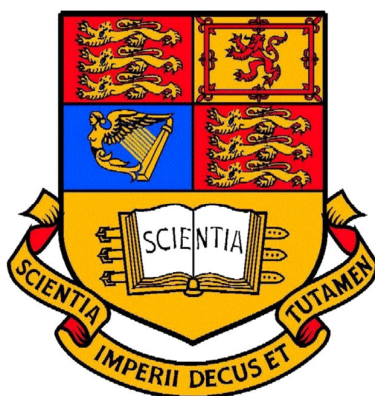




**I κ B α inhibits apoptosis at the outer
mitochondrial membrane through a novel,
NF- κ B-independent, interaction with VDAC1**

by

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Declaration of Originality

I, Evangelos Pazarentzos, hereby declare that the work presented in this thesis is my own and to the best of my knowledge is original. The content of this thesis has not, is not and will not be submitted in any form for another degree or diploma at any university or other institute of tertiary education. Any contents in this thesis, including figures, data, ideas and material obtained from other sources are appropriately cited and acknowledged in the text and a full list of references is given at the end of this thesis.



Abstract

The inducible transcription factor NF- κ B is tightly regulated by the inhibitory I κ B-family of proteins that associate with the transcription factor and act in response to stress stimuli. The best studied inhibitory protein is I κ B α which resides in the cytosol where it retains NF- κ B. Our study shows that I κ B α also associates with the outer mitochondrial membrane (OMM) and exerts an unexpected novel anti-apoptotic function, independent of NF- κ B inhibition. *I κ B α ^{-/-}* cells become refractory to apoptosis when I κ B α is specifically reconstituted at the OMM. We found that cancer cells with constitutively active NF- κ B accumulate I κ B α at the OMM and when its expression is down-regulated these cells are sensitised to apoptosis. At the OMM I κ B α associates with VDAC1 and hexokinase II (HKII). Our findings show that I κ B α inhibits the dissociation of HKII from VDAC1 and prevents Bax-mediated cytochrome c release. Deletion mutants of I κ B α reveal a domain necessary for apoptosis inhibition that is different from the domain for NF- κ B retention, thereby separating the two functions. These results reveal an unexpected activity of I κ B α in guarding the integrity of the OMM against apoptosis induction and open possibilities for more specific interference in diseases involving deregulated NF- κ B.



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Publications

Mahul-Mellier, A.L., Datler, C[#], **Pazarentzos, E[#]**, Lin, B., Chaisaklert, W., Abuali, G. & Grimm, S. (2012) De-ubiquitinating proteases USP2a and USP2c cause apoptosis by stabilising RIP1. *Biochim Biophys Acta*. (#equal contribution)

Mahul-Mellier, A.L., **Pazarentzos, E.**, Datler, C., Iwasawa, R., AbuAli, G., Lin, B. & Grimm, S. (2012) De-ubiquitinating protease USP2a targets RIP1 and TRAF2 to mediate cell death by TNF. *Cell Death Differ*, **19**, 891-899.

Iwasawa, R., Mahul-Mellier, A.L., Datler, C., **Pazarentzos, E.** & Grimm, S. (2011) Fis1 and Bap31 bridge the mitochondria-ER interface to establish a platform for apoptosis induction. *EMBO J*, **30**, 556-568.

Lemarie, A., Huc, L., **Pazarentzos, E.**, Mahul-Mellier, A.L. & Grimm, S. (2011) Specific disintegration of complex II succinate:ubiquinone oxidoreductase links pH changes to oxidative stress for apoptosis induction. *Cell Death Differ*, **18**, 338-349.

Manuscripts in preparation

I κ B α inhibits apoptosis at the outer mitochondrial membrane through a novel, NF- κ B-independent, interaction with VDAC1 (first author)

CKMT1 inhibits apoptosis and acts as a gatekeeper of mitochondria permeability transition pore opening

KaiI is a dependence receptor



Abbreviations

ADP	: Adenosine Diphosphate
AIF	: Apoptosis Inducing Factor
ANK	: Ankyrin Repeat
ANT	: Adenine Nucleotide Translocator
Apaf-1	: Apoptotic Peptidase Activating Factor 1
APS	: Ammonium Persulfate
ATP	: Adenosine Triphosphate
BA	: Bongkreic Acid
Bcl-2	: B-cell Lymphoma 2
BH domain	: Bcl-2 Homology Domain
bp	: Base Pairs
BSA	: Bovine Serum Albumin
CHX	: Cycloheximide
CsA	: Cyclosporin A
Cyp-D	: Cyclophilin D
Cyt-C	: Cytochrome C
Da	: Dalton
DAPI	: 4,6-diamidino-2-phenylindole



DIABLO	: Direct IAP binding protein with low PI
DioC	: 3,3-dihexyloxacarbocyanine iodide
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethylsulfoxide
dNTP	: Deoxy nucleotide triphosphate
EDTA	: Ethylenediaminetetraacetic acid
EGTA	: Ethylene Glycol-bis(2-aminoethylether)-N-N-N-N-tetraacetic acid
ER	: Endoplasmatic Reticulum
FACS	: Fluorescent activated cell sorter
Gag	: Group Antigen
GFP	: Green Fluorescent Protein
GRP-75	: Glucose regulating protein 75
HBS	: HEPES buffered saline
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HXK	: Hexokinase
HRP	: Horse Radish Peroxidase
IκBα	: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IKK	: I κ B kinase
IMM	: Inner Mitochondrial Membrane
IMS	: Inter-membrane space



IP	: Immunoprecipitation
IVT	: In-Vitro Translated
MOMP	: Mitochondrial Outer Membrane Permeabilisation
mRNA	: Messenger RNA
NF-κB	: Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	: Nuclear Localisation Signal
OMM	: Outer Mitochondrial Membrane
PAGE	: Polyacrylamide Gel Electrophoresis
PARP	: Poly ADP-Ribose Polymerase
PCR	: Polymerase Chain Reaction
PI	: Propidium Iodide
Pol	: Reverse Transcriptase
PT-pore	: Permeability Transition pore
PVDF	: Polyvinylidene Fluoride
Rev	: Reverse Transcriptase
RIP	: Receptor Interaction Protein
ROS	: Reactive Oxygen Species
RT	: Room Temperature
SC	: Scramble
S.D.	: Standard Deviation
SDS	: Sodium Dodecyl Sulfate



shRNA	: Short Hairpin RNA
Smac	: Second mitochondria derived activator of caspase
STS	: Staurosporine
TMRE	: TetraMethylRhodamine Ethyl ester
TNF	: Tumour Necrosis Factor
TRAIL	: TNF-related apoptosis inducing Ligand
VDAC	: Voltage Dependent Anion Channel
VSVG	: Vesicular Stomatitis Virus Glycoprotein
WT	: Wild Type
β-gal	: beta-galactosidase
ΔΨ_m	: Mitochondrial Electrochemical protein gradient



A. Introduction



A.1. Cell Death

Homeostasis is a term referring to a property of a system, which controls its internal structure and stabilises any parameter that may affect the balance so as to keep the performance of the system high. Typically, the term is used in biology to describe anything from a cell to a multicellular organism. It was first used by Claude Bernard and Walter Cannon and extensive research has commenced on the molecular factors that might influence homeostasis. Cell death is a critical process for the control of homeostasis and tissue integrity in multicellular organisms. Two major, yet fundamentally different forms of cell death have been identified that accomplish homeostasis: necrosis and apoptosis.

A.1.1. Necrosis

The origin of the word necrosis comes from the ancient Greek “nekros”, whose literal translation is “a dead body”. This form of cell death is a spontaneous, passive process resulting in an early disruption of the plasma membrane and the progressive breakdown of cellular organelles accompanied by organelle swelling (oncosis). The swelling leads to the lysis of the cell and the leakage of cellular contents into the extracellular space, which provokes an inflammatory response (Figure A.1.) (Leist & Jaattela, 2001). Neighbouring cells can also undergo necrosis upon lysis of an individual cell, thus necrotic cell death can affect a group of cells (Stoica & Faden, 2010). Cellular necrosis is caused by several external causes,



mostly strong perturbation of the environment (extreme high or low temperatures for example), mechanical trauma, infections and poisons. Clinically, hypoxic cells or tissues that underwent ischemic injury often show necrotic cell death. Additionally, venoms produced by certain animals or infections by particular bacteria can cause rapid and severe necrosis, for example Group A streptococcus in necrotizing fasciitis (Roje *et al.*, 2011). As mentioned above, necrotic cell death is generally caused by extreme stress or a major failure in homeostatic regulation (Raffray & Cohen, 1997; Syntichaki & Tavernarakis, 2003). Consequently, it is essentially the accidental death of a cell and can be caused by conditions that are so extreme in nature that the cell has not the opportunity to respond (Syntichaki & Tavernarakis, 2003). However, recently the view that necrosis is not merely an accidental cell death is gaining support. Cells whose apoptosis system is inhibited or compromised, can still die following an apoptotic stimulus but show all the characteristics of necrosis (Edinger & Thompson, 2004). Whether this type of cell death is physiologically relevant or whether it occurs only in very distinct circumstances in nature is a field of intense investigation.



A.1.2. Apoptosis

In the early 1970s, John Kerr and his group noticed a peculiar form of cell death in acute liver injury in rats and named this process shrinkage necrosis (Kerr *et al.*, 1972). Later, during the same decade, having studied the features of shrinkage necrosis in comparison with necrosis, this form of cell death was given the name “apoptosis”. The term ‘apoptosis’ is often used to describe the process of programmed cell death (PCD); also referred to as cell suicide. However, some authors have pointed out that the terms “apoptosis” and “PCD” should not be deemed interchangeable since during development, when most of the cell death is ‘programmed’ in some way, there is some cell death that does not fit the defining characteristics of apoptosis. Some of these features will be mentioned later. Nevertheless, many authors have not adopted this distinction in their writing (Haanen & Vermes, 1995; Kiechle & Zhang, 2002; Slovirer, 2002) and in this text, unless declared otherwise, the terms ‘apoptosis’, ‘programmed cell death’, and ‘cell suicide’ will also be used synonymously.

Apoptosis is a mode of cell death quite distinct from necrosis, not just because it is a programmed and regulated mechanism but also in terms of its morphology and biochemistry (Bohm & Schild, 2003). It can be characterized by special morphological (Figure A.2., Table A.2.) and biochemical features (Figure A.3., Table A.2) such as blebbing of the plasma membrane, chromatin condensation, cell shrinkage, nucleus fragmentation, energy dependence,



containment of lysosomal enzymes and the absence of an inflammatory response (Bohm & Schild, 2003; Kiechle & Zhang, 2002).

