as neurodegenerative diseases, autoimmune disorders, ischemic damage or many types of cancer [3-5].

The term apoptosis was first used to describe a morphologically distinct form of cell death [6]. The authors of that report observed a group of characteristic features during the process of death of hepatocytes, which were repeated in other tissues and cell types, and were substantially different from events observed during pathological cell death or necrosis. In this context, death by necrosis triggers an inflammation response after intracellular proteins are released at the extracellular matrix when the membrane of dying cells is disrupted [7]. Cells dying by apoptosis retain its membrane integrity and are later recognized and engulfed by phagocytes for recycling of their contents, without triggering an immune response [8].

There are several morphological characteristics that distinguish apoptosis from other ways of cell death [9]. The main morphological features characterizing apoptosis are chromatin condensation and nuclear fragmentation, formation of bulges in the plasma membrane or “blebbing” that leads to formation of the apoptotic bodies, and loss of adhesion [9].

Apoptotic cells also exhibit characteristic biochemical modifications such as protein cleavage, DNA breakdown and phagocytic recognition [10]. Most of the proteolytic events that occur during apoptosis are achieved by the action of a family of proteases called caspases (cysteinyl aspartate proteinases) [11]. Although they are able to cleave proteins at aspartic acid residues, different

caspases have different specificities involving recognition of neighboring amino acids. Once effector caspases are activated, there seems to be an irreversible commitment towards cell death. DNA breakdown by Ca^{2+} and Mg^{2+}dependent endonucleases also occurs, resulting in DNA fragments of 180 to 200 base pairs, as resulting of DNA cleaveage at internucleosomic regions [8]. Thus, a characteristic “DNA ladder” can be visualized by agarose gel electrophoresis. The last biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue. This phagocytic signal usually consists on the exposure of the normally inward-facing phosphatidylserine at the external plasma membrane [12].

Mechanisms of apoptosis

During the past years, apoptosis became a major research field for biologists and biochemists. The mechanisms of apoptosis are highly sophisticated, and there are constantly appearing at the bibliography novel proteins and pathways, so the full map of molecular routes implied is still in expansion.

To date, the most accepted view acknowledges two main apoptotic pathways; the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway [13]. There is now evidence that the two pathways are linked, and that molecules in one pathway can influence the other. There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cell [14], but is very cell-type specific and will not be discussed in this work.

Extrinsic pathway

Multicellular organisms have developed a mechanism to actively kill individual cells in the organism [15]. This pathway involves the activation of transmembrane receptors, the so called Death Receptors (DRs), by binding of their ligands. This mechanism of apoptosis is called the extrinsic pathway and has been studied most extensively in the immune system. However, it is acquiring important relevance in other systems such as the nervous system, where it has a role during normal development and pathogenic situations [16].

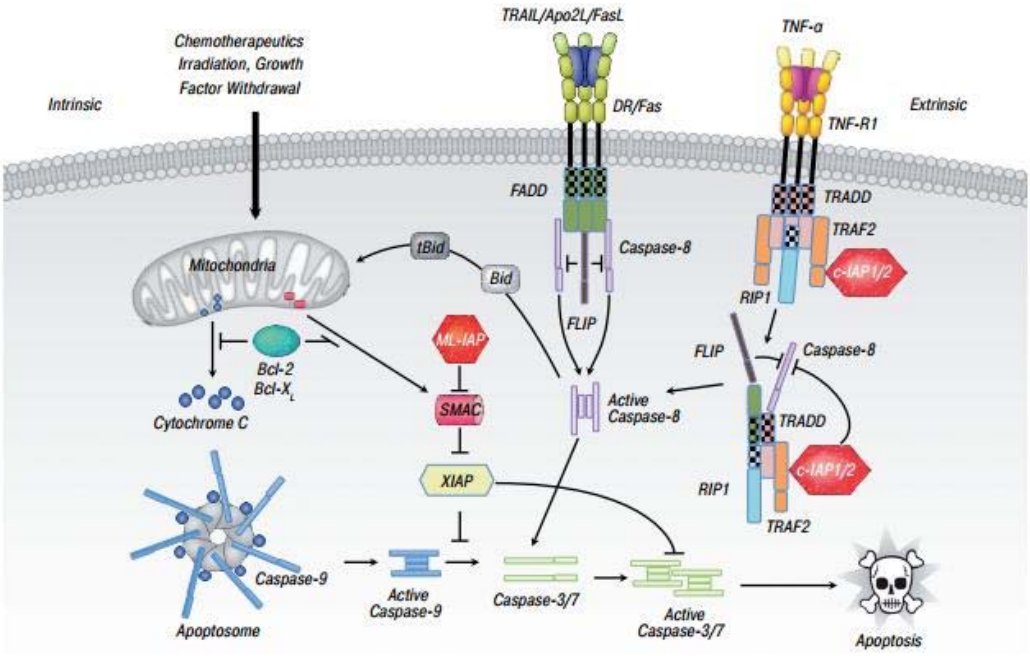

Figure I1. Schematic representation of extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated through DR stimulation, leading to caspase-8 activation that directly activates caspase-3. Intrinsic pathway is initiated by stimuli that causes Bax and Bak induced mitochondria outer membrane permeabilization, releasing pro-apoptotic factors to the cytosol that ultimately activate caspase-3. Note the cross-talk between the two pathways in the Bid cleavage by caspase-8, generating tBid that is able to induce mitochondria permeabilization. (Adapted from Almagro et al, 2012)

The most studied systems in the extrinsic pathway are the FasL/Fas and the TNF-α/TNFR1 systems. In these models, binding with the homologous trimeric ligand must occur, leading to clustering of the receptors in the plasma membrane. After this, cytoplasmic adapter proteins are recruited by interaction with specific regions of the intracellular tails of the death receptors. These adapters serve as a docking point for the formation of the Death Inducing Signaling Complex (DISC). Caspase-8 is a member of the DISC, and its recruitment leads to its self-activation by auto-catalytic cleavage of procaspase-8 [17].

Caspase-8 is able to directly activate the executioner caspase-3, which in turn is able to activate other molecules of the same protein, leading to an amplification of the signal and initiating the execution phase of apoptosis.

Intrinsic pathway

The intrinsic pathway of apoptosis can be initiated by an extensive array of stimuli that produce intracellular signals within the cell. The stimuli that can activate this pathway are very diverse, and wide, from loss of pro-survival factors like growth factors, hormones, to more active stimuli like radiation, toxins, hypoxia, free radicals or viral infections, among many others [18].

All of these stimuli cause changes in the inner mitochondrial membrane that result in a permeabilization of the outer mitochondria membrane, loss of the mitochondrial transmembrane potential and release of apoptogenic factors from the intermembrane space of the mitochondria to the cytosol [19]. Finally, these pro-apoptotic proteins lead to the activation of executioner caspases, initiating the execution of apoptosis.

In some cellular contexts the separation between the extrinsic and intrinsic pathways of apoptosis is clear, but in most cell types there is a highly regulated interaction between them. The most recognized link between these two pathways is the protein Bid. Bid is a BH3-only pro-apoptotic protein, which is normally located at the cytosol. It can be cleaved by caspase-8 into an active fragment, tBid, which can translocate to the mitochondria and provoke its permeabilization [20, 21]. Thus, this is a perfect example of interaction between these two apoptotic pathways, since caspase-8 can be activated through the extrinsic pathway and then tBid will trigger the intrinsic pathway activation.

Apoptosis Machinery

Characterization of the molecular components of the apoptotic pathway was initiated in studies from programmed cell death in the nematode *Caenorhabditis elegans*. As demonstrated by Sulston and Horvitz in 1977, 131 out of 1090 somatic cells invariantly undergo programmed cell death during normal development of *C. elegans* [22]. The discovery of cell lineage dependent programmed cell death paved the way for genetic characterization of its molecular machinery.

The central components of the apoptotis machinery in *C. elegans* are three CED (cell death abnormal) proteins, namely CED-3, CED-4 and CED-9 [23]. CED-9 functions as an inhibitor of apoptosis by preventing CED-4 from

interacting with CED-3 [24], whereas CED-4 is a pro-apoptotic adapter molecule required for the activation of CED-3 [25], a cysteine protease responsible for the execution of cell death program [26]. In mammals, the homologs of CED-9, CED-4 and CED-3 are present in the Bcl-2 family, Apaf-1/NOD-like receptor family and caspase family, respectively [27].

Caspases

In 1993, it was demonstrated that the CED-3 gene encodes a cysteine protease highly homologue to the human interleukin-1β-converting enzyme (latter renamed as caspase-1), which later was demonstrated to induce apoptosis when overexpressed [28]. This lead to the development of research activity focused on characterization of caspase family members in mammals. To date, 11 genes were found in the human genome to encode for 11 human caspases [29].

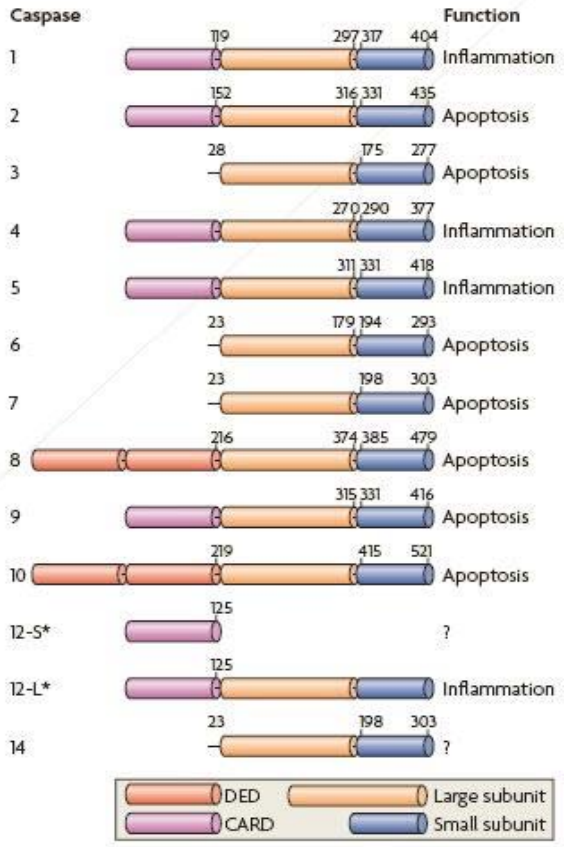

Figure I2. Schematic representation of caspase structure. All caspases share a similar structure, comprising a propeptide followed by a large and a small subunit. The pro-peptide is usually used to recruit the enzyme to activation scaffolds such as the APAF1 apoptosome. Two distinct, but structurally related, propeptides have been identified; the caspase recruitment domain (CARD) and the death effector domain (DED), and these domains typically facilitate interaction with proteins that contain the same motifs.(Adapted from Taylor et al, 2008)

Caspases share a number of features distinguishable from other proteases. They are initially expressed as inactive zymogens or procaspases, containing a prodomain, a large subunit (p20) and a small subunit (p10). Proteolytic cleavage of the zymogen removes the prodomain and separates the subunits, rendering caspases active. Another characteristic feature of caspases is that they normally cleave after an Aspartate (Asp) residue [30].

Caspases can be classified by two methods. By the basis of their known major function, caspases can be classified in two sub-families; pro-

apoptotic and pro-inflammatory caspases. However, this classification can be outdated soon as increasing evidences indicate that caspases participate in other processes different from cell death or inflammation. An alternative classification method is among the length of its prodomains, which also correspond to their position in the apoptotic pathway. So, caspases can be divided in initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) and effector or executioner caspases (caspase-3, -6, -7).

The prodomains of the inititator procaspases correspond to their protein-protein interaction motifs, which are named death effector domain (DED) and caspase recruitment domain (CARD), and involve their interaction with upstream molecules such as FADD or Apaf-1 [31]. Effector procaspases with short pro-domains are responsibles of most of the substrate proteolysis seen at the downstream phase of apoptosis. They are also usually cleaved by upstream caspases [32].

The catalytic domain of caspases is at its p20 subunit and consists of an active site formed by two conserved aminoacid positions; Cys258, which is part of a QACXG conseved motif, and His237 [30]. Caspases recognize at least four contigous aminoacids in their substrate and cleave after the C-ter residue, usually an Aspartic one.

Active caspases are homodimers, each monomer compress a small subunit (p10) and a large subunit (p20). Six β-strands in each monomer conform a central 12-stranded β-sheet, with several α-helices and small β-sheets in the sides of the central β-sheet, giving rise to a globular fold. Four loops (L1-L4) at the opposite ends of the central β-sheet form the active site, and determine the specificity of caspase substrates [33]. The binding pockets for the positions of the substrate are named S1-S4 subsites, respectively to each position in the substrate sequence. S1 and S3 are the most conserved among all caspases, whereas S2 and S4 show more degree of variation. S1 is formed by two Arg and a Gln residue [34]. Thus, it presents a very basic pocket structure ideally shaped for the Asp residue that has to be hydrolyzed [35].

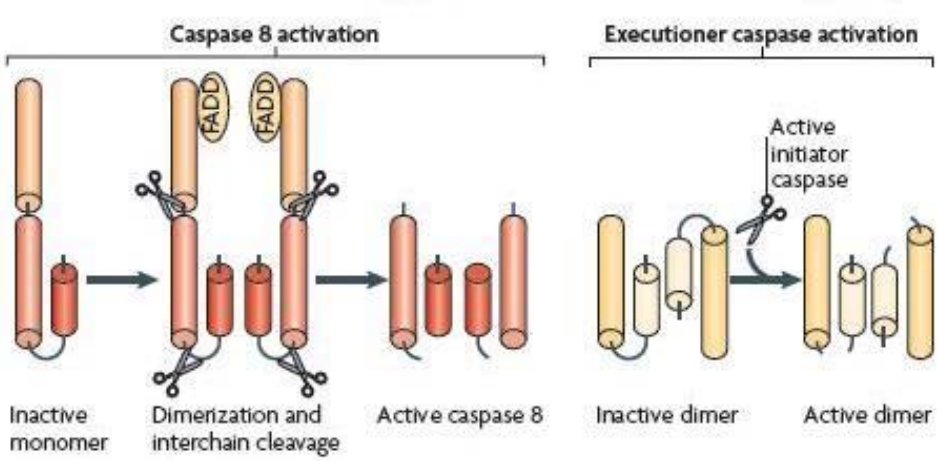

Figure I3. Caspase activation mechanism. Initiator caspase activation requires first dimerization of inactive caspase monomers. After DR stimulation, caspase-8 monomers are recruited to the DISC by binding to FADD, which allows its dimerization and interdomaincleveage. Executioner caspases are present as inactive dimers and require processing by initiator caspases to undergo conformational changes that lead to the formation of the enzymatically active dimer. (Adapted from Tait et al, 2010)

The S4 subsites confer major specificity to different caspases. In this context, caspases can be classified according to its preferred substrates [36, 37]. The group I have the widest S4 subsite and favors accommodation of large hydrophobic residues. Group II comprises caspase-3 and -7, and posses the narrowest pocket which has more affinity for an Asp residue in the P4 position. Caspase -8 and -9, among others, are part of the group III and have S4 subsites with intermediate position, favoring small hydrophobic substrates like Val or Ile.

All in all, caspases are the major effectors of the apoptotic process, and they induce irreversible changes in the cell. Therefore, is very important to have a tight regulation of the apoptotic pathway, and in fact there is a severe regulation of caspase activity through different mechanisms at different levels in the apoptotic signaling cascade.

Inhibitors of Apoptosis Proteins (IAPs)

IAPs are a family of proteins able to inhibit apoptosis in response to diverse stimuli, although they also play an important role in various biological processes such as cell proliferation, migration and regulation of inflammation, among

others [38, 39]. The *IAP* gene was first identified in insect cells infected with baculovirus [40]. It was shown that this viral gene encoded a protein that protected the infected cells from apoptosis. Subsequent studies have identified IAP family proteins in a wide variety of species ranging from virus to mammals. The human genome encodes eight IAP family members.

The characteristic feature of the IAP family is the presence of one to three tandem specific motifs called BaculovirusIap Repeat (RIP) at the *N*-ter side of the protein. The core component of the BIRs is a consensus Cys/His motif that coordinates a single zinc ion [41, 42]. BIRs are protein interacting domains with specific binding properties, and can be classified in three types [43, 44]. BIR type II domain is responsible of the interaction with the caspases, and is essential for IAP anti-apoptotic activity. It forms a highly hydrophobic core that interacts with a conserved motif found in the majority of caspases and IAP inhibitors, the IAP Binding Motif (IBM). Type I and III BIRs do not bind IBMs but interact with other proteins involved in cell signalling.

Another important conserved motif for IAPs anti-apoptotic activity is its *C*-ter RING (Really Interesting New Gene) domain. It is a zinc-finger motif with E3-ligase activity [45, 46]. This enables the ubiquitination of target proteins to induce its degradation via K48-linked poly-ubiquitination, or regulate its cellular distribution through a K63 or K11-linked mono-ubiquitination [47]. IAPs can also mediate their own ubiquitination [48]. The RING domain also enables hetero or homo-dimerization of IAPs to control its stability [49].

In addition to the BIRs and RING, other conserved protein domains can be found in IAPs, including a central caspase recruitment domain (CARD) that regulates E3-ubiquitin ligase activity [50], or an Ub associated (UBA) domain that can recognize mono- and poly-Ub chains and therefore allows the recruitment of IAP in protein complexes [51].

There are eight IAP family members encoded in the human genome and, among them, X-linked IAP (XIAP), and cellular IAPs 1 and 2 (cIAP1 and cIAP2) are the ones able to bind caspases via the BIR domain and regulate its activity through RING ubiquitination or neddylation [52]. cIAP1, cIAP2 and XIAP have three tandem BIR domains, one UBA and one *C*-ter RING domain. In addition,

cIAPs contain a central CARD domain. The BIR1 is a type I BIR [53], while the BIR2 and BIR3 domains are type II BIR and thus able to bind IBMs in caspases or IAP antagonists [54].

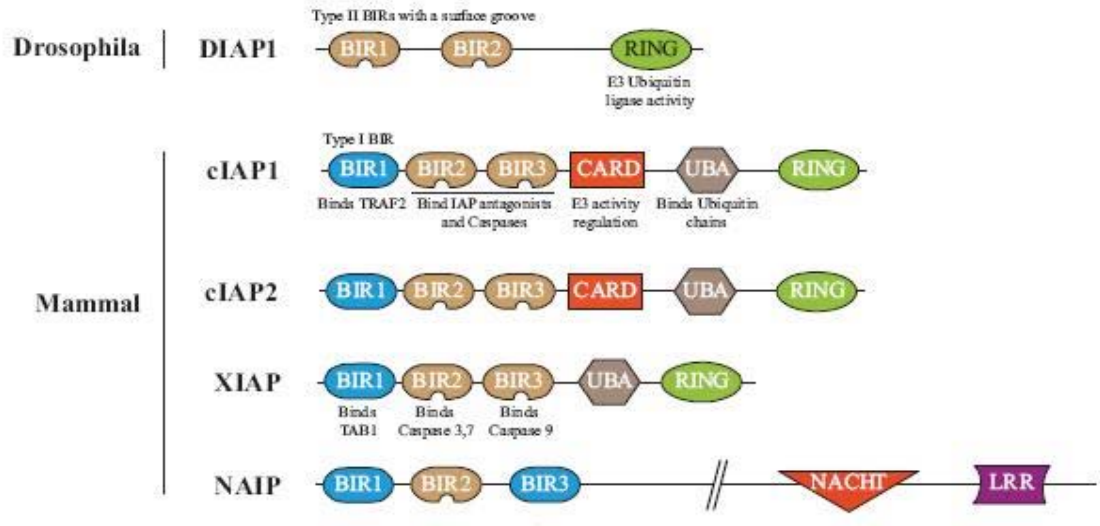

Figure I4. Schematic representation of Inhibitors of Apoptosis. All IAPs share similar domains with specific functions. BIR domains mediate interactions with other proteins involved in signaling. The UBA domains bind Ubiquitin chains. The CARD domain has the function of shutting down the E3 ligase activity of the IAP. RING domains provide E3 ligase activity to IAPs, and also serve as a docking point for E2 activating enzymes. (Adapted from Berthelet et al, 2013)

Although XIAP, cIAP1 and cIAP2 are all able to bind caspases, only XIAP is able to inhibit them by direct interaction [55]. XIAP inhibits caspases -3, -7 and -9. Overexpression of XIAP efficiently inhibits apoptosis caused by both the extrinsic and the intrinsic apoptotic pathway [56, 57], although down-regulation or deletion does not trigger apoptosis but sensitizes cells to an apoptotic insult [58]. BIR1 and BIR2 of XIAP bind to the active site of caspase-3 and -7 and inhibit their activity by occluding the substrate entry and also inducing its K48 poly-ubiquitination leading to their degradation. BIR3 binds caspase-9 and disturbs its self-dimerization [59]. On the other way, BIR domains of cIAP1 and cIAP2 can bind to caspases but cannot inhibit them, because of critical substitutions of aminoacids in the region targeting caspase inhibition in XIAP. It is possible to mutate these residues in the BIR domains of cIAPs to render them fully effective to caspase inhibition [60].

Apart from caspases, there are a large number of proteins, which comprise a potential IBM motif. The most studied are Smac/DIABLO and HtrA2 [61, 62].

They are synthetized as a precursor in the mitochondria and are released at the cytosol during apoptosis. Then, they bind to IAPs preventing their inhibitory action upon caspases activation. Smac/DIABLO is able to bind to BIR2 and BIR3 of XIAP and antagonize XIAP inhibition of caspase-3 and -9, and is also able to induce auto-ubiquitination and degradation of IAPs [63]. Omi/HtrA2 is a serine protease that binds and inactivates cIAPs and XIAP by an irreversible proteolytic cleavage [64]. The characterization of these molecules enabled the development of pharmacological inhibitors of IAPs, called Smac Mimetics, which helped in the study of the role of IAPs and are showing promising therapeutic effects [65, 66].

Bcl-2 family proteins

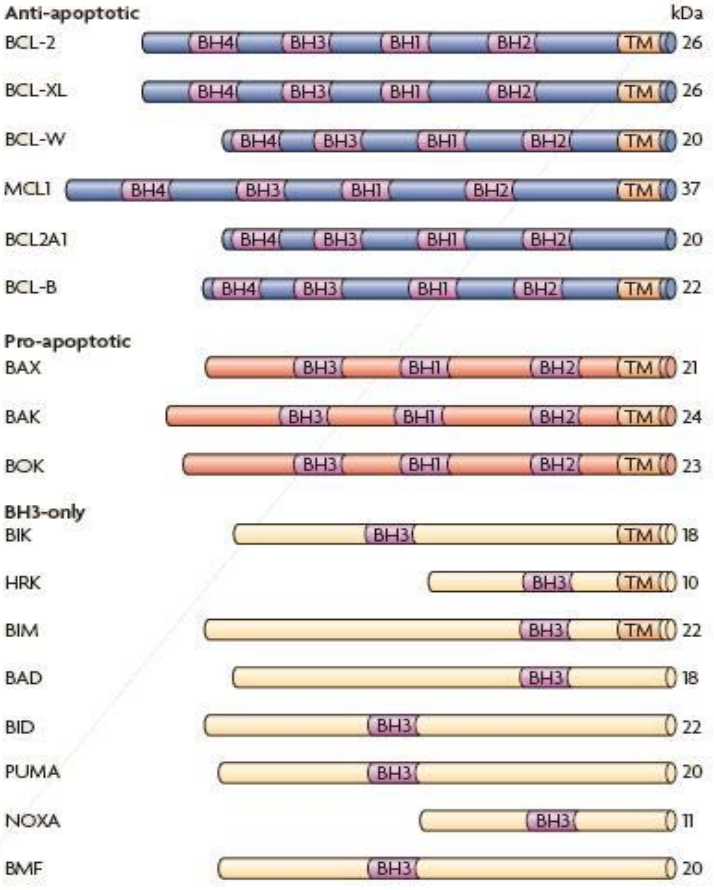

Figure I5. Schematic representation of Bcl-2 family proteins. Bcl-2 family proteins contain at least one type of BH domains. Ant-apoptotic family members have all the four BH domains. Pro-apoptotic subfamily lacks the BH4 domain. The BH3-only subfamily is a structurally diverse group of proteins that only display homology within the small BH3 motif. (Adapted from Taylor et al, 2008)

The *Bcl-2* gene was the first anti-apoptotic and anti-tumorogenic gene to be discovered. It was identified from a breakpoint region of recurrent chromosomal translocation in the human follicular lymphoma [67, 68]. Later on, Bcl-2 was shown to be able to repress apoptosis in *C.elegans*, replacing the function of CED-9, and highlighting that Bcl-2 is its human homologous [69, 70]. Thus, the Bcl-2 family proteins were the first evolutionary conserved system to control apoptosis to be discovered.

Since then, the Bcl-2 family has been studied

extensively. They are classified among its functionality, pro-apoptotic or anti-apoptotic. All of them contain at least one of the four conserved α-helix motif known as Bcl-2 homology (BH1-BH4) domains.

Anti-apoptotic members contain all four BH domains. The most important members are Bcl-2-related-gene A1 (A1), Bcl-2, Bcl-2-related-gene long isoform (Bcl-xL), Bcl-w and myeloid cell leukemia 1 (MCL-1). They all adopt globular structures with a helical bundle surrounding a central hydrophobic core [71].

Pro-apoptotic members can be divided between effector proteins and BH3-only proteins. The most important effector proteins are Bcl-2 antagonist killer 1 (Bak) and Bcl-2-associated x protein (Bax). Both share the BH4 domains with the anti-apoptotic members and its structure is very similar. BH3-only proteins are subdivided based on their ability to interact with the anti-apoptotic members of the Bcl-2 family or both the anti-apoptotic and pro-apoptotic effector proteins repertoire. Bcl-2 antagonist of cell death (BAD), Noxa and Puma correspond to the first group and are referred as “sensitizer” BH3-only proteins. Bcl-2-interacting domain death agonist (BID) and Bcl-2-interacting mediator of cell death (BIM) are part of the second group called “direct activators”. Unlike its globular multi-BH domain partners, BH3-only proteins are intrinsically structurally disordered, and their BH3 region becomes an amphipathic helix upon interaction with protein partners [72].

In mammals, the most recognized function of the Bcl-2 family proteins is to regulate the integrity of the outer mitochondrial membrane (OMM) upon its permeabilization. The interactions between the anti-apoptotic members, BH3-only proteins and effectors determine the mitochondria permeabilization and apoptosis [73]. However, Bcl-2 family proteins are also related to other cellular functions like regulation of Ca^{2+} influx from the Endoplasmic Reticulum (ER) and control of the Unfolded Protein Response (UPR) [74].

Structural and functional considerations of Bcl-2 family proteins

The 3D structures of the Bcl-2 family members have been elucidated either by X-ray or NMR studies [75-77]. All the anti-apoptotic and effector Bcl-2 proteins share a “Bcl-2 core”. This core represents a ~20kDa globular domain with

seven or eight amphipathic α-helices, arranged around a central buried helix [78]. The BH1 domain on one side and the BH3 domain on the other delimit a hydrophobic groove that serves as binding point for BH3 domains, and is often called “BC groove”. The BH4 domain stabilizes the BH1-BH3 region. The *C*-ter part of the protein is the transmembrane (TM) region and in some cases is accommodated in the BC groove [79, 80]. The major differences between anti-apoptotic and effector proteins are in structural features of the individual Bcl-2 family members, such as the amino acid composition and the degree of occlusion of the BC groove.

BH3-only proteins carry their pro-apoptotic functions either by inhibiting anti-apoptotic Bcl-2 family members or by activating Bax or Bak. Both of its effects involve direct interaction with target proteins [81, 82]. Neutralization of anti-apoptotic partners has been well characterized and has led to the development of novel therapeutics called BH3 mimetics [83]. The BH3 amphipathic helix of BH3-only proteins binds to the BC groove of pro-survival proteins mainly by the insertion of four hydrophobic residues in the pocket and by ionic interactions between conserved Asp and Arg in the sequence of both proteins [84].

Pro-apoptotic effector proteins can bind either its anti-apoptotic partners or BH3-only proteins. During resting conditions, Bak resides in the outer membrane of the mitochondria while Bax is cytosolic with its TM domain enclosed in its BC groove. After an apoptotic signal, the union of a BH3-only protein promotes the release of its TM domain from the BC groove and its translocation to the mitochondrial membrane [85]. After translocation, Bax and Bak form homo-oligomers that can permeabilize the mitochondria outer membrane [77], although the exact mechanism is still undefined.

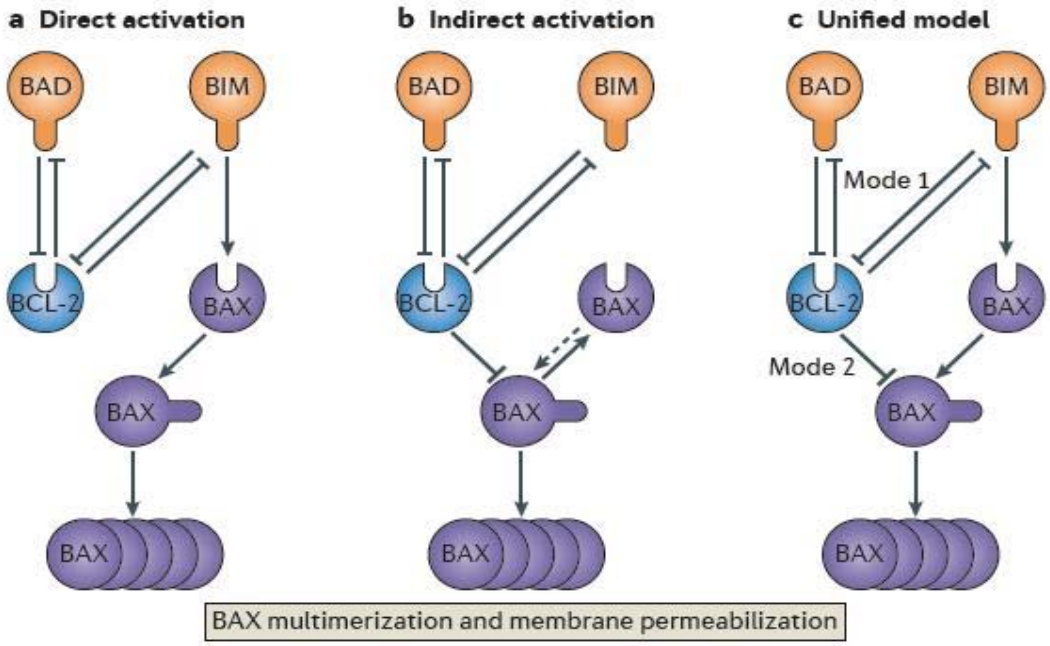

Figure I6. Proposed models of regulation of apoptosis by Bcl-2 family proteins. a. In the direct activation model certain BH3-only proteins, particularly the truncated form of BID (tBID) and BIM, directly engage and activate the pro-apoptotic effectors BAX and BAK. **b.** In the indirect activation model, Bax and Bak are constitutively inhibited by Bcl-2 anti-apoptotic members. Bax and Bak become active only after BH3-only proteins neutralize the function of Bcl-2 anti-apoptitic members. **c.** In the unified model, BH3-only proteins not only directly activate Bax and Bak, but also neutralize Bcl-2 anti-apoptotic family members in order to induce mitochondria permeabilization. (Adapted from Czabotar et al, 2014)

The interaction between BH3 domains of effector pro-apoptotic proteins and anti-apoptotic Bcl-2 family members restrain their activity. This suggests a simple mechanism for apoptotic regulation, where BH3 domains of anti-apoptotic Bcl-2 family members and BH3-only compete for binding to Bax or Bak [86]. Another model is the indirect activation, in which pro-survival proteins prevent apoptosis by neutralizing the BH3-only proteins that directly activates Bax or Bak [87]. At the end, evidences suggest that both models operate together to orchestrate a network of interaction between all three subgroups of the Bcl-2 family [88].

To sum up, the most important feature in the Bcl-2 family is the interaction of a BH3 domain of a pro-apoptotic family member, either BH3-only or effectors, with the BC groove of a multi domain relative. Whereas the interaction of BH3-only proteins with anti-apoptotic members yields a stable complex, the interaction with Bax or Bak induces a conformational change that induces the

release of their own BH3 domain, which can then engage another molecule to form the oligomers that induce the permeabilization of the mitochondria.

Bcl-2 in the mitochondria

The contribution of Bcl-2 family proteins to the regulation of OMM permeabilization is undisputed. The activation of pro-apoptotic proteins Bax and Bak induce cytochrome c release from the mitochondria, and has been stated that conformational changes in Bak and translocation of Bax to the OMM occurs at the same time that cytochrome c release [89]. However, the exact nature of the pore formation in the mitochondria membrane, as well as its protein composition and its interactions is still not fully understood.

The finding that globular Bcl-2 proteins structure is similar to bacterial pore-forming toxins led to the hypothesis that Bax and Bak could form channels in the membrane directly [75, 90]. In fact, it has been shown in experiments with synthetic membranes *in vitro* that both anti-apoptotic and pro-apoptotic members of Bcl-2 family can form membrane channels [91]. Incubation of Bax with isolated mitochondria induces cytochrome c release and allows the release of large dextran molecules from lyposomes, while Bcl-xL is able to inhibit both processes [92]. Bid synergizes with Bax in promoting cytochrome c release, either by activating Bax or by inhibiting Bcl-xL [93]. So, a model where Bax and Bak directly can form pores at the OMM can be postulated. Although there are some caveats to this model, as some discrepancies in studies *in vitro* or in cultured cells has been reported [94, 95]. Moreover, while these pores have been able to accommodate cytochrome c, there are much larger proteins that are released from the mitochondria.

Rather than Bax and Bak forming pores themselves, it has been proposed that they may modulate existing mitochondrial channels, such as Voltage Dependent Anion Channel (VDAC) and Adenine Nucleotide Traslocase (ANT). Bcl-2 family proteins have been shown to be able to interact with ANT and modulate its activity in some situations [96]. It has been shown that ANT is able to form pores on lyposomes [97], and this process is inhibited by Bcl-2 and Bcl-xL. However, the role of ANT in mitochondria permeabilization has been ruled out since some studies have shown that all three isoforms of ANTs are

dispensable for cytochrome c release [98]. VDACs are the main pathway for metabolite diffusion across the mitochondria. Early studies in yeast and in synthetic lipidic membranes suggested a role for a hypothetic VDAC-Bax channel in the OMM [99], and additional findings established that Bcl-2 family proteins could interact and regulate the opening of VDACs [100]. However, the contribution of VDAC in apoptosis has been discarded, since know-out mice for all three VDAC isoforms displays normal apoptosis [101]. Nonetheless, the VDAC2 isoform was found to be essential for Bak import into the mitochondria outer membrane [100].

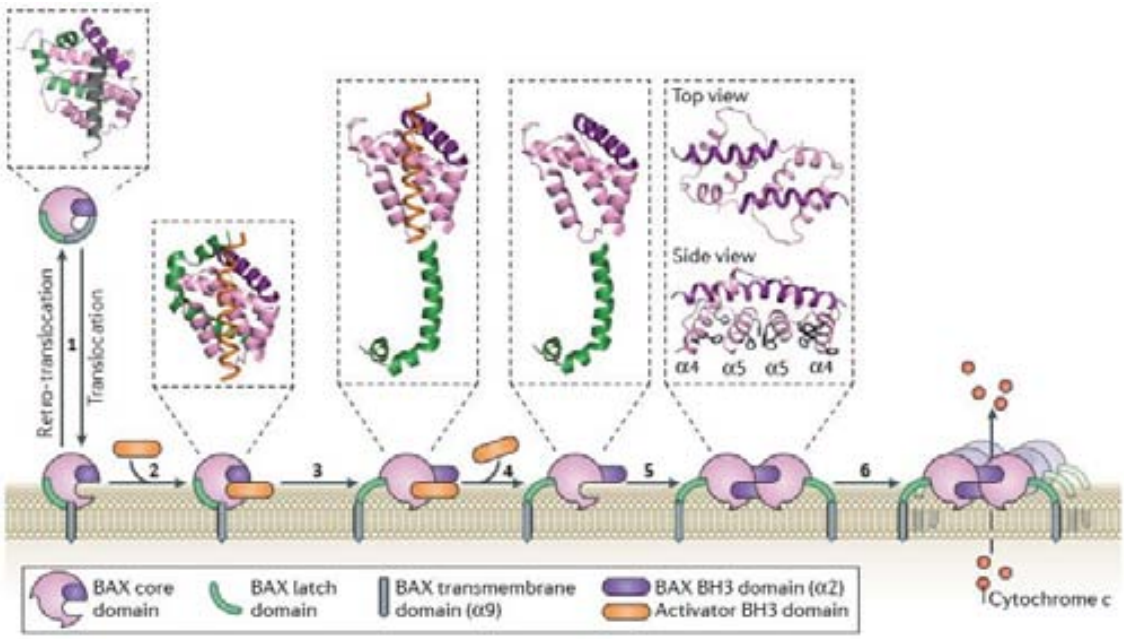

Figure I7. Model for the activation and oligomerization of Bax and Bak. First step is the translocation of Bax to the mitochondria outer membrane after the release of its transmembrane domain from its BC groove. This step is triggered by the interaction with a BH3-only protein. After translocation to the MOM, The initiating activator BH3 domain then disengages. This allows BH3 domain from another Bax molecule to bind to its BC groove, thus forming a dimer. (Adapted from Czabotar et al, 2014)

Apart of the permeabilization of OMM during apoptotic processes, the Bcl-2 family also modulates the balance between mitochondrial fission and fusion [102]. Mitochondria are dynamic organelles that undergo fission and fusion to yield an interconnected tubular mitochondrial network. Mitochondrial fission is regulated by Drp-1, a soluble factor recruited to mitochondria during fission [103]. Bax and Bak have been shown to influence the rates of fussion and fission, but the mechanics of this process remains unclear [104]. The finding that Drp-1 colocalizes with Bax during apoptosis, led to the hypothesis of its

implication in cell death [105]. Indeed, both Drp1 and its fly analog overexpression in *C.elegans* and flies enhance cell death, and its downregulation delays mitochondria permeabilization and cytochrome c release in mammals [106, 107]. However, other studies point in opposite directions by showing no direct effect of Drp-1 in OMMP [108]. Bcl-xL interacts with Drp-1 and this interaction also has a role in synapse formation in neurons [109].

Bcl-2 in the ER

Despite a vast majority of research efforts have been focused on studying the Bcl-2 family functions at the mitochondria, it's well known their localization and anti-apoptotic functions at the ER. Representatives of all subgroups of the Bcl-2 family have been found localized at the ER, where they display a broad functionality, controlling Ca^{2+}signalling during ER stress, regulating the UPR response, modulating ER-stress induced autophagy and mediating the ER-mitochondria crosstalk, among others [74, 110].

It is known that high cytosolic Ca^{2+} concentrations are cytotoxic for cells, and various cell processes are modulated by Ca^{2+} signalling [111]. Ca^{2+} oscillations in the cell are tightly regulated, and the majority of Ca^{2+} in the cell is either bound to specialized proteins or compartmentalized in cell organelles. The ER represents the main Ca^{2+} reservoir in the cell [112], and calcium diffusion across the membranes it's regulated by two main channels; the sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA), that actively imports Ca^{2+} from the cytosol into the ER lumen, and the inositol trisphosphate (IP_3) receptor (IP_3R), which mediates the release of Ca^{2+} into the cytosol.

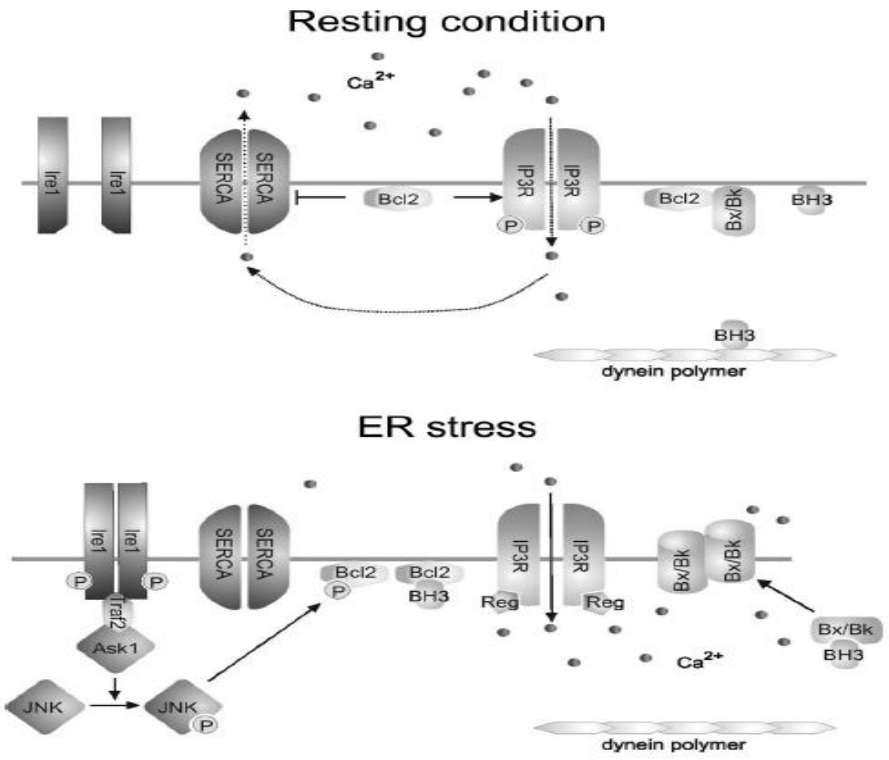

Figure I8. Modulation of ER calcium by Bcl-2 family proteins. Bcl-2 and Bcl-xL promote the leak of calcium from the ER lumen into the cytosol by binding to IP_3R. In parallel, they attenuate the active import of calcium into the ER, thus lowering ER calcium concentration. After ER stress, Bcl-2 and Bcl-xL activity over IP_3R is compromised by the action of Bax, Bak and BH3-only proteins that can disrupt its interaction. (Adapted from Szegezdi et al, 2009)

The release of Ca^{2+} from the ER is a critical point in apoptosis triggered by various stimuli, including DNA damage, production of free radicals or, in some instances, during death receptor engagement [113, 114]. Bcl-2 and Bcl-xL can interact with IP_3R and modulate its activity, by a mechanism not fully understood [115]. While some studies show that this interaction enhances the conductance of the channel, leading to lower Ca^{2+} concentrations in the lumen of the ER and thus diminishing Ca^{2+} oscillations, others point out at a direct inhibitory effect from Bcl-2 over IP_3R [116, 117]. Nonetheless, both models are coherent with the anti-apoptotic effects of Bcl-2 and Bcl-xL in the ER, acting by abrogating Ca^{2+} apoptotic signals. Other evidences found in favor as IP_3R as the main effector of Ca^{2+} release from the ER during apoptosis is the finding that cytochrome c is able to bind to IP_3R and increase its calcium conductance [118], thus creating a positive feed-back between ER and the mitochondria.

Bcl-xL is also part of a complex in the ER formed by Bap31 and procaspase-8 [119]. During cellular stress, caspase-8 is activated and cleaves Bap31,

generating its p20 cleaved form [120, 121]. It has been shown that overexpression of the truncated product of Bap31 induces apoptosis by promoting mitochondrial fission and Ca^{2+} release from the ER to the mitochondria, leading to cytochrome c release [122].

Bax and Bak also localize at the ER, although their roles there have not been fully explored. It has been found that they oligomerize at the ER under apoptotic situations [123], although the exact nature of this multimers is not understood. Studies in MEFs deficient for Bax and Bak have shown lower ER Ca^{2+} concentration and protection from apoptosis that involve Ca^{2+} signals [124]. Since they fail to interact with any ER Ca^{2+} channel, their proposed mode of action is through inhibition of Bcl-2 and Bcl-xL function at the ER.

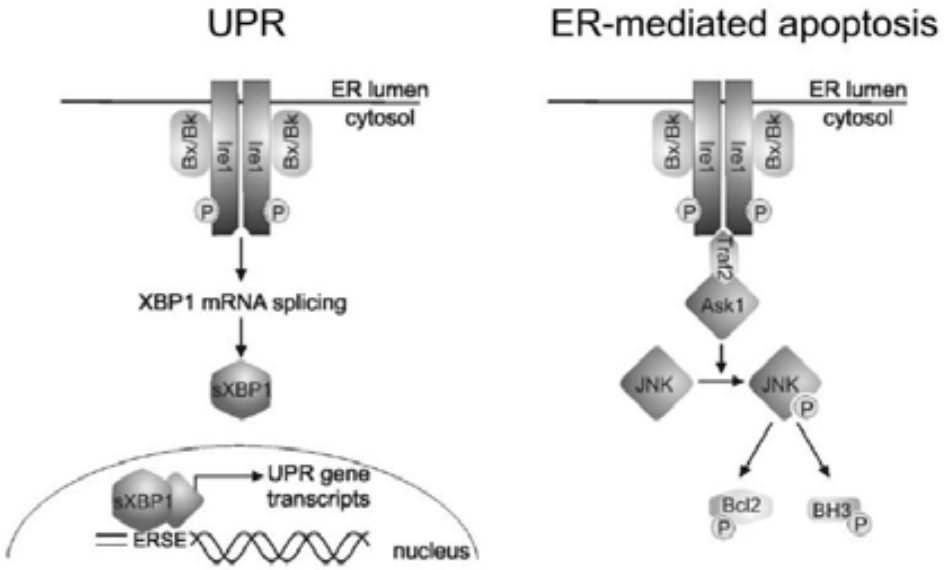

Figure I9. Modulation of UPR by Bcl-2 family proteins. Upon activation of IRE1, IRE1 dimerizes and cross-auto-phosphorylation occurs by a mechanism that is thought to be promoted by Bax and Bak. The endoribonuclease activity ofIRE1 then cleaves the mRNA of the transcription factor XBP-1, producing a frame shift spliced variant of XBP1 (sXBP1), that translocates to the nucleus and induces the transcription of ER stress response element (ERSE)-containing UPR genes. During prolonged ER stress, the UPR initiates apoptotic signaling pathways via differential modulation of Bcl-2 family proteins. Bax and Bak regulate the activation of the IRE1/TNF receptor-associated factor 2 (TRAF2)/ASK1 arm of the UPR, leading to JNK activation. JNK positively regulates the proapoptotic BH3-only proteins Bim and Bad and negatively regulates Bcl-2. (Adapted from Szegezdi et al, 2009)

Another partner for Bcl-2 and Bcl-xL in the ER is Bax Inhibitor 1 (BI-1). It is an ER located transmembrane protein part of the Trans-Membrane Bax-Inhibitory Motif (TMBIM) family proteins [125, 126]. Despite its name, it doesn't interact with Bax, but with Bcl-2 and Bcl-xL, and thus its inhibitory effects are linked to

regulate Bcl-2 and Bcl-xL activity in the ER [127]. In this regard, modulation of BI-1 expression alters ER Ca^{2+} concentration in a similar fashion that Bcl-2 and Bcl-xL [128]. In fact, Bcl-xL and Bcl-2 down-regulation of ER Ca^{2+} concentration is dependant on BI-1 expression. BI-1 directly interacts with IP_3R, but the exact mechanism of action of BI-1 remains to be identified [129, 130].

Cellular stress can cause problems in protein folding. Disruption of ER function induces activation of an adaptive response called the Unfolded Protein Response (UPR). The UPR is an attempt to increase the folding capacity of the ER through enhancing the expression or activity of proteins that would help in reducing the misfolded proteins load, such as chaperones and proteases [131] . However, if the ER stress bypasses a certain threshold, apoptosis is induced.

UPR response is controlled by three ER resident transmembrane proteins that act as sensors of ER stress; inositol-requiring enzyme 1 (IRE1α), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [132, 133]. They all contain luminal domains that sense the accumulation of unfolded proteins in the ER lumen. They are bound to glucose-regulated protein 78 (GRP78), which maintains them in an inactive state. ER stress induces the dissociation of GRP78 and the UPR signal transduction begins [134].

IRE1α is the most conserved ER stress sensor, and its activity is strictly regulated by interaction with other proteins [135]. The first steps of the UPR are destined to alleviate the unfolded protein charge and reduce the ER stress, but when this is prolonged, IRE1α activity is turned off and apoptotic signal transduction begins. Bax and Bak have a role in activating IRE1α. In Bax/Bak deficient cells, the UPR response is impaired [136]. It was described that Bax and Bak interact with the cytosolic region of IRE1α and stabilize its active form. Thus, is surprising that Bax and Bak may have bifunctional roles in the UPR, a pro-survival role in the first steps of the process by activating the IRE1α sensor, and then engage mitochondrial mediated apoptosis when homeostasis cannot be restored. This view is supported by the fact that Bax/Bak deficient cells are resistant to most forms of ER-stress induced apoptosis [137].

Furthermore, BI-1 regulates IRE1α inactivation, most likely by direct binding at IRE1α and inhibition of the effect of Bax and Bak on the UPR. In this regard, BI-

1 interacts with the ER branch of IRE1α, and BI-1 deficient cells are more sensible to ER stress and have impaired IRE1α inactivation [138, 139]. So, BI-1 appears to have also a bifunctional role in the UPR, like Bax and Bak. Bcl-2 anti-apoptotic family members doesn't appear to have a direct role in the control of UPR, but an indirect effect by binding and modulating the function of Bax, Bak and BI-1 can't be ruled out.

Finally, a role for Bcl-2 family proteins in the regulation of ER-stress induced apoptosis is demonstrated. Bcl-2 and Bcl-xL have been shown to directly interact with Beclin-1, an essential cofactor for autophagy initiation [140], and this interaction is attributed to the expression of Bcl-2 and Bcl-xL at the ER membrane [141]. This interaction appears to inhibit the function of Beclin-1, and can be displaced by the action of BH3-only proteins or by Bax and Bak. Similarly, pharmacological BH3 mimetic disturb Bcl-xL and Beclin-1 interaction, promoting autophagy [142].

ER stress can trigger autophagy through two pathways that require either IRE1α or IP_3R activation [143]. Overexpression of Bcl-2 and Bcl-xL is able to inhibit the last, but has no effect on the former [144]. Although elevated cytosolic calcium concentrations promote autophagy, for the IP_3R-dependent autophagy Beclin-1 was shown to be essential, and its activation doesn't involve calcium release from the ER [145, 146]. So, is likely that IP_3R may act in this context as a scaffold protein rather than a channel. It remains to be explored how Bcl-2 family proteins exactly regulate ER-stress induced autophagy and if IRE1α activation may have an impact in Bcl-2/Beclin-1 interaction.

Death receptors

Death receptors are a subgroup of membrane receptors of the Tumor Necrosis Family (TNF) superfamily [15, 17]. They are type I transmembrane proteins, which are characterized by the presence of its N-ter exposed to the extracellular space. DRs have up to six cysteine-rich domains in their *N*-ter extracellular region, which defines their ligand specificity, an intermembrane domain and a *C*-ter intracellular domain [147]. In the *C*-ter region of the DRs, there is a conserved sequence called the Death Domain (DD), which is essential for binding of factors related to apoptosis signaling [148].

Eight members of the death receptor family have been characterized so far: TNF-receptor 1 (TNFR1; also known as DR1, CD120a, p55 and p60), CD95 (also known as DR2, APO-1 and Fas), DR3 (also known as APO-3, LARD, TRAMP and WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1; also known as DR4 and APO-2), TRAILR2 (also known as DR5, KILLER and TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR) [149-152]. Apart from these, a group of similar transmembrane receptors but without the DD domain has been characterized, called Decoy Receptors. The role of these proteins is thought to be the regulation of DR activity by competitive ligand binding.

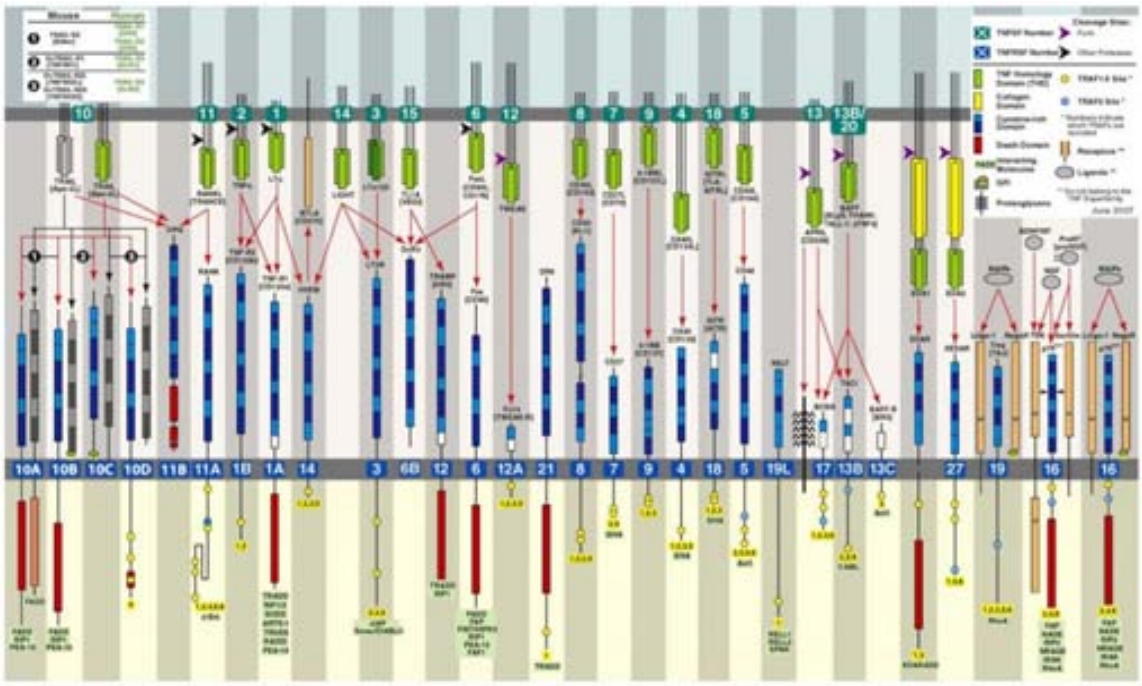

Figure I10. Members of the TNF superfamily and their ligands. Alternate blue and light blue boxes represent the Cys-rich domains characteristic of this superfamily of proteins. Red boxes represent Death Domains characteristic of the Death Receptors. (Adapted from Taylor et al, 2009)

DRs are activated by their cognate ligands, a group of cytokines that belong to the TNF protein superfamily, with the exception of NGF that belongs to neurotrophins family. These proteins are mainly expressed as type II transmembrane proteins, which are characterized by having its *C*-ter exposed to the extracellular space [153]. In some cases, DR ligands are released by proteolytic cleavage, although the apoptosis-inducing effects seem to be higher in the case of the membrane-bound forms.

Two types of DR signaling complex can be distinguished between the protein complexes that recruit after ligand binding [154]. The first group recruits the death-inducing signaling complexes (DISCs) that are formed at the CD95 receptor, TRAILR1 or TRAILR2. The second comprises TNFR1, DR3, DR6 and EDAR. These recruit a different set of molecules, which transduce both apoptotic and survival signals. The DRs better characterized are TNFR1 and CD95/Fas.

TNFR1 signaling

The TNF/TNF-receptor system consists of two receptors, TNFR1 and TNFR2, and three ligands, the lymphocyte-derived cytokine LT-α, and the soluble and membrane-bound forms of TNF-α [155]. TNFR1 is ubiquitously expressed and posses an intracellular death domain (DD), while TNFR2 expression is restricted to specific neuronal subtypes, to olygodendrocytes, microglia and astrocytes in the brain, to endothelial cells, and some types of T-cell subpopulations such as lymphocytes, cardiac myocytes and thymocytes. TNFR2 does not have a DD [149]. TNFR1 mediates the apoptotic signaling induced by TNF-α, however it can also activate pro-survival, differentiation or proliferation signaling pathways . In fact, TNFR1 is primarily mediating inflammation and not cell death. In order to induce apoptosis by TNFR1 activation, inhibition of pro-survival signals is essential. This can be accomplished by inhibition of protein synthesis by treatment with Actinomycin D or cycloheximide, by inhibition of NF-κB, or by silencing the expression of anti-apoptotic genes such as $FLIP_L$ or $FAIM_L$ [156, 157].

TNF-α binding to the receptor causes the recruitment of proteins to the intracellular tail of TNFR1. At first stages after receptor engagement, RIP1, TRAF2, TRAF5, TRADD and cIAPs 1 and 2, along with TNFR1, form the complex I [158, 159]. In this complex, RIP1 is polyubiquitinated by TRAFs and cIAPs and serves as a docking point for the formation of complexes such as LUBAC or IKK [160]. This finally leads to the degradation of the inhibitor of κB (IκB) and the translocation of NF-κB to the nucleus, where it promotes the expression of pro-survival genes such as c-FLIP or XIAP [161, 162].

If prolonged TNFR1 activation, endocytosis of complex 1 by clathrin-dependent vesicles occur [163]. NF-κB signaling is terminated by the action of deubiquitylation enzymes A20 and CYLD [164, 165]. This leads to the dissociation of the components of complex I. Dissociation of RIP1 and TRADD allows its interaction with FADD, which in turn, recruits caspase-8 and -10 [158, 166]. This newly formed complex, containing RIP1, FADD, TRADD, TRAF2 and caspase-8/-10 is the complex IIa, and initiates the apoptotic cascade. In situations when caspase-8 is inhibited, RIP1 kinase activates and binds to RIP3 to form the complex IIb that will lead to activation of necroptosis [167].

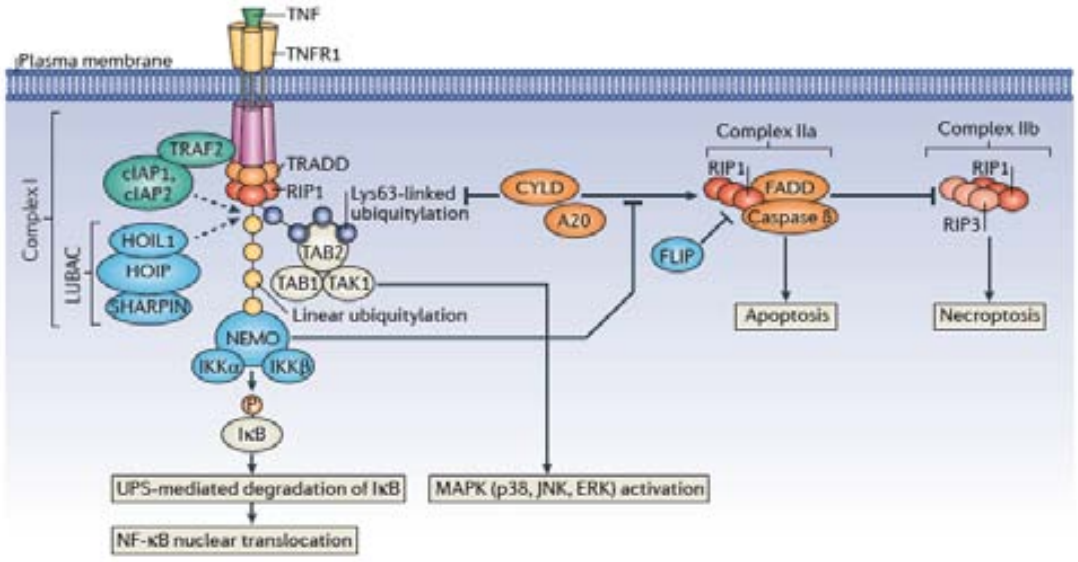

Figure I11. TNFR1 signaling pathways. Upon binding of TNFα to its receptor, the cytosplasmatic detah domain of trimerized TNFR1 recruits TRAF2, RIP1 and TRADD to form the complex I. Additional protein and complexes like cIAPs and LUBAC can be recruited that mediate activation of pro-survival signaling pathways. If prolonged TNF stimulation, complex I dissociates by the action of deubyquitlating enzymes, allowing RIP1 and TRAF2 to migrate to cytosol an form other complexes with either FADD (compex IIa) or RIP3 (complex IIb), which will promote cell death. (Adapted from Ofengeim et al, 2013)

All in all, the TNF/TNF-receptor system is responsible of the regulation of a wide variety of cellular functions, and its regulation and switching from one process to another is an extensive field of research. RIP1 status is very important to regulate the formation of different TNFR1 complexes. Further investigation will provide new insights into the mechanisms by which RIP1 controls a multitude of cellular pathways through TNFR1 and the implications for human diseases.

CD95 signaling

CD95 is the most well characterized DR so far. Its best-known function is the induction of apoptosis, and during years it was thought that this was its main function. However, now it's clear that CD95 signaling also participates in a wide range of functions like synapse formation or motility, among many others [168, 169].

CD95 is constitutively expressed in most tissues, while CD95L expression is restricted to activated T cells or natural killer cells, in which it plays a pivotal role in the elimination of transformed and infected cells, and in immune homeostasis. Membrane bound CD95L (mCD95L) can be cleaved by proteases of the extracellular matrix to release a soluble form of CD95L, termed sCD95L [170]. Only mCD95L is responsible of triggering the apoptotic signal, while sCD95L role is linked to the signaling of other cellular processes like proliferation or invasion in tumors [171].