In multicellular organisms, apoptosis plays an important role in, among other scenarios, the immune response, development, and tissue homeostasis (Bohm & Schild, 2003; Haanen & Vermes, 1995; Janssen *et al.*, 2000; Kerr *et al.*, 1972). In particular apoptosis is important for the elimination of unnecessary neurons during the development of the nervous system, the removal of auto-immune lymphocytes in the thymus, and the maintenance of the stability in cell numbers in dynamic tissues such as the skin and those of the gut (Haanen & Vermes, 1995). Apoptosis exerts its effect even in unicellular organisms that seem capable of employing this mode of cell death for their suicide. Bacteria as unicellular organisms will commit to an altruistic death in order to prevent damage or promote survival of the total population. For instance, a viral infection would promote the activation of the cell death machinery of this bacterium in order to prevent the spread of the infection. Another example would be the autolytic pathway of the bacteria which serves as a programmed suicide mechanism and allows the exchange and acquisition of the lysed bacteria DNA from other surrounding bacteria (Lewis, 2000). Though PCD may look detrimental from the perspective of an individual cell, it can have a positive effect in a more complex multicellular community – namely the elimination of defective cells in order to aid the survival of those that function properly. Removal of DNA-damaged cells for

reducing the mutation rate is another aspect of apoptosis that multicellular organisms can benefit from and this response is also observed in both unicellular prokaryotes and eukaryotes (Lewis, 2000; Pereira *et al.*, 2008).

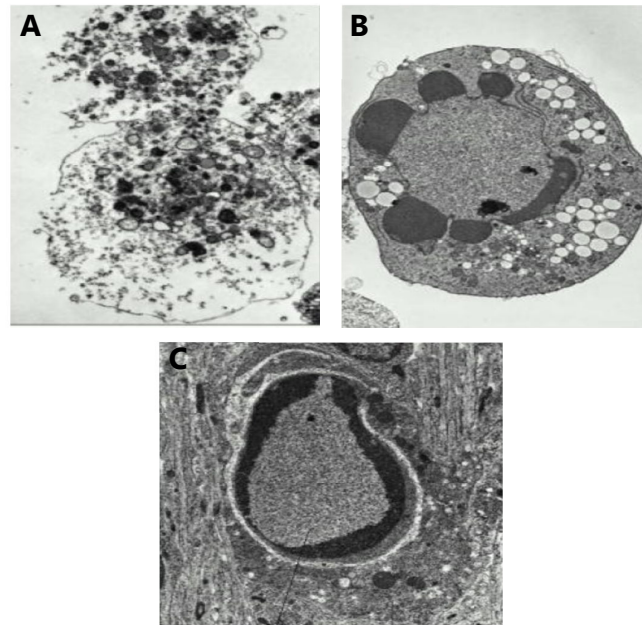


Figure A.1. Necrotic and apoptotic cell death. **(A)** Necrotic cell undergoing cell lysis and the contents of the intracellular organelles are released into the extracellular environment. **(B)** Late stage apoptotic cell forming several membrane enclosed vesicles as depicted by the presence of small vacuolar structures in the cytosol. **(C)** An apoptotic cell is engulfed for clearing. *Figure was taken from thesis of Dr A-L Mahul Mellier*



Apoptosis is an integral part of cell homeostasis with several distinct or interconnected aspects. Morphological changes, cell cycle alterations, DNA and phenotypical variability, although important, are not central to the findings of this study and hence will not be extensively covered. However, those aspects of apoptosis that link molecular signalling pathways to disease will be discussed and extensively analysed with special emphasis on genes and proteins affecting the signalling pathways of apoptosis inhibition or execution and its connection to tumourigenesis.

Apoptosis	Necrosis
Affects individual cells	Affects group of neighbouring cells
No inflammatory response	Major inflammatory response
Cell shrinkage	Cell swelling
Membrane blebbing with maintained integrity	Loss of membrane integrity
Increase of mitochondrial membrane permeability, release of proapoptotic factors and formation of apoptotic bodies	Organelle swelling and lysosomal leakage
Chromatin condensation and controlled DNA fragmentation	Random DNA degradation
Apoptotic bodies ingested by neighbouring cells	Macrophage phagocytosis of lysed cells

Table A.1. Differences between apoptosis and necrosis

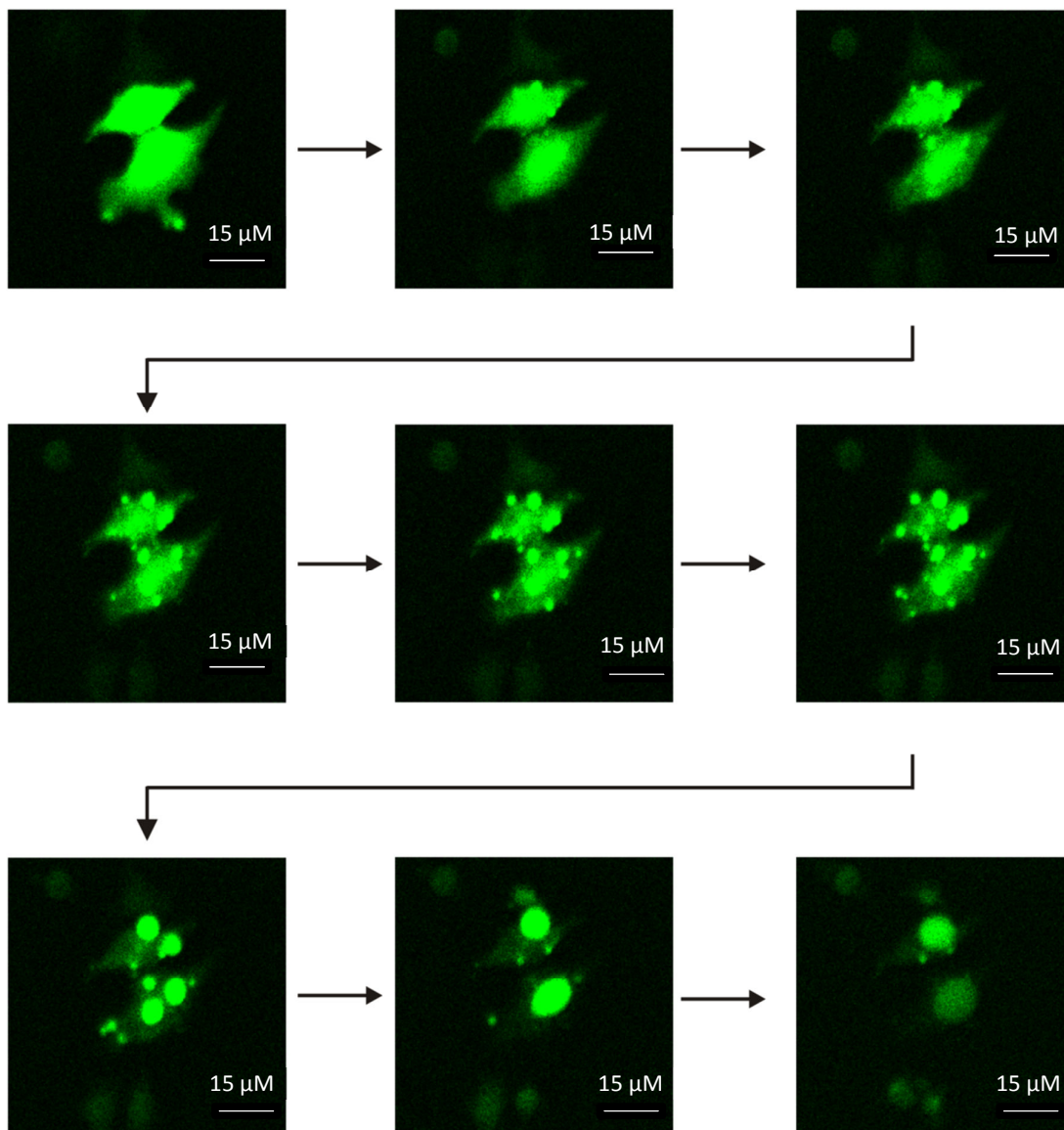


Figure A.2. Time lapse images of apoptotic cells: morphological features.

Hela cells were transfected with eGFP and cells undergoing spontaneous apoptosis were captured. Initially cells present equal cytoplasmic staining and gradually spherical structures start appearing when cells begin to die (second pane onwards). As apoptosis progresses, numerous apoptotic bodies appear and eventually cells round up and lose focal adhesion. *Pictures were taken using a Leica scanning confocal microscope in intervals of 2 hours.*

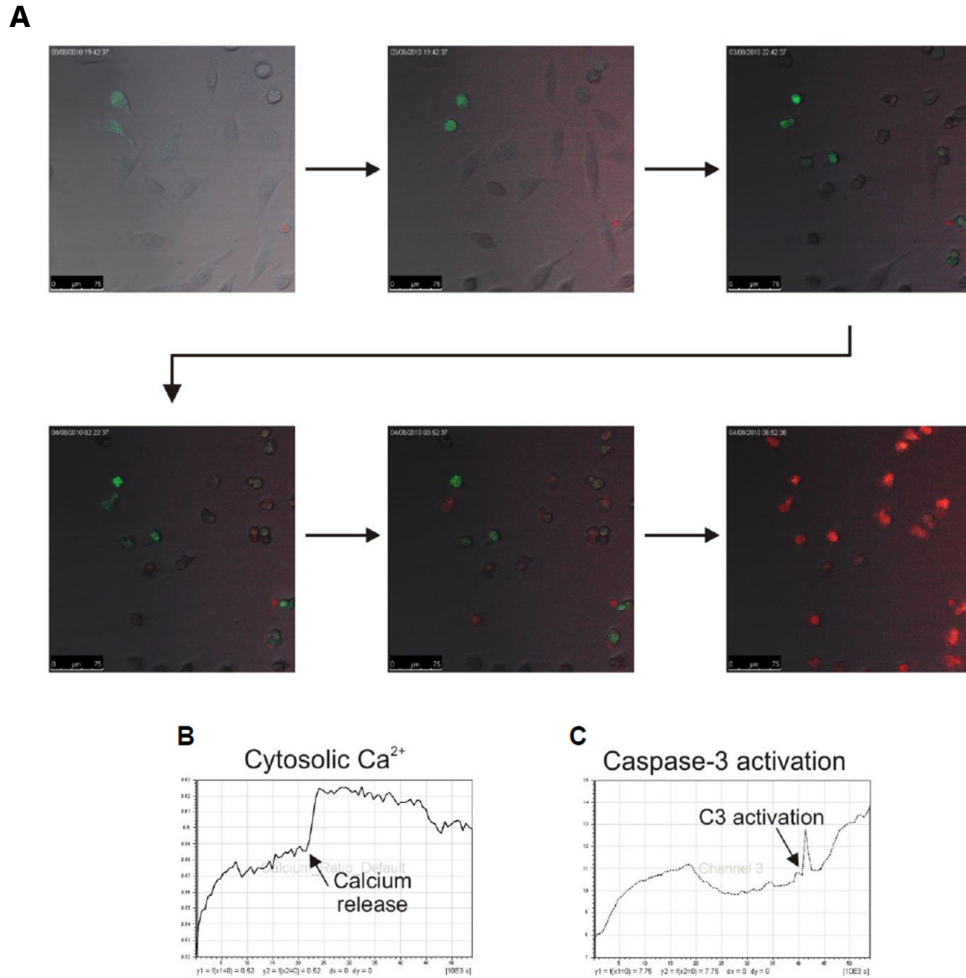


Figure A.3. Time lapse images of apoptotic cells to visualise biochemical features. HeLa cells were transfected with the cytosolic calcium indicator YF3.6. Cells were subsequently treated with doxorubicin ($2\mu\text{M}$) and CaspaTag caspase 3/7 sulforhodamine (a dye that only binds to activated caspase-3 or/and -7 and emits red fluorescence upon binding). The top 6 panels show the time course of cytosolic calcium increase (as indicated by the increased green fluorescence) and the activation of caspase 3/7 (as indicated by the red fluorescence). The bottom two figures show the cytosolic calcium elevation and the caspase 3/7 activation of a representative cell. *Images and graphs were taken from the thesis of Dr R. Iwasawa*



Morphological Features	Biochemical Features
Cell shrinkage	Free $[Ca^{+2}]_c$ rise
Chromatin condensation	DNA degradation
Nuclear fragmentation	Caspase activation
Membrane blebbing	Loss of mitochondrial membrane potential
Cell detachment	Phosphatidylserine externalisation
Production of apoptotic bodies	
Phagocytosis of cell corpses	

Table A.2. Morphological and biochemical features of apoptotic cells.



A.1.3. The two major pathways of apoptosis initiation

Two major pathways that lead to the induction of programmed cell death are known: the extrinsic and intrinsic pathway. Apoptosis can be instigated by the activation of death receptors (DR) on the cell surface or can be induced by signaling processes that are initiated within the cell as a result of stress and/or damage by, say, exposure to particular drugs (Elliott & Elliott, 2009; Kiechle & Zhang, 2002). The former type of apoptosis is termed receptor-mediated or extrinsic while the latter is called mitochondria-mediated or intrinsic (Figure A.5.)

Apoptotic pathways involve many proteins that are required to orchestrate the complex signalling pathways. The two apoptosis pathways share a basic signaling cascade: the initiation phase, the integration/decision phase and the execution phase. The initial reception of the apoptotic signal is highly heterogeneous and depends on the nature of the death-inducing signals. In the extrinsic pathway, it is the ligand interacting with a “death receptor” and in the case of the intrinsic pathway, it is damage inflicted on cellular organelles (such as the nucleus, endoplasmic reticulum, lysosomes, Golgi apparatus or mitochondria). The integration/decision phase involves the formation of a caspase-activation complex and initiation of the enzymatic activity of the caspase proteases. Cells then pass the point of no return and are destined to die. In the last, the execution phase, morphological and biochemical changes occur due to the cleavage of



cellular proteins by caspases. The biggest difference between the extrinsic and the intrinsic pathway is the initial reception of the death signals. In the extrinsic pathway the cell obtains its first apoptotic signal from plasma membrane receptors, whereas BH3-only proteins of the Bcl-2 family usually sense the signals in the intrinsic pathway.

In essence, the receptor-mediated, extrinsic pathway is initiated by DRs, situated at the cell's plasma membrane, when binding to their ligands. This process, via specific mediators, goes on to trigger a proteolytic cascade that leads to the cleavage of specific proteins in the cell, thus committing the cell to apoptosis (Ashkenazi, 2008).

The mitochondria-mediated, intrinsic apoptotic pathway does not require the binding of ligands to receptors at the cell surface but rather is a stress-induced process involving mitochondria. In particular, specific cellular events take place under conditions of cell stress that lead to the disruption of the outer mitochondria membrane (OMM). This allows the release of several pro-apoptotic mitochondrial factors, which trigger a cascade of effector caspases that can then, as in the extrinsic pathway, commit the cell towards apoptosis (Green & Kroemer, 2004).



A.1.3.1. Extrinsic apoptotic pathway

As mentioned above the extrinsic or death receptor-dependent pathway of apoptosis receives its signal from plasma membrane receptors (Ashkenazi & Dixit, 1998). These receptors belong to the tumour necrosis factor (TNF) receptor superfamily and are characterized by the presence of two to five copies of cysteine-rich extracellular repeats. This superfamily includes Fas (also known as Apo-1 or CD95), TNF-receptor-1 (TNF-R1), death receptor-3, TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R1 or DR4), TRAIL-R2 (DR5) and death receptor 6. Of all the plasma membrane receptors that can mediate a signal for apoptosis the TNF-R1- and the Fas receptor are the most intensively studied and hence will be further discussed.

A.1.3.1.1. TNF receptor-mediated extrinsic pathway

Tumour necrosis factor (TNF) is a multifunctional pro-inflammatory cytokine mainly produced by macrophages. TNF is involved in the progression of many diseases such as rheumatoid arthritis and Crohn's disease and inhibitors of TNF are used for therapeutic purposes. TNF can bind two kinds of TNF receptors (TNFR), TNFR1 and TNFR2 (de Jong *et al.*, 2001).



On the plasma membrane, TNF-R1 forms, upon binding its ligand, a homotrimer. This brings the death domain (DD) in the cytosolic moiety of the proteins in close proximity, which facilitates the recruitment of several adaptor proteins to form a death-inducing signaling complex (DISC) leading to the activation of initiator caspases. In the absence of TNF the receptor is bound to Silencer of DD (SODD), which inhibits the recruitment of the adaptor proteins for caspase activation thus acting as a negative regulator of apoptosis (Figure A.5.) (Jiang *et al.*, 1999). Upon binding of the ligand, SODD dissociates from the TNF-R1 complex exposing the DD and facilitating the recruitment of another DD-containing protein, the “TNF-R1-associated death domain protein” (TRADD) (Figure A.5.) (Hsu *et al.*, 1995)). Following the binding of TRADD to the TNF-R1 complex, there are two possible consequences.

One scenario is that the DD of TRADD recruits the “receptor interaction protein (RIP)” and the “TNF receptor-associated factor-2” (TRAF2) (Chen & Goeddel, 2002; Hsu *et al.*, 1996). This tetra-protein complex consisting of TNF-R1, TRADD, RIP1, and TRAF2 is known as complex I (Figure A.5.), (TNF receptor mediated pathway, left). This complex can lead to one of three different responses (Figure A.5.). In one case the “nuclear factor- κ B” (NF- κ B) is activated and promotes survival of the cells (Lin *et al.*, 1999). This is triggered and regulated by the phosphorylation of its inhibitor I κ B α . This begins with the recruitment of the heterodimer of I κ B α kinases (IKK α and IKK β) and the regulatory protein NEMO



(also known as IKK γ) to the TNF-R1 signaling complex I by TRAF2 (Devin *et al.*, 2003). RIP1 seems to stabilise the IKK complex. In the second response, c-Jun N-terminal kinase (JNK) is activated through complex I. Upon activation of JNK, it translocates to the nucleus and activates transcription factors, for example, c-Jun and ATF-2, by phosphorylation. These transcription factors control the regulation of several genes including those involved in apoptosis regulation, for example c-IAP and c-FLIP (Hu *et al.*, 1997; Srinivasula *et al.*, 1997)) thereby preventing the induction of apoptosis. In the last response, “RIP1-associated ICH-1/CED-3 homologous protein with death domain” (RAIDD) or synonymously known as “caspase and RIP adapter with death domain” (CRADD) associates with RIP (Ahmad *et al.*, 1997; Duan & Dixit, 1997). A domain similar to the caspase recruitment domain (CARD) of RAIDD is able to activate initiator procaspase-2 to a mature caspase-2 for the induction of cell death (Baud & Karin, 2001; Duan & Dixit, 1997; Shearwin-Whyatt *et al.*, 2000).

The second, fundamentally different scenario of TNF-R1-mediated apoptosis involves a different set of proteins that are recruited to the receptor. TRADD associates with TNF-R1 complex through its DD, which then recruits “Fas-associated death domain” (FADD). In addition to a DD, FADD also contains a death effector domain (DED) for the recruitment of DED-containing initiator caspases (procaspase-8 and -10). The complex of TNF-R1, TRADD, FADD and procaspases-8 or -10 is known as “complex II” (Figure A.5.). This recruitment of procaspases



results in a temporal elevation of caspases concentration around the TNF-R1, which leads to their mutual cleavage and activation (Kim *et al.*, 2000) via the proximity-induced dimerization model. The activated caspase-8 or -10 then cleave and activate effector caspases such as procaspase-3 or -7 for the execution of cell death (Figure A.5.)

Consequently, the TNF-R1 activation does not uniformly induce cell death as it can both result in a pro-survival pathway or in a pro-apoptotic pathway. The NF- κ B pathway and the JNK pathway lead to the expression of pro-survival proteins (Stehlik *et al.*, 1998; Wajant *et al.*, 2003). A study in mice indicated that the loss-of-function of proteins involved in the NF- κ B activation pathway are either embryonic lethal or results in neonatal fatality (Beg *et al.*, 1995b). This indicates that the ability of TNF to induce apoptosis is concomitantly suppressed *in vitro* by a constitutive activation of NF- κ B. Research I conducted during this PhD thesis as part of another project revealed that the ubiquitin-specific protease USP2a is a component of the TNFR1 complex and has a pivotal role in the conversion of complex I into complex II and hence the decision for cell death over survival (Mahul-Mellier *et al.*, 2011).

A.1.3.1.2. Fas receptor-mediated extrinsic pathway

Fas is a heavily glycosylated 40-45kDa plasma membrane protein involved in cytotoxic T-cell killing. The Fas receptor homotrimerizes at the plasma



membrane upon ligand binding similarly to the TNF-R1 (Ashkenazi & Dixit, 1998; Pitti *et al.*, 1996; Smith *et al.*, 1994). Association of the Fas ligand with the Fas receptor forms a platform for caspase activation known as “death inducing signalling complex (DISC)” via the DD of the Fas receptor (Figure A.5.) Fas receptor mediated pathway (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995; Kischkel *et al.*, 1995; Nagata, 1997). The adaptor protein FADD is first recruited to the Fas-receptor, and the DED of the FADD allows the association of procaspases-8 or -10 (Chinnaiyan *et al.*, 1995; Medema *et al.*, 1997). The formation of the DISC quickly increases the local concentration of pro-caspases, which in turn cleave each other and thereby become mature, active caspases (Boatright *et al.*, 2003; Donepudi *et al.*, 2003; Walczak & Krammer, 2000). As is the case of the TNFR complex, activated caspase-8 or -10 then activate downstream effector pro-caspases such as procaspases-3 or -7 (Stennicke *et al.*, 1998), which execute apoptosis by substrate cleavage (Figure A.5.)

A.1.3.1.3. Caspases and execution of apoptosis in the extrinsic pathway

The caspases that bind the adaptor proteins of the DISC are called initiator (or activator) caspases and comprise caspases-8 and -10 (Ashkenazi, 2008; Budihardjo *et al.*, 1999). All caspases involved in apoptosis in a physiologically normal cell are present as inactive precursors (“pro-caspases”) which, when



brought into close proximity of one another, are, due to the low intrinsic protease activity possessed by pro-caspases, able to cleave each other to form a large and small subunit that associate as tetramers to make up the active form of the enzyme. Once activated, the caspases can go on to cleave other pro-caspase molecules into their active states. Active caspases are thus able to activate even more caspases to ultimately result in a caspase cascade (Budihardjo *et al.*, 1999; Elliott & Elliott, 2009; Kiechle & Zhang, 2002; Mancini *et al.*, 1998).