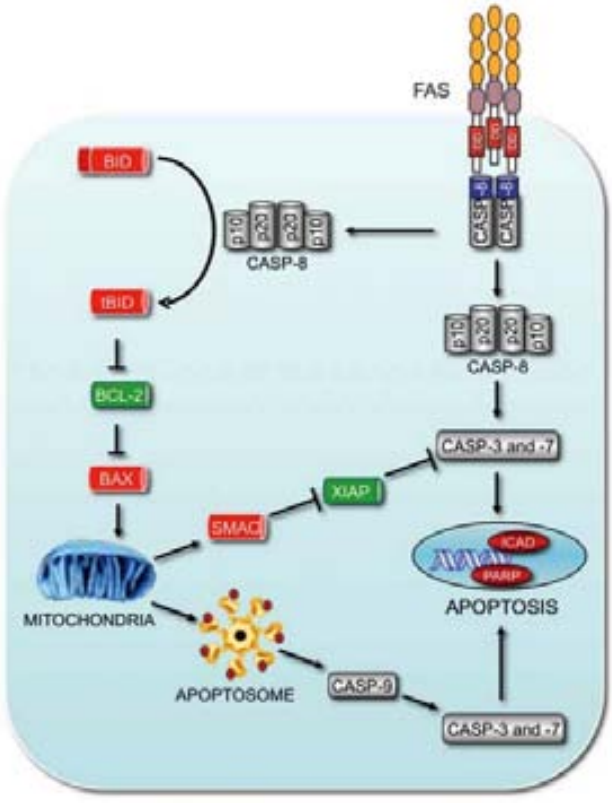

Figure I12. CD95 apoptotic signaling pathways. Caspase-8 is recruited at the DISC after FasL binding to its receptor. In type I cells, caspase-8 directly processes and activates caspase-3, inducing apoptosis. In Type II cells, caspase-8 cleaves the BH3-only protein Bid, generating its truncated form, tBid, which activates Bax and Bak. Upon mitochondria permeabilization, pro-apoptotic factors released conform the apoptosome, which is able to activate caspase-9 which, in turn, activates caspase-3 and induces apoptosis. **(**Adapted from Kaufmann et al, 2012)

CD95 is expressed in the plasma membrane as a homotrimer [172]. This interaction is mediated by the *N*-ter part of the protein, within the first Cys-rich domains, and essential for correct signal transduction. It has been shown that mutations or deletions on this domain that led to disruption of the receptor agreggation cause reduced apoptotic potency by binding of agonist [173].

According to this, mutations in these genes in mice models result in the development of autoimmune disorders like autoimmune lymphoproliferative syndrome (ALPS).

Binding of CD95L cause receptor trimerization and the recruitment of proteins to the *C*-ter intracellular domain of CD95, forming the DISC. The adaptor molecule Fas-associated DD containing protein (FADD) binds to the DD of CD95 by homotypic interactions [174]. Apart of this DD, FADD also comprises another domain at its *N*-ter called the death effector domain (DED) [175], required for the recruitment of caspase-8 to the DISC. Once this occurs, the high concentration of procaspase-8 in the DISC leads to autoproteolytic cleaveage of the zymogens, and its activation [176]. Then, active caspase-8 is dissociated from the DISC and is released to the cytosol in a homodimeric form, although some reports show that it can also manifest its caspase activity still bound to the DISC [177].

After caspase-8 activation in the DISC, the apoptotic program is initiated. There are two CD95-associated apoptotic signaling pathways [178, 179]. In the first one, called type I, caspase-8 activation is sufficient to directly activate the effector caspase-3 leading to apoptosis of the cells. In the other one, termed type II, caspase-8 activation in the DISC is less than in Type I cells and not sufficient to directly cleave caspase-3, thus signal is amplified through the mitochondria. This is accomplished by the cleavage of the BH3 only protein Bid by caspase-8, forming its truncated form tBid [20]. tBid translocates to mitochondria and induces Bax and Bak heterodimerization, leading to mitochondria permeabilization. Cytochrome c released to the cytosol binds to Apaf-1 and procaspase-9 to form the apoptosome [180]. Once the complex is formed, caspase-9 is activated. Finally, caspase-9 activates caspase-3 and apoptosis is initiated.

These two separated signaling pathways were first described by Scaffidi et al., in studies where they demonstrate that some cell lines can be rendered resistant to CD95-induced apoptosis by overexpression of Bcl-2 while other cell lines were not affected [179]. So, in Type II cells, overexpression of Bcl-2 or Bcl-xL protects the mitochondria from permeabilization and thus inhibits the

apoptotic signal. Since Type I cells are not reliant on the mitochondria for activation of caspase-3, neither Bcl-2 or Bcl-xL overexpression has no protective effect in these cells.

The authors also describe differences in the kinetics of caspase-3 and caspase-8 activation within type I or type II cells. In type II cells, apparition of the processed, active fragments of caspase-8 and caspase-3, as well as the cleavage of the different substrates, is delayed. Moreover, differences on the way of how active caspase-3 is generated were also reported. In type I cells, initial cleavage generate a p20 subunit that represents the active p17 plus the short prodomain, while in Type II cells the active p17 subunit is directly formed, without any detectable p20. This is consistent with the notion that in type I cells caspase-3 is activated by another different caspase, namely caspase-8, which will generate the p20 subunit first, and then p17 is generated through auto-processing.

Despite these differences, both cell types appear to be equally sensitive to CD95-induced apoptosis. It is important to note that in both cell types mitochondria is permeabilized, but only in type II cells is an essential requirement for apoptosis induction. Thus, caspase-8 activation in type I cells occurs upstream of the mitochondria, while in Type II cells it is downstream.

These differences were later demonstrated *in vivo*. Knock-out mice for Bid, Bax and Bak, and transgenic mice for Bcl-2 were used, and the hepatocytes from these mice were shown to be resistant to CD95-antibody injection, while thymocytes activated apoptosis normally [137, 181]. Since then, these two cellular types were considered as a prototype for each one of the pathways, thymocytes are Type I cells while hepatocytes are Type II cells.

DISC formation is also reduced in type II cells, compared to type I cells [182]. At first, several groups hypothesized that these differences in DISC formation between cell types were due to the presence of a protein bound to CD95 and competing for FADD, thus inhibiting DISC formation. However, later studies have shown that these differences are linked to impaired receptor internalization in type II cells [183].

After CD95 stimulation, aggregation of the receptor occurs at lipid rafts, followed by clathrin-dependent internalization in endosomal vesicles [183, 184]. Colocalization of Fas with endosomal markers can be observed as soon as after 3 min of stimulation, at a time correlating with caspase-8 activation. In fact, inhibition of endocytosis blocks DISC recruitment of essential factors and apoptosis. However, it has been shown that FADD and caspase-8 recruitment precedes the receptor internalization, suggesting that they participate in other non-apoptotic signaling pathways [185]. Interestingly, caspase-8 activity is required for CD95 internalization in type I cells, as co-treatment with the caspase-8 inhibitor z-VAD blocked receptor endocytosis [186].

While there is a general agreement above the importance of receptor internalization in type I cells, its relevance in type II cells is controversial. Studies show that CD95 internalization in type II cells occur as a later event, not before 3 hours after receptor stimulation [187]. This correlates with caspase activation in these cells, although more experiments need to be performed to demonstrate its direct relation with apoptosis induction, as happens in type I cells. Other studies claim that the differences between type I and type II cells rely, at least in part, in the quantity of CD95 exposed on the membrane.

More recently, XIAP has been described as a discriminator between type I and type II CD95-induced apoptosis [188]. The authors describe that XIAP is downregulated following CD95-antibody injection in mouse thymocytes, but is upregulated in the hepatocytes . Then, using *in vivo* experiments with Bid/XIAP double deficient mice, and its single counterparts, they show that hepatocytes from Bid deficient mice were resistant to CD95-induced apoptosis, and this sensitivity was restored in Bid/XIAP double deficient mice.

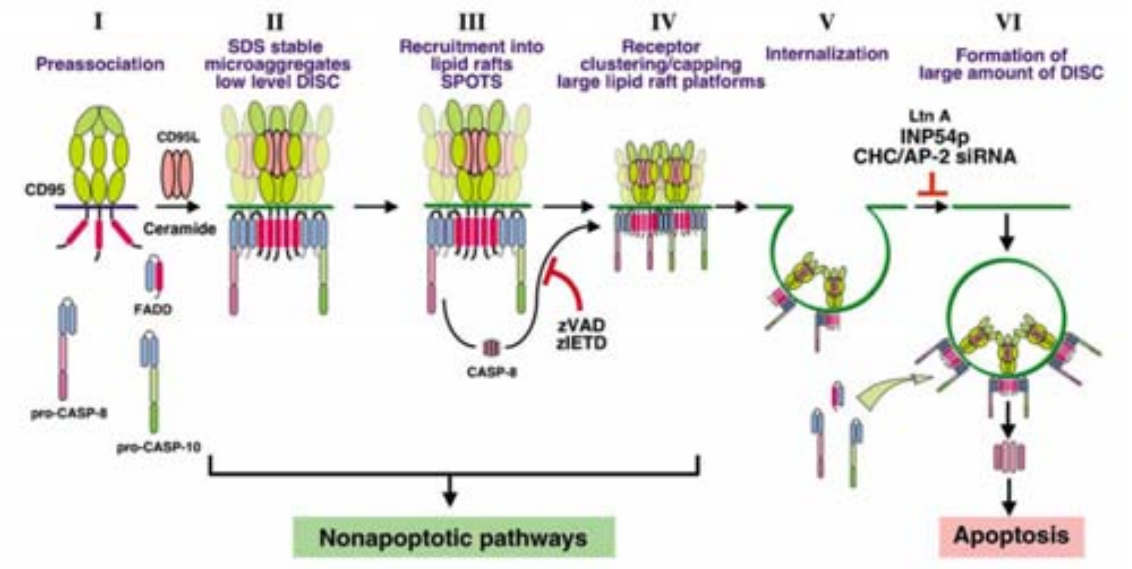

Figure I13. Early CD95 signaling. Upon ligand binding, receptor clustering aggregation occurs in lipid rafts. Internalization of the clustered receptors by clathrin-dependent endocytosis is required for efficient DISC formation and apoptosis induction. (Adapted from Lee et al, 2006)

Hepatocytes from XIAP deficient mice also displayed more sensitivity towards CD95-induced apoptosis as compared to wild type, but that does not happen in thymocytes, indicating that XIAP is a critical attenuator in type II cells. In accordance, pre-treatment with Smac mimetics on the wild type mice also sensitizes its hepatocytes, and those from Bid deficient mice, which are resistant to CD95-induced apoptosis, can be sensitized in the same manner. All in all, this data highlights the importance of XIAP in regulating CD95-induced apoptosis in type II cells, and predicts that the endogenous IAP antagonists SMAC and/or HTRA2 may have critical roles in this process.

In summary, it is still unclear why CD95 activates different apoptotic pathways in different cell types. The results accumulated to this point suggest that various factors contribute to whether one signaling pathway is favored above the other, like the extent of CD95 aggregation/internalization, or the ratio between activated effector caspases and the caspase inhibitor XIAP.

Involvement of calcium in CD95 signaling

CD95 activation has been shown to induce a rise in intracellular calcium, although its role in Fas-apoptosis induction is still not well understood [189]. Studies in Jurkat cells, which behave like type II cells since Bcl-xL

overexpression abrogates Fas-induced apoptosis, have shown a dependence on that rise of intracellular calcium for DNA degradation after CD95-induced apoptosis, but not for caspase activation [190]. Thus, some morphological features of apoptosis could depend on calcium signaling. Further investigations demonstrated that the rise in cytosolic calcium after CD95 engagement was dependant on depletion of intracellular stores [114]. Specifically, CD95 triggering induces PLCγ activation and formation of IP_3, which in turn stimulates IP_3R receptors and diffusion of ER calcium to the cytosol. Activation of PLCγ was also shown necessary for cytochrome c release from mitochondria. After its release from mitochondria, cytochrome c can bind to IP_3R and further stimulate its conductance to calcium, leading to an amplification feedback loop for calcium mobilization from ER.

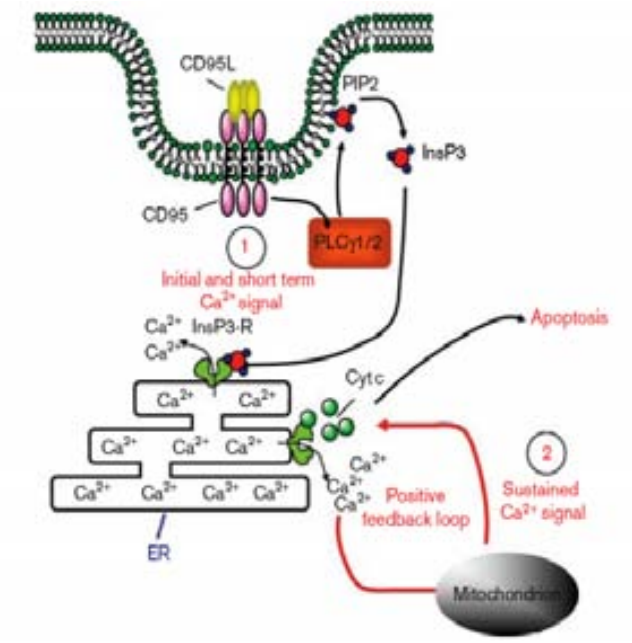

Figure I14. Calcium involvement in CD95 signaling. CD95 stimulation induces a cytolosic rise in calcium concentration. CD95L ligation activates PLCγ, which generates IP_3 that binds and activates IP_3R to generate a first calcium wave. After cytochrome c release from mitochondria, it is able to bind to IP_3R and further promote calcium release from ER, leading to a second, more prolonged wave of cytosolic calcium. (Adapted from Vacher et al, 2011)

Further studies of this pathway led to the characterization of the ER protein BAP31. It forms a complex in the ER membrane bound to caspase-8 and Bcl-xL. After CD95 stimulation, caspase-8 cleaves BAP31, releasing a p20 BAP31 form that induces cytochrome c release from mitochondria [120]. Inhibition of this processing by point mutations in BAP31 or caspase-8 inhibition inhibits cell death, while overexpression of the p20 form causes apoptosis. The pro-apoptotic function of BAP31 has been linked to calcium efflux from ER to the mitochondria [122]. BAP31 has been shown to interact with Fis1 in the ER-mitochondria microdomains and mediate the connections between these two organelles.

Apart from induction of apoptosis, calcium mobilization after CD95 activation has been linked to other non-apoptotic functions of CD95, like T-cell activation. Thus, calcium signaling in the CD95/CD95L system has not been explored in detail, and questions remain still unanswered.

Non-apoptotic functions of CD95

Apart from its apoptotic functions, there are growing amount of evidences that indicate that CD95 has non-apoptotic functions as well. These functions would include control of maturation and proliferation of T-cells, promotion of liver regeneration and neurite outgrowth, among many others [191, 192].

The first suggestions that CD95 may have non-apoptotic functions came by observations of the CD95L forms used in experimentation. The reagents used normally to study CD95 apoptotic function are compounds designed for its ability to trigger apoptosis, and are not the exact molecule that is expressed physiologically. It has been shown that the soluble form of mCD95L, sCD95L does not trigger apoptosis, and thus it should promote other signaling pathways [193, 194].

In T cells, loss of CD95 does not cause an ALPS-like syndrome, as should be expected considering that it would cause a deregulation in apoptosis [195]. Now it has been demonstrated that CD95 regulates activation and proliferation of T-cells during acute immune responses [196]. Specifically, it can either inhibit the action of T-cell receptor (TCR) or stimulate anti-CD3 activity by regulating the opening of calcium channels in the membrane [197], although it's likely that other mechanisms are also involved.

In liver cells, which have the higher constitutive expression of CD95, injection of agonistic antibody causes massive apoptosis. Surprisingly, it was reported that after partial hepatectomy, injection of agonistic antibody had actually the opposite effect, it accelerated the rate of liver regeneration [198]. Liver damage is associated to the activation of anti-apoptotic pathways like NF-κB or Akt, so is likely that this may help switch from apoptotic to non-apoptotic signaling after CD95 stimulation [199].

CD95 is also widely expressed in the central nervous system [200, 201]. Neurons express CD95, although they are not sensitive to CD95 mediated apoptosis. Studies *in vivo* have shown that, after sciatic nerve crush injury, injection of CD95 agonistic antibodies accelerated the rate of recovery [202]. In the same context, it was demonstrated that CD95 ligation induced neurite outgrowth in sensory neurons through activation of MAP kinases [203]. Thus, there is data suggesting a physiologic role for CD95 in regulating neuron development, differentiation and regeneration in the CNS.

Still, little is known about the molecular links that connect CD95 stimulation with activation of pro-survival pathways like ERK or NF-κB. The type of ligand used, as well as the cellular type, seems to be relevant. Another possible switch between pro-apoptotic and pro-survival functions could be differential expression of CD95 forms by alternative splicing of the Fas mRNA [204]. Morever, several post-translational modifications on CD95 that regulate its ability to be internalized have been documented. In this context, it has been shown that inhibition of CD95 internalization after CD95L binding may promote alternative pro-survival signaling pathways [182]. The involvement of other molecules, such as FADD, caspase-8 or c-FLIP is also well documented [205]. Several studies show that all three proteins are important for the regulation of survival and activation of T-cells, and FADD and caspase-8 are required for liver regeneration after injury [168, 206]. So, there are an important number of reports indicating that the CD95/CD95L system may be more like TNF/TNFR1, where apoptotic and non-apoptotic functions coexist.

Death receptor antagonists

Death receptors antagonists are defined as molecules that interfere with the apoptotic signaling cascade generated after engagement of the death receptors, conferring the cells protection and survival against these death-inducing stimuli. Various death receptors have shown to be relevant in the regulation of alternative signaling pathways triggered by ligand binding to its receptor.

There are several death receptors antagonists identified to date. The list includes cFLIPs, FAIM, Lifeguard, A20, SODD, PEA-15, SUMO-1, BAR, FAP-1 or TOSO

FLIP

FLIP was initially identified in a genome screening searching for DED containing proteins that would interact with the DISC [207]. In addition to FADD and caspase-8, another group of viral and cellular DED-containing proteins are also an integral part of the DISC, the FLICE-like inhibitory proteins (FLIPs) [208, 209].

The cellular version of FLIP has three isoforms, two short ones ($FLIP_S$ and $FLIP_R$), which contain two DED domains in its *N*-ter part and a short *C*-ter domain, and the longer isoform ($FLIP_L$), which in addition to the two DED *N*-ter domains, contains a catalytically inactive protease domain at its *C*-ter, with an overall structure highly homologous to procaspase-8 and procaspase-10 [210].

Viral FLIPs (vFLIPs) and $FLIP_S$ have been described as caspase-8 inhibitors. $FLIP_L$, on the other hand, has been described to have both pro-apoptotic and anti-apoptotic functions. The mechanism of apoptosis inhibition of vFLIPs and $FLIP_S$ relies on its homotypic interactions with FADD and caspase-8 through its DED. They can form heterodimers with caspase-8, preventing its processing and activation [211, 212].

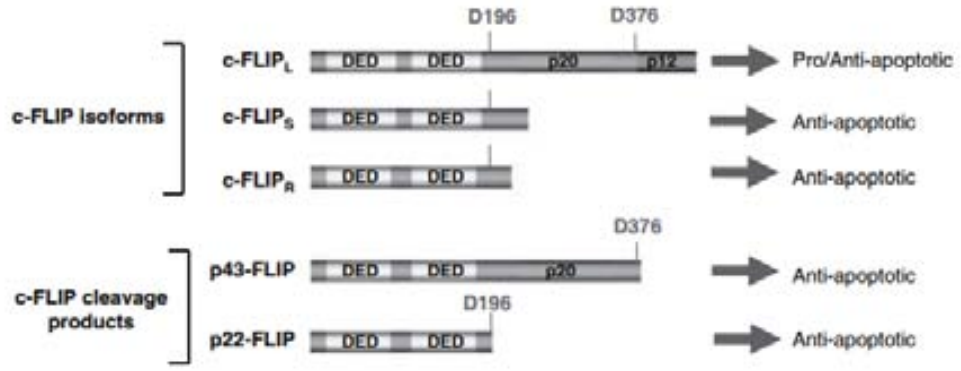

Figure I15. Schematic structure of human cFLIPs and their cleavage products. DED represent the death effector domains, while p20 and p12 are the caspase-like subunits. Putative function of each isoform is also shown. (Adapted from Lavrik et al, 2012)

$FLIP_L$ can also exert both anti-apoptotic and pro-apoptotic functions. Studies on this topic supported the notion that $FLIP_L$ could either promote or inhibit apoptosis depending on its expression levels [211]. Although the anti-apoptotic role of $FLIP_L$ was demonstrated by the observation that fibroblasts from knock-out mice for $FLIP_L$ were more sensitive to DR-induced apoptosis [213], other reports showed in certain models that $FLIP_L$ overexpression induced apoptosis [214]. The characterization of a heterodimer formed by an active form of caspase-8 and $FLIP_L$ added more controversy [215]. A recent study show that $FLIP_L$ only exerts pro-apoptotic functionality when overexpressed and in presence of strong DR stimulation [216]. On the other hand, when expressed at lower levels, closer to the physiological, both $FLIP_S$ and $FLIP_L$ have an anti-apoptotic effect [217].

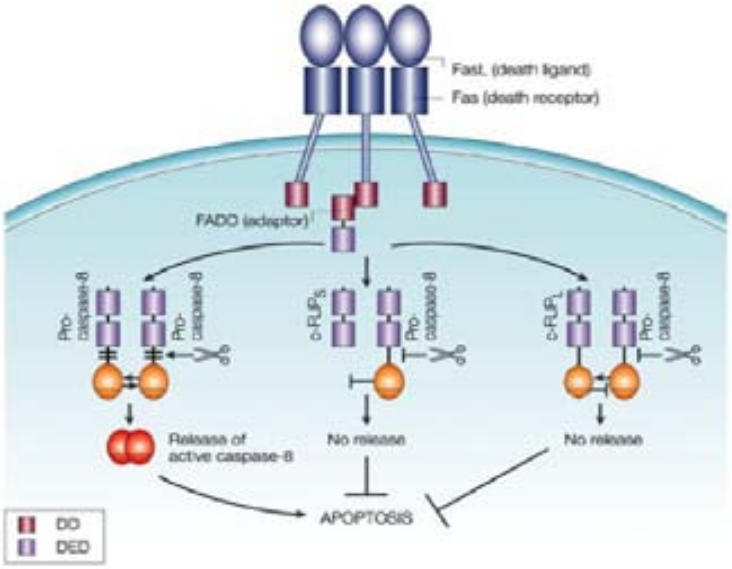

Figure I16. Differential mechanism of caspase-8 inhibition by $FLIP_S$ and $FLIP_L$. $FLIP_S$ inhibits caspase-8 activity by preventing its dimerization. $FLIP_L$ heterodimerizes with caspase-8 and permits the first cleavage, but not the second, rendering an inactive heterodimer. It is thought that that heterodimer and the p10 fragments released after the first cleavage may have roles in another signaling pathways,. (Adapted from Thome et al, 2001)

Caspase-8 activation occurs in two steps. After dimerization, there is first an autocatalytic processing of the procaspase-8 that renders the p43 fragment. Then, a transcatalytic cleavage follows and the p18 and p10 fragments formed are able to form the active heterotetramer. $FLIP_L$ and caspase-8 form heterodimers at the DISC that are only able to undergo the first step of the

caspase-8 activation, but unable to perform the second step of cleaveage, thus inhibiting the apoptotic signal [207, 211, 218].

The pro-apoptotic effects of $FLIP_L$ have been linked to regulation of NF-κB and ERK signaling pathways. It has been shown that $FLIP_L$ overexpression in T-cells promotes ERK and NF-κB activation after TCR stimulation [219, 220]. Moreover, its interaction with other proteins necessary for the activation of these signaling pathways has been demonstrated, like Raf-1, TRAF1, TRAF2 or RIP1. Additional evidence also shows that the initial caspase-8 cleavage of $FLIP_L$ generates a truncated form that has more affinity for interaction with TRAF2, so that highlights a potential mechanism of regulation [221].

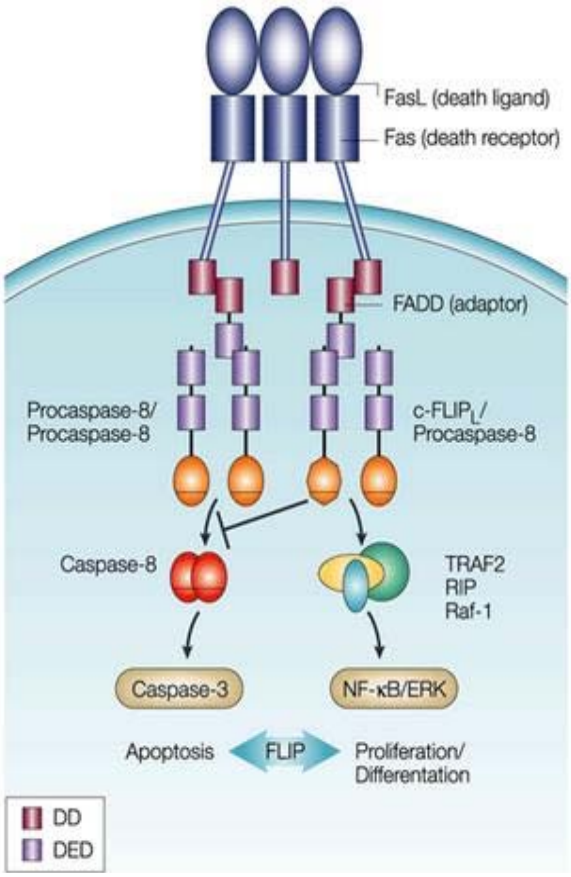

Figure I17. Regulation of death receptor-induced gene expression by FLIP.$FLIP_L$ inhibits caspase-8 at the DISC. It can also recruit molecules like TRAF2, RIP1 or Raf-1 to promote activation of pro-survival pathways like NF-κB or Erk, thus controlling expression of genes regulated by these pathways.(Adapted from Thome et al, 2001)

In the nervous system, $FLIP_L$ has been shown to be cytoprotective in models of ischemia *in vivo* [222]. Apart from its roles in death receptor signaling, results from our group highlight another functions of cFLIPs. In this context, we demonstrated that $FLIP_L$ can interact with Trk receptor and is essential for neurite outgrowth mediated by neurotrophins [223]. The paper shows that $FLIP_L$ is expressed in different regions of the mouse embrionary nervous system, although its expression decreases at later stages. Overexpression of $FLIP_L$ enhances neurotrophin-mediated neurite outgrowth, while RNAi downregulation significantly reduces it.

More recently, we also described that $FLIP_L$ is required for MAPK/Erk activation after TNFα treatment in cortical neurons [157]. TNFα induces the

activation of NF-κB, which induces $FLIP_L$ expression. Increased expression levels of $FLIP_L$ are essential for the activation of the MAPK/Erk pathway, since downregulation of $FLIP_L$ expression or inhibition of NF-κB block the MAPK/Erk pathway. Therefore, a novel essential role of $FLIP_L$ in the activation of MAPK/Erk pathway was described.

FAIM

Fas Apoptotic Inhibitory Molecule, FAIM, was discovered using a differential display strategy in B cells to detect cDNAs that rendered resistance to CD95-induced apoptosis and that were absent in cells sensitive to Fas [224]. FAIM was cloned from two different sources, thymocytes and the brain. The study of the genomic organization of FAIM described two putative transcription initiation points, indicating that two isoforms are generated. Indeed, there is one shorter, $FAIM_S$, that is expressed ubiquitously, and one with 22 more aminoacids, termed $FAIM_L$, which expression is restricted to the central nervous system [225].

Its anti-apoptotic role was further assessed after the generation of FAIM deficient mice. These mice are viable, but showed a more sensitive response to CD95-induced apoptosis in the thymocytes and hepatocytes [226]. Moreover, it was found that caspase-8 and caspase-3 activity was enhanced in FAIM deficient mice, caspase-8 and Fas association was increased, and a downregulation in $FLIP_L$ levels was also reported. Thus, the mechanism of action of FAIM was explained trough inhibition of caspase-8 and regulating the expression of $FLIP_L$ to exert its anti-apoptotic functions.

Results from our group have previously shown that $FAIM_L$ is expressed in neurons during development, and that endogenous $FAIM_L$ is able to protect mice cortical neurons from DR-induced apoptosis, both by CD95 and by TNFR1 stimulation. It was also shown that $FAIM_L$ binds to Fas and inhibits caspase-8 activity [227].

Recent work in our lab unraveled a novel mechanism by which $FAIM_L$ could exert its anti-apoptotic effects [228]. At first, it was shown that $FAIM_L$ could only protect type II cells from Fas-induced apoptosis, but not type I cells. $FAIM_L$ can

also interact with XIAP and inhibit its auto-ubiquitinylation, protecting it from proteasome-mediated degradation. Endogenous levels of XIAP are required for $FAIM_L$ to exert its anti-apoptotic functions, moreover, disruption of XIAP and $FAIM_L$ interaction by mutagenesis or deletion of interacting domains also abrogates $FAIM_L$ protection from CD95-induced apoptosis. Thus, we described a novel pathway by which XIAP stability is regulated by a DR antagonist.

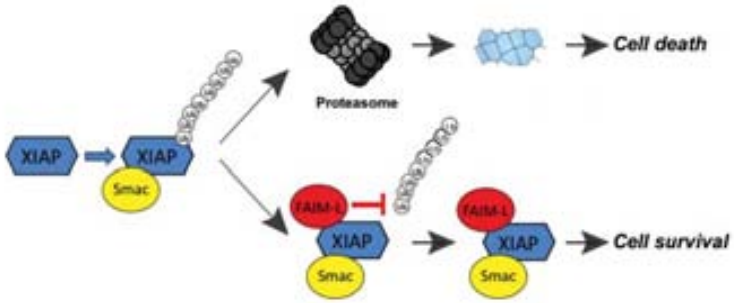

Figure I18. Mechanism of regulation of XIAP stabilty by $FAIM_L$. $FAIM_L$ directly binds to and stabilizes XIAP by preventing its auto-ubyquitilation. When XIAP is ubyquitilated, it is targeted for the proteasome for degradation. $FAIM_L$ mediated inhibition of its autoubyquitilation prevents its degradation and increases XIAP levels, thus promoting cell survival. (Adapted from Moubarak et al, 2013)

A part from its anti-apoptotic effects, we also described its role in regulating NGF-induced neurite outgrowth in the neuronal model PC-12 cell line. Specifically, it was demonstrated that $FAIM_S$, but not $FAIM_L$, is required by neuritogenesis induced by NGF, and that this process is dependent on ERK and NF-κB signaling.

LFG

Lifeguard (LFG), also called Fas Apoptotic Inhibitory Molecule (FAIM2) or Neural Membrane Protein 35 (NMP35), is a Fas receptor antagonist that was discovered in a screening searching for genes that conferred resistance to Fas-induced cell death, in a pool of cells transfected with a cDNA library [229]. In the same work it is described that LFG overexpression confers resistance only to CD95-induced apoptosis, but not to TNFα or other stimuli like staurosporine.

Sequence analysis of LFG revealed that it belongs to the TMBIM family proteins [230]. It contains six members in humans identified to date; TMBIM-1 or GAAP,

TMBIM2 or LFG, TMBIM3 or GRINA, TMBIM4 or GAAP, TMBIM5 or GHITM, and TMBIM6 or BI-1. This family is characterized by a putative conserved structure of seven transmembrane domains with a *N*-ter cytosolic domain. However, it has been proved that both GAAP and BI-1 have six transmembrane domains instead of seven [231]. Among them, BI-1 is the most studied member. As explained before, it's an ER protein that has anti-apoptotic effects in the intrinsic pathway by inhibiting the action of Bax and Bak. All of the members of the family have roles in regulation of apoptosis, and most of them have cytoprotective effects when overexpressed in various modes of apoptosis.