Activated initiator caspases proceed to cleave and activate a group of caspases known as effector caspases (e.g. caspases-3, -6, and -7). These effector caspases can also cleave and activate further caspases, to enhance the caspase cascade (Ashkenazi, 2008; Budihardjo *et al.*, 1999; Mancini *et al.*, 1998). Effector caspases are able to process key proteins such as ICAD (inhibitor of caspase activated DNase), thereby facilitating DNA degradation by caspase-activated DNases (CAD), and cleaving intermediate filament proteins, which generate nuclear lamins that are involved in the composition of the cytoskeleton, ultimately producing the cellular phenotype of apoptosis (McConkey *et al.*, 1996; Mow *et al.*, 2001; Nicholson & Thornberry, 1997).

A.1.3.2. The intrinsic pathway of apoptosis induction

As its name suggests, the intrinsic pathway or the mitochondria-dependent pathway of apoptosis is initiated from an organelle within the cell. This is usually in



response to cellular signals resulting from irreparable DNA damage, presence of toxic levels of oxidative stress, endoplasmic reticulum stress, growth factor deprivation, calcium flux, UV irradiation or treatment with chemotherapeutic drugs. All these signals converge on mitochondria (Figure A.5.) (Green *et al.*, 2004) for the induction of mitochondrial outer membrane permeabilisation (MOMP (Green & Kroemer, 2004)).

For years mitochondria have solely been considered a chemical powerhouse and a cellular compartment that hosts several biosynthetic and biodegrading pathways. It still is the organelle where the vast majority of ATP is produced. So its role in the survival of the cell is considered critical. Now this organelle is also regarded as the major player in the intrinsic pathway of apoptosis. Proteins that are involved in the respiratory chain are major players in the apoptotic machinery. Cytochrome c (Cyt-c) or complex II proteins are some examples (Lemarie & Grimm, 2011; Lemarie *et al.*, 2011). The architecture of mitochondria as revealed through biochemical and electron microscopy studies possess a rather complex structure. (Newmeyer & Ferguson-Miller, 2003). Outer membrane and intermembrane space proteins proved to be major players in the apoptotic machinery such as Cyt-c. During apoptosis, the permeabilisation of the OMM often coincides with the reduction of the mitochondrial membrane potential ($\Delta\Psi_m$) over the inner mitochondrial membrane (Goldstein *et al.*, 2000). Maintaining the $\Delta\Psi_m$ appears now as an important prerequisite for cell survival.



Details of the induction of MOMP will be discussed in later sections, but the activation of Bax or Bak seems to be fundamentally required for MOMP. The signalling cues for the activation of the intrinsic pathway either directly promote Bax/Bak activation or achieve this indirectly by modulating/inhibiting the activity of anti-apoptotic Bcl-2 family members, such as Bcl-2 or Bcl-X_L (Figure A.5.).

A.1.3.2.1. The Bcl-2 family members

Bcl-2 was first identified as a proto-oncogene by a common translocation in human follicular B-cell lymphoma and the overexpression of Bcl-2 in transgenic animal models mimicked the human disease (McDonnell *et al.*, 1989; McDonnell & Korsmeyer, 1991; Tsujimoto *et al.*, 1985). Unlike other oncogenes that promote cell proliferation to produce cancer, Bcl-2 was found to inhibit cell death induced by multiple physiological and pathological stimuli (Hockenbery *et al.*, 1990; Kinloch *et al.*, 1999; Vaux *et al.*, 1988). There are over twenty Bcl-2 family members identified and examples are shown in Table A.3.. The sequence alignment of Bcl-2 family members revealed that they share at least one of the possible four conserved Bcl-2 homology domains (BH domain) and often contain trans-membrane domains (TM) at the C-terminus for membrane anchorage (Figure A.4.). Functionally, the Bcl-2 family members can be divided into three groups: anti-apoptotic, pro-apoptotic, and BH3-only pro-apoptotic (Table A.3.). The anti-apoptotic members, such as Bcl-2 and Bcl-X_L (Boise *et al.*, 1993), contain all four BH domains (BH1, BH2, BH3 and BH4 domains, Figure A.4.). The pro-apoptotic members, Bax (Oltvai



et al., 1993) and Bak (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995) for example, contain only three BH domains (BH1, BH2 and BH3 domains, Figure A.4.. Lastly, BH3-only members, such as Bim and Bid possess only the BH3 domain (Figure A.4.) and act by either suppressing the activity of anti-apoptotic Bcl-2 family members or by activating/promoting the activity of pro-apoptotic members of the Bcl-2 family.

The founding family member, Bcl-2, is a 26kDa protein localized to mitochondria (Hockenbery *et al.*, 1990), the endoplasmic reticulum (ER) and other peri-nuclear membranes (Korsmeyer, 1995). It is involved in the maintenance of mitochondrial membranes. It balances the interaction between other members of Bcl-2 family members such as Bax. Together with other anti-apoptotic Bcl-2 family members or with other mitochondrial apoptosis modulators, Bcl-2 works to prevent MOMP and the release of cytochrome c from mitochondria, and thereby promotes cell survival.

On the contrary, pro-apoptotic members promote the release of cytochrome c from mitochondria, and this decision is regulated by the ratio between anti-apoptotic and pro-apoptotic Bcl-2 members present in the cell (Danial & Korsmeyer, 2004). Bax was the first pro-apoptotic member of Bcl-2 family to be identified through its interaction with Bcl-2 (Oltvai *et al.*, 1993). In its inactive state, Bax is localized largely in the cytosol as a monomer although some Bax molecules are loosely attached to the outer mitochondrial membrane. Upon reception of



apoptosis signals, however, Bax undergoes an allosteric conformational change and gets inserted into the outer mitochondrial membrane (OMM) (Desagher *et al.*, 1999). The activated Bax then undergoes homo-oligomerization or hetero-oligomerization with Bak, another pro-apoptotic Bcl-2 member (Gross *et al.*, 1998). This oligomerization has been postulated to form a pore to allow the release of inter-membrane space (IMS) proteins (discussed later in detail). The double knockout of Bax and Bak makes cells resistant to almost all death-stimuli for the intrinsic pathway of apoptosis (Lindsten *et al.*, 2000; Wei *et al.*, 2001) and hence, these two proteins constitute a required gateway for the intrinsic pathway to be operative in mitochondria (Wei *et al.*, 2001). BH-3 only members trigger apoptosis in response to various stimuli by acting as upstream sentinels, which selectively respond to specific signals by first receiving the death signals and transmitting them to other members of Bcl-2 family proteins (Cheng *et al.*, 2001).

Anti-apoptotic members							
Bcl-2	Bcl-X _L	Bcl-W	Bfl-1	Boo/Diva	Mcl-1	Bcl-B	
Pro-apoptotic members							
Bax	Bak	Bok		Bfk	Bcl-X _S		
BH3-only pro-apoptotic							
Bim	Bik	Bad	Bmf	PUMA	NOXA	Nip3	Bid

Table A.3. The Bcl-2 family members

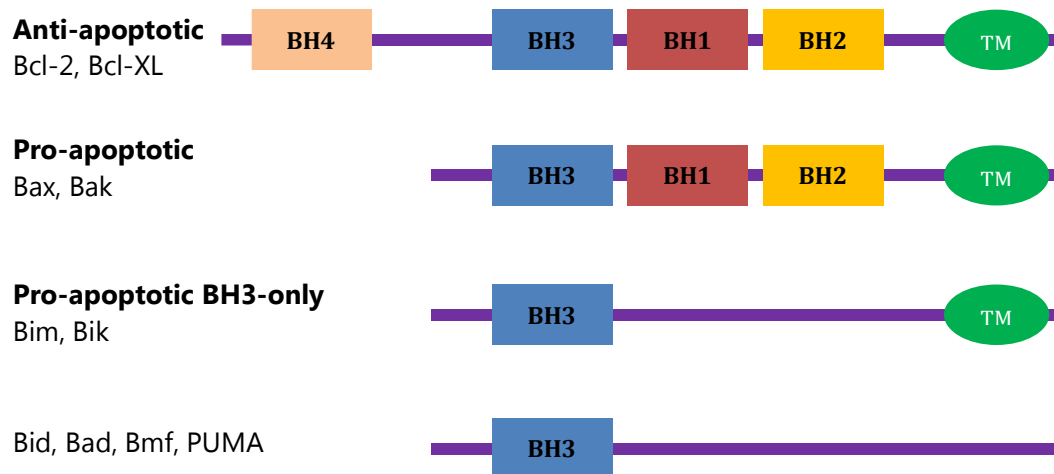


Figure A.4. Bcl-2 family members. The Bcl-2 family members can be divided into three functional groups: anti-apoptotic, pro-apoptotic and pro-apoptotic BH3-only. Their functions vary but at least one of the Bcl-2 homology domains (BH domains) is present in all members. Anti-apoptotic members contain all four BH domains (BH1,2,3 and 4) whereas pro-apoptotic members possess only three BH domains (BH1,2 and 3). Many family members also contain a trans-membrane domain for membrane anchorage. Bcl-Xs contains a BH3 and BH4 domain and NOXA contains two BH3 domains. Mcl-1 contains only BH1 and BH3 domains.



A.1.3.2.2. The intrinsic pathway and commitment to apoptosis

Following the activation of Bax and the induction of MOMP, several pro-apoptotic proteins are released from mitochondria into the cytosol (Figure A.5.). These include endonuclease G (endoG), Omi/Htra2, apoptosis inducing factor (AIF), Smac/Diablo (second mitochondria-derived activator of caspases), and most importantly cytochrome-c (Halliwell & Gutteridge, 2007; Leung & Halestrap, 2008; Liu *et al.*, 1996a; Pereira *et al.*, 2007). AIF translocates to the nucleus and brings about apoptosis in a caspase-independent manner by causing chromatin condensation and DNA fragmentation (Kang *et al.*, 2010). Smac/Diablo is believed to act by inhibiting the activity of caspase through blocking proteins belonging to the inhibitors of apoptosis (IAP) family (Adrain *et al.*, 2001; Halliwell & Gutteridge, 2007). Released cytochrome-c forms a complex comprising itself, an adaptor protein called "apoptotic proteinase activating factor-1" (Apaf-1), procaspase-9, and ATP as a heptamer known as the apoptosome. The apoptosome is capable of activating caspase-9 (Acehan *et al.*, 2002; Li *et al.*, 1997; Srinivasula *et al.*, 1998). Caspase-9 can then cleave effector caspases -3, -7 and -6, in a similar way as the initiator caspases of the receptor-mediated pathway, and initiate a caspase cascade leading to apoptosis (Adrain *et al.*, 2001; Beurel & Jope, 2006; Brown & Borutaite, 2008; Halliwell & Gutteridge, 2007; Kang *et al.*, 2010).



MOMP can also occur in a manner independent of Bax activation, namely through the opening of the permeability transition pore that will be extensively discussed later.