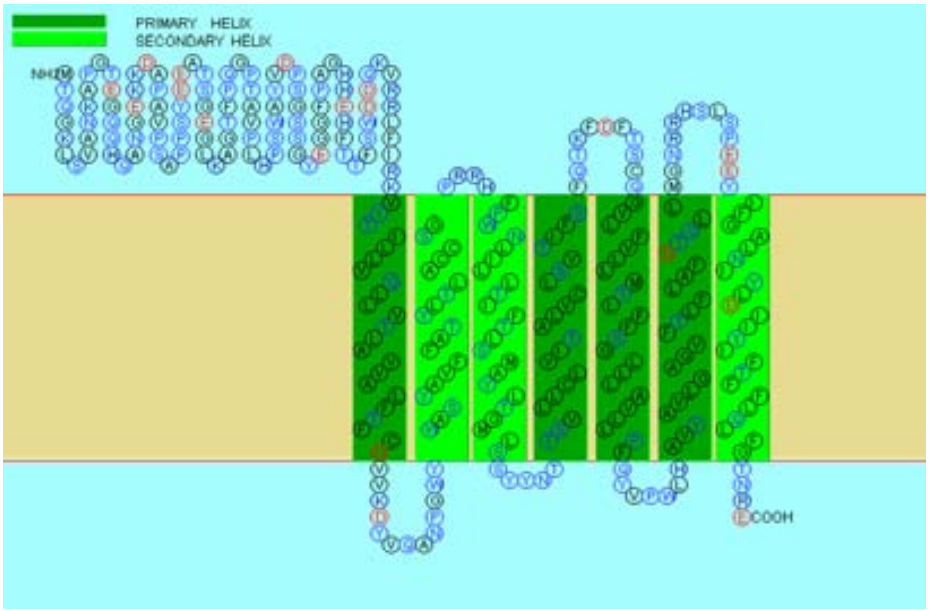

Figure I19. Schematic representation of putative structure of LFG. Using structure prediction software like SMART or TOPPRED, LFG structure was predicted. Presumably, LFG has seven transmembrane domains, and a *N*-ter tail that is predicted to be exposed at the cytosol.

By Northern blot analysis, mRNA transcripts of LFG can be widely found around the organism, although there is a much higher expression in neurons, especially at the hippocampus [232]. In fact, later reports confirmed that LFG is expressed at the postsynapstic sites and dendrites of several neuronal types [233]. It was reported LFG colocalization with the glutamate receptor Glu2 and synaptophysin, and the authors of the study suggested that it is localized at the presynpatic membrane, and hypothesize that apart from its anti-apoptotic functions, LFG may have other role in synapses.

Subsequent studies on LFG protein further confirmed its Fas-antagonist role. Several groups confirmed its anti-apoptotic effects in different cell lines, such as Jurkat, SH-SY5Y, or human keratinocytes [234, 235]. Its expression and function in primary neuronal cultures was also studied, and it was found that cortical neurons, which are not sensitive to CD95L, express high levels of LFG. This expression is dependent on the PI3K/Akt pathway, as chemical inhibition of the pathway downregulated LFG expression and sensitized neuronal cultures to CD95-induced apoptosis [236]. Moreover, granular neurons, which are sensitive to FasL, can be protected by overexpression of LFG. Additionally, it has been demonstrated that LFG expression is required for survival and maintenance of Purkinje and granular cells [237]. Thus, LFG inhibitory role of CD95-induced apoptosis in neurons was confirmed.

In the other hand, LFG has been found to be upregulated in several types of tumors, and its expression protects these tumors from Fas-mediated cell death. This transactivation of LFG gene is associated with the activation of the Akt-/LEF-1 in human breast cancer cells. It was demonstrated that LFG also confers resistance in models of ischemia. Mice deficient for LFG were found to be more sensitive to ischemia *in vivo*, as measured by infarct volume and caspase-8 and -3 activation. Cultured neurons from these mice also presented more cell death when subjected to oxigen-glucose deprivation (OGD), as compared to wild type. Although not analyzed in detail, the authors attribute its protective effects to its Fas-inhibitory function, since they find that CD95 expression is upregulated after brain ischemia, and there are other reports describing implications of Fas in ischemia [238]. Interestingly, it has been shown that LFG downregulation in these tumors sensitizes cells to perifosine, a PI3K/Akt inhibitor used in chemotherapy that kills cells by engaging the intrinsic pathway [239]. So, there are evidences that LFG may have anti-apoptotic effects in other modes of cell death apart from CD95-induced apoptosis

The mechanism of LFG protection from Fas-induced cell death is still unknown. LFG has been shown to interact directly with CD95, but this interaction doesn't affect the ligand binding to the receptor or DISC formation, so is unlikely that LFG may exert its anti-apoptotic effects at the receptor level [229]. However, caspase-8 is inhibited when LFG is overexpressed, so LFG effects should be

upstream of caspase activation. There is still a lack of understanding on how LFG confers resistance to CD95-induced cell death.

Apart from its anti-apoptotic functions, recently it has been shown that LFG also has a role in axon growth. LFG can be synthetised locally in the axons, where it supports axon growth. LFG mRNA 3'UTR sequence drives its localization to the axons in granular neurons, and deletion of this sequence restricts its localization to the cell body and impairs axon growth [240]. Thus, is likely that LFG may also have a role in neural development. In this regard, another recent study shows that LFG expression is regulated during the course of bacterial meningitis, and although its knockdown sensitized to cell death in the acute phase of the bacterial meningitis, LFG deficient mice had a better hippocampal neurogenesis after antibiotic treatment [241]. This is surprising, and highlights a possible differential modulation of LFG expression during the course of pathological situations, that may aid LFG to regulate neuronal plasticity in a time-dependent manner.

More recently, in our lab we have discovered a new role for LFG in tumors. LFG is downregulated in the most aggressive forms of neuroblastoma, a solid tumor very common in infants [242]. LFG downregulation enhances cell migration, increases sphere formation and reduces adhesion. All of these factors may aid the tumor to increase its aggressiveness by increasing its potential metastatic activity. Furthermore, we report that LFG expression is repressed by MYCN at the transcriptional level, by regulating histone deacetylation and LFG DNA promoter methylation. All in all, LFG non-apoptotic roles are began to be described and it's likely that, like other DR antagonists such as FLIP, other roles in signaling may arise apart from its apoptosis inhibitory effects.

Ubiquitination regulation of apoptosis

Ubiquitin (Ub), is an 8-kDa protein, which can be covalently attached to a target protein. It can bind either as a single moiety or as a polyubiquitin chain. The covalent attachment of Ub to a protein influences diverse cellular processes, such as protein degradation, endocytic trafficking, DNA repair, and many others.

Ub-conjugation requires the action of three different types of enzymes; Ub-activating enzymes (E1), Ub-conjugating enzymes (E2) and Ub-protein ligases (E3) [243]. E1 activates an ubiquitin molecule by ATP-dependent formation of a thioester. E2 receives the Ub from E1 and interacts with E3 to transfer Ub to the target molecule. E2 usually determines the type of ubiquitin linkage, whereas E3 ligases provide substrate recognition specificity. The different types of Ub-linkage have diverse effects. K48-linked chains usually promote degradation of the target protein through recognition by the proteasome, thus regulating the steady-state of proteins. MonoUb and K63-linked chains are implicated in non-degradative processes. In this context, monoUb is often linked to regulation of cellular localization, whereas K63-linked chains have normally a scaffolding role in signal transduction events [244].

Over the past years, E3 Ub-ligases and deubiquitilating enzymes have emerged as key regulators of several cellular processes, including apoptosis. Protein levels of many pro- and anti-apoptotic molecules are controlled by Ub-dependent degradation. However, non-degradative ubiquitylation events also have important roles in the regulation of apoptosis.

The numerous E3 enzymes can be categorized into two distinct groups, based on their binding features: the homologous to E6-AP carboxyl terminus (HECT) and the RING groups [245]. The RING family of E3 Ub-ligases is the largest of the two. The RING domain is distinguished as a motif consisting of eight highly conserved cysteine or histidine residues, that bind two zinc ions, and serves as a scaffold that brings E2 and substrate together. The HECT domain of E3 Ub-ligases, is located at the *C*-ter of these enzymes, whereas their *N*-ter domains are diverse and mediate substrate targeting. The HECT domain consists of an *N*-ter region that interacts with the E2 and a *C*-ter region that contains the active-site cysteine that forms the thioester with ubiquitin and the substrate.

As we have discussed previously, the most prominent E3 ligases implicated in the modulation of apoptosis are the IAPs. XIAP is the only IAP that is able to directly inhibit caspase activation by direct interaction. However, some reports have challenged this view, since it has been found that the RING domain, essential for the E3 ligase activity of XIAP, was needed to maintain its anti-

apoptotic activity in vivo [246]. cIAPs modulate the activity of caspases by ubiquitylation, although the exact consequences of Ub attachment on caspases is not fully understood.

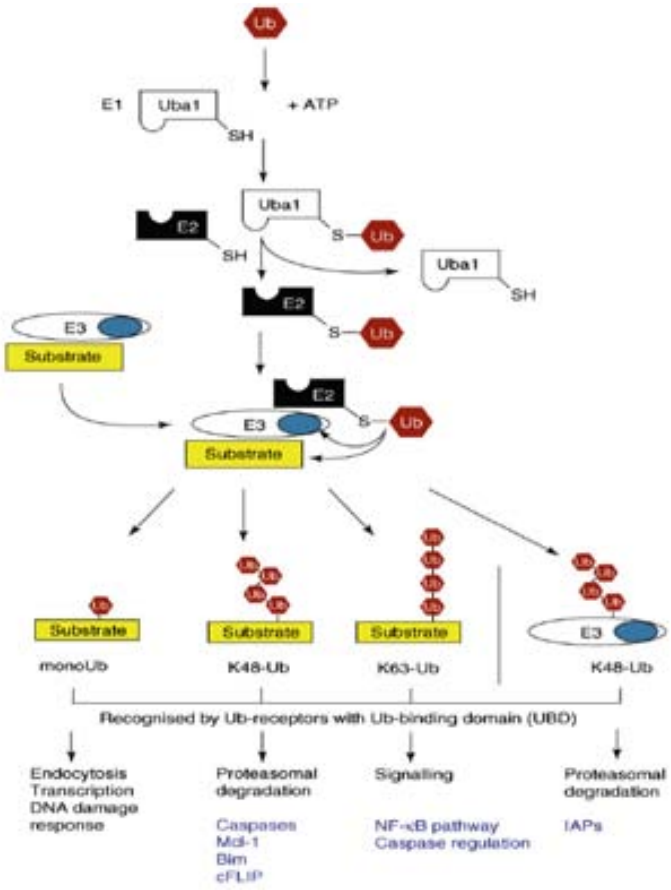

Figure I20. Conjugation of Ub to target proteins is mediated by E1, E2 and E3 enzymes. The Ub-activating enzyme E1 (ubiquitin-like modifier activating enzyme 1 [Uba-1] in mammals) activates Ub through ATP-dependent thiolester formation. The Ub-conjugating enzyme (E2) receives Ub from the E1 and interacts with the E3 to transfer Ub to the target molecule. The E2 usually determines the type of Ub chain linkage, whereas E3 ligases provide substrate recognition. Ub chains are synthesized by multiple rounds of Ub attachments, using internal lysine residues of Ub as conjugation sites. Depending on the type of modification, protein ubiquitylation has distinct cellular outcomes. Ub E3 ligases can also be subject to auto-modification to regulate their levels. (Adapted from Meier et al. 2009)

Studies in Drosophila have shed light to the question on how ubiquitylation modulate caspase activity. DRONC, the Drosophila homologue of caspase-9, is polyubiquitinated by DIAP1 and targeted for proteasomal degradation, as increased levels of DRONC can be found in DIAP1 mutant cells [247, 248]. Non-degradative ubiquitylation of caspases also plays a prominent role in its regulation. Caspase -3 has been found to be polyubiquitylated by XIAP, and cIAP2 also monoubiquitylates caspase -3 and -7 in vitro. However, the functional implications of these modifications remain unexplained. Studies in Drosophila found that drICE, the homologue for caspase -3 and -7, is

polyubiquitylated by non-degradative K63-linked chains, reducing its proteolytic activity. This is caused because Ub chains sterically occlude the catalytic pocket of the caspase, thereby interfering with substrate entry [249]. This is an example on how the mere binding of Ub to a target molecule modulates its activity. drICE levels are also regulated by degradative ubiquitilation, caused by DIAP2 E3 ligase activity.

IAPs also modulate cell survival by controlling the signalling events that lead to activation of the NF-κB pathway [160]. As mentioned in previous sections, cIAPs 1 and 2 facilitates TNFR1 induced signalling to NF-κB, leading to expression of pro-survival genes. Upon receptor stimulation, cIAPs are recruited to TNFR1 signalling complex by interaction with TRAF1 and TRAF2, and along TRADD and RIP1, form the complex I. There, they mediate the K63-linked polyubiquitilation of RIP1. This polyUb chains serve as a docking point for the recruitment of factors that will lead ultimately to induction of NF-κB signalling. cIAPs are also crucial in the prevention of TNF-α mediated apoptosis. Loss of cIAP1 renders cells more sensitive to TNF-α because complex-II formation occurs more efficiently in the abscence of cIAP1. Likewise, depletion of IAPs can also be achieved by treatment with SMAC-mimetics. These compounds cause rapid auto-ubiquitilation of cIAPs 1 and 2, leading to its proteosomal degradation, and inducing apoptosis in a wide variety of cells.

Activity of the proteins of the Bcl-2 family is also tightly regulated by ubiquitylation. In healthy cells, Bcl-2 levels are high, while levels of BH3-only proteins are low. Upon exposure to cellular stress this balance shifts in favour of BH3-only proteins, with specific type of stresses activate distinct sets of BH3-only proteins. During ER stress, the BH3-only protein Bim is activated. Bim levels are maintained in check during normal conditions by a mechanism dependent on Bim phosporilation, that renders the protein marked to ubiquitylation and proteasomal degradation. After induction of ER stress, Bim levels increase via the combination of transcriptional events and dephosphorylation by the phospatase PP2A, which results in Bim stabilization. Moreover, Bim-deficient cells reconstituted with a non-ubiquitylatable mutant form of Bim die, whereas wt Bim reconstitution is well tolerated [250]. Thus, Ub-dependent regulation of Bim is very important. In a more classic way, Mcl-1

levels are also tightly regulated by ubiquitlyation. Mcl-1 has a low half-life, and can be targeted by E3 ligases to degradative ubiquitylation. Phosphorilation of Mcl-1 at conserved residues influences protein stability by disturbing Mcl-1 interaction with E3 ligases, thus lowering its ubiquitylation [251].

Another level of regulation of apoptosis by ubiquitylation events can be found in DR antagonists. During TNF-α mediated apoptosis, $FLIP_L$ levels are down-regulated by activation of the E3 ligase Itch, which ubiquitylates $FLIP_L$ and targets it for proteasomal degradation [252]. Itch activation is mediated by JNK1 phosporylation, as mice deficient for JNK1 and Itch are resistant to TNF-α induced apoptosis and display higher $FLIP_L$ levels. Moreover, $FAIM_L$ stabilizes XIAP by direct interaction with its BIR2 domain and disruption of its auto-ubiquitylation [228]. In summary, ubiquitylation is an essential factor in the regulation of apoptosis, and unravelling how the ubiquitin-signal works is crucial for the understanding of many cellular processes.

OBJECTIVES

OBJECTIVES

Death receptors are known to induce a wide range of biological processes. Induction of apoptosis has been the most characterized so far. However, the effects of death receptor activation on proliferation, survival and differentiation have gathered a large amount of interest in the last years.

Death receptor antagonists are known to mediate the effects of death receptors. They have been initially named for its ability to antagonize the apoptotic activation by death receptors. However, recent reports also link their function to many other processes, such as neuritogenesis, in the case for $FLIP_L$, or synapse formation, in the case of LFG.

Understanding the mechanisms by which death receptor antagonists exert its function may help to understand how the different effects of death receptor activation are modulated. Moreover, it will open the gates to new therapeutic approaches and contribute to a better understanding of cellular processes.

Among the death receptors, LFG is one of the less studied. It is characterized by being able to inhibit Fas-induced apoptosis, without effect on TNF or TRAIL-induced apoptosis. In the last years, the role of this protein as a critical regulator of apoptosis in the nervous system has been characterized. However, a mechanism of action is still lacking. In this study we tried to elucidate the mechanism by which LFG protects from Fas-induced apoptosis, by exploring two facts we have previously identified; its ubiquitination and its subcellular localization. In addition, I'll also explain in this work the generation of transgenic mice for $FAIM_S$, $FAIM_L$ and LFG.

The objectives of this study can thus be defined in this manner:

1 – Create conditional knock-in mice for $FAIM_S$, $FAIM_L$ and LFG, with conditional adult neuronal specific expression pattern.

2 – Study LFG ubiquitination, trying to identify a role for the Ub-attachment and its localization inside LFG sequence.

3 – Study LFG subcellular localization and the mechanism involved in its antiapoptotic effect.

MATHERIALS AND METHODS

Materials and methods

Cell lines

HEK293 cell line

HEK 293 cells were generated in the early 70s by transformation of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA. Despite that the cells originated from cultured kidney cells, embryonic kidney cultures may contain small numbers of almost all cell types of the body, including neural crest cells, neurons and glia. In fact, it has been shown that HEK293 have many properties of immature neurons, suggesting that the adenovirus was taken up and transformed a neuronal lineage cell in the original kidney culture.

HEK293 cells are very easy to work with, and are normally used in experiments in which the behavior of the cell itself is not of interest. Usually these experiments involve overexpression of a certain gene to analyze the expressed protein, so the cell is used as a mere test tube with a membrane.

Regarding its behaviour when treated with FasL, HEK293 cells can be classified as type I cells. They were cultured in 100mm culture dishes in DMEM medium supplemented with 10% FBS, 20U/ml penicillin and 20µg/ml streptomycin.

HEK293T cell line

HEK293T cell line is derived from HEK293, transformed with E1A adenoviral gene and overexpressing the T antigen of SV40, allowing for the episomal replication of reporter plasmids carrying SV40 promoter. Thus, HEK293T cell line is a good tool for the overexpression of proteins and to obtain good amounts of secreted proteins. In this work, they are used for lentiviral production, cytokine production (Fc-FasL or LZ-FasL), or for co-inmunoprecipitation experiments.

It's worth saying that HEK293T does not express FasR, and thus are insensitive to FasL treatment. They were cultured in 100mm culture dishes in DMEM medium supplemented with 10% FBS, 20U/ml penicillin and 20µg/ml streptomycin.

HeLa cell line

HeLa cell line was derived from cervical cancer cells taken on February 8, 1951, from Henrietta Lacks, a patient who eventually died of her cancer. It is the oldest and most commonly used human cell line. They have been used in a vast variety of research fields, ranging from vaccine testing, to virus infection and apoptosis.

HeLa cells are sensitive to Fas induced apoptosis, and have been used to study its signaling. Since it has been proved that Bcl-xL overexpression renders them resistant to FasL induced apoptosis, they behave like type II cells in this regard. They were cultured in 100mm culture

dishes in DMEM medium supplemented with 10% FBS, 20U/ml penicillin and 20µg/ml streptomycin.

SK-N-AS cell line

SK-N-AS cell line is derived from a female patient with neuroblastoma from the metastasis of the bone marrow. They are used as a neuroblast model, and in *in vivo* experiments too since they form tumors in nude mice. Unlike other neuroblastoma derived cell lines, retinoic acid treatment doesn't differentiate them to a "neuron-like" morphology.

SK-N-AS are sensitive to FasL and behave like type II cells. They were cultured in 100mm culture dishes in DMEM medium supplemented with 10% FBS, 20U/ml penicillin and 20µg/ml streptomycin.

Mice cortical neurons

Female C57BL/6 mice were killed and manipulated following the experimental protocol approved by the Vall d'Hebron Institutional Review Board. Embryonic cerebral cortices were dissected from mouse embryos at day 16 (E16). Cells were counted and resuspended in DMEM with glutamine supplemented with 5% heat-inactivated FBS and 5% heat-inactivated fetal horse serum, 20U/ml penicillin, and 20µg/ml streptomycin. Cells were seeded in 25mg/ml poly-L-lysine-coated plates at a density of 1.6 × 105 cells/cm^2.

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

Cell transfection

Cationic liposoluble reagents

Liposomes can be used to deliver a molecular cargo such as DNA to a target cell. The lipids used to form the liposomes can be cationic, anionic, neutral, or a mixture of all, and the structure and charge of the lipids forming the liposome will dictate its characteristics. In the case of DNA, which has a polyanionic nature, cationic liposomes are favoured and typically used for gene delivery. Cells can engulf these liposomes-DNA complexes which results in gene transfection with high efficiency in most cell lines. The downsides of this technique are that cationic liposomes are unstable in the presence of serum and upon storage. They also show some degree of citotoxicity.

In this work, we used Lypofectamine 2000 (Invitrogen), which is a comercially available cationic liposoluble reagent. It has been optimized for the transfection of nucleic acids in a wide variety of eukaryotic cells. In this work, it has been used to transfect HeLa cells and HEK293 cells in transient overexpression experiments. For this, manufacturer's instructions were followed, but the ratio between Lypofectamine 2000 and DNA was optimized and we used half the quantity recommended by the manufacturer, both in DNA and Lypofectamine 2000. The day before transfection, cells were seeded at 90% confluency in OptiMem I Reduced Serum Medium. The

day of the transfection, the amounts of DNA and Lypofectamine were diluted separately in OptiMem Reduced Serum Medium. After 5min of incubation, DNA and Lypofectamine were mixed and incubated for 30min at room temperature. After this time, DNA-Lypofectamine 2000 complexes are formed and are added to the cell culture, drop-wise. After 4h of incubation, medium was changed with fresh culture medium to avoid toxicity. Normally, after 24h you can verify correct overexpression and cells can be used for experiments.

Lypofectamine 2000 was also used to transfect HEK293T cells to produce lentivirus, the details of the protocol will be discussed in the lentiviral production section.

Calcium phosphate method

This transfection method is based on the formation of DNA-containing soluble aggregates. DNA is mixed with $CaCl_2$ in a buffered saline phosphate solution, and the mixture is incubated at room temperature to form precipitates which are taken up by the cells by endocytosis of phagocytosis. This method is cheaper than cationic liposoluble reagents, but its efficiency is not so high and it's very cell-type dependant. Moreover, reagents integrity is critical, since small pH changes (±0,1) will compromise transfection efficiency.

In this work, this method was used to transect HEK293T cells for transient overexpression experiments. The reagents needed for transfection with the calcium phosphate method are as follows:

· DNA of interest

· MilliQ water (buffered with 2,5mM Hepes, pH 7.3)

· $CaCl_2$ 250mM

· Hank Buffered Saline Buffer 2X (280mM NaCl, 50mM Hepes, 0,5mM Na_2HPO_4)

Cells were seeded the day before transfection at 90% confluency in normal culture medium. DNA and $CaCl_2$ are diluted in water at the desired proportions. Formation of the precipitates is attained by adding the DNA-$CaCl_2$ mixture to the same volume of HBS 2X solution in a drop-wise manner while vortexing. After 30min of incubation at room temperature, the mixture is added to the cell culture. Medium is changed after 6h and replaced with fresh culture medium. Usually, after 24h high efficiency of overexpression is achieved and cells can be used for experiments.

Lentiviral transduction

Lentiviral vectors derived from the human immunodeficiency virus (HIV-1) have become major tools for gene delivery in mammalian cells. The advantageous feature of lentivirus vectors is the ability to mediate potent transduction and stable expression into dividing and non-dividing cells both in vitro and in vivo. Essential lentiviral (HIV-1) genes must be expressed in these cells to

allow the generation of lentiviral particles. These genes are usually expressed by several plasmids.

In our work, we used a method described by Naldini and colleagues at 1996. They used plasmids developed by Dr Trono, who designed three generations of plasmids. In each generation, biosafety was improved by dividing the genes of HIV-1 in different plasmids. The plasmids used in our work are from the second generation, suitables for the work *in vitro*, and are described as follow:

· pEIGW/pLVTHM: This plasmid allows for overexpression or down-regulation of gene expression. It contains the genetic material to be transferred to the target cell, flanked by *cis* elements necessary for reverse transcription and insertion in the genome. It also contains GFP to track succesful infection.

· psPAX2: This vector codifies for viral packaging proteins, under the control of CAG promoter, a very potent one. Among the proteins it codifies, DNA polymerase, reverse transcriptase and TAT binding protein are the most relevant.

· pM2G: Is the vector codyfing for the virus envelope. The viral protein comes for viral stomatitis virus, allowing for infection of a great variety of tissues and cell lines.

Lentivirus are generated in HEK293T cells. Cells are seeded at a density of 2,5 millions of cells per 100mm plates. Plasmids are transfected using Lypofectamine 2000, using 20µg of pEIGW/pLVTHM, 13µg of psPAX2 and 7µg of pM2G. When the medium is changed after transfection, cells are left producing virus for 48h. Then, the medium is harvested and centrifuged at 2500*g* for 5min, and the supernatant filtered through a 0,45µm filter, to eliminate cells and particles in suspension. This conditioned medium contains lentiviral particles, and can be aliquoted and stored at -80ºC. The title of the virus can be calculated by infecting HEK293T cells with different virus concentrations and calculating the percentage of infection.

Cell death assays

Hoechst 33258 bis-benzimide staining

Hoechst stains are part of a family of blue fluorescent dyes that stain DNA. These dyes are cell permeable and thus can penetrate cell membranes and can bind to DNA in live or fixed cells. The dyes bind to the minor groove in the structure of the double-stranded DNA, with preference for Adenine-Thymidine rich sequence regions. Once bound to DNA, it is excited by ultraviolet (UV) light (350nm) and emits light in the blue spectrum (450nm).

It is used as a death assay because allows for the visualization of the condensed chromatin and fragmented nuclei typical in apoptosis. Using ImageJ software, we counted both cells with fragmented nuclei and intact cells, and then calculated the percentatge of apoptotic cells.

For death assays, cells were fixed with 2% paraformaldehyde (PFA) for 30min at room temperature. Then, cells were stained with 0,05µg/ml of Hoechst 33258 and incubated for another 30min at room temperature. After staining, cells can be kept at 4ºC protected from light for up to 48h.

Caspase-3 activity assay

Caspase activation during apoptosis can be used as a biochemical hallmark of the cell status. Compunds have been developed that mimics the structure of caspase substrates, and are cleaved by caspases in the same way. When cleaved, these substrates are fluorescent. This allows for quantification of caspase activity, since the amplitude of the fluorescent signal measured will correlate with the state and amount of caspase activation. Ac-DEVD-AMC is a synthetic tetrapeptide fluorogenic substrate for caspase-3 and contains the amino acid sequence of the PARP cleavage site at Asp-216. The tetrapeptide substrate can be used to identify and quantify the caspase-3 activity in apoptotic cells.

After the treatment, cells are rinsed once with PBS and then lysed in caspase assay buffer (20mM Hepes/NaOH pH 7.2, 10% sucrose, 150mM NaCl, 10mM DTT, 5mM EDTA, 1% Nonidet P-40, 0,1% CHAPS, EDTA free 1X protease inhibitor cocktail (Roche)). Lysates are cleared by centrifugation at 16000*g* for 5min and the protein was quantified using the Bradford method. Assays were performed at least in triplicate using 20µg of protein and 20µM of the fluorogenic substrate. After 1h of incubation at 37ºC, plates were read in a fluorimeter using a 360nm for excitation filter and a 530nm emission filter.

Protein extraction

In order to study the cell state after a certain treatment or conditions, proteins must be extracted from the cell efficiently and without degradation or modifications, to ensure an accurate representation of the cell state prior to protein extraction. According to the uses we want for the protein extract, different methods can be used. Normally, harshest ones like SDS solubilization yield high efficiency but disturb interaction between proteins and desnaturalize them. In some applications, proteins interactions and structure must be preserved and thus milder methods are used.

After each experiment, cells were rinsed once with ice-cold phosphate saline buffer (PBS) pH 7.2, for immediate obtaining of cellular extracts. When the proteins were to be analyzed by Western Blot to check its expression, they were resuspensed in denaturing lysis buffer (2% SDS, 125nM Tris-HCl pH 6.8). Cell lysis is done at 95ºC during 10min, and then samples can be kept at -20ºC for further analysis.

In some cases, like in co-inmunoprecipitations experiments, a more soft lysis was needed since the interactions between proteins should be conserved, thus proteins needed to maintain its native conformation. In this case, non-ionic detergents were used, along with protease inhibitors

to impede degradation of the sample. Thus, after rinse the cells with PBS, they were resuspended in native lysis buffer (20mM Tris pH 7.4, 140mM NaCl, 10%glycerol, 2mM EDTA, 1mM EGTA, 1% Triton X-100, supplemented with 1XEDTA-free Complete protease inhibitor mixture (Roche)). Lysis was left for at least 30min on ice and then lysates were cleared by centrifugation at 16000*g* during 30min and the supernatants harvested. Protein concentration was quantified by a modified Lowry assay (DC protein assay, Bio-Rad). Supernatants could be kept storaged at -20ºC for further analysis.

Western Blot

The western blot is an analytical technique used to detect specific proteins in a sample of complexes mixtures of proteins. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein.

In our case, we used gels with a polyacrilamide matrix in the presence of SDS (SDS-PAGE), a method described by Laemmli in 1970. Samples were prepared mixing 10-40µg with Laemmli loading buffer (2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0,003% bromophenol-blue, 62,5mM Tris pH 6.8). They were incubated at 95ºC for 5min and then loaded onto polyacrilamide gels.

The gel used had two parts. The first one, called stacking gel, has 4% of acrylamide and it's prepared with the stacking buffer (125nM Tris-HCl pH 6.8). This first part allows for migration of all the proteins without separation to stack all the sample loaded onto the gel. The second part is the resolving gel, which has a variable percentatge of acrylamide depending on the size of the proteins you want to resolve. Larger proteins need lower acrylamide percentatges to be separated better, and smaller molecules need higher percentatge of acrylamide gels. In our case, 8-12% gels were used. The resolving gel is prepared with resolving buffer (375mM Tris-HCl pH 8.8), and the higher pH is important for SDS-protein complexes to have net negative charge and be able to migrate through the gel.

Electrophoresis is performed in a MINI-PROTEAN cuvette (Bio-Rad) at a constant intensity (25mA/gel) in running buffer (25mM Tris base, 192mM glycine, 0,1% SDS, pH 8.3).

Once all the proteins have migrated, they were transferred electrophoretically to the surface of a polyvinyl difluoride (PVDF) membrane. These membranes have to be activated prior using, by treating them with pure methanol and then rinsing with miliQ water several times to hydrate them. The method used for transference was the "submarine method", the polyacrilamide gel and the membrane were kept together with a cassete, covered in sponge and submerged in transfer buffer (48mM Tris-HCl, 39mM glycine, 20% methanol). The voltage of the transference is always constant at 100V and it's done during 90min.

After transference, the membrane has to be blocked with a solution of BSA or milk to avoid unspecific binding of the antibodies to the membrane. In our case, we used a solution of 5% dry milk in TBS-T buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 0,05% Tween-20). The membrane is incubated with the blocking solution for at least 1h at room temperature and then washed at least three times with TBS-T buffer. Then, primary antibodies are added at their aproppiate dilution and incubated either at 4ºC overnight or for 1h at room temperature.