A.1.3.2.3. Bid connects the extrinsic and intrinsic pathway

The extrinsic and intrinsic pathways of apoptosis are initiated in a fundamentally distinct manner. Thus, the two pathways were thought to be completely separable. However, they both depend in their execution on effector caspases activation. The knockout of either caspase-8 (Varfolomeev *et al.*, 1998) or FADD (Yeh *et al.*, 1998) abrogates the responses mediated by death receptor ligand-induced apoptosis whereas the mitochondrial pathway of apoptosis remains intact. Conversely, the knockout of either Apaf-1 (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998) or caspase-9 (Hakem *et al.*, 1998; Kuida *et al.*, 1998) abolishes apoptosis induced by stimuli affecting the intrinsic pathway like cell stress but the cells still execute apoptosis through death receptor ligands. A controversy arose when expression of Bcl-2 was found to alleviate Fas-induced apoptosis (Scaffidi *et al.*, 1998). This led to the consideration that mitochondrial Bcl-2 family members may be involved in the extrinsic pathway. It was later found that a BH3-only protein, named Bid, bridges the two pathways. Recruitment and the activation of procaspase-8 in the DISC complex provides sufficient concentration of activated caspase-8 for the execution of apoptosis in certain cell types (type I cells). However, in type II cells activation of caspase-8 in the DISC complex is insufficient



to fully induce apoptosis and requires the presence of Bid. Bid is a substrate of caspase-8 (Gross *et al.*, 1999; Li *et al.*, 1998; Luo *et al.*, 1998) and the truncated Bid (tBid-after activation from caspase-8) translocates to mitochondria where it triggers the release of Cyt-c by interacting with Bax (Figure A.5.) (Desagher & Martinou, 2000; Eskes *et al.*, 2000; Zamzami *et al.*, 2000). This promotes the formation of the apoptosome and the activation of procaspase-9. The ectopic expression of Bid was also found to enhance the activation of procaspase-3 induced by TNF indicating the importance of Bid in linking the extrinsic and the intrinsic pathways of apoptosis (Luo *et al.*, 1998)

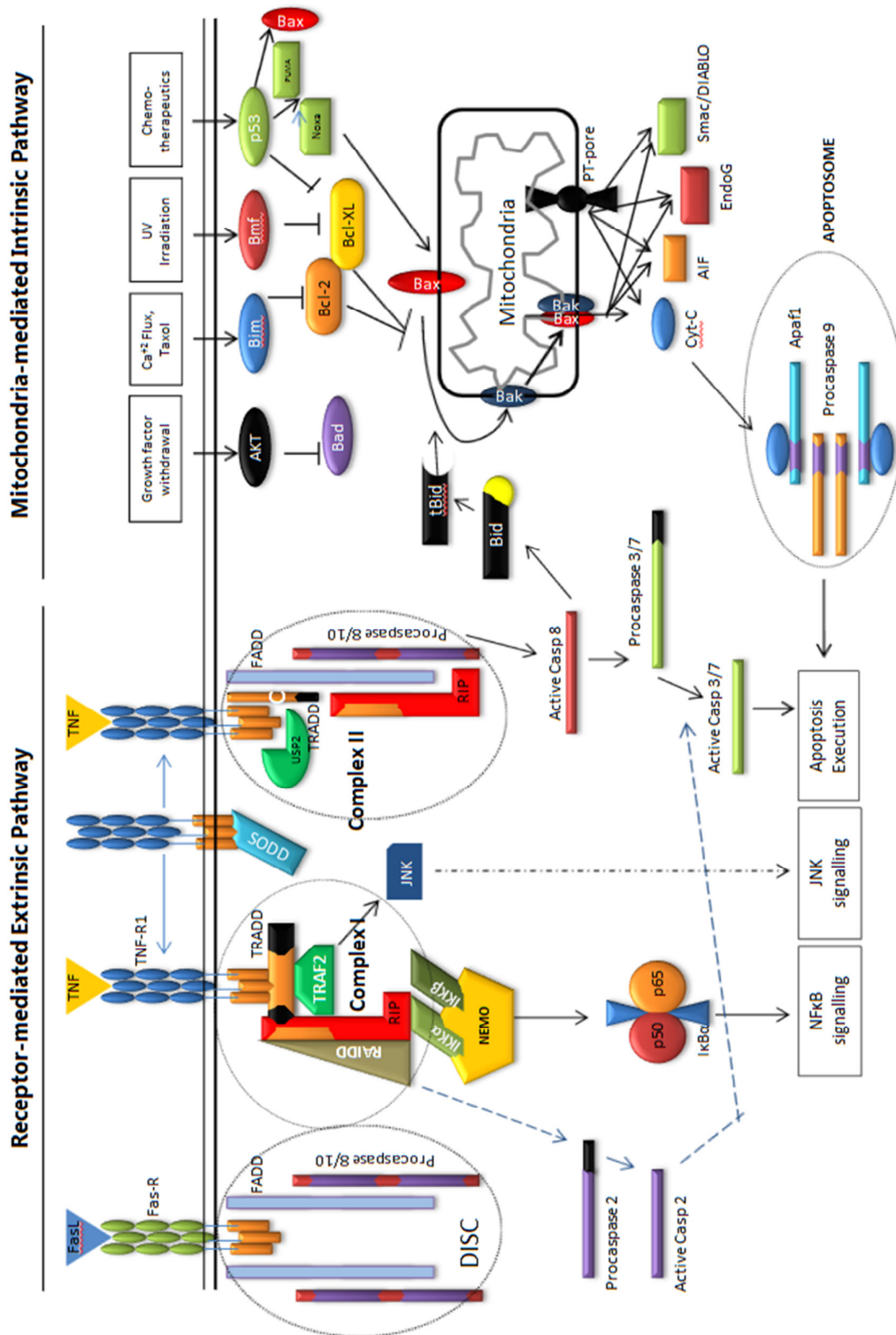




Figure A.5. Overview of extrinsic and intrinsic apoptosis pathways.**Receptor-mediated extrinsic pathway**

Upon binding of TNF to the TNF-R1, SODD dissociates and the receptor forms either complex I or complex II. Complex I consists of TNF-R1, TRADD, TRAF2 and RIP. It can either activate NF- κ B, recruit RAIDD to activate procaspase-2 or activate the JNK pathway. USP2a levels are important for the decision of formation of complex II. The latter is constituted of TNF-R1, TRADD, FADD and either procaspase 8 or -10. These caspases are activated at this complex. The ligation of Fas-L to the Fas-R results in the homo-trimerization of Fas-R and recruitment of FADD along with the pro-caspases-8 or -10. Those group together to form the DISC where procaspases-8 or -10 are activated and in turn they activate downstream caspases.

Mitochondria-mediated intrinsic pathway

Cellular stress such as DNA damage, calcium influx, UV irradiation, chemotherapeutic drug treatment, growth factor deprivation, ischemia or ER-stress, signal to BH3-only members of the Bcl-2 family. They either suppress the anti-apoptotic Bcl-2 family proteins or promote the activation of Bax to induce the permeabilisation of the outer mitochondrial membrane. The permeabilisation occurs either by Bax forming pores or via the activation of the PT-pore. Both lead to the rupture of the OMM and release of pro-apoptotic proteins from the mitochondria intermembrane space such as cytochrome-c, AIF, endonuclease G or Smac/DIABLO. Released cytochrome-c forms a complex with Apaf-1 and procaspase-9 that is termed apoptosome where caspase-9 is activated and alone or through caspases 3/7 lead to apoptosis.



A.1.3.3. Alternative forms of cell death

As mentioned earlier, classic apoptosis is defined by biochemical and morphological changes which include caspase activation, mitochondrial dysfunction, membrane blebbing, and DNA fragmentation. There are, however, some forms of cell death that cannot easily be categorised. An irreversible inhibitor of caspases (zVAD-fmk), that binds to the catalytic site of a caspase through an aspartate residue by mimicking the cleavage site and a fluoromethyl ketone (fmk) group forming a covalent inhibitor-enzyme complex (Thornberry *et al.*, 1994), should be able to inhibit all apoptotic pathways. However, not all cell death pathways are inhibited by this compound. Ectopic expression of Bax without another death stimulus triggers cell death leading to the breakdown of mitochondrial function (loss of membrane potential, release of Cyt-c, AIF and other pro-apoptotic proteins), which was reported not to be inhibited by zVAD-fmk (Xiang *et al.*, 1996). Moreover, other proteases like granzymeA/B, cathepsins and calpains, seem to play a role in apoptosis (Johnson, 2000; Leist & Jaattela, 2001). Caspase-independent apoptosis is thus triggered by these proteases with many apoptotic characteristics still present.

Upon MOMP apoptosis inducing factor (AIF) is released from mitochondria. As mentioned before, AIF translocates to the nucleus when released and induces chromatin condensation and DNA fragmentation (Susin *et al.*, 2000). This process is also promoted by another inner mitochondrial protein, endonuclease G, which



likewise translocates to the nucleus upon its release (Li *et al.*, 2001). This apoptotic function is pivotal for mouse morphogenesis during embryoid body cavitation (Joza *et al.*, 2001). Of note, this death-inducing pathway also cannot be inhibited by zVAD-fmk (Green & Reed, 1998; Vermeulen *et al.*, 2002; Xiang *et al.*, 1996).

Virally infected cells are eliminated by T cells and natural killer cells by a process of granule exocytosis pathway. The cytotoxic granules deliver a pore-forming protein, perforin, and a family of serine proteases. These granzymes have two isoforms and one of them, granzyme B, is able to activate caspase-3 and -9, which leads to the cleavage of Bid and iCAD resulting in apoptosis (Alimonti *et al.*, 2001). Statins can induce apoptosis via activation of caspases but without the release of cytochrome c but with Smac and Omi/HtrA2 (Ghavami *et al.*, 2010). Calpains represent another example of cell death proteases that initiate a type of cell death that is difficult to be classified with the known characteristics of apoptosis. It is known that calpains share some substrates of caspases but the fine line between necrotic and apoptotic cell death resides in the balance of these cysteine proteases (Harwood *et al.*, 2005). During development and in neuronal cells, another type of cell death is observed. It is termed “paraptosis” and it is characterised by vacuolation and mitochondria swelling (as observed in necrosis) but also it requires energy and new protein synthesis (as observed in apoptosis). Although caspase 9 is required for paraptosis, its function is different between



apoptosis and paraptosis since BAF (Boc-Asp(O-methyl)CH₂F) caspase inhibitor is incapable of inhibiting caspase 9 during paraptosis (Sperandio *et al.*, 2000).

A.1.3.4. The mitochondrial membrane potential

Permeabilisation of the OMM leading to a loss of $\Delta\Psi_m$ is regarded as a critical event in the mitochondria-mediated apoptosis pathway, but whether loss of $\Delta\Psi_m$ is the decisive mechanism for apoptosis induction or a consequence is still under intense investigation. There are two significant ways in which loss of $\Delta\Psi_m$ can take place (Green & Kroemer, 2004). Bax proteins can associate with the OMM and form pores that lead to its permeabilisation as well as sequester Bcl-2 proteins, which suppress apoptosis thereby contributing towards the initiation of the disruption of the OMM, and ultimately the release of pro-apoptotic factors into the cytoplasm (Beurel & Jope, 2006; Green & Kroemer, 2004; Kiechle & Zhang, 2002) Beurel & Jope, 2006). Another route for the loss of $\Delta\Psi_m$ is through the activation of the mitochondrial permeability transition pore complex (Grimm & Brdiczka, 2007).

A.1.3.4.1. Dissecting the molecular mechanism of MOMP

The mitochondria have been presented so far in this thesis as pivotal organelles for the intrinsic pathway of apoptosis. One of the key regulatory events in the mitochondria-dependent apoptosis pathway is the mitochondrial outer membrane permeabilisation (MOMP), which results in the release of proteins from



the intermembrane space (IMS) of mitochondria (Kroemer *et al.*, 2007) with Cyt-C being an example. The MOMP is regarded as the “point of no return” in the cascade of events leading to apoptosis (Kroemer & Reed, 2000) and precedes apoptotic cell death both *in vitro* (Zamzami *et al.*, 1995a) and *in vivo* (Zamzami *et al.*, 1995b). The molecular mechanism of OMM permeabilisation remains not fully understood, although it has been observed universally during apoptosis. As mitochondria release all of their Cyt-c within minutes following the apoptotic stimulus in a temperature-independent manner, it was thought that an enzymatic transport is not involved in this process (Goldstein *et al.*, 2000). It is now widely accepted that an enzymatic process of transport is not responsible since the transporter proteins of the pore do not function as such but become unspecific channels. There are now two dominant theories to explain the mechanism of MOMP. One involves the pro-apoptotic Bcl-2 family member proteins, Bax and Bak, and their oligomerization and insertion into the OMM to form a pore (see above). The other theory involves a mega-protein complex known as permeability transition (PT) pore that connects the inner and outer mitochondrial membranes and is converted into an unspecific channel to allow membrane permeabilisation.

A.1.3.4.2. Bax oligomerization and pore formation

Structural similarities of Bcl-2 family members with bacterial toxins that get inserted into the membrane were the initial reason why Bcl-2 family members were thought to be involved in MOMP (Suzuki *et al.*, 2000). It was made clear later



that they are indeed major regulators of MOMP (Kroemer, 1997; Susin *et al.*, 1996; Vaux & Korsmeyer, 1999; Wei *et al.*, 2001): Anti-apoptotic members of Bcl-2 family were shown to prevent the release of Cyt-c from mitochondria indicating that Bcl-2, regulates the integrity of the OMM (Gross *et al.*, 1999; Kluck *et al.*, 1997; Yang *et al.*, 1997; Zamzami & Kroemer, 2001). On the contrary, pro-apoptotic members of the Bcl-2 family, such as Bax or Bak, were reported to induce Cyt.-c release (Rosse *et al.*, 1998). In healthy cells, Bax is localized mainly in the cytosol as a non-active monomer but there are some Bax molecules loosely attached to mitochondrial membrane (Hsu *et al.*, 1997; Wolter *et al.*, 1997). It was discussed earlier that upon an apoptotic stimulus Bax undergoes an allosteric conformation, which is required for a Bax-induced MOMP (Antonsson *et al.*, 2000; Desagher *et al.*, 1999). This activated form of Bax is then believed to homo-oligomerize to create selective protein-permeable pores (Figure A.6.) that allow Cyt-c to be released into the cytosol (Dewson & Kluck, 2009). Bax activation possibly coincides with Bak activation, which exists inserted into the OMM in healthy cells (Griffiths *et al.*, 1999) and likewise undergoes an allosteric conformational change so it becomes activated to homo-oligomerize contributing to the pore formation (Green, 2005; Mikhailov *et al.*, 2003). Growing evidence allows supports that the BH3 domain of Bax and/or Bak is exposed and the oligomerization takes place between BH3 and the hydrophobic groove of another activated Bax or Bak molecule (Dewson & Kluck, 2009). This pore allows proteins in the IMS to be released into the cytosol



without affecting the function of the inner mitochondrial membrane (IMM) and/or the mitochondrial matrix. However, how these factors, especially Bax, “find their way” to the OMM or how they interact with the OMM to form a macromolecular protein-permeable pore remains an on-going conundrum. It is possible that the lipid-protein interaction is important (Lucken-Ardjomande & Martinou, 2005; Zamzami & Kroemer, 2003) and it is also possible that Bax or Bak do not form a pore but simply destabilize the lipid bilayers instead (Basanez *et al.*, 2002).

Transgenic double knockout Bax/Bak $-/-$ cells are resistant to apoptosis induced by UV irradiation, staurosporin (STS), growth factor deprivation, etoposide, and ER stress by thapsigargin and tunicamycin (Wei *et al.*, 2001). Ectopic expression of anti-apoptotic Bcl-2 proteins can likewise inhibit the apoptosis induced by overexpression of BH3-only proteins (Gross *et al.*, 1999), which induce apoptosis via Bax or Bak. Vesicles composed of purified mitochondrial outer membranes can be permeabilized in response to the activated form of recombinant Bax and vesicles made of mitochondrial lipids without the presence of any mitochondrial proteins were permeabilized by the introduction of recombinant monomeric Bax in the presence of Bid or Bid-derived BH3 peptide (Antonsson *et al.*, 2000; Eskes *et al.*, 1998; Jurgensmeier *et al.*, 1998; Narita *et al.*, 1998).

Bcl-2 family members are very important for the regulation and initiation of MOMP as explained above. Yet, more signals contribute to MOMP upstream of



Bcl-2 members. Bim, Bid and PUMA which are BH3-only proteins directly associate with Bcl-2 family and act upstream of Bax or Bak activation, whereas Bad or Bik inhibit the activity of anti-apoptotic Bcl-2 therefore favouring the process of MOMP (Cheng *et al.*, 2001; Kuwana *et al.*, 2002; Letai *et al.*, 2002). There are other non-Bcl-2 family proteins that possess similar properties. The tumour suppressor p53 regulates the expression of BH3-only members such as Puma and Noxa by certain stimuli (Vousden & Lu, 2002), and Puma-deficient cells display resistance to p53-induced apoptosis (Jeffers *et al.*, 2003). However, p53 can also trigger MOMP and apoptosis in the absence of its transcription factor activity through direct activation of Bax (Chipuk *et al.*, 2004) or Bak (Leu *et al.*, 2004). Additionally, many other nuclear proteins function in the cytosol leading to MOMP. An example is histone 1.2, which is released upon X-ray-induced DNA damage and can trigger apoptosis (Konishi *et al.*, 2003). Hexokinases, which reside in the cytosol can interact with VDAC, and this interaction can inhibit the MOMP induced by Bax (Majewski *et al.*, 2004a). VDAC-Hexokinase interaction will be extensively covered in other sections of this study.



A.2. The mitochondrial permeability transition pore (PTP)

In late 80s research conducted on calcium (Ca^{2+}) led to the discovery of the permeability transition (PT) concept (Hunter *et al.*, 1976). It was observed that Ca^{2+} addition to isolated mitochondria led to a transition of mitochondria with an increase in the permeability of the inner mitochondrial membrane (IMM). This led to matrix swelling and eventually to a catastrophic collapse of the mitochondria membrane potential ($\Delta\Psi_m$) and the shutdown of the energy synthesis. Patch-clamp experiments showed that a megachannel or PT-pore as it was named, mediated high conductances, which were attenuated by cyclosporine A (CsA), Mg^{2+} and adenosine di-phosphate (ADP). However, the same molecules could inhibit the effects observed on isolated mitochondria after calcium administration. This observation led to the proposal that the megachannel was responsible of the Ca^{2+} -dependent permeability transition (Broekemeier *et al.*, 1989; Petronilli *et al.*, 1989; Szabo & Zoratti, 1991, 1992).

The PTP complex is composed of several, albeit not clearly defined, proteins. It is currently believed that the major PTP complex consists of “voltage-dependent anion channel” (VDAC), an OMM protein; “adenine nucleotide translocator” (ANT), which spans the IMM; and “Cyclophilin D” (CypD), which associates with ANT and resides in the matrix (Figure A.6.) (Beutner *et al.*, 1996;



Crompton *et al.*, 1998). In addition, hexokinase-II (HK), peripheral benzodiazepine receptor (PBR) associate with the OMM and creatine kinase1 (CK1) is localized in the IMS, all of which have been reported to interact with the PTP complex (Verrier *et al.*, 2004). The above mentioned PT-pore components constitute the classical PT-pore although the exact composition of the PTP is still the subject of scientific debate, since there are several reports that describe contradictory information about the composition of the pore. Nevertheless, the classical PT-pore fits the results of the present study and further insight will be given at later chapters. Irrespective of any dispute on the pore composition there is agreement on the localisation of this structure at contact sites between the outer and the inner mitochondrial membrane (Brdiczka, 1991; Crompton, 2000). A consensus on the approximate size of this megachannel also exists with the PTP being a multi-protein complex of 600 kDa (Beutner *et al.*, 1996; Faustin *et al.*, 2004). PTP proteins can also be part of other sub-complexes that control energy metabolism or initiation of cellular death (Crompton *et al.*, 2002; Zoratti *et al.*, 2005). The outer mitochondrial membrane has a semi-permeable nature due to VDAC which allows the passage of small solutes of up to 1.5kDa allowing the exchange of respiratory-chain substrates such as NADH, FADH, and ATP/ADP between the IMS and the cytosol. In contrast, the IMM is almost impermeable and this sustains an electrochemical proton gradient ($\Delta\Psi_m$) by the oxidative phosphorylation by complex I to IV of the respiratory chain (Colombini, 1983; Mitchell & Moyle, 1965a,



b). Under physiological conditions, $\Delta\Psi_m$ ranges between 120 to 180mV, with the intra-mitochondrial side being negative. This polarization attracts lipophilic cations and they accumulate at 100 to 1000 fold higher concentration in the matrix than in cytosol. As a result, several different cationic fluorochromes are employed to measure the $\Delta\Psi_m$ (Castedo *et al.*, 2002).

A.2.1. PT-Pore under physiological conditions

It is common knowledge that the mitochondria are the energy factories of every cell and in order to maintain the normal functions such as the $\Delta\Psi_m$, the biosynthesis of molecules and the movement of metabolites, mitochondria have to be tightly controlled. ADP/ATP channelling is one of the main functions of the PT-pore. Matrix, IMS and cytosol are all compartments that are involved in this process including ANT and VDAC. CK and hexokinase are feeding this process according to cellular energy demand (Brenner & Grimm, 2006).

The pool of adenine nucleotides, the matrix pH, the redox state and the $\Delta\Psi_m$ are all parameters that need to be controlled and in turn regulate the physiological function of the PT-pore (Halestrap *et al.*, 1997). A transient permeability of the IMM to protons, water and solutes can stabilise the mitochondria homeostasis under certain conditions but more extended alterations can also affect the cell fate by changing the physiological function of PT-pore (Le Bras *et al.*, 2005).