Before incubating with the secondary antibody, membranes have to be washed with TBS-T buffer to eliminate the excess of unbound primary antibody. Secondary antibodies are conjugated to the horsedarish peroxidase enzyme (HRP), which can oxide a substrate that, when using hydrogen peroxide as the oxidizing agent, will yield a characteristic change that is detectable by spectrophotometric methods. In our case, detection was performed using the EZ-ECL (Biological Industries) kit, and membranes were exposed to SuperRX films (Fujifilm).

Antibody	Antigen MW	Source	Working dilution	Supplier
$FAIM_L$	24kDa	Rabbit	1:2000	own production
GFP	27kDa	Mouse	1:20000	Clontech
Ubiquitin	8kDa	Mouse	1:2000	Santa Cruz
FLAG	---	Mouse	1:40000	Sigma
HA	---	Rat	1:2000	Roche
Mcl-1	14kDa	Rabbit	1:2000	BD Pharmigen
Bcl-2	28kDa	Mouse	1:2000	Cell Signalling
LFG	35kDa	Rabbit	1:500	Santa Cruz
Bcl-xL	30kDa	Rabbit	1:2000	BD Transduction
Secondary Antibodies				
Ig Rabbit		Goat	1:20000	Sigma
Ig Mouse		Rabbit	1:5000	Sigma
Ig Rat		Goat	1:20000	Sigma

Table M1. Antibodies used in this study for Western Blot

Coinmunoprecipitation

Immunoprecipitation involves the use of an antibody for a specific protein to isolate that particular protein out of a solution containing many different proteins. This technique can be used to study intact protein complexes, and then it's called co-immunoprecipitation. It is used to analyze interactions between proteins utilizing a whole cell extract where proteins are present in their native conformation in a complex mixture of cellular components. It is critical to use non-denaturing conditions in order to maintain any interactions that occur.

In this work, the majority of co-inmunoprecipitation experiments were performed in HEK293T cells expressing the proteins we wanted to study fused with tags. Specifically, we used FLAG-tagged proteins as bait. After transfection and cell lysis, 1µg of total protein extract was incubated at 4ºC overnight with 20µl of ANTI-FLAG M2 affinity gel, which is a monoclonal antibody against FLAG covalently bound to agarose. After incubation, the agarose beads are pelleted by centrifugation and washed with lysis buffer for at least three times. Elution of the protein bound to the beads was done with 3XFLAG peptide. Incubation of the agarose beads with a solution of 5µg/ml 3XFLAG peptide in TBS induced the release of the FLAG-tagged target protein by competition with the 3XFLAG peptide. Then, supernatants can be harvested and Laemmli buffer added to analyze the eluate by Western Blot. Along with the eluate, normally a sample of the original protein extract must be analyzed as an input control.

We also performed co-inmunoprecipitation of overexpressed proteins with endogenous ubiquitin. In this case, 1µg of total protein extract was incubated with 5µg of primary antibody against ubiquitin at 4ºC overnight. Then, inmunocomplexes were collected with protein A bound to sepharose beads by orbital shaking at 4ºC for 1h. Beads were pelleted by centrifugation and washed at least three times with lysis buffer. Elution was done by resuspending the beads with Laemmli buffer and boiling them for 5min. Then, samples are ready to be loaded on a SDS-Polyacrilamide gel. This method of elution is very harsh and will also denature the light and heavy chains of the primary antibody used for inmunoprecipitation, which will be present at the sample. This can yield problems with the inmunodetection after the Western Blot, since they will cross-react with secondary antibodies of the same species and unspecific bands at ~20 and ~50kDa will be observed.

Inmunofluorescence

This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. It is used in fixed cells, since antibodies can't cross the plasma membrane. First, the sample is incubated with an antibody against the target molecule, named primary antibody, and then another antibody is added, which carries the fluorophore, recognises the primary antibody and binds to it. These are called the secondary antibodies. After that, samples are ready to be visualized in a fluorescent mycroscope.

In this work, we performed inmunofluorescence of cortical neurons. E15 cortical neurons at 1 DIV were seeded and 72h later were rinsed with PBS at room temperature and fixed in 4% paraformaldehyde/PBS for 30min at room temperature. Then, they were washed twice with PBS and subsequently permeabilized and blocked with 5% bovine serum albumin and 0.1% Triton X-100 in PBS for 90 min at room temperature. The cells were incubated overnight with the primary antibodies, rinsed three times with PBS, and incubated with secondary antibodies for 1h at room temperature protected from light. Confocal micrographs were obtained using an espectral confocal microscopy FluoView1000.

Antibody	Source	Working Dilution	Supplier
FLAG	Mouse	1:2000	Sigma
LFG	Rabbit	1:100	Anaspec
calnexin	Rabbit	1:20	Cell Signalling
COXIV	Rabbit	1:50	Cell Signalling
Rab5	Rabbit	1:100	Cell Signalling
GM130	Rabbit	1:25	BD Pharmigen
EEA1	Mouse	1:200	BD Pharmigen
Rab11	Mouse	1:50	BD Pharmigen
Rab7	Mouse	1:250	Sigma
TGN38	Mouse	1:200	Affinity Bioreagents
Secondary Antibodies			
Alexa Fluor 488 anti-mouse	Donkey	1:300	Invitrogen
Alexa Fluor 488 anti-rabbit	Donkey	1:300	Invitrogen
Alexa Fluor 568 anti-rabbit	Donkey	1:300	Invitrogen
Alexa Fluor 594 anti-mouse	Donkey	1:300	Invitrogen
Alexa Fluor 594 anti-rabbit	Donkey	1:300	Invitrogen

Table M2. Antibodies used in this study for Inmunofluorescence

Colocalization analysis

In inmunofluorescence, colocalization refers to observation of the spatial overlap between two or more different fluorescent labels, each having a separate emission wavelength, to see if the different molecules are located in the same area of the cell. This technique is important in the study of cell biological and physiological processes to demonstrate a relationship between pairs of bio-molecules.

Colocalization studies often relied in visual observance of colocalization between two pairs of fluorophores. For example, when using green and red labels, when two of these pixels colocalize would form a yellow pixel, meaning there is colocalization in that area. However, in the last years methods have been developed in order to enhance the reproducibility and reliability of colocalization experiments. Using mathematic aproaches, Erik Manders introduced the Pearson's Correlation Coefficient, along with other coefficients of which the overlap coefficients M1 and M2 have proved to be the most popular and useful. The calculation of these coefficients still relied on the setting of a certain thresholds for pixels intensity, in which pixels below a given intensity would not be get into account. An approach was introduced by Sylvain Costes, who utilized Pearson's Correlation Coefficient as a tool for setting the thresholds required by M1 and M2 in an objective fashion

In our work, colocalization was quantified in 10–15 randomly chosen cells from each sample using ImageJ (Coloc2) colocalization Software to calculate the Manders' Colocalization Coefficients (M1 and M2) and the Pearson Correlation Coefficient (Rr) . Threshold values were determined using Costes method, incorporated at Coloc2 software. Only pixels whose red and green intensity values were both above their respective thresholds were considered to be pixels with colocalized probes. Manders Colocalization Coefficients were then calculated as the fractions of total fluorescence in the region of interest that occurs in these “colocal” pixels (with a higher value representing more colocalization), and Pearson Correlation Coefficients. M1 coefficient values represented as mean percent colocalization are shown.

Maldi-Toff MS analysis

Maldi is the abreviation for “Matrix Assisted Laser Desorption/ionization”. The basis of this technique were built in 1985 when it was found that the alanine aminoacid could be ionized when mixed with tryptophan and irradiated with a pulsed 266nm laser. Since then, a great effort of research has been made to improve the method to ionize larger biomolecules. The development of organic matrix and inexpensive nitrogen laser technology brought MALDI available to a vast number of researchers.

Tof MS is the abreviation of “Time Of Flight Mass Spectrometry”. When a sample is ionized, charged fragments with different size are formed. If you apply a potential difference V0 between the sample, it attracts the ions in the opposite direction. As the potential difference V0 is constant with respect to all ions, ions with smaller m/z value (lighter ions) and more highly charged ions move faster through the drift space until they reach the detector. Consequently, the time of ion flight differs according to the mass-to-charge ratio (m/z) value of the ion. The method of mass spectrometry that exploits this phenomenon is called Time of Flight Mass Spectrometry.

In proteomics, the major application of Maldi-Tof MS is to identify proteins isolated from a sample, with a method called peptide mass fingerprint. In this technique, the protein of interest is cleaved by proteases into smaller peptides. Then, the peptide masses are compared to either a database containing known protein sequences.

In this work, Maldi-Toff MS was used to identify putative ubiquitylation sites in the protein LFG. It is achieved by purifying recombinant LFG by SDS-PAGE electrophoresis and digested with trypsin or proteinase K. Trypsin cuts proteins after Arg or Lys residues, but it is unable to cleave after ubiquitinated lysins due to steric hydrance. Proteinase K is a protease that cleaves proteins inespecifically. By comparing peptides resultant from both digestions, we can identify hypothetic ubiquitylation sites in a protein by identifying the lysines which were not cleaved by trypsin.

The Maldi-Tof MS experiments were performed with the Proteomics Group of Dr. Canals in the Vall d’Hebron Institut de Recerca. Briefly, LFG-FLAG was overexpressed in HEK293T cells and

purified by FLAG inmunoprecipitation. Then, it was run in a 12% acrylamide gel. After electrophoresis, the gel was stained with Comassie Blue and bands corresponding to LFG and its ubiquitinated forms were cut and digested. The proteolysis is typically carried out overnight and the resulting peptides are extracted with acetonitrile and dried under vacuum. The peptides are then dissolved in a small amount of distilled water or further concentrated and purified, and then are ready for mass spectrometric analysis.

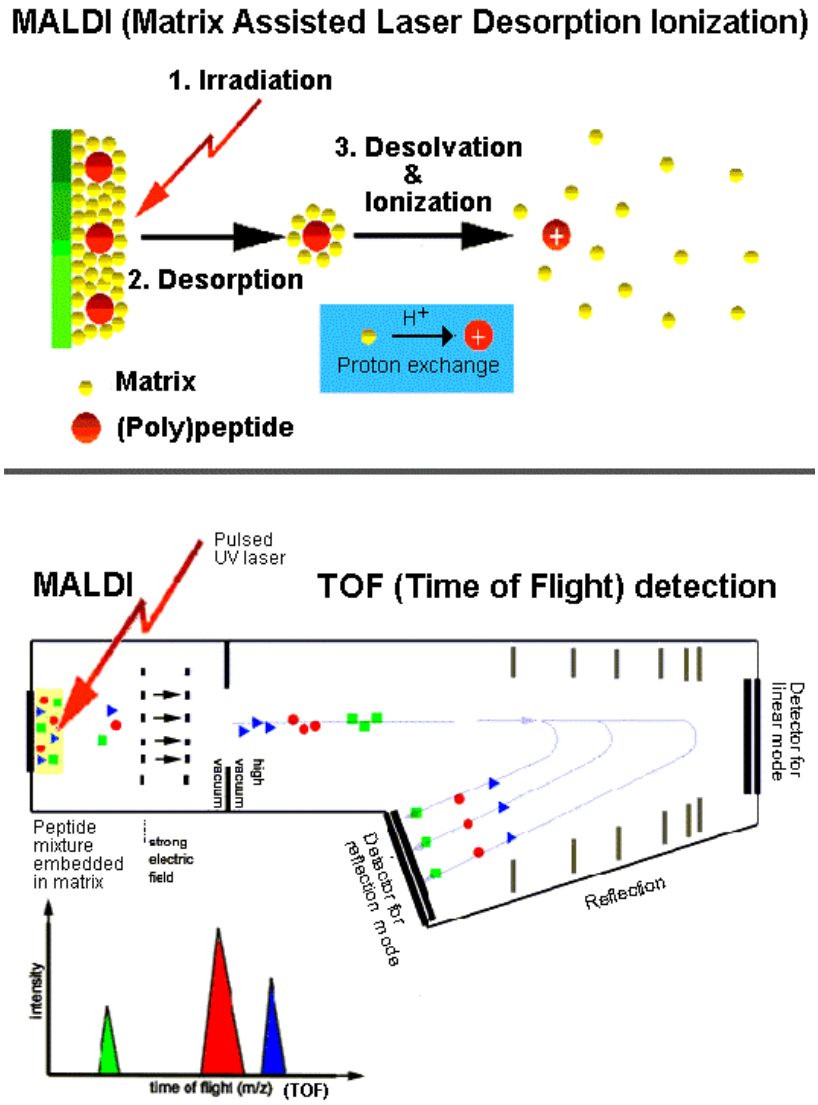

Figure M1. Principles of Maldi-Toff. Sample is adsorbed into a organic matrix and laser-irradiated. This ionizes the sample and generated charged peptide fragments, which can be separated applying a strong magnetic field according to its m/z ratio.

Molecular cloning

Molecular cloning is a set of experimental methods in molecular biology that are used to assemble recombinant DNA molecules and to direct their replication within host organism. Cloning refers to the fact that the method involves the replication of a single DNA molecule

starting from a single living cell to generate a large population of cells containing identical DNA molecules.

Generally, the DNA we want to be cloned is obtained from an organism of interest and combined with a vector DNA. This recombinant DNA is introduced in a host organism, normally an easy-to-grow one like the bacteria *Escherichia Coli.* This will generate a population of organisms in which recombinant DNA molecules are replicated along with the host DNA. This single cell can then be expanded exponentially to generate a large amount of bacteria, each of which contain copies of the original recombinant molecule. Then, the recombinant DNA can be extracted and purified, to use it for future applications like cell transfection or subsequent clonings.

Plasmid extraction from bacterial cultures and purification

DNA plasmids are circular molecules of double-stranded DNA with a variable lenght around 1-200kb. Inside the bacteria they behave like additional genetic units and are replicated and hinerited indepently of the host genome. Most of the times, the plasmids contain additional genes that aid the bacteria to live under determined circumstances, like antibiotic resistance, and can be used to select bacteria which express our plasmid of interest.

Bacteria were grown in LB BROTH medium (10g/l tryptone, 5g/l yeast extract, 5g/l NaCl) with proper antibiotics added. Plasmids were extracted and purified using comercially available kits. These kits extract the plasmidic DNA by alcaline lysis of the bacteria. Briefly, bacterial colonies of interest are grown at 37ºC overnight the day before the plasmid purification. Then, bacterial cells are pelleted by centrifugation and resuspended in a solution containing NaOH/SDS, supplemented with RNAase A. The lysate is neutralized with potassium acetate and cleared by centrifugation. The supernatant contains the plasmidic DNA dissolved. In order to purify it, alcoholic precipitation of the DNA is performed. Isopropanol is added to the supernatant to precipitate DNA and then it's pelleted by centrifugation. An additional wash with ethanol 70% is needed, and then the plasmidic DNA is left to dry at room temperature for several minutes. Finally, plasmid DNA is resuspended in buffer TE (10mM Tris-HCl pH 8, 1mM EDTA). The kits used in this work were Qiaprep Spin Miniprep Kit (QIAGEN) and the Nucleobond Xtra Maxi kit (Macheley-Nagel), depending on the amount of plasmid we needed to prepare.

Transformation of DNA plasmids into competent bacteria

Cells able to incorporate DNA are called competents. Several methods exist to render *E.coli* bacteria competent, although in our work we used comercially available ones.

We used XL-2 Blue Ultracompetent Cells (Stratagene) and followed manufacturer's instructions for transformation. Briefly, DNA is added to 50µl of bacteria, the amount depends on the nature of the DNA. If it's a plasmid in its native state, small amounts are sufficient, however, when it is a ligation, amount has to be augmented to enhance the yield of the transformation. The DNA

and the bacteria are incubated for 30min on ice, and then a thermic shock is performed, by exposing the mixture to 42ºC for 1min and rapidly to ice 1min more. Then, 500µl of LB BROTH is added and the bacteria are incubated at 37ºC for 30min. Finally, the mixture is plated on LB agar plates with the apropiate antibiotic for selection.

DNA analysis in agarose gels

Agarose gels allows for separation of acid nucleics based on their migration by charges. In an electric field, migration is determined by the size of the molecule of DNA. Percentatge of agarose in the gel is also important, since bigger molecules have more difficulty to migrate in tighter porus, and depending on the size of the molecules we want to separate, differents concentrations of agarose may be used.

Agarose gels are prepared dissolving the necessary amount of agarose (Amershan Biociences) in buffer TAE (40mM Tris-Acetate pH=8.0, 20mM sodium acetate, 1mM EDTA). 1ng/ml of SYBR Safe is added after dissolve the agarose, which will allow for detection of DNA. DNA samples are mixed with Loading buffer (1mM EDTA, 5% glycerol, 0,01& Bromophenol blue) prior loading into the gel. Electrophoresis was performed at constant voltage of 100V during 30-90min. Gels are visualized by irradiation with UV (280nm) in a UV chamber.

Cloning in mammal expression vectors

Every cloning requires a specific strategy adecuated to the techniques disposed and sequence of the DNA involved. There are some basic tools in cloning that are widely used and are explained in detail below:

PCR amplification

In order to obtain the desired fragment for a cloning, using as a starting material an expression plasmid or genomic DNA, the best approach is to amplify it with PCR. We use specific primers that recognize the start (forward) and the end (reverse) of the target sequence, adding sequences for restriction enzymes to recognize, to use after in the ligation.

Digestion by restriction enzymes and generation of blunt ends

Restriction enzymes recognize DNA and cut it at specific sequences, which are generally short and palindrome. There is a wide variety of restriction enzymes and each one recognizes a different sequence, so are very useful tools when creating cloning strategies. Depending on the type of sequence cut, they generate blunt or cohesive ends in the DNA. Cohesive ends are also called “sticky” and require less time to be ligated, and the target sequence for a restriction enzyme gets conserved if the same one was used in both ends of the DNA to ligate. Generally, digestions are done incubating for 1h at 37ºC, adding the apropiate buffer depending on the restriction enzyme, although some enzymes require different reaction temperatures. We followed always manufacturer’s instructions.

Sometimes, it is needed to generate a blunt end after digestion with a restriction enzyme that yields cohesive ends, by eliminating the additional bases that get impaired. To do that, we use Klenow fragment of DNA Polymerase I (New England Biolabs), following manufacturer's instructions. Briefly, the DNA is mixed with the appropiate buffer and 33µM of each dNTP. 1 unit of Klenow enzyme is added and DNA is incubated at 25ºC for 15min. Reaction is stopped by adding 10mM EDTA and heating at 75ºC for 30min.

Ligation

To obtain new molecules of DNA, fragments of the vector and the insert should be united by the action of the DNA ligase. Ligations are performed with 50ng of the vector, and the proportional quantity of insert to have a molar ratio of 1:2 between the vector and the insert. We use T4 DNA ligase (New England Biolabs), following manufacturer's instructions. Before ligation, normally the 5' phosphate groups of the vector should be eliminated to avoid religation of the vector. Thus, the linearized vector is incubated with phospatase alkaline (Roche) at 37ºC during 1h, using the apropiate buffer.

RESULTS

CHAPTER I. LFG inhibits Fas ligand-mediated endoplasmic reticulum-calcium release and prevents type II apoptosis in a Bcl-xL-dependent manner

LFG localizes mainly at the ER and Golgi membranes

LFG has been reported to localize at the plasma membrane and Trans-Golgi network membrane [233, 254]. However, a more detailed study of its distribution inside the cell is needed. To determine LFG subcellular localization, we examined its colocalization with markers of cellular organelles. To do so, mice cortical neurons were transduced with lentivirus carrying overexpressing LFG-FLAG particles for 3d. Then, cells were fixed and permeabilized, and cells were stained using antibodies against FLAG and each of the subcellular markers. Hoechst staining was used as a marker of the nucleus. Images were taken in a confocal microscope.

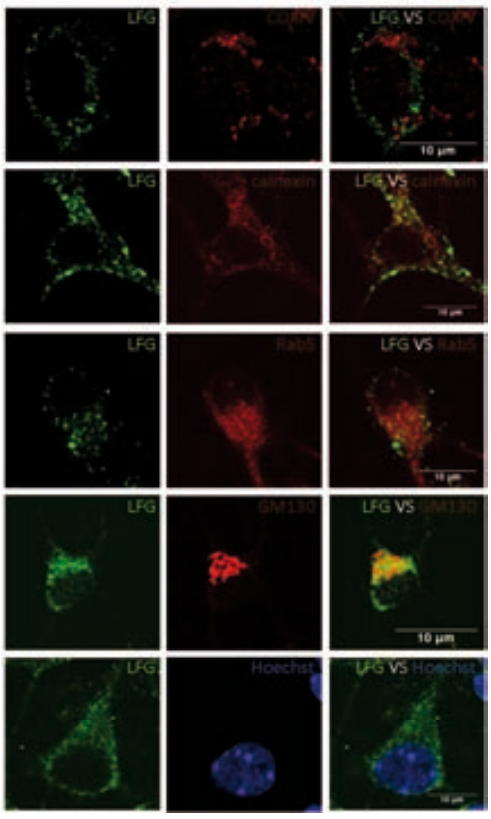

Figure R1. LFG colocalization with subcellular markers. Representative confocal images of cortical neurons. Cortical neurons were transduced with LFG-FLAF for 3d. Cells were fixed, permeabilized, and immunostained with anti-FLAG (green), anti-COXIV (Mitochondria marker), anti-calnexin (Endoplasmic Reticle marker), anti-Rab5 (Early Endosome marker), anti-GM130 (Golgi marker), and Hoechst (Nucleus marker) (red). Third column shows merge of green and red channels

LFG colocalized with the ER marker calnexin, the Golgi marker GM130, and the early endosome marker Rab5 (Figure R1). Moreover, LFG presented a finely punctate pattern with some aggregation in the perinuclear region, which suggests an endoplasmic and vesicular localization. In contrast, no significant colocalization was observed with COXIV, a mitochondrial marker, or Hoechst staining, a nuclear marker. To study more in depth LFG colocalization with cellular markers, the colocalization was quantified.

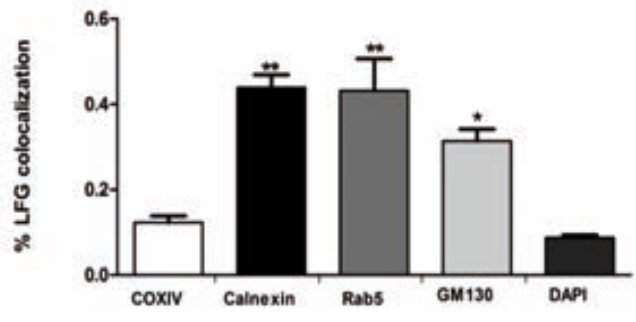

Figure R2. Percentage of LFG colocalization with subcellular markers. Colocalization was measured using the ImageJ Coloc2 software. Manders' colocalization coefficients for LFG with each of the subcellular markers are shown. One-way ANOVA test was performed comparing the coefficient value for calnexin, Rab5, and GM130 against that of COXIV **p≤0.005, *p≤0.05.

Channels		M1	M2	Rr
Ch1 :LFG	Ch2: COXIV	0.123±0.014	0.248±0.018	0.175±0.016
Ch1: LFG	Ch2: Calnexin	0.439±0.027	0.345±0.045	0.401±0.049
Ch1: LFG	Ch2: Rab5	0.421±0.083	0.262±0.057	0.386±0.055
Ch1: LFG	Ch2: GM130	0.314±0.025	0.6±0.052	0.531±0.009
Ch1: LFG	Ch2: DAPI	0.091±0.02	0.055±0.016	0.081±0.031

M1: Colocalization coefficient for Channel 1.

M2: Colocalization coefficient for Channel 2.

Rr: Pearson correlation coefficient for each Channel colocalization.

Table R1. Colocalization analysis of LFG with subcellular markers. Confocal images of cortical neurons were obtained, and 10-15 randomly chosen cell bodies per condition were analyzed using the ImageJ Coloc2 software. Manders' colocalization coefficients and Pearson's correlation coefficients for LFG with subcellular markers are expressed as mean ± SD.

M1 values are represented as percentage of LFG colocalization with the subcellular markers (Figure R2). R_r values were also statistically significant ($p<0.05$) for the colocalization analysis with calnexin, Rab5, and GM130 (Table R1), and were above the >0.5 value, which indicates positive correlation of

signals. No significant colocalization was detected for COXIV or Hoechst staining when submitted to Pearson's correlation test. Thus, these observations suggest that LFG localizes at the membranes of the ER.

LFG is localized along the endocytic pathway

Since the localization of LFG appears to be vesicular, we sought to study the localization of LFG along the endocytic pathway in greater depth. LFG colocalization with markers of proteins of early, late, and recycling endosomes, lysosomes, as well as markers of the ER and Trans-Golgi Network, was analyzed and quantified. Mice cortical neurons were fixed and permeabilized, and then stained with antibodies against LFG and each of the protein markers. Images were taken in a confocal microscope.

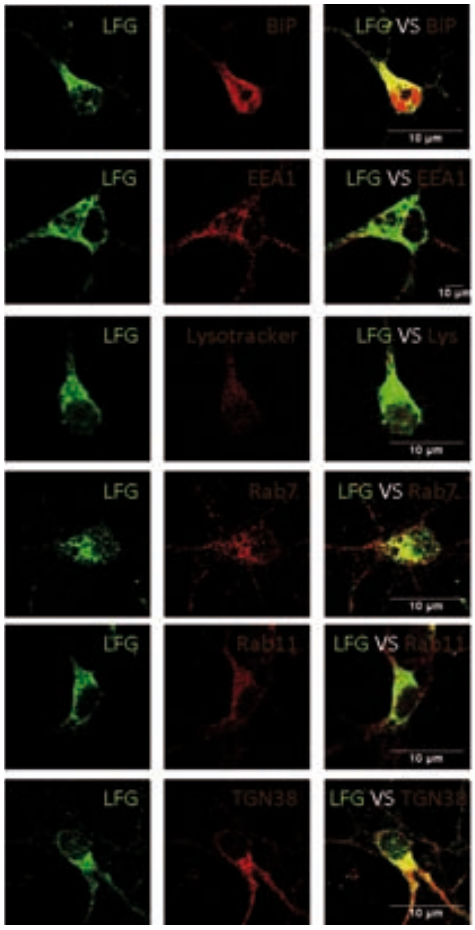

Figure R3. LFG is localized in the endocytic pathway. Representative confocal images of cortical neurons. Cells were fixed, permeabilized, and immunostained with anti-LFG (green), and different elements of the endocytic pathway; anti-BiP (Endoplasmic Reticle marker), anti-EEA1 (Early Endosomes marker), Lysotracker (Lysosomal marker), anti-Rab7 (Late Endosome marker), anti-Rab11 (Recycling Endosome marker) and anti-TGN38 (Trans-Golgi Network marker)(red). Thirdcolumn shows merge of green and red channels.

LFG preferentially colocalized with BiP and TGN38—ER and Golgi markers respectively. It also showed around 20% colocalization with the vesicular stainings (EEA1, Rab7, Rab11 and Lysotracker) (Figure R3 and R4). R_r values were far greater in the first case (Table R2), indicating that LFG staining has a more robust pattern with ER and Golgi structures. All together, these results indicate that most cellular LFG is located at the ER and the Golgi.

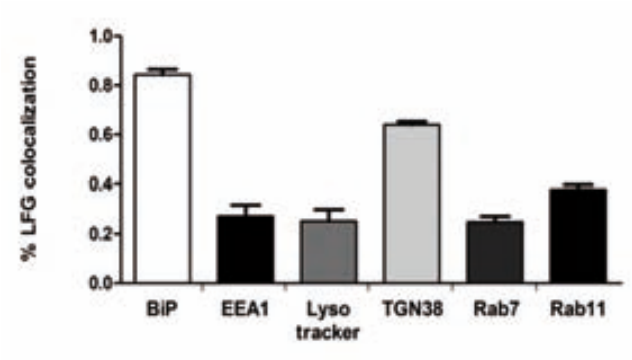

Figure R4. Percentage of LFG colocalization with proteins of the endocytic pathway. Colocalization was measured using the ImageJ Coloc2 software. Manders' colocalization coefficients for LFG with each of the subcellular markers are shown.

Channels		M1	M2	Rr
Ch1 :LFG	Ch2: BiP	0.878±0.017	0.346±0.019	0.675±0.008
Ch1: LFG	Ch2: EEA1	0.379±0.016	0.388±0.013	0.459±0.021
Ch1: LFG	Ch2: Lysotracker	0.255±0.004	0.457±0.052	0.441±0.011
Ch1: LFG	Ch2: Rab7	0.272±0.042	0.313±0.023	0.36±0.029
Ch1: LFG	Ch2: Rab11	0.252±0.041	0.477±0.085	0.423±0.059
Ch1: LFG	Ch2: TGN38	0.639±0.012	0.6±0.09	0.686±0.042

M1: Colocalization coefficient for Channel 1.

M2: Colocalization coefficient for Channel 2.

Rr: Pearson correlation coefficient for each Channel colocalization.

Table R2. Colocalization analysis of LFG with proteins of the endocytic pathway. Confocal images of cortical neurons were obtained, and 10-15 randomly chosen cell bodies per condition were analyzed using the ImageJ Coloc2 software. Manders' colocalization coefficients and Pearson's correlation coefficients for LFG with endocytic pathway markers are expressed as mean ± SD.

LFG interacts with Bcl-xL

LFG has been proposed to be a member of the TMBIM family of proteins. Some of these members have the capacity to modulate the activity of Bcl-2 family members, such as Bcl-xL and Bcl-2, which are found in mitochondria but also in the ER. We hypothesized that LFG also modulates the activity of Bcl-2 family members in a similar manner. In this regard, we determined LFG interaction with Bcl-2 family proteins by co-immunoprecipitation experiments. LFG-FLAG was co-expressed with HA-tagged Bcl-2 family proteins in HEK293T cells. After cell lysis, FLAG-tagged proteins wereimmunoprecipitated and HA-tagged proteins were visualized by Western blotting.

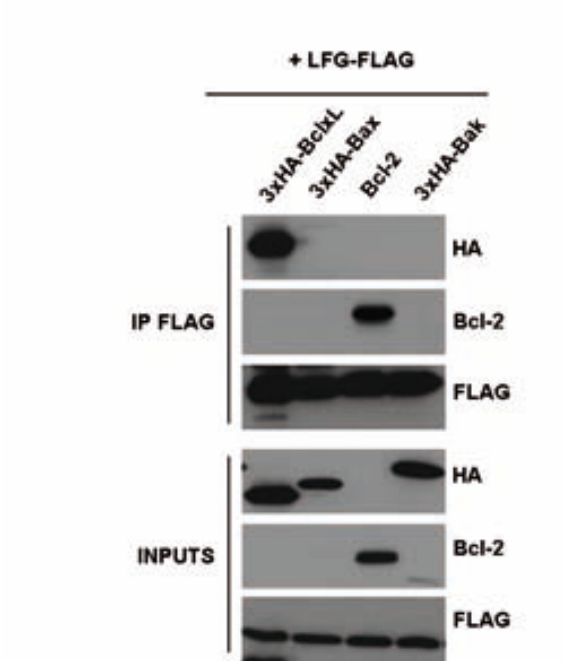

Figure R5. LFG interacts with Bcl-2 and Bcl-xL, but not with Bax or Bak. HEK293T cells were transfected with LFG-FLAG expression plasmid and co-transfected with 3xHA-Bcl-xL, Bcl-2, 3xHA-Bax, or 3xHA-Bad, as indicated. FLAG-tagged LFG was immunoprecipitated and interaction was determined by immunoblotting with HA, FLAG, and Bcl-2 antibodies.