A.2.2. PT-Pore under apoptotic/necrotic conditions

Cellular stress such as excessive production of reactive oxygen species (ROS) or the deregulation of calcium homeostasis lead to the opening of the PTP (Bernardi, 1999; Duchen, 2000; Zoratti & Szabo, 1995). Persistent PTP opening initiates a bioenergetic crisis accompanied by depletion of ATP, calcium deregulation and influx of solutes and H₂O. Ultimately, these changes result in the loss of the mitochondrial membrane potential ($\Delta\Psi_m$), a distorted balance of osmotic pressure leading to swelling of the mitochondrial matrix and cristae remodelling (Figure A.6.) (Kroemer & Reed, 2000; Susin *et al.*, 1998). The matrix swelling induces the swelling of the IMM and finally the rupture of the OMM leading to the release of IMS proteins such as Cyt-c and AIF (Figure A.6.), resulting in the loss of mitochondrial structure and functional integrity (Green & Kroemer, 2004; Zamzami & Kroemer, 2001). Apoptosis through calcium-mediated mitochondrial PT has been observed in variety of treatments such as calcium ionophores, thapsigargin, neurotoxins, chemotherapeutic agents and pro-oxidants. Such calcium-mediated apoptosis can often be suppressed by the repression of calcium uptake into mitochondria (treatment with ruthenium red or its derivative Ru360) or by the inhibition of PTP by cyclosporine A, sangliferin or bongkreic acid (Waldmeier *et al.*, 2002). The opening of PTP does not necessarily result in the permeabilisation of membranes. Transient opening ("flickering") of the PTP is physiological and provides mitochondria with a fast calcium release, which



helps to regulate calcium homeostasis and signalling thereby preventing the induction of apoptosis. Calcium is released from ER and is taken up from mitochondria but it can still be released without affecting the mitochondrial integrity through the PT-pore “flickering” (Petronilli *et al.*, 1999; Zoratti & Szabo, 1995). In addition, although the loss of the mitochondrial membrane potential has been regarded as one of the characteristics of apoptosis, this - especially if it happens only for a short duration - does not necessarily originate from persistent PTP opening as many different signals can induce a transient loss of $\Delta\Psi_m$. As it will be discussed later, the duration and the strength of the stimulus determines the involvement of PT pore in necrosis or apoptosis (Kroemer *et al.*, 1998).

It has been proposed that there are three different possibilities about how the permeabilisation of the OMM is accomplished in cooperation with the PT pore during apoptosis and they all deal with the kinetics of Bax. The first scenario proposes that PT-pore opening precedes the Bax translocation from the cytosol to the mitochondria (Precht *et al.*, 2005). In the second scenario the PT-pore opens only after Bax has translocated to the OMM (Eldering *et al.*, 2004; Weaver *et al.*, 2005), whereas according to the third scenario Bax and Bak co-operate on the OMM forming pores and allowing permeabilisation without the involvement of the IMM or the PT-pore (Eskes *et al.*, 1998; Piret *et al.*, 2004). An explanation for the difficulty to determine the scenario involved in OMM permeabilisation could lie in the different detection sensitivities of the methods used. The detection of one



pathway or protein activation is not enough to determine the sequence of events since another, more sensitive method can be utilized and the activation of another factor or signalling pathway would appear earlier.

Apoptosis is not the only type of cell death that the PT pore can regulate. Numerous examples of cell damage show that the PT-pore is also involved in necrosis (Grimm & Brdiczka, 2007). When comparing the energy dependence of the apoptosis signals a general conclusion can be drawn: if ATP is involved the cell will undergo apoptosis and where energy is not needed the passive, necrotic procedure will be activated (Leist *et al.*, 1997). Ischemia of cells with subsequent reperfusion represents an instance of PT-pore involvement in necrosis. During this process free ADP and Ca^{+2} increase and the subsequent reperfusion is characterised by an imbalance of high intra-mitochondrial substrates for the respiratory chain versus low ADP, which in turn leads to superoxide production and oxidative stress (Crompton & Costi, 1990). Oxidative stress then activates the PT-pore and results in further increase of the free ADP. In agreement with this example, cyclosporin A, which is a PT-pore inhibitor, was found to protect cardiomyocytes from $\Delta\Psi_m$ collapse and cell death induced by ischemia/reperfusion (Grimm & Brdiczka, 2007).

Cyclophilin D as a pore component was also seen to be involved in necrosis and in some cases the respective experimental procedure determined the involvement of cyp-D only in necrosis and not apoptosis. One study (Nakagawa *et*



al., 2005) showed that upon H₂O₂ administration the PT-pore was involved via cyp-D and claimed that only necrosis is regulated by PT-pore. On the other hand H₂O₂ was also used in a study in which Cyp-D was absent and it was found that apoptosis was affected (Baines *et al.*, 2005). The difference in those studies is probably due to the different concentrations of the drugs that were used to induce cell death. Consequently, the duration and the strength of the signal for the induction cell death can determine which type of cell death is induced but conclusion for the exclusive involvement of the PT-pore in either of the pathways cannot be drawn.

Hence, depending on the experimental model, the PT-pore can be consequence or cause of the fatal signalling pathway and only after the characterization of the cell death type can its real contribution be defined.

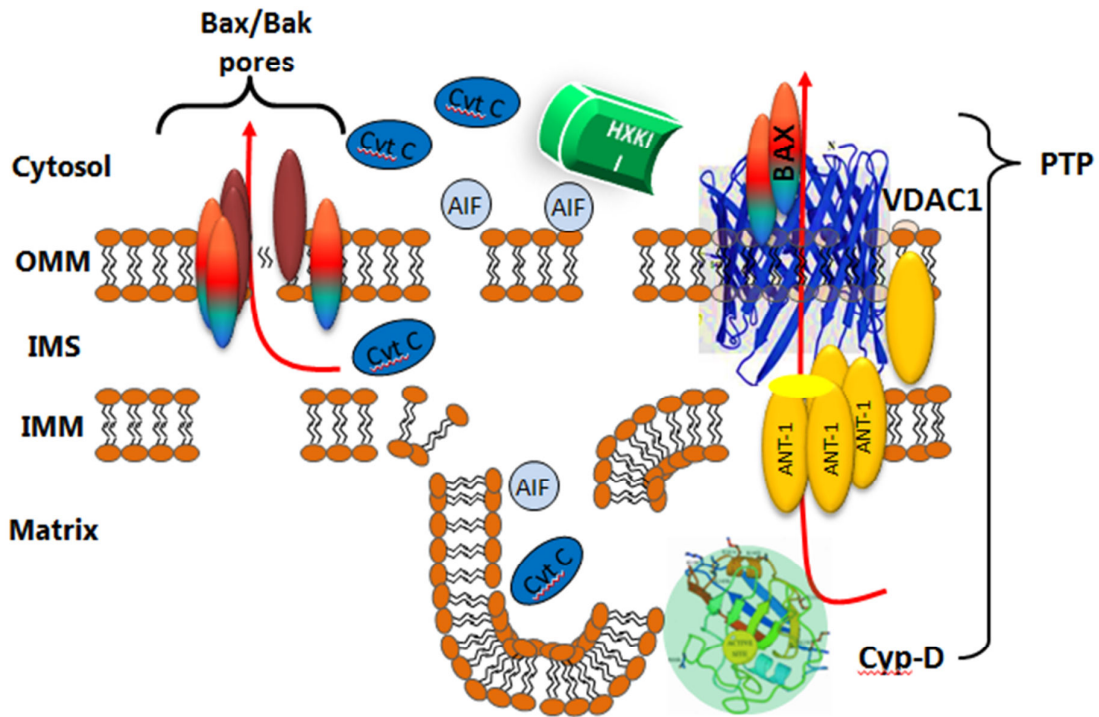
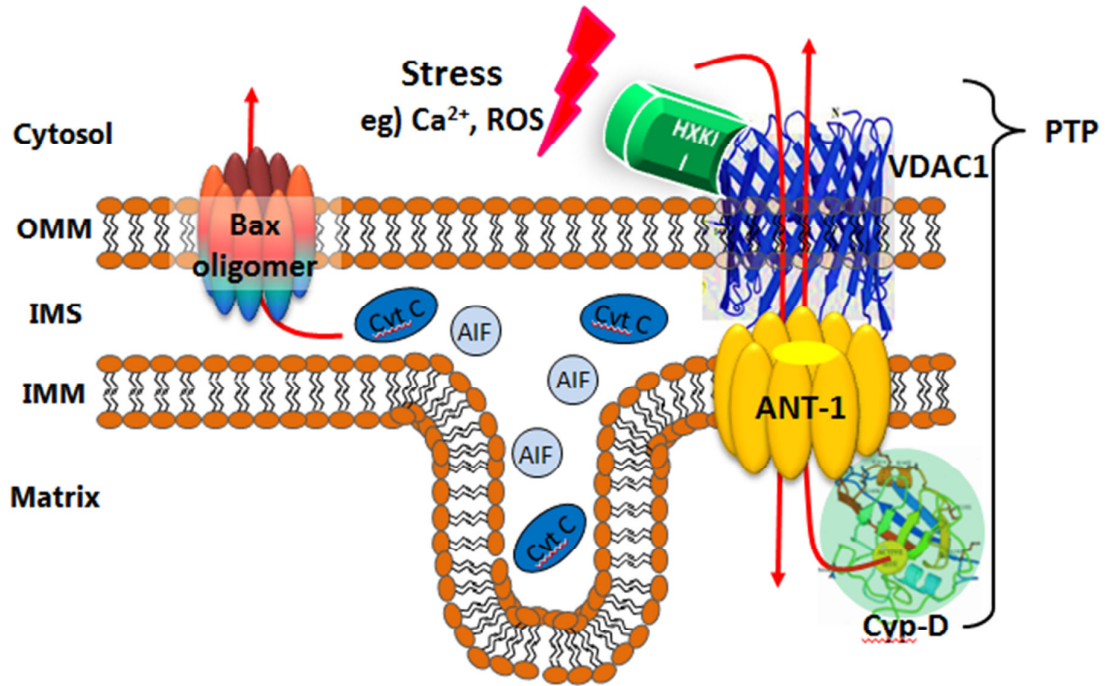




Figure A.6. Mechanism of MOMP and apoptosis induction.

Bax/Bak pores (left side). Pro-apoptotic members of Bcl-2 family member, Bax and Bak, form a macromolecular protein permeable pore after Bax has been activated and translocated to the outer mitochondrial membrane by homo-oligomerization. The pore allows the egression of intermembrane space proteins, such as Cyt-c and AIF.

PT-pores (right side). The classical permeability transition pore (PTP) complex which is comprised of VDAC1, ANT1, cyclophilin D (cyp-D) and Hexokinase, regulate the MOMP. In healthy cells (upper panel), PTP complex allows small molecules such as NADH and FADH to pass between cytosol and mitochondrial matrix. However, upon stress such as calcium influx or ROS production, the PTP becomes leaky resulting in the influx of water into the matrix. This causes matrix swelling, cristae remodelling and eventually rupture of the OMM allowing pro-apoptotic proteins to get released into the cytosol. Hexokinases bind to VDAC at the OMM and occupy the binding site where Bax can bind. Upon apoptosis, hexokinases are released and Bax can bind to VDAC allowing the opening of a mega pore which leads in the release of pro-apoptotic proteins from the IMS.



A.2.3. VDAC and hexokinase as PT-pore components and modulators

VDAC was characterized as a hexokinase binding protein at the OMM and soon it became apparent that hexokinase I or II bind to the OMM and this binding is enhanced via their interaction with VDAC1 (as one of the most abundant OMM proteins) (Fiek *et al.*, 1982). Hexokinases are differentially expressed in most tissues but in cancer cells hexokinase II (HXKII) is highly overexpressed and bound to mitochondria contributing to high tumour glycolysis, also known as the Warburg effect (Pedersen *et al.*, 2002). There are four isoforms of hexokinases identified so far but HXKI and HXKII are mostly involved in mitochondria binding, have greater glucose affinity and possess a 15aa N-terminal sequence that allows them to bind to VDAC1 (Azoulay-Zohar *et al.*, 2004).