LFG co-immunoprecipitated with Bcl-xL and Bcl-2 but not with Bax or Bak (Figure R5). Since Bcl-xL and LFG are highly expressed in the CNS [232, 255], we focused our attention on the LFG-Bcl-xL relationship. In order to further characterize the Bcl-xL-LFG interaction, we constructed truncated forms of LFG. The first 101 amino acids of the LFG sequence, corresponding to the N-terminus unfolded domain, were subcloned and fused to a 3xFLAG tag in a

pcDNA3 expression vector to form the 3xFLAG-ΔC-LFG constructs. The rest of the sequence, corresponding to the transmembrane domain with the C-terminal tail, was subcloned along with a FLAG tag and with a methione residue at the start to initiate the open reading frame, forming the ΔN-LFG-FLAG vector. A schematic representation of these constructions is shown below.

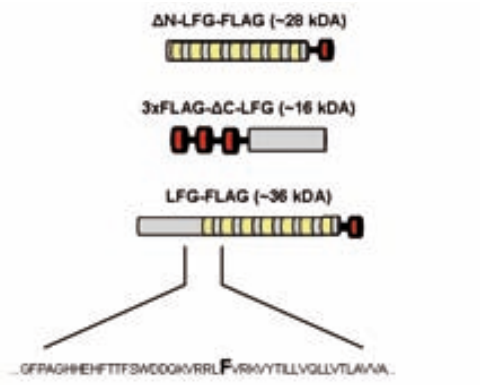

Figure R6. Schematic representation of LFG truncated forms. Schematic representation of LFG-FLAG, ΔN-LFG-FLAG, and 3xFLAG-ΔC-LFG truncated forms. Green boxes represent putative transmembrane regions, and red boxes the FLAG-tag. A fragment of the primary sequence of LFG is shown highlighting the specific amino acid where ΔN-LFG-FLAG ends and 3xFLAG-ΔC-LFG starts.

HEK293T cells were co-transfected with the LFG constructs and 3xHA-Bcl-xL. FLAG-tagged proteins were inmunoprecipitated and Bcl-xL interaction was assessed by HA detection.

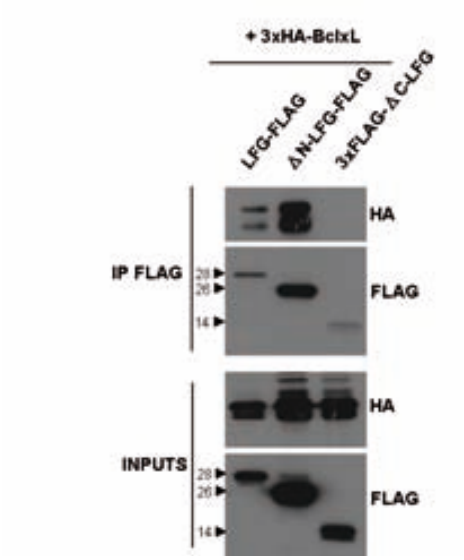

Figure R7. LFG interacts with Bcl-xL through its *C*-ter side. HEK293T cells were transfected as indicated, and FLAG-tagged proteins were immunoprecipitated. The interaction was assessed by immunoblotting against HA and FLAG tags.

FLAG immunoprecipitation and HA detection revealed that LFG interacts with Bcl-xL through its C-terminal region (Figure R7). To further improve our results, we wanted to study the colocalization of LFG and Bcl-xL endogenously in mice cortical neurons. To do so, cells were fixed and permeabilized, and then stained with LFG and Bcl-xL antibodies. Hoechst staining was used as a negative control of colocalization.

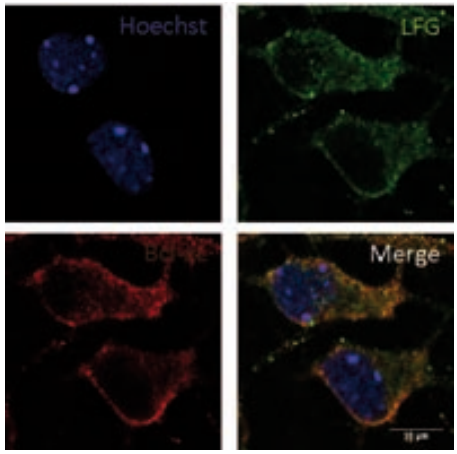

Figure R8. LFG colocalizes with Bcl-xL. Representative confocal images of cortical neurons showing LFG and Bcl-XL colocalization.

Channels		M1	M2	Rr
Ch1 :DAPI	Ch2: LFG	0.002	0.025	0.058
Ch1: LFG	Ch2: BclxL	0.638	0.875	0.74

M1: Colocalization coefficient for Channel 1.

M2: Colocalization coefficient for Channel 2.

Rr: Pearson correlation coefficient for each Channel colocalization.

Table R3. Colocalization analysis of LFG with Bcl-xL. Confocal images of cortical neurons were obtained, and 10-15 randomly chosen cell bodies per condition were analyzed using the ImageJ Coloc2 software. Manders' colocalization coefficients and Pearson's correlation coefficients for LFG with Bcl-xL and DAPI are expressed as mean ± SD.

We observed LFG colocalization with Bcl-xL, located at the cytosol excluding the nucleus, with a stronger staining in the perinuclear region, thereby suggesting ER colocalization (Figure R8). The values of Manders' correlation coefficients and Pearson's correlation coefficients were also positive, indicating a robust measure of the degree of colocalization (Table R3). In summary, all these data indicate that LFG interacts with Bcl-xL through its C-terminal region.

LFG protects type II cells, but not type I, from Fas-induced apoptosis

LFG prevents FasL-induced cell death in cortical neurons, granular neurons, SH-SY5Y and HeLa cells, among others. All these cells are classified as type II cells. Nonetheless, there is no direct evidence that LFG protects type I cells against FasL-induced apoptosis. To address this question, we performed a comparative study of LFG inhibition of Fas-induced apoptosis in type I and type II cells. To this aim, we overexpressed this protein in parallel with other anti-apoptotic proteins in HEK293 and SK-N-AS cells as models of type I and type II cells, respectively. Bcl-xL inhibits Fas-induced apoptosis in type II cells but not in type I, and FLIP, which competes with caspase-8 for recruitment to the DISC after DR stimulation, protects both type I and II cells against DR-induced apoptosis. Thus, Bcl-xL and FLIP were used as positive controls of the two models.

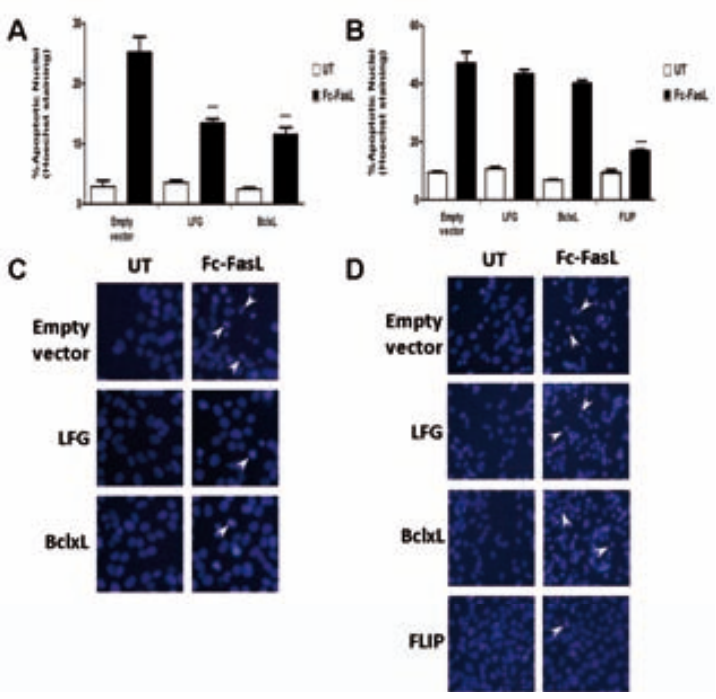

Figure R9. LFG protects type II cells from nucleic fragmentation after FasL treatment. A. SK-N-AS cells were transduced with empty plasmid or LFG-, or Bcl-xL- overexpressing lentiviral particles for 3 days. Cells were then treated with Fc-FasL (200 ng/ml) or left untreated for 24 h. Percentage of apoptosis was assessed, and a two-way ANOVA test was performed, comparing LFG- and Bcl-xL-transduced cells with their empty vector counterparts. ***p≤0.001.**B.** HEK293 cells were transfected with pcDNA3-, LFG-, 3xHA-Bcl-xL-, and 3xHA-FLIP-overexpressing plasmids for 48 h and treated with Fc-FasL (200 ng/ml) or left untreated for 6 h. Percentage of apoptosis was assessed, and a two-way ANOVA test was performed, comparing LFG-, Bcl-xL- and FLIP-transfected cells with their empty vector counterpart. ***p≤0.001 **C.** Representative images of SK-N-AS cells with Hoechst staining, transduced as **A.** and treated with Fc-FasL (200 ng/ml) or left untreated for 24 h. White arrows highlight apoptotic nuclei. **D.** Representative images of HEK293 cells with Hoechst staining, transfected as **B.** and treated with Fc-FasL (200 ng/ml) or left untreated for 6 h. White arrows highlight apoptotic nuclei.

FasL-induced cell death in SK-N-AS cells, as measured by apoptotic nuclei counting, was inhibited by Bcl-xL and LFG overexpression (Figure R9A and R9C). Such inhibition was not observed in HEK293 cells, where only FLIP overexpression prevented apoptosis (Figure R9B and R9D). To further assure that LFG overexpression protected type II cells from Fas-induced apoptosis, we also quantified the caspase activity of cells after FasL treatment.

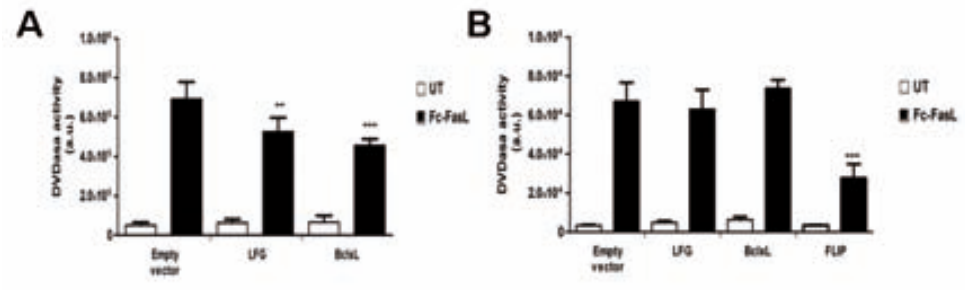

Figure R10. LFG inhibits caspase-3 activity in type II after FasL treatment. A. SK-N-AS cells were transduced as in **FigR23**and were treated with Fc-FasL (200 ng/ml) or left untreated for 6 h. DVDase activity was assessed, and a two-way ANOVA test was performed, comparing LFG and Bcl-xL transduced cells with their empty vector counterpart. ***p*≤0.005, ****p*≤0.001. **B.** HEK293 cells were transfected as **FigR23**,then treated with Fc-FasL (200 ng/ml) or left untreated for 4 h. DVDase activity was assessed, and a Student's test was performed, comparing LFG-, Bcl-xL-, and FLIP-transfected cells with their empty vector counterpart. ***p*≤0.005, ****p*≤0.001.

LFG overexpression, as well as that of Bcl-xL, decreased caspase-3 activation in SK-N-AS cells (Figure R10A). These results are in agreement with the apoptotic nuclei counting, and confirm the notion that HEK293 behave like type I cells, since only FLIP overexpression is able to abrogate caspase activation in this cell line after FasL treatment (Figure R10B).The efficiency of overexpression of the DR antagonists was assessed by Western blot (Figure R11A and R11B). All together, these data demonstrate that LFG overexpression protects type II cells, but not type I, against Fas-induced cell death.

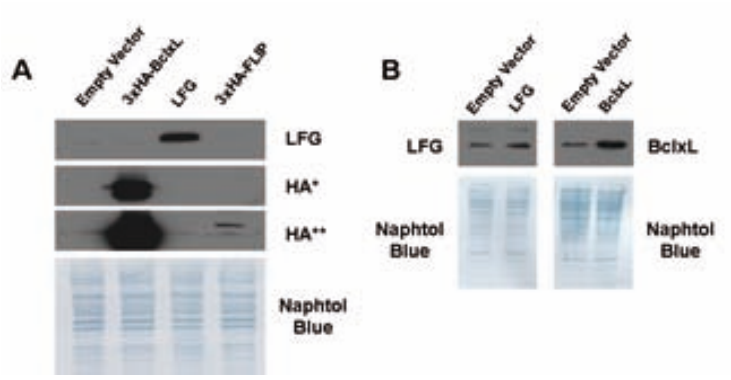

Figure R11. Overexpression of anti-apoptotic proteins in SK-N-AS and HEK293 cells.A. Immunoblot analysis was performed to assess LFG, Bcl-xL and FLIP overexpression in HEK293 cells. *Short exposure, **Long exposure **B.** Immunoblot analysis followed by Naphthol Blue staining confirmed LFG and Bcl-xL overexpression in SK-N-AS cells.

Requirement of endogenous Bcl-xL for LFG to exert its anti-apoptotic effects

Our findings that LFG protects type II cells from Fas-induced cell death and that it interacts with Bcl-xL suggested that one of these molecules might depend on the other to exert its anti-apoptotic effects. In order to study this hypothesis, we used SK-N-AS cells, which express both LFG and Bcl-xL endogenously. The idea was to down-regulate the expression of one of the proteins while overexpressing the other, to study the effects of its partner downregulation in its anti-apoptotic activity. Thus, cells were transduced with lentiviral particles carrying LFG, Bcl-xL, or Scrambled (Scr) shRNA, and after 3 days of transduction they were infected again with overexpression lentiviral particles for LFG and Bcl-xL for 3 additional days.Knockdown efficiency was determined by Western blot (Figure R12A and R12B).

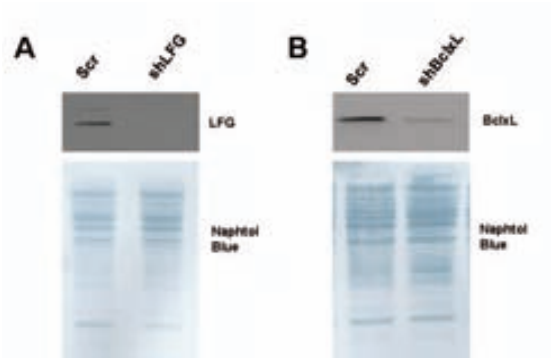

Figure R12. LFG and Bcl-xL down-regulation in SK-N-AS cells. Immunoblot analysis was performed to assess**A.** LFG and **B.** Bcl-xLdown-regulation in SK-N-AS cells

LFG and Bcl-xL overexpression reduced caspase 3 activity and the percentage of apoptotic nuclei after Fas treatment (Figure R13A and R13B), in consonance with previous results. However, when Bcl-xL was downregulated, LFG overexpression was unable to protect SK-N-AS cells. This was not the case in the opposite situation, thus Bcl-xL overexpression maintained its anti-apoptotic activity despite endogenous LFG down-regulation. On the basis of all these observations, we conclude that endogenous levels of Bcl-xL are required for LFG to exert its anti-apoptotic action, while protection from Fas-induced apoptosis by Bcl-xL overexpression does not require LFG.

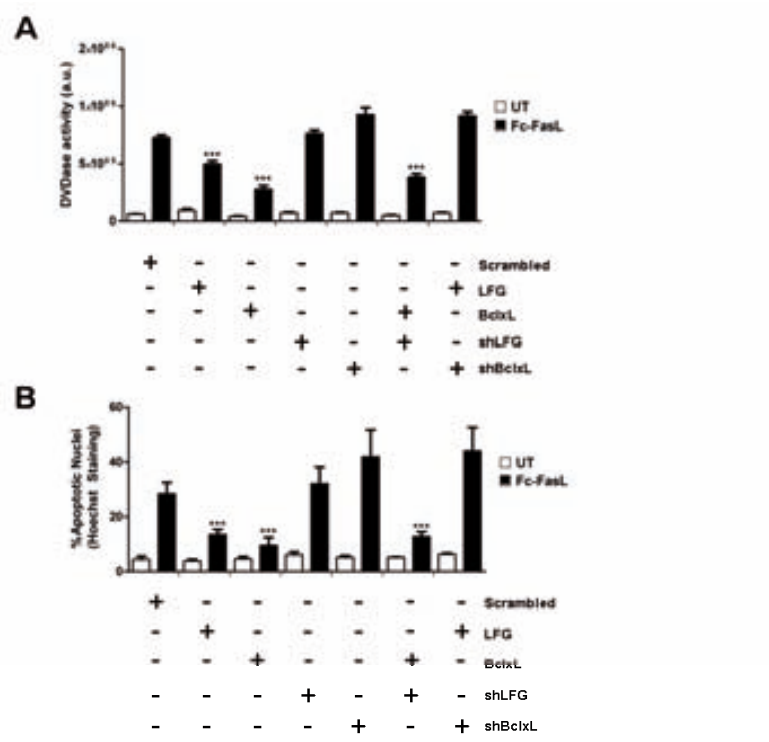

Figure R13. LFG protection from Fas-induced apoptosis is dependent on Bcl-xL endogenous expression. A. SK-N-AS cells were transduced with lentiviral particles carrying shRNA against LFG, Bcl-xL, or scrambled for 3 days and then transduced with empty, LFG- or Bcl-xL-overexpressing lentiviral particles for 3 additional days. SK-N-AS cells were treated or not with Fc-FasL (200 ng/ml) for 6 h. DVDase activity was assessed, and a two-way ANOVA test was performed, comparing all conditions against Scrambled ***p≤0.001. **B.** SK-N-AS cells were transduced as indicated and treated or not with Fc-FasL (200 ng/ml) for 24 h. Percentage of apoptosis was assessed by Hoechst staining, and a two-way ANOVA was performed, comparing all conditions against Scrambled ***p≤0.001.

LFG overexpression downregulates calcium release from the ER after Fc-FasL stimulation

Calcium mobilization from the ER to mitochondria is a crucial step in various forms of cell death, including Fas-induced apoptosis. Bcl-xL exerts anti-apoptotic effects in the ER by blocking calcium release [115]. In the same way, BI-1, a TMBIM family member, modulates calcium release from the ER and has anti-apoptotic effects. Since LFG is a member of the TMBIM family and we have demonstrated that it exerts its action in a Bcl-xL-dependent manner, we hypothesized it is involved in the regulation of calcium release from the ER after Fas engagement. To study the calcium response after FasL treatment, SK-N-AS cells were transduced with lentiviral overexpression particles carrying LFG, Bcl-xL or empty vector for 3d. Then, cells were treated with FasL and calcium measurement experiment was performed.

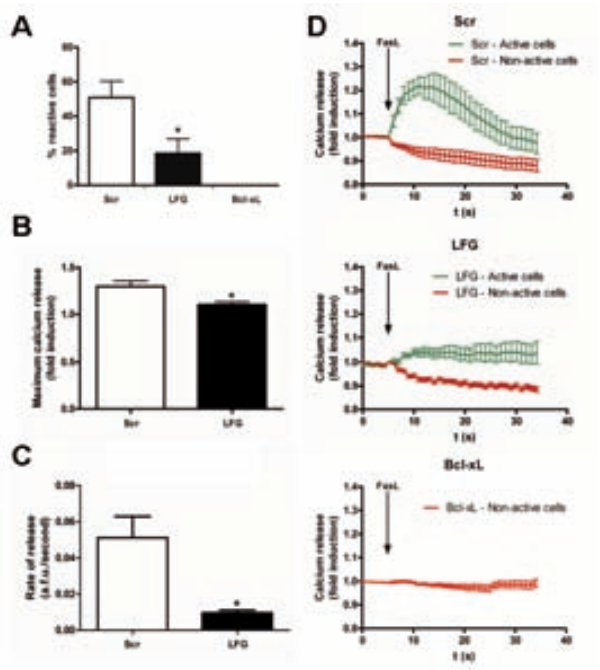

Figure R14. LFG inhibits calcium release from the ER after FasL treatment. SK-N-AS cells were transduced with empty vector or LFG- or Bcl-xL-overexpressing lentiviral particles for 3 days. Cells were then treated with Fc-FasL (50 ng/ml) and calcium response was measured **A.** Percentage of cells that responded to Fc-FasL by releasing calcium during the time measured. Calculated as the mean of the percentage of reactive cells in each individual experiment (n=4), a one-way ANOVA test was performed, comparing LFG- and Bcl-xL-transduced cells with empty vector *$p \leq 0.05$ **B.** Maximum calcium release of empty vector and LFG transduced cells, calculated as the mean maximum calcium release of at least 10 individual cells. Student test was performed *$p \leq 0.05$ **C.** Rate of calcium release of empty vector and LFG-transduced cells, calculated as the mean rate of calcium release of the same cells as **B.**, Student test was performed *$p \leq 0.05$ **D.** Representation of calcium release dynamics after Fc-FasL treatment in empty vector, LFG-, or Bcl-xL-transduced cells. Cytosolic calcium measures were performed and calculated for at least 10 individual cells for each condition.

After FasL treatment, around the 50% of control cells released calcium from the ER, and this percentage was significantly decreased in LFG-overexpressing cells, where only 18.5% of the cells reacted to FasL stimulation (Figure R14A). This reduction was not attributable to the efficiency of overexpression in SK-N-AS cells, as the transduction rate was close to 100%. When all the cells were overexpressing Bcl-xL, no calcium release occurred during the time window of our experiment. Moreover, maximum calcium release was significantly lower in LFG-overexpressing cells than in control cells (Figure R14B). In addition, the rate of release was dramatically reduced in the former (Figure R14C). These observations indicate that modulation of calcium channel opening may be the mechanism by which LFG inhibits calcium release, rather than by lowering resting calcium concentration in the ER. No calcium mobilization was detected in Bcl-xL-overexpressing cells (Figure R14A and R14D). In summary, our data suggest that LFG overexpression impairs calcium release from the ER after FasL treatment.

CHAPTER II. LFG ubiquitination

LFG is ubiquitinated

Previously in our lab, a two hybrid screening assay was performed to search for proteins that directly interact with LFG. The *N*-ter part of LFG was used as the bait, and the screening was performed with and adult mouse cDNA library. After the screening, 30 proteins were isolated with a positive interaction.

Nº	Gene Name	nº repet.	% of total
1	Ubiquitin B	44	35,77
2	Ubiquitin A-52	18	14,63
3	Ribosomalprotein S27a	9	7,32
4	Ubiquitin C	8	6,50
5	Ubiquilin 2	7	5,69
6	Tax 1 bindingprotein	5	4,07
7	UBIN (Ubiquitinlikeprotein)	3	2,44
8	Traf& TNF-R associatedprotein (TTRAP)	2	1,63
9	Chsme 17 clone rp23-114o8strain C57BL/6J	2	1,63
10	Ubiquitinassociateddomain…	2	1,63
11	Ubiquilin 1	2	1,63
12	EGF pathwaysubstrate 15	2	1,63
13	Splicin Factor SC35	2	1,63
14	Brain MY042 protein	1	0,81
15	AncientUbiquitousProtein	1	0,81
16	BAC clone RP24-322M5	1	0,81
17	Ring Finger protein 126 (E3 Ubiquitin ligase)	1	0,81
18	KIAA0312 protein	1	0,81
19	Hetero nuclear ribonucleoprot. A2/B1	1	0,81
20	Jun coactivator Jab1	1	0,81
21	BAC clone RP23-212H18 Cezanne 2	1	0,81
22	BAC clone RP23-198J15	1	0,81
23	Quinescin Q6-like 1	1	0,81
24	Epsin 1	1	0,81
25	MKK7 bindingprotein 2	1	0,81
26	Myelinproteolipidprotein gene	1	0,81
27	Hypoth.Ubiquitin-associateddomainprotein...	1	0,81
28	Similar to tyr kinase, non-receptor, 2;	1	0,81
29	GTP-binding protein (drg2 gene)	1	0,81
30	Cox II	1	0,81

Table R4. List of proteins identified after yeast colonies screening. nº repeat: Number of independent colonies that encoded the same protein. % of total: Percentage of the all colonies screened.

Most proteins obtained are from ubiquitin-related proteins. Although it is known that ubiquitin is one of the major false positives in two hybrid assays, there is a large number of these proteins obtained. Thus, we couldn't discard a possible role of the ubiquitin system in LFG function.

To study the relationship between LFG and the ubiquitin system, first we had to confirm the interaction between ubiquitin and LFG found by the two hybrid assay. To do so, we performed a co-inmunoprecipitation assay. HEK293T cells were transfected with LFG-FLAG and FLAG-tagged proteins were inmunoprecipitated. As ubiquitin is expressed in high levels endogenously, it's not needed to co-transfect it. In parallel, YFP was transfected as a negative control of the inmunoprecipitation, because it doesn't carry the FLAG tag, and $FAIM_L$-FLAG was transfected as a negative control of the ubiquitination, since previous results in our lab shown that it doesn't interact with ubiquitin. After Western Blot, interaction with ubiquitin was assessed by detection with ubiquitin antibody.

As shown in Figure R5, detection with ubiquitin antibody yielded a classic ubiquitination pattern in the LFG-FLAG lane. This pattern is characterized by the presence of several high MW bands that start at the position of the protein that was inmunoprecipitated, in an equidistant manner. This is caused by the presence of several forms of our target protein bound to ubiquitin, as ubiquitin chains can be formed with different number of ubiquitin molecules associated. Thus, each band corresponded to LFG bound to different number of ubiquitin molecules. As expected, no ubiquitination pattern was observed in the YFP and $FAIM_L$-FLAG lanes.

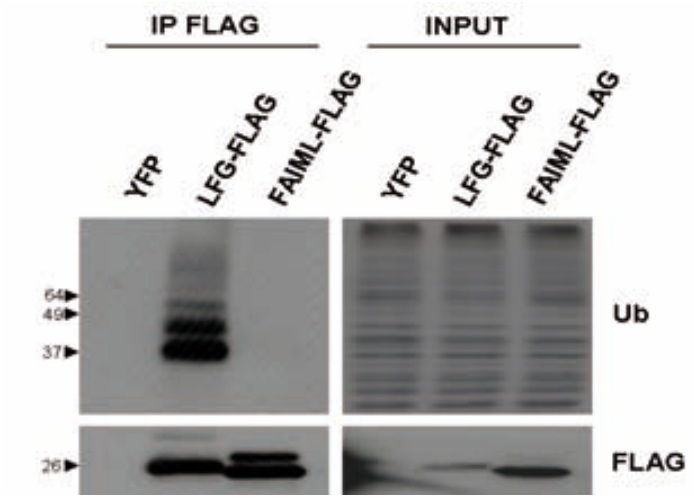

Figure R15. LFG is ubiquitinated. HEK293T cells were transfected as indicated. FLAG-tagged proteins were inmunoprecipitated and ubiquitination was assessed by inmunodetection with antibody against ubiquitin. FLAG inmunodetection was performed to assess correct overexpression and pull-down.

To further assess the LFG-ubiquitin interaction, we performed a co-inmunoprecipitation assay with three LFG plasmids, carrying different tags and yielding protein chimeras of different MW. HEK293T cells were transfected with LFG-FLAG, HA-LFG-FLAG and GFP-LFG-FLAG expression plasmids, and FLAG-tagged proteins were inmunoprecipitated. After Western Blot, interaction with ubiquitin was assessed by detection with ubiquitin antibody.

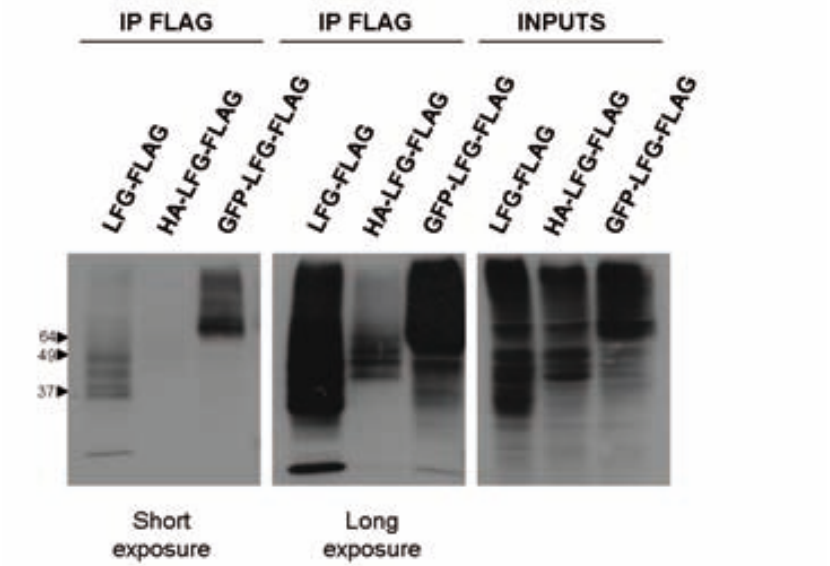

Figure R16. Ubiquitination pattern of LFG changes according to the MW of the LFG construction overexpressed. HEK293T cells were transfected as indicated. FLAG-tagged proteins were inmunoprecipitated and ubiquitination was assessed by inmunodetection with antibody against ubiquitin.

The classic ubiquitination pattern was detected in all three lanes (Figure R16). However, we can see a shift in the MW of the pattern. HA-LFG-FLAG and GFP-LFG-FLAG lanes had an ubiquitination pattern that starts at a higher MW than LFG-FLAG, as expected by the higher MW of the chimera due to the additional tags, and GFP-LFG-FLAG presents a higher MW pattern of ubiquitination that HA-LFG-FLAG too.

SDS lysis is a harsh protein solubilization procedure that denatures proteins and destroys non-covalent interactions between them. When a protein is ubiquitinated, ubiquitin bounds covalently to the target protein. Thus, ubiquitination cannot be displaced by SDS treatment, and can be used as an additional method to prove ubiquitination of a protein. Previous results in our lab have shown that LFG interacts with the protein SoxN. This interaction is mediated by electrostatic bounds and can be displaced by SDS treatment, so we used it as a positive control of SDS treatment. HEK293T cells were transfected with LFG-FLAG, and then co-transfected with SoxN-HA or empty plasmid. In parallel, $FAIM_L$-FLAG was transfected too as a negative control. A fraction of each sample was treated with 2%SDS at 95ºC during 5min, and then FLAG-tagged proteins were inmunoprecipitated. Interactions were analyzed after WB by HA and ubiquitin detection.

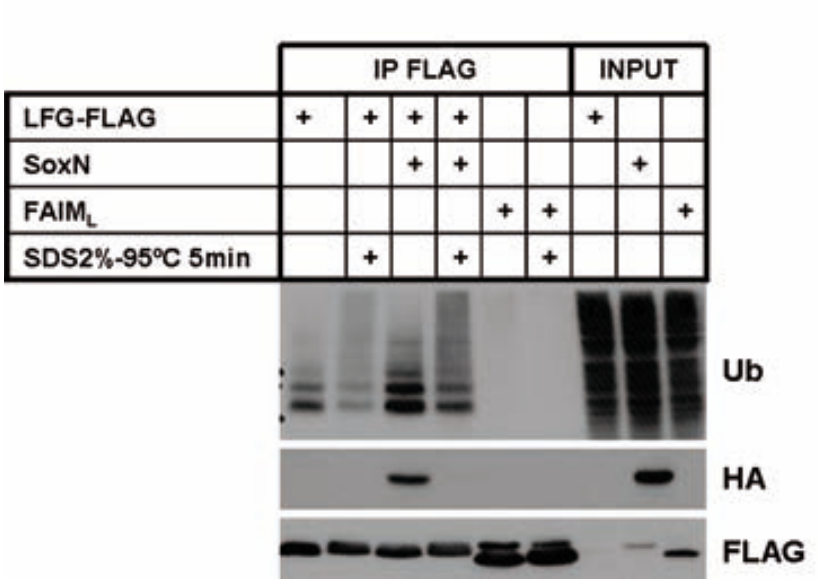

Figure R17. LFG ubiquitination pattern is maintained despite denaturing conditions. HEK293T cells were transfected as indicated. SDS treatment on lysates was performed or not, and then FLAG-tagged proteins were inmunoprecipitated. Ubiquitination was assessed by inmunodetection with antibody against ubiquitin, FLAG and HA inmunodetection was perfomerd to assess correct overexpression and pull-down.

LFG ubiquination pattern is maintained despite SDS treatment of the sample previous to co-inmunoprecipitation (Figure R17). Moreover, SoxN-LFG interaction is lost after SDS treatment, as seen by HA detection. $FAIM_L$-FLAG lanes doesn't show ubiquitination pattern as expected. Thus, this indicates that LFG-ubiquitin interaction is mediated by a covalent bound. In summary, these results show that LFG is ubiquitinated.

LFG ubiquitination doesn't induce its degradation

One of the major functions of protein ubiquitination is to mediate protein processing by the proteasome. Ubiquitin bound to a protein "marks" it, so it can be recognized by the proteasome and target it for degradation. MG132 is a chemical compound that inhibits proteasome activity and can be used to study protein degradation by the proteasome. In order to study whether LFG ubiquitination mediates its degradation, we transfected HEK293T cells with LFG-FLAG and treated them with different concentrations of MG312 during 24h. Mcl-1 is an anti-apoptotic protein with a high turnover rate that can only be detected by immunobloting when proteasomal activity is impaired, thus we used it as a positive control.

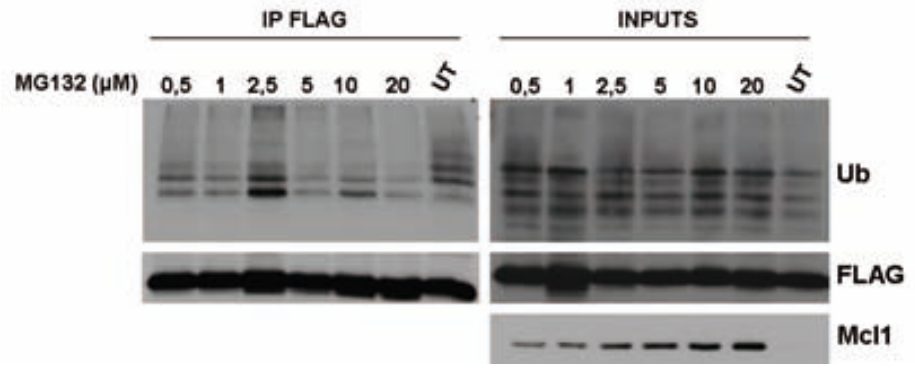

Figure R18. LFG ubiquitination doesn't induce its degradation by the proteasome. HEK293T cells were transfected with LFG-FLAG. 24h after transfection, cells were treated with MG132 as indicated for 24h. FLAG-tagged proteins were inmunoprecipitated and ubiquitination was assessed by ubiquitin inmunodetection. Mcl-1 inmunoblot confirmed correct effect of MG132.

As seen in Figure R8, LFG ubiquitination pattern is not altered by MG132 treatment, even at high concentrations. We were able to detect Mcl-1 up-

regulation, so the treatment was successful. This data indicates that LFG ubiquitination does not induce its degradation by the proteasome.

Apart from the proteasome, there are other proteases in the cell than can mediate degradation of proteins, like cathepsins or serine-proteses, a family of proteases found in the lysosome. Classically, ubiquitination mediates the targeting to the proteasome of the protein, but in some instances, it can also mediate the degradation of the protein by other proteases. Thus, in order to be sure that wasn't the case of LFG we studied its ubiquitination using different protease inhibitors. HEK293T cells were transfected with LFG-FLAG and then treated with the protease inhibitors shown in Table R5 for 24h. Then, FLAG-tagged proteins were inmunoprecipitated and ubiquitin was detected after Western Blot.

Drugs inhibitors	Targets	Dose (μM)
E64	Cysteine proteases	10
Pepstatin A	Aspartic proteases /Cathepsin D	100
Z-FA.fmk	Cathepsin	10
Z-FF.fmk	Cathepsin B and L	10
TPCK	Serine proteases	10
TLCK	Serine proteases	10

Table R5. Description of protease inhibitors used. Drug inhibitors used to study LFG ubiquitination.

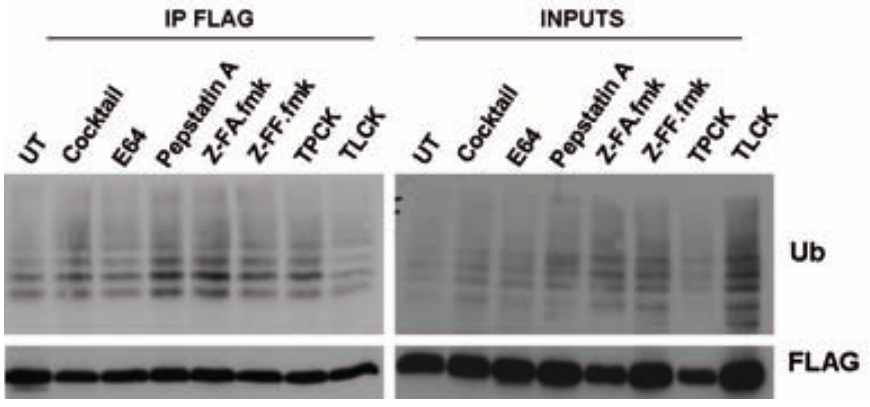

Figure R19. Inhibition of proteases doesn't alter LFG ubiquitination. HEK293T cells were transfected with LFG-FLAG. 24h after transfection, cells were treated with the indicated protease inhibitors as indicated for 24h. Cocktail refers to a combination of all the protease inhibitors. FLAG-tagged proteins were inmunoprecipitated and ubiquitination was assessed by ubiquitin inmunodetection.

LFG ubiquitination pattern didn't change by treatment with any of the protease inhibitors used (Figure R19). The signal of the ubiquitin pattern was constant in all lanes, indicating that the amount of LFG ubiquitinated doesn't change when proteases are inhibited. All in all, these data indicates that LFG ubiquitination does not induce its degradation.

LFG ubiquitination is not modulated after DR stimulation or induction of ER stress

In order to study LFG ubiquitination, we raised the question whether LFG ubiquitination could be modulated by apoptotic stimuli. We hypothesized that LFG ubiquitination could have a role in its anti-apoptotic function, so we wanted to study if there were any change in the pattern of ubiquitination of LFG after DR stimulation. To do so, HEK293 cells were transfected with LFG-FLAG and treated with 100ng/ml LZ-FasL/LZ-CD95L, 100ng/ml TNF-α and/or 1nM Actinomycin D, a compound that inhibits protein expression and is needed in some cell types to render them sensitive to DR stimulation.

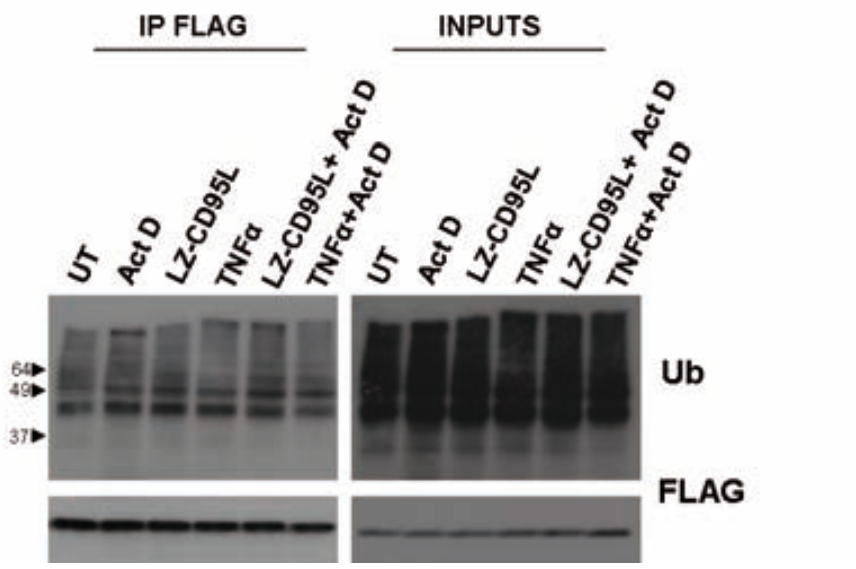

Figure R20. DR stimulation does not induce any changes in LFG ubiquitination. HEK293 cells were transfected with LFG-FLAG. 24h after transfection, cells were treated as indicated for 24h. FLAG-tagged proteins were inmunoprecipitated and ubiquitination was assessed by ubiquitin inmunodetection.

LFG treatment with LZ-FasL or TNF-α doesn't have any impact on LFG ubiquitination (Figure R20). Co-treatment with ActD didn't cause any change in the ubiquitination pattern either. The intensity of the ubiquitination pattern in

each condition is the same, indicating that LFG ubiquitination is not modulated by any of the stimuli used.

To further study if apoptotic stimuli could have any impact in LFG ubiquitination, we treated cells with tunycamicin, an inhibitor of *N*-glycosilation and thapsigargin, a SERCA-inhibitor. Tunycamicin induces activation of the UPR, and thapsigargin impairs calcium release from the ER. Both compounds lead, ultimately, to induction of apoptosis by ER stress. Since ER stress apoptotic pathway shares features with Fas-apoptotic signaling, we treated LFG-FLAG transfected HEK293 cells with them, and checked for changes in the LFG ubiquitination pattern.

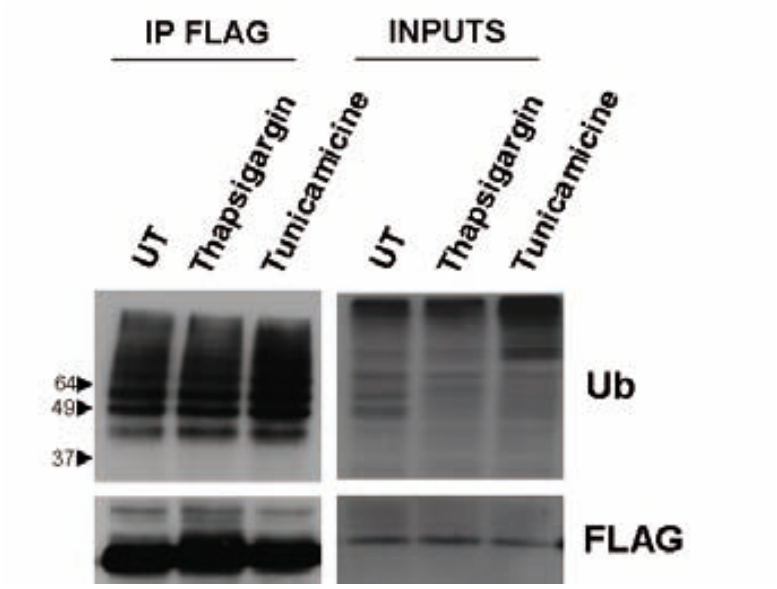

Figure R21. Induction of ER stress does not induce any changes in LFG ubiquitination. HEK293 cells were transfected with LFG-FLAG. 24h after transfection, cells were treated as indicated for 24h. FLAG-tagged proteins were inmunoprecipitated and ubiquitination was assessed by ubiquitin inmunodetection.

When cells were treated with tunycamicin or thapsigargin, no changes in the ubiquitination pattern of LFG could be seen (Figure R21). The intensity of the bands in the treated cells was the same as in the control lane, indicating that the amount of ubiquitinated LFG is the same in all the samples. Thus, induction of ER stress doesn't modulate LFG ubiquitination. Alltogether with the previous results, we can conclude that induction of apoptosis by DR stimulation or ER stress doesn't yield any changes in LFG ubiquitination.

LFG ubiquitination can be located at *N*-terminal region lysins

In order to study the location of LFG ubiquitination inside the protein, we performed a Maldi-Toff analysis. Ubiquitin normally bounds to target proteins by covalent union with Lys residues, although it can also bind to Ser or Thr residues. One way to elucidate the location of the aminoacid ubiquitinated is by the use of proteases. Some proteases like trypsin, cleave proteins at the *C*-terminal side of Lys or Arg aminoacids. However, when an ubiquitin is present at a Lys residue, trypsin can't cut it due to steric hydrance. By analyzing the peptides resultant of trypsin digestion, we can identify putative candidate lysins that can be ubiquitinated.

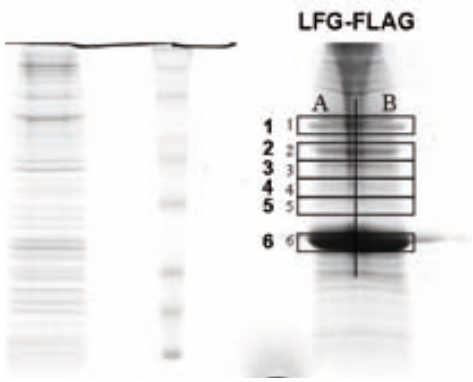

Figure R22. LFG-FLAG was purified and analyzed by Maldi-Toff analysis. HEK293T cells were transfected with LFG-FLAG. 24h after transfection, FLAG-tagged proteins were inmunoprecipitated. Eluated protein was run in a 12% acrymalide gel and then it was stained with Comassie Blue to check protein loading. Bands 1-6 were cut separately and then two fractions (A,B) of each band were used for different protease digestion and Maldi-Toff analysis.

Maldi-Toff analysis was performed by the Proteomics group of Dr.Canals in Valld'Hebron. Briefly, LFG-FLAG was purified by inmunoprecipitation, and then ran an acrylamide gel. After electrophoresis, the gel was stained with Blue Comassie and bands corresponding to LFG-FLAG and its ubiquitinated forms were cut and purified (Figure R22). Samples were fractionated and digested with trypsin, which cleaves at *C*-ter site of Lys and Arg residues of proteins, or proteinase K, a protease that cuts proteins inespecifically. Then, resultant peptides were analyzed by phase-reverse nano-liquid chromatography coupled to a mass spectroscopy electrospray-ionic trap. Peptides were identified by comparing to LFG sequence.

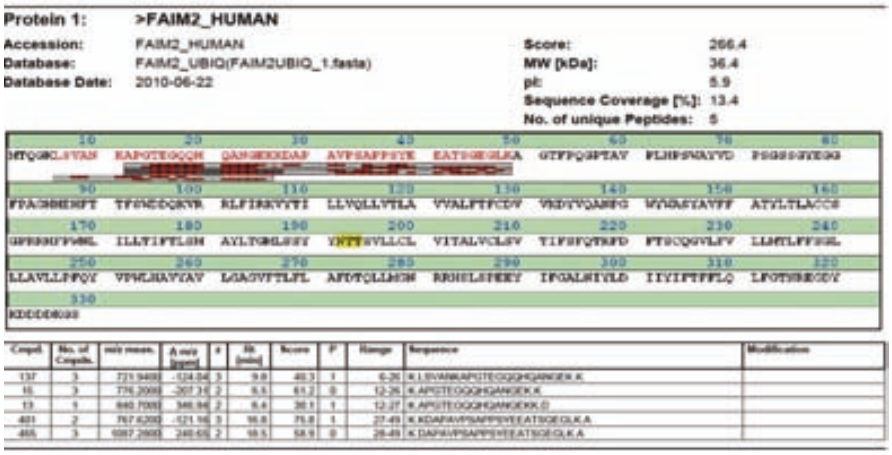

Figure R23. LFG results after Maldi-Toff analysis. Report for LFG after mass spectrometry analysis of the digested bands. Residues detected by mass spectrometry are marked in red under the LFG sequence. The table below shows the full peptides detected.

LFG could be identified in all bands analyzed, and ubiquitin could be identified in 1-5 bands, but not in band 6. We could identify some peptides from the *N*-terminus side of LFG, and that allows for discarding some Lys residues that are next to the resultant peptides, because trypsin could cleave on that position (Figure R23). No peptides from the *C*-terminus side of LFG were detectable. That may be due to high hydrophobicity of the resultant peptides that difficult its detection, or that the peptides were too large. It was probably a combination of both, since LFG high hydrophobic sequence also difficults proteases to reach cleaving points, and thus generates larger peptides from the digestion. So, we can identify peptides from the *N*-terminus side of LFG corresponding to all the Lys of that region, except for Lys98 and Lys106. Thus, these two positions are the candidates to be ubiquitination sites of LFG.

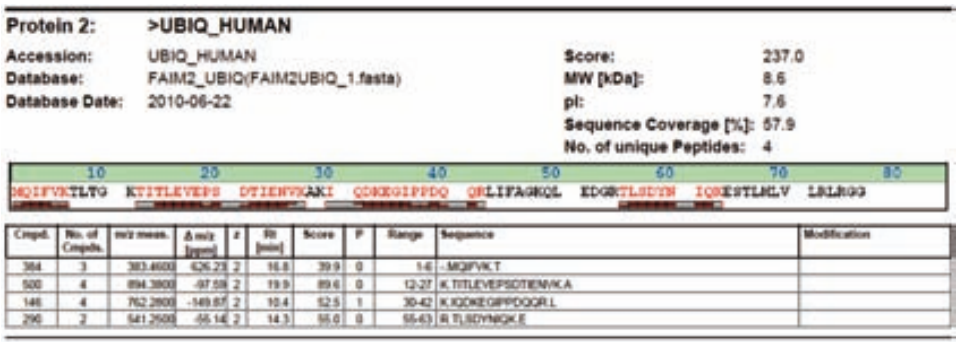

Figure R24. Ubiquitin results after Maldi-Toff analysis. Report for ubiquitin after mass spectrometry analysis of the digested bands. Residues detected by mass spectrometry are marked in red under the ubiquitin sequence. The table below shows the full peptides detected.

The analysis of ubiquitin peptides revealed that peptides from all the Lys could be identified except from Lys48 (Figure R24). Thus, that suggests that LFG poliubiquitination could be through K48 linkage. Although K48 ubiquitination has been usually linked to proteasome degradation, we have proved that LFG ubiquitination does not induce its degradation. Nonetheless, some reports have linked K48 to other cellular functions apart from protein degradation. Further experiments should be done in order to characterize the implications of LFG ubiquitination.

DISCUSSION

DISCUSSION

LFG is ubiquitinated

Ubiquitination of proteins has an outstanding role in the modulation of several cellular processes, such as apoptosis. The levels of several pro- and anti-apoptotic proteins are determined by degradative ubiquitination, and the activity of some proteins, like caspases, can be modulated too by non-degradative ubiquitylation [256]. Thus, understanding how E3 ligases and modulator proteins are regulated, as well as, the changes ubiquitin can induce when it is attached to target substrates, is of major importance.

In our study characterizing the DR antagonist LFG, we found that it interacts with several proteins of the ubiquitin system by Yeast Two Hybrid assay. So, we sought to confirm this interaction by co-inmunoprecipitation. In our experiments, LFG showed a marked ubiquitination pattern. When different LFG constructs were analyzed, the ubiquitination pattern changed its Molecular Weight (MW) in coherency with the different MW of the constructs used. Thus, our results prove that LFG is ubiquitinated.

This finding led us to investigate its possible role in LFG function. The most common function of protein ubiquitination is to target proteins for degradation. In order to search if that was the case for LFG, we treated cells with a well characterized inhibitor of the proteasome, the compound MG132. Our results show no changes in the levels of LFG ubiquitination after MG132 treatment, neither after treatment with a cocktail of protease inhibitors that included inhibitors of cathepsins and calpains. All in all, we can conclude that LFG ubiquitination is non-degradative.

To further investigate the role of LFG ubiquitination, we wanted to search whether it was modulated after apoptotic stimuli. Again, no changes in the pattern of LFG ubiquitination were observed when cells were treated with different apoptosis inductors, such as FasL, TNF-α, thapsigargin or tunycamicin. In summary, we have demonstrated that LFG ubiquitination are not modulated after DR stimulation or ER stress induction.

Our hypothesis was based on the role of ubiquitination in another well-characterized DR antagonist, $FLIP_L$. It has been found that $FLIP_L$ is ubiquitinated after TNF-α treatment, and that leads to proteasomal degradation of $FLIP_L$ [252]. Lower levels of $FLIP_L$ sensitize to apoptosis, as it is unable to get recruited to DISC and inhibit caspase-8 activity. Moreover, $FLIP_L$ levels of ubiquitination are upregulated as soon as 20min after TNF-α treatment, and total levels of $FLIP_L$ began to fall after 4h of treatment. We couldn't observe any of these phenomena with LFG in our model, thus indicating that ubiquitination of LFG and $FLIP_L$ play very different roles.

Other functions of ubiquitination are related to regulation of protein activity and/or localization inside the cell . These processes are linked to K63 polyubiquitination or monoubiquitination. However, monoubiquitination is not likely since the pattern of LFG ubiquitination is related to polyubiquitinated proteins, as it shows presence of high MW forms.

In order to investigate the location of LFG ubiquitination, as well as the Ub-linkage, we performed a Maldi-Toff analysis of ubiquitinated LFG. With this method, we could study the location of ubiquitinated lysines inside LFG sequence, and also the lysine used as a linkage in the ubiquitin.

The results of this assay were not as clear as expected. Due to the high hydrophobic nature of LFG protein, the majority of the peptides of the *C*-terminal side of the protein, equivalent to its seven putative transmembrane domains, couldn't be detected by mass spectrometry. However, peptides of the *N*-terminal side of LFG and from ubiquitin could be analyzed.

From LFG analyzable peptides, we could conclude that ubiquitin is located either at the lysines K98 or K106. The analysis of peptides from ubiquitin showed that the linkage to LFG was through K48.

K48-linked polyubiquitin chains primarily target proteins for proteasomal degradation. By contrast, we have previously demonstrated that LFG ubiquitination does not induce its degradation so, apparently, our results are contradictories. However, we cannot exclude the possibility that we are in front of a non-orthodox K48 polyubiquitylation, which does not induce degradation,

as has been reported in some instances. It can also be possible that LFG is ubiquitinated by a mixed Ub-chain, in which K48 and K63-linked ubiquitins associate. This type of Ub-linkage is linked to signaling processes. In resume, it is likely that LFG may be ubiquitinated in a non-classical way.

On the other way, a recent study used di-glycine antibodies to enrich ubiquitinated proteins from tissue extracts, and then analyzed them by mass spectroscopy [257]. This method allowed the authors to identify around 20.000 putative ubiquitination sites in a vast sunset of proteins. Among them, it was LFG, and they identified hypothetic ubiquitination sites at its lysines K26, K27, K49, K11 and K5. These results are in contradiction with our mass spectroscopy analysis, which identified possible ubiquitylation sites at K98 and K106. These differences could be explained by the methods used. In our analysis, we purified recombinant LFG, and ubiquitylation sites were identified using different proteases. One of these proteases was trypsin, which cleaves sequences after Lysines residues only if there is not an ubiquitin molecule bound to that aminoacid. In the study reported, they first do an enrichment ubiquitinated protein extract from a whole tissue, and then identify the peptides by mass spectroscopy. This first step of enrichment and being a whole tissue extract is more prone to false positives, and it's likely that the peptide identified could be mistaken by another protein with similar sequence. Nonetheless, more experiments should be done to verify the location of LFG ubiquitylation sites, and to give a functional approach to LFG ubiquitination.

An analysis of LFG sequence reveals that it contains a PPXY motif, formed by the conserved sequence of aminoacids (Proline – Proline – any aminoacid - Tyrosine), which is comprised in the 35-38 aminoacids of its sequence. The PPXY motif is a consensus sequence for interaction with WW-domains, also known as the rsp5-domain or WWP repeating motif, which are present in HECT-E3 ligases [258]. The most studied protein of this family is the E3 ligase Nedd4. The PPxY motif is present in factors regulating the activity of HECT-E3 ligases, and interactions between WW-domains and PPxY have been shown to be important in the regulation of the Hippo signaling pathway. Moreover, the PPxY motif is present in the capsid of some adenovirus and, along with Nedd4 ligase activity, facilitates adenovirus entry to the cell.

Although not explored in detail, this is in coherency with the finding that LFG is ubiquitynated. Since LFG have a consensus sequence for interaction with HECT-E3 ligases, it is easy to think that it may be ubiquitylated by one E3-ligase of this family. In fact, in the thesis project of Tatiana Hurtado de Mendoza, it is mentioned that LFG is able to interact with the E3-ligase AIP4. This ligase, also known as Itch, is a member of the HECT-E3 family of ligases and, among other functions, regulates $FLIP_L$ stability in TNF-α induced apoptosis. However, in the thesis project of Tatiana they fail to demonstrate that AIP4 ubiquitylates LFG.

In summary, a great portion of data suggests that LFG is ubiquitylated, and that it can interact with E3-ligases from the HECT family. Whether LFG ubiquitylation has a role, or it is just a collateral effect of its interaction with proteins from the ubiquitin family, as has been suggested for other proteins, is unknown. The location of ubiquitylation sites in LFG sequence and the type of ubiquitylation has to be explored more in depth, since the number of studies done at the moment is low and they yield contradictory results on top of it. Nonetheless, the evidences found till now are promising and more investigation on LFG function will shed some light on the ubiquitylation processes in which LFG is involved.

LFG localizes at the ER and Golgi membranes, and in the vesicles of the endocytic pathway

Early studies on LFG addressed its localization in mice brain layers, and showed that it is expressed in postsynaptic sites and dendrites. Moreover, its localization in the plasma membrane, more specifically in the lipid rafts where it interacts with Fas receptor, has been demonstrated [235]. However, a clear punctate pattern not only in the plasma membrane but also in cell body is detectable when using LFG antibodies for immunofluorescence. Thus, a more detailed study on its intracellular localization was lacking. Here, we report that LFG localizes at the ER and Golgi membranes, and to a lesser extent, in the endosomes.

Upon Fas stimulation, the receptor is internalized and DISC is formed in early endosomes [163, 259]. Given that LFG interacts directly with the Fas receptor, it

is not surprising to find that this DR antagonist colocalizes with early endosome markers. Some studies attribute the rate of internalization of the Fas receptor as one of the mechanisms of protection/sensitization to Fas-induced cell death. LFG may participate in controlling the endocytosis of the receptor after stimulation, hence indirectly regulating the amplitude of the apoptotic signal.

Interestingly, we have previously mentioned that LFG carries a PPxY motif, thus suggesting that it interacts with HECT-E3 ligases. Nedd4 subfamily of ligases, the most characterized one from HECT family, contains a C2 domain that interacts with molecules such as phospholipids. Its function is to reinforce adapter-mediated recruitment of proteins to the specific membranes, hence being responsible of intracellular targeting and protein sorting to MVBs (multivesicular bodies) and endosomes. The role of Nedd4 in the internalization of several plasma membrane receptors is well characterized [260, 261]. Taking into account the capacity of LFG to interact with these molecules, and its localization in the plasma membrane and the endosomes, we can speculate that it may play a role in receptor internalization, maybe as a docking point for E3 ligases to ubiquitinate the receptors, or aiding in the internalization process by an unknown mechanism.

Our results prove that the bulk of LFG inside the cell is localized at the ER and Golgi membranes. Given that LFG is a member of the TMBIM family proteins, whose most studied members have been found to localize at ER and Golgi membranes, it is not surprising that LFG itself localizes there too. In fact, in agreement with our findings, a study addressing other TMBIM family members reported that LFG also localizes at the membranes of the Trans-Golgi complex [254].

LFG protects type II, but not type I cells, from Fas-induced apoptosis

To date, the mechanism of LFG protection from Fas-induced apoptosis is still unknown. It has been proposed to act at the level of caspase-8 activation, since it has been shown that it is able to inhibit its activity. In addition, its location at plasma membrane and its interaction with Fas receptor led to the hypothesis that it may act at the level of DISC formation, thus inhibiting caspase-8 activity

[229, 235]. However, no changes in the FADD expression or interaction with Fas were found in LFG overexpressing cells, so this is a flaw to that hypothesis.

In all the reports where LFG protection from Fas-induced apoptosis was studied, not attention was taken whether the cell lines used were type I or type II. The cell lines used in these studies, such as HeLa, SH-SY5Y, cortical or granular neurons, behave as type II cells. Thus, no data was available on LFG overexpression impact on the type I mode of Fas-induced apoptosis. Our results show for the first time that LFG is only able to protect type II cells from apoptosis induced after FasL treatment.

Intriguingly, we found that LFG is not localized at the mitochondria. Given that LFG has the capacity to block apoptosis only in type II cells, it would be reasonable to assume that it should be found in the mitochondria where it would participate in inhibiting mitochondrial membrane permeabilization. This suggests that LFG must be doing its anti-apoptotic function elsewhere, and thus there are other organelles implicated in the signaling cascade of Fas-induced apoptosis.

Bcl-xL is a discriminator between type I and type II modes of Fas-induced apoptosis. Like LFG, it is able to protect only from type II induced cell death. In the case of Bcl-xL, it is because it inhibits mitochondria permeabilization, thus blocking the release of apoptogenic factors and halting the apoptotic signaling cascade [262, 263]. However, it is also able to exert anti-apoptotic functions in the ER. Since we have demonstrated that LFG localizes at the ER, we sought to study if LFG it interacts with Bcl-xL, and in that case, the ER would be a good candidate for being the missing piece implicated in Fas-apoptotic signaling.

LFG-Bcl-xL interaction

Bcl-2 family proteins are key mediators of the apoptotic signaling. Their main function is to regulate the permeabilization of the mitochondria membrane through the balance of the protein levels between its anti- and pro-apoptotic members. Moreover, Bcl-2 family proteins also play a prominent role in regulating calcium homeostasis within the cell, by controlling the ER calcium release [110, 264]. As high cytosolic levels of calcium are linked to apoptosis

induction, Bcl-2 and Bcl-xL also exert anti-apoptotic functions in the ER by lowering the amount of calcium release after an apoptotic insult. In this context, BI-1 also plays a role. It can interact with Bcl-2 and Bcl-xL and control its activity, thus modulating calcium release from the ER [127].

Here, we demonstrate that LFG, which has high homology with BI-1 [265], can interact with Bcl-2 and Bcl-xL, but not with Bax or Bak. In addition, using truncated forms of LFG, we identified its C-terminal as the site of interaction. Finally, endogenous colocalization was assessed by inmunofluorescence. All in all, our results demonstrate that LFG interacts with Bcl-xL and Bcl-2.

Given the high resemblance between BI-1 and LFG, one could think that the capacity of LFG to interact with Bcl-xL and Bcl-2 may be a reminiscent of its function. There is evidence that link the function of TMBIM family proteins in regulating Bcl-2 family members, through different mechanisms. It can be possible that LFG exerts its anti-apoptotic effects by modulating the activity and/or stability of Bcl-xL.

LFG protection from Fas-induced apoptosis is dependent on endogenous expression of Bcl-xL

During ER stress-induced apoptosis, depending on the apoptotic stimuli used and the state of the cell, various signaling pathways are involved that ultimately lead to cell death. Calcium release from the ER causes a rise in cytosolic calcium concentration that activates calpains, which in turn are able to activate the ER-localized initiator caspases -4 and -12, which will initiate the apoptotic signaling cascade [111]. Other mechanisms suggested involve the ER-mitochondria calcium transmission to induce cytochrome c release. Nonetheless, all this events depend firstly on calcium mobilization from the ER to occur. All this is inhibited by Bcl-2 and Bcl-xL at the point of calcium release, thus blocking apoptosis. However, the action of Bcl-2 anti-apoptotic members on ER calcium release is dependant on the expression of BI-1, and it has been shown that BI-1 is essential for resistance to ER-stress-induced apoptosis. Thus, BI-1 expression is required for Bcl-xL protection from ER-stress-induced apoptosis.

In our work, we have shown that LFG and Bcl-xL protect type II cells from Fas-induced apoptosis, and that these two proteins directly interact. Therefore, we tested the hypothesis that the action of one of them might depend on the presence of the other, as in the BI-1-Bcl-xL binomy in the ER. We demonstrate that LFG protection from FasL-induced cell death is dependant on Bcl-xL endogenous expression. This is opposite to the case from BI-1-Bcl-xL in ER-stress-induced apoptosis, where Bcl-xL protection is dependant on BI-1 expression.

The fact that Bcl-xL endogenous expression is required for LFG protection from Fas-induced apoptosis can be explained by several ways. First of all, it can be that Bcl-xL acts upstream than LFG in the apoptotic signaling cascade. If Bcl-xL exerts its anti-apoptotic effects before LFG, then its action would not be affected by LFG downregulation. However, in that case LFG would still be able to act, thus our data indicates that this is likely not the case.

Another possible option would be that LFG could be regulating Bcl-xL activity and/or stability, and exert its anti-apoptotic effects through the stabilization or potentiation of Bcl-xL activity. And example of this mechanism of action was described recently by our lab, in the case for $FAIM_L$ and XIAP. $FAIM_L$ was shown to stabilize XIAP by reducing its auto-ubiquitylation and proteasomal degradation, thus upregulating XIAP levels and increasing the pool of XIAP able to interact and inhibit caspases. In this case, XIAP endogenous expression is required for $FAIM_L$ protection from Fas-induced cell death, since $FAIM_L$ exerts its anti-apoptotic effects through XIAP stabilization. Since that's the case also for LFG with Bcl-xL, we are inclined to think that's the most pausible mechanism of action.

Finally, we know that Bcl-xL acts at several levels of Fas-induced apoptosis signaling. Normally, its anti-apoptotic effects during Fas-induced cell death are linked to its prevention of mitochondria permeabilization. In fact, it has been shown that downregulation of Bcl-xL is required to sensitize some cell lines to DR-induced apoptosis, and in our work, the downregulation of Bcl-xL causes an increment in the apoptosis caused by FasL treatment. Thus, we can't rule out that the downregulation of Bcl-xL renders the cell unable to react to apoptotic

stimuli, and thus Bcl-xL downregulation would be too hard to overcome, even overexpressing other anti-apoptotic proteins like LFG.

Additional experiments should be performed in order to elucidate the implications of the interaction between LFG and Bcl-xL. Since LFG downregulation didn't have any effect on Fas-induced apoptosis in our cell model, while Bcl-xL sensitized cells to FasL treatment, we can conclude that Bcl-xL has a major impact than LFG in the Fas apoptotic signaling.

LFG inhibits calcium release from the ER after FasL stimulation

The last step in our work was to try to elucidate the mechanism of LFG anti-apoptotic activity. So far, we have described that LFG is localized at the ER, and is able to protect only type II cells from Fas-induced apoptosis, in a Bcl-xL-dependent manner. On the other hand, LFG has high homology with BI-1, the most characterized protein of the TMBIM protein family. BI-1 regulates the intrinsic cell death pathway that protects cells from DNA-damaging agents and ER stress inducers. It interacts with Bcl-xL in the ER and regulates calcium signaling.

Fas-mediated apoptosis shares some features with intrinsic death pathways, such as ER stress, and promotes a rise in intracellular calcium, which mediates some apoptotic features attributed to Fas-induced cell death [114, 238]. On the basis of all these observations, we hypothesized that LFG plays a role in this context.

We propose that LFG exerts its anti-apoptotic effects via an ER-dependent mechanism. Indeed, we show that LFG overexpression impairs calcium release from the ER after FasL stimulation. Both the number of cells reacting to FasL by mobilizing calcium and the rate of calcium release were lowered when LFG was overexpressed. As expected, Bcl-xL overexpression totally abrogated calcium release. Bcl-xL has been reported to modulate cell death by inhibiting calcium release from the ER. In the same way, BI-1 also modulates calcium release by controlling the activity of Bcl-2 and/or Bcl-xL. Since LFG has a very similar structure to BI-1 and also interacts with Bcl-xL, we consider that LFG may exert its anti-apoptotic effect by impairing the ER calcium release induced by FasL.

The crosstalk between the ER and mitochondria, and the calcium efflux between them are important steps in Fas apoptotic signaling. However, the ER contribution to Fas apoptotic signaling has not been studied extensively. Calcium release from the ER after Fas stimulation has been described for several cell lines, but a clear role for its contribution in Fas apoptotic signaling is lacking.

There isn't a high amount of data regarding calcium mobilization on Fas-induced apoptosis, and only few papers about the topic can be found in the bibliography. It has been shown that abrogation of calcium release from the ER after FasL treatment protects cells from apoptosis cell death assessed by measuring condensation and fragmentation of the nuclei [190]. However, caspase activity is not inhibited. Thus, it seems that calcium mobilization is required for some features of apoptosis, but has no effect on others. In this context, calcium may be important for activation of the apoptogenic factors released after mitochondria permeabilization, specifically endonucleases, since DNA condensation and fragmentation is inhibited, but not caspase-3 activity.

Another report goes further and describes that after Fas stimulation, there is an activation of PLC-γ that results in generation of IP_3, which will interact with IP_3 receptors (IP_3R) in the ER and induce calcium release [114]. This first wave of calcium release is required for mitochondria permeabilization. Moreover, cytochrome c is able to bind to IP_3R and induce calcium release from the ER, generating a second wave of calcium mobilization that works as a positive feed-back loop between the ER and the mitochondria.

Our results point out directly at this step as the point at where LFG exerts its action. Bcl-xL has been shown to interact with IP_3R and modulate its activity. Moreover, BI-1 also interacts with IP_3R and controls calcium release from the ER under stress conditions. On the basis of our observations, we propose that LFG may be inhibiting calcium release from the ER after FasL treatment in a similar manner. Since LFG anti-apoptotic activity is dependant on Bcl-xL endogenous expression, we can speculate that its mechanism of action would be either by modulating the activity of Bcl-xL or by stabilizing it. More

experiments need to be performed in order to elucidate the involvement of LFG-Bcl-xL interaction in the calcium release induced by FasL.

Another step in the ER-mitochondria calcium conundrum during Fas apoptotic signaling has been described that involves the protein Bap31. It's and ER-located protein that forms a multi-protein complex in association with Bcl-xL, BI-1, procaspase-8 and BAR, among other proteins. During Fas-induced apoptosis, caspase-8 cleaves Bap31, generating a smaller fragment (p20). Inhibition of Bap31 cleavage inhibits apoptosis, and the p20 fragment induces apoptosis when overexpressed [121, 122]. Bap31 cleaved form is relevant for the formation of ER-mitochondria microdomains that generate a calcium efflux between the two organelles, which is required for mitochondria permeabilization. These observations allow us to hypothesize another possible mechanism for LFG protection from Fas-induced apoptosis. Since LFG has been shown to inhibit caspase-8 activity in type II cells, it could be possible that inhibition or impairment of Bap31 cleavage is another consequence of LFG activity, which will lead to a reduction of calcium mobilization.

Finally, there is a report linking LFG to other modes of cell death different from Fas-induced apoptosis [239]. Specifically, LFG downregulation has been shown to sensitize cells to perifosine-induced apoptosis. Perifosine is a chemical compound that acts as an Akt inhibitor and PI3K inhibitor. The cell death induced by this chemical has been linked to alteration of multiple signaling pathways, like inhibition of NF-κB pathway, or activation c-Jun N-ter Kinases and ER stress pathways. Given our data about LFG inhibition of calcium release from the ER, is reasonable to think that the results of that report are caused by impairment of calcium mobilization during ER stress. Thus, it would be interesting to explore the effects of LFG on other models of cell death, such as ER stress or DNA-damaging agents.

CONCLUSIONS

CONCLUSIONS

First: LFG is ubiquitinated.

Second: LFG ubiquitination is located at the *N*-terminal lysines.

Third: LFG ubiquitination is through K48-linkage, although it doesn't induce its degradation.

Fourth: LFG ubiquitination cannot be modulated by apoptotic stimuli such as DR stimulation or ER stress induction.

Fifth: LFG localizes to ER and Golgi membranes, and to a lesser extent, to the endosomes.

Sixth: LFG is able to protect only type II cells from Fas-induced apoptosis.

Seventh: LFG interacts with Bcl-xL through its *C*-terminal region. This interaction is most likely located at the ER.

Eighth: LFG protection from Fas-induced apoptosis is dependant on Bcl-xL expression.

Ninth: LFG is able to inhibit calcium release from the ER after FasL treatment.

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ANNEX

Transgenic mice generation

Cloning of $FAIM_S$, $FAIM_L$ and LFG in transgenic vector

Genetically modified mice are extensively used in biomedical research. They can be used as models of human diseases, and have proven to be a crucial tool for the understanding of the mechanism of various pathologies. For example, mice carrying cloned oncogenes or knockout mice for tumour suppressor genes are good models for human cancer.

The most common type is the knockout mice, where the activity of a gene (or various genes) is removed. However, several types of transgenic mice can be developed, like knock-in mice, where a target gene is overexpressed. Conditioned knock-out or knock-in of a gene can be achieved to restrict the expression of our target gene in specific tissues, or to temporally regulate the expression of the target gene.

To generate the transgenic mice, the DNA is injected first into embryonic stem (ES) cells. ES cells that have undergone homologous recombination are identified and injected into a 4 day old mouse embryo, which is called a blastocyst. It is implanted in the uterus of a fertilized female mouse, and later the offspring is tested for presence of the gene. Finally, heterozygous offspring is mated again to produce homozygous transgenic strain.

Our objective was to create conditional knock-in mice for our desired genes with conditional adult neuronal specific expression pattern.

To regulate the expression of our target genes, we are going to use the Tet-off/on system. In fact, these are two different systems, Tet-off and Tet-on. In each system, a recombinant tetracycline controlled transcription factor (tTA or rtTA) interacts with a tTA/rtTA responsive promoter, P_{tet}, to drive expression of the gene under study. Expression is regulated by the effector substance tetracycline. rtTA requires a tetracycline ligand for DNA binding and hence, transcription. In contrast, the interaction between tTA and DNA is prevented by tetracycline. Thus, the two versions of the Tet system respond to tetracycline in opposite ways. In our model, we are going to use the Tet-off version. Hence, our target gene will be constitutively overexpressed and we can down-regulate its expression by feeding the transgenic mice with tetracycline. Actually, doxycicline is the compound used instead of tetracycline, as they structure is similar and doxycicline has higher affinity for both tTA and rtTA, so can be used at lower concentrations and thus is likely it will generate less secondary effects.

At the same time we want our transgenic mice to overexpress our target gene only in the adult neurons. To do so, the expression of the tTA protein will be under the control of the promoter of the CamKIIα, a protein that is expressed specifically in the cortex and the hippocampus [253]. Thus, to start we need two separate transgenic mice; one carrying our target gene under the control of the P_{tet} promoter, and other with the tTA protein under the control of CamKIIα expression. Then, these two mice will be crossed and the inbred mice carrying our desired genes will be characterized.

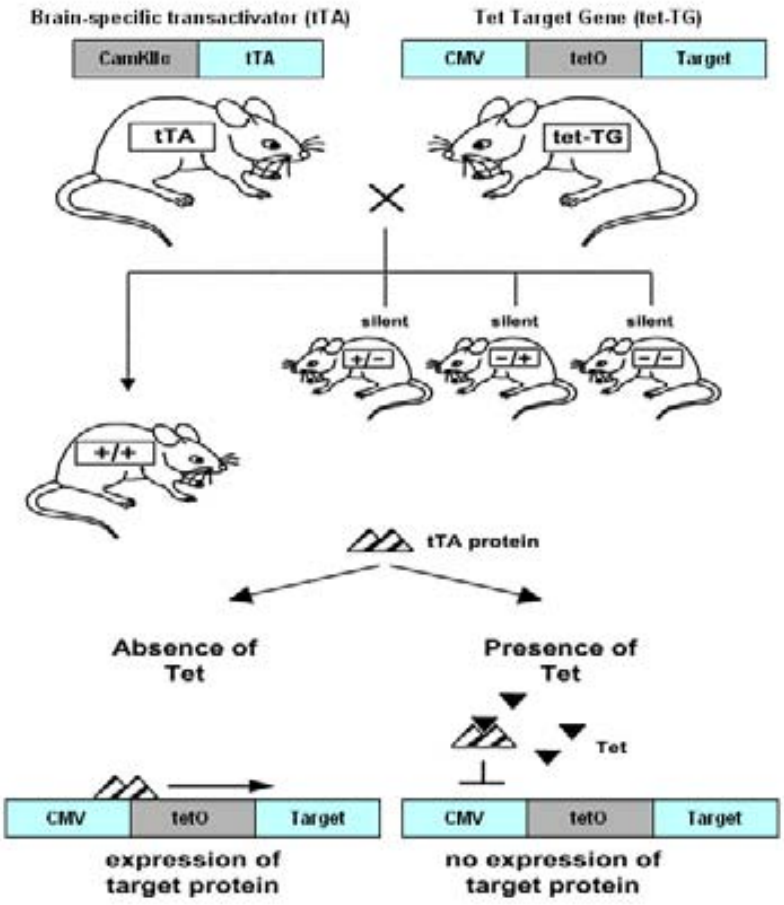

Figure A1. Transgenic mice generation strategy for $FAIM_L$, $FAIM_S$ and LFG. To produce transgenic mice with conditional overexpression of our target genes in the adult CNS, we use the TetOff system with the tTA protein under the control of CamKIIα promoter. Crossing of mice with our target gene under TetOff system, and mice with tTA under CamKIIα promoter, will generate transgenic mice with our desired characteristics.

Our aim is to generate transgenic mice for $FAIM_S$, $FAIM_L$ and LFG. These proteins have been cloned previously in our lab. Before the injection of the DNA in the embryonic stem cells, we have to construct the plasmid with our target gene under the control of the P_{tet} promoter. Additionally, we are going to insert an IRES-GFP sequence in the 5' end of our target gene, so GFP fluorescence can be used as a marker to monitor the expression of our vector.

Here, I am going to describe the construction of the transgenic expression plasmids. I will be referring to the construction of the $FAIM_L$ one as an example, since all of them were generated in the same way and the strategy for cloning and testing was the same.

Cloning of $FAIM_L$ into transgenic vector

Rat $FAIM_L$ was previously cloned in our lab into a pcDNA3 expression vector, and fused to a 3xFLAG tag at his 5'. That was the plasmid used as a template for generating the insert cDNA for $FAIM_L$. We wanted to generate our transgenic plasmid with a GFP marker to be able to monitor the expression of our gene. So, before cloning the $FAIM_L$ sequence into our transgenic vector, we had to fuse it to an IRES-EGFP sequence. To do so, we had to perform two cloning steps. In the first one, we had to clone the $FAIM_L$ sequence in-frame in a vector containing the IRES-EGFP sequence (pIRES2-EGFP). Then, the $FAIM_L$-IRES-EGFP sequence could be substracted from that plasmid and cloned into the transgenic vector PMM400, under the control of P_{tet} promoter. A scheme of the cloning strategy it's represented below.

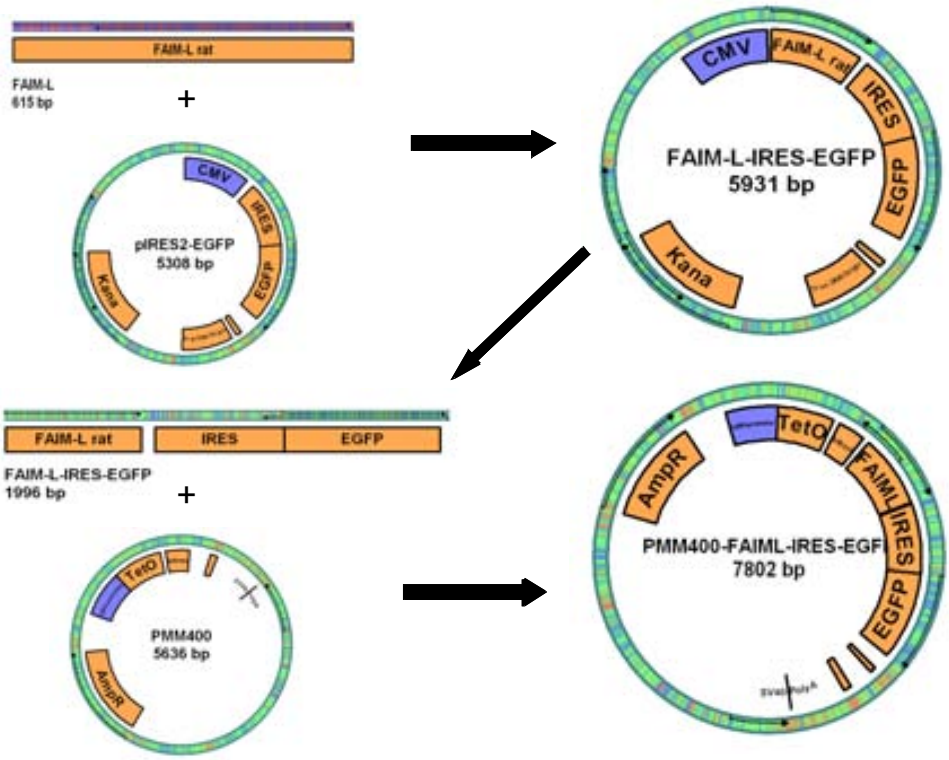

Figure A2. Cloning strategy of the transgenic plasmid for $FAIM_L$. $FAIM_L$ sequence was extracted from a previously cloned plasmid in our lab, and cloned inside the pIRES-GFP plasmid. Then, $FAIM_L$-IRES-GFP sequence is extracted and cloned inside the PMM400 plasmid, which carries the TetO, or P_{tet}, promoter upstream our target sequence.

When $FAIM_L$ was cloned in our lab, one BamHI target site at the 5' and aXhoI target site at the 3' were added in order to be able to extract $FAIM_L$ sequence without the FLAG-tags easily. Thus, we digested the pcDNA3-3xFLAG-$FAIM_L$ plasmid with BamHI and XhoI, and the 615bp band, corresponding to $FAIM_L$ cDNA, was purified. After purification, the 3' and 5' ends were blunted. In parallel, the pIRES-EGFP vector was digested with XhoI, blunted and dephosphoriled. After this, 50ng of the vector was mixed with 150ng of the insert and ligation was performed. The ligation was transformed into XL-Blue competent cells, and after 24h of incubation of the bacteria plates at 37ºC, 10 colonies were selected for analysis. To verify which colonies were positive for our plasmid, we did digestion with different restriction enzyme. Depending on the size of each plasmid and the enzyme restriction sites in its sequence, they generate a different pattern of bands when digested with a specific restriction enzyme and then ran in an agarose gel. Thus, this can be used as a "fingerprint" for our plasmid, since our construction will have a specific pattern of digestion with some enzymes that will be different from the religated vector or other aberrant forms of our plasmid that may have formed.

After this, we selected a positive colony, the one which yielded a better plasmid expression as seen by intensity of the band after electrophoresis in an agarose gel. The plasmid obtained in this first round of cloning was called $FAIM_L$-IRES-GFP. We cloned the sequence of $rFAIM_L$ in-frame with the IRES-GFP sequence of our vector, so we generated a construction that will express $FAIM_L$ and GFP, and then green fluorescence can be used as a marker for expression of our gene.

The last step to generate our transgenic vector is to insert the $FAIM_L$-IRES-EGFP sequence into the PMM400. It has a P_{tet} promoter with a multi-cloning site on his 3' end, so it's designed to clone a target gene under the control of that promoter. We used XhoI/XbaI digestion to purify our insert from $FAIM_L$-IRES-EGFP plasmid, and the same digestion was performed in the PMM400 vector, followed by dephosphorilation. 50ng of the vector was mixed with 150ng of the insert and ligation was performed. The ligation was transformed into XL-Blue competent cells, and after 24h of incubation of the bacteria plates at 37ºC, 8 colonies were selected for analysis. Digestion of the colonies with NotI, BglII, BamHI and HindIII restriction enzymes was performed to check for our target plasmid.

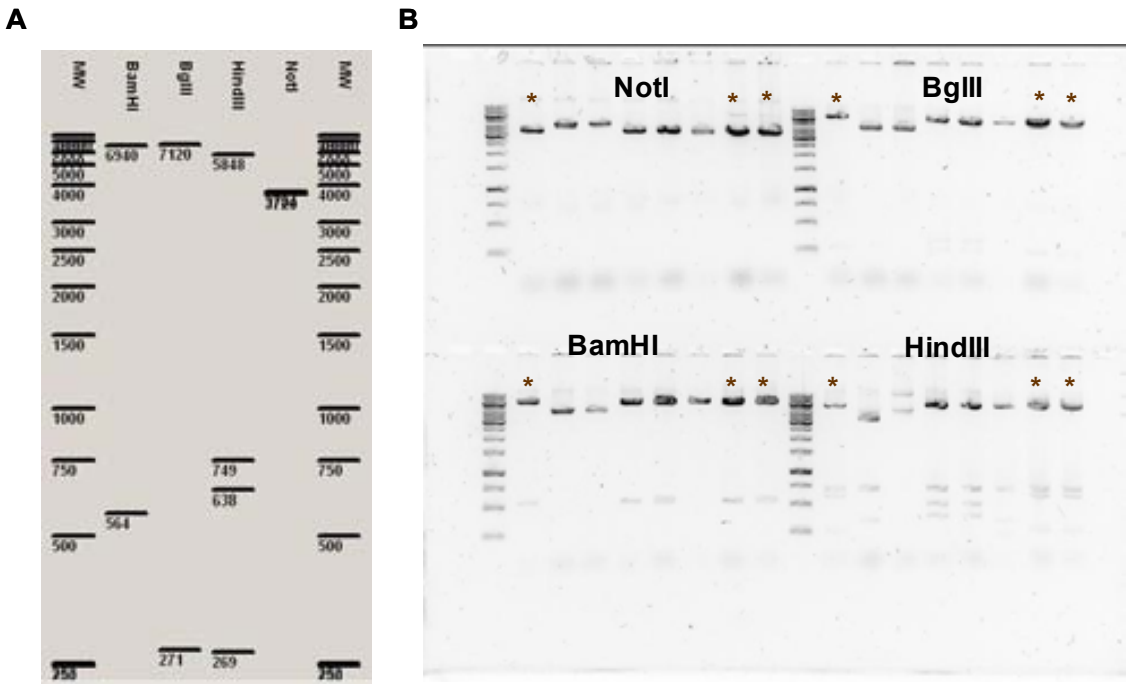

Figure A3. Cribling of colonies from PMM400-FAIM$_L$-IRES-EGFP plasmid.A. Theoric MW of the bands obtained when digesting the PMM400-FAIM$_L$-IRES-GFP vector with the indicated restriction enzymes (using pDraw software). **B.**Agarose gel of colonies digested and sustained to electrophoresis. Red asterisks mark positive colonies.

From the 8 colonies initially selected, the numbers 1, 7 and 8 were positive for our construction (Figure A3). So, these clones were purified and sent to the UCTS of Vall d'Hebron for sequenciation. We selected one of the validated clones and named this plasmid PMM400-FAIM$_L$-IRES-EGFP. Next, we verify the expression of our target gene, in this case FAIM$_L$, and its regulation by Doxycicline treatment.

Regulation of FAIM$_L$ expression by Doxycicline treatment

To study the regulation of FAIM$_L$ expression by doxicycline, we need cells that carry the recombinant tetracycline controlled transcription factor, namely tTA. In our case, we had U87-tTA expressing cells. Before transfecting the cells, the transgenic plasmid has to be digested with NotI restriction enzyme and the band of 3720bp should be purified and transfected. This is because the plasmid PMM400-FAIM$_L$-IRES-EGFP carries a CMV promoter upstream of the P$_{tet}$ promoter, which is stronger and thus controls the constitutive expression of our target gene. The plasmid is designed in such a way that by NotI digestion we can substract the sequence of the P$_{tet}$ promoter with our target gene, and use it for subsequent transfections or for injection.

U87-tTA cells were transfected with PMM400-FAIM$_L$-IRES-EGFP, PMM400-FAIM$_L$-IRES-EGFP-NotI digested, empty PMM400 and TetO-GFP as a positive control for doxicycline treatment. 24h after transfection, cells were treated or not with 10µM

doxycicline for 24h, and then cells were harvested and lysed. $FAIM_L$ and GFP expression was analyzed by Western Blot

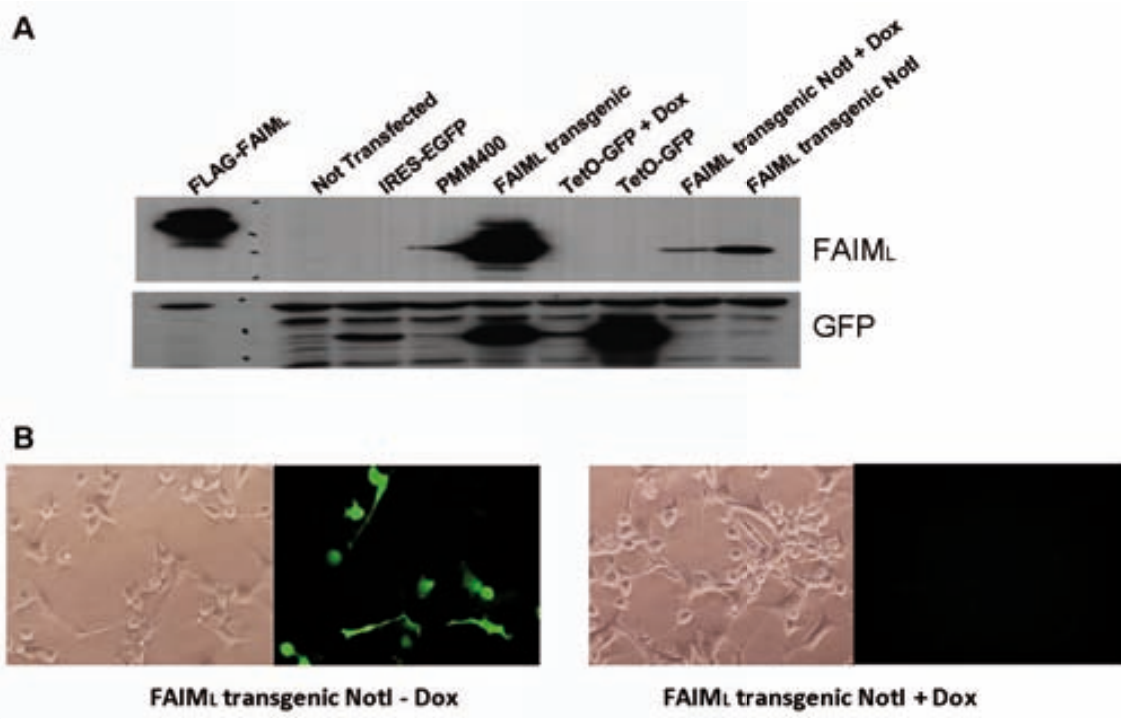

Figure A4. Regulation of $FAIM_L$ expression by doxycicline. A. U87-tTA expressing cells were transfected with the indicated plasmids. 24h after transfection, cells were treated with 10μM doxycicline for 24h. Expression of $FAIM_L$ and GFP was determined by inmunoblotting. **B.** Representative images of U87-tTA expressing cells transfected with PMM400-$FAIM_L$-IRES-GFP digested with NotI, treated or not with 10μM doxycicline as indicated. Images on the left boxes are taken with transmitted light, images on right boxes are taken with green fluorescent light.

$FAIM_L$ expression was detected in the PMM400-$FAIM_L$-IRES-EGFP overexpressing cells, confirming the integrity of our gene. In comparison, in PMM400-$FAIM_L$-IRES-EGFP-NotI digested cells the expression of $FAIM_L$ was lower (Figure A4A). However, this is likely due to differences in the efficiency of transfection, since the percentage of cells transfected for a linear DNA is lower than for a circular DNA. When PMM400-$FAIM_L$-IRES-EGFP-Not I digested cells were treated with doxycline, the expression of $FAIM_L$ was downregulated (Figure A4A). These data proves that the plasmid is well constructed, and that $FAIM_L$ expression can be modulated by doxycline. As expected, GFP expression could be detected in PMM400-$FAIM_L$-IRES-EGFP overexpressing cells, as well that in the TetO-GFP overexpressing cells, when they were not treated with doxycicline (Figure A4A). That was not the case for $FAIM_L$-IRES-EGFP-Not I digested cells, in which we could not detect GFP expression by Western Blot. However, green fluorescence could be detected by visualization in an inverted microscope in $FAIM_L$-IRES-EGFP-Not I digested cells, and that was abrogated by treatment with doxycicline (Figure A4B). Thus, $FAIM_L$ expression could be monitorized

by green fluorescence visualization, despite not being able to check GFP expression by Western Blot. This can be due to lower expression of proteins that are after the IRES sequence, which has been reported in some cases, and also the expression of our transgenic gene in PMM400-$FAIM_L$-IRES-EGFP-Not I digested cells is lower than in not digested plasmid. All in all, our data concludes that the PMM400-$FAIM_L$-IRES-EGFP was successfully generated and can be used for the injection in the ES cells.

By now, transgenic mice of the 3 genes have been generated, and the $FAIM_L$ Knock-in mice is being further characterized. We are in the process of verifying the correct overexpression of $FAIM_L$ in the mice brain, and its regulation by doxycicline treatment. The first round of LFG and $FAIM_S$ transgenic mice, without the P_{tet} promoter, is already generated too. However, it is still in process to cross these mice with CamKIIα-tTA mice. These transgenic mice are going to be a very helpful tool in the study of $FAIM_L$, $FAIM_S$ and LFG function in the CNS, and can be crossed with transgenic mice models for diseases, to have insights in the role of these proteins in the pathogenesis studied.