Recent studies demonstrate that the VDAC1-HXKII interaction is critical and when stabilized, prevents apoptosis induction in tumours (Pastorino & Hoek, 2003; Vysokikh & Brdiczka, 2004). Conversely, disruption of this protein complex can induce apoptosis or sensitise tumour cells to cell death (Zaid *et al.*, 2005). Hexokinase as an anti-apoptotic molecule promotes the “closed” conformation of VDAC, leading to inhibition of Ca^{+2} -induced apoptosis through the PT-pore. (Azoulay-Zohar *et al.*, 2004). Binding of HXKII to VDAC1 restricts the availability of free VDAC sites that can interact with Bax and also prevents Bad to associate with the OMM and induce apoptosis (Capano & Crompton, 2002; Pastorino *et al.*,



2002). Another model of how the VDAC1-HXKII complex affects the PT-pore could be through ANT-1. Cyp-D alters the conformation of ANT-1 which in turn alters the VDAC1 conformation that allows strong binding of hexokinase so that the permeability transition is inhibited and so that ultimately the PT-pore cannot open. According to that model the level of Cyp-D and its interaction with ANT-1 stabilises VDAC1 through enhanced hexokinase binding (Crompton, 1999; Machida *et al.*, 2006; Vyssokikh & Brdiczka, 2003).

As mentioned above, Cyclophilin D (CypD) is a member of the PT-pore megachannel localized in the matrix of mitochondria. Overexpression, knockout and knock-down studies conclude that CypD appears to be a critical component of the PT-pore with anti-apoptotic or pro-apoptotic characteristics (Baines *et al.*, 2005; Schinzel *et al.*, 2005; Schubert & Grimm, 2004). Recent studies demonstrate that mitochondrial binding of HXKII is affected by Cyp-D and the anti-apoptotic effects of Cyp-D maybe exerted via the stabilized binding of HXKII to mitochondria (Chiara *et al.*, 2008; Machida *et al.*, 2006) thereby contributing to the high glycolytic rate that is observed in cancers and enhancing the Warburg effect (see below).



A.3. The NF- κ B system

A.3.1. The inducible transcription factor NF- κ B

The “nuclear factor κ -light-chain-enhancer of activated B-cells” (NF- κ B) family of transcription factors is expressed in a diverse number of cell and tissue types and is understood to play an important role in the inflammatory response (Baeuerle & Henkel, 1994; Baichwal & Baeuerle, 1997). NF- κ B is in its active state dimeric; this can be a homodimer or heterodimer of five known subunits: NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel (Gilmore, 2006). All the subunits possess a structurally conserved region of about 300 amino acids, termed the Rel homology domain (RHD), which contains the domains necessary for subunit dimerization, nuclear localisation, and also DNA binding. The subunits also feature a region called the transactivation domain (TD), which is necessary to promote the transcription of target genes. However, two subunits, NF- κ B1 and NF- κ B2, when they form homodimers, can only act as repressors of transcription since they do not have the TD (Li & Verma, 2002). An example of their ability to positively and negatively regulate transcription is exemplified with the KAI1 metastasis suppressor gene. In this case p50 is converted into a suppressing complex with the help of the β -catenin oncoprotein (Kim *et al.*, 2005). All the subunits can be classified in two groups. The “NF- κ B protein” group contains p50 and p52, which is generated after removal of the c-terminus of p105 and p100



respectively, and the “Rel group” of proteins consisting of p65, RelB and c-Rel. In most cells NF- κ B dimers are mainly, but not exclusively, composed of NF- κ B1 and RelA (p50/p65) (Aggarwal, 2000; Bauerle *et al.*, 2010).

The site where NF- κ B transcription factors bind is a highly variable 9-10 base pair DNA sequence (κ B site) with the following consensus 5'-GGGRNWYYCC-3' where R=A or G, N=any nucleotide, W=A or T and Y=C or T. With the exception of RelB, all the subunits from both groups can form homodimers. This high combination capacity of the NF- κ B subunits gives the cell, dependent on its protein expression profile, the ability to activate specific genes since the various dimers exhibit differential binding affinities to promoters (Gilmore, 2006)

In most cells NF- κ B is present with its subunits sequestered in the cytosol by a family of inhibitory proteins called “inhibitors of κ B” (I κ B), which will be further covered below (Bauerle & Henkel, 1994; Li & Verma, 2002). In stimulated cells a sequence of molecular events take place that lead to the degradation of these inhibitory proteins, allowing the NF- κ B dimer to enter the nucleus and carry out its function as a transcriptional activator or repressor. The cytokines interleukin-1 (IL-1) and TNF- α , the latter of which we discussed earlier as being involved in apoptosis induction, are two of the most important activators of NF- κ B (Li & Verma, 2002). In fact, almost every cytokine of the TNF family and also chemotherapeutic apoptosis-inducing agents activate NF- κ B (Aggarwal, 2000). Figure A.7. shows the structural organization of Rel and NF- κ B protein families.



A.3.2. Inhibitors of NF- κ B

I κ Bs are primarily cytoplasmic proteins and function by sequestering and maintaining the inactivity of NF- κ B (Gilmore, 2006). The I κ B family, as the NF- κ B transcription factor family, has several members such as I κ B α , I κ B β , I κ B ϵ and Bcl-3, all of which are identifiable by the presence of several copies of a structural motif known as ankyrin repeat – the motif is involved in protein-protein interactions (Hayden & Ghosh, 2012). Different I κ Bs bind differentially to NF- κ B dimers and their expression levels also vary in different tissues. From biochemical studies and after their structures were determined it became apparent that I κ Bs are directly interacting with NF- κ Bs by making multiple contacts (Chen & Ghosh, 1999). Generally, the contact sites cover the nuclear localization signal (NLS) and sequences responsible for DNA binding.

The general structural organisation of I κ Bs is shown on Figure A.7. The best characterised I κ B family member is I κ B α (Figure A.8). The structural analysis of the I κ B α :NF- κ B complex revealed that I κ B α contains 5 or 6 ankyrin repeats (depending on the classification of the sixth ankyrin repeat as independent or as part of the C-terminus domain of the I κ B α protein (Jacobs & Harrison, 1998; Sachdev *et al.*, 1998b). For this study the five ankyrin repeat structure was followed as described in the protein database Uniprot. The I κ B α is comprised of three distinct regions: The N-terminal regulatory domain for signal dependent degradation, the ankyrin



repeat domain and the C-terminal part, which contains a PEST sequence (Haskill *et al.*, 1991).

Bacterial infections, cytokines, or stress signals, all lead to the phosphorylation of the N-terminal part of I κ B α at position 32 and 36. This phosphorylation allows the ubiquitination of the protein at lysines 21 and 22 by K48 ubiquitin chains, leading to its proteasomal degradation (DiDonato *et al.*, 1996; DiDonato *et al.*, 1997; Lee *et al.*, 1997; Lee *et al.*, 1998; Palombella *et al.*, 1994; Rodriguez *et al.*, 1996; Roff *et al.*, 1996; Scherer *et al.*, 1995). Proteolytic removal of I κ B α leads to the exposure of the NLS of p65 or similar subunits, which in turn allows the translocation of NF- κ B into the nucleus. A mutation on residues 32 and 36 substituting the serines with alanines converts I κ B α into a super-repressor (I κ B α -SR) since proteolytic degradation cannot take place and NF- κ B is permanently inhibited (Van Antwerp *et al.*, 1996).

The ankyrin repeat domain (ARD) allows the interaction of I κ B α with NF- κ B subunits. For example, there are several contact sites on I κ B α that are responsible for the interaction with p65 or p50. ANK1 and ANK2 bind to the NLS of p65 allowing I κ B α to adopt the correct conformation to bind to the rest of p65. ANK3 has the fewest sites for p65 binding and ANK4 and ANK5 also facilitate binding of p50 and the dimerization domain of p65 (Jacobs & Harrison, 1998).



It is believed that the ankyrin repeats of I κ B α bind directly to the NLS of only one of the two NF- κ B dimer subunits – this is enough to prevent NF- κ B's transcriptional activity (Li & Verma, 2002). The NLS of the other, unbound, subunit allows entry of an inactive I κ B α :NF- κ B complex into the nucleus while a nuclear export sequence (NES) found on the I κ B α molecule at aa45-54, facilitates the export of this complex out of the nucleus. In this way a balance is maintained by continually shuffling the inactive complexes between the two compartments (Baeuerle & Henkel, 1994; Li & Verma, 2002; Turpin *et al.*, 1999). The export process has been found to be much more efficient than the import process, thereby creating a much higher quantity of the inactive complex in the cytoplasm. Mutagenesis of I κ B α within the second ankyrin repeat (I κ B α -110A3) at residues 115,117 and 120 render I κ B α completely incapable to enter the nucleus in this complex and inhibit DNA binding and transcriptional activation (Sachdev *et al.*, 1998b). The precise reason why a cell would carry out the energy dependent process of moving the inactive I κ B α :NF- κ B complex in and out of the nucleus is not clear.

There are several functions attributed to I κ B proteins. In the cytoplasm, I κ Bs prevent the translocation of NF- κ B to the nucleus, whereas in the nucleus, I κ B α can dissociate DNA-bound NF- κ B from the DNA and also export the NF- κ B back to the cytoplasm as an inactive complex (Baeuerle & Henkel, 1994; Ghosh *et al.*, 1998; Gilmore, 2006; Li & Verma, 2002). Responsible for the dissociation of DNA-



bound NF- κ B is the C-terminal part of I κ B α . When in the nucleus, I κ B α , through its acidic residues at the c-terminus (aa282-308), is able to remove DNA-bound NF- κ B and export it to the nucleus. Deletion analysis of I κ B α C-terminus revealed that deletion of aa280-317 inactivated the ability of I κ B α to inhibit p65, p50 (Hatada *et al.*, 1993b) and c-Rel Δ 282-308 (Luque & Gelinas, 1998). Deletion of the PEST-like sequence within the C-terminus of I κ B α completely abrogated the ability of I κ B α to release NF- κ B from the DNA (Ernst *et al.*, 1995).

Generation of I κ B α ^{-/-} mice gave further insight into the properties of I κ B α . These mice show constitutive NF- κ B activation, enhanced granulopoiesis and neonatal lethality, suggesting I κ B α to be the prototype I κ B that can primarily control the p50/p65 complex (Beg *et al.*, 1995a; Hayden & Ghosh, 2012). TNF stimulation of these cells extensively activated the NF- κ B pathway and due to the absence of I κ B α the kinetics of NF- κ B termination of transcription was very slow. The expression of I κ B β under the promoter of I κ B α reconstituted the kinetics of NF- κ B signalling (Beg *et al.*, 1995a; Cheng *et al.*, 1998; Klement *et al.*, 1996). The mechanism by which I κ B α and other I κ Bs control the NF- κ B response became more complicated through the finding that the model of cytoplasmic sequestration is only partially true since the absence of I κ B α , I κ B β and I κ B ϵ does not alter the distribution of p65 between cytosol and nucleus but confirmed that stimulus-dependent NF- κ B activation requires the above mentioned I κ Bs (Tergaonkar *et al.*, 2005).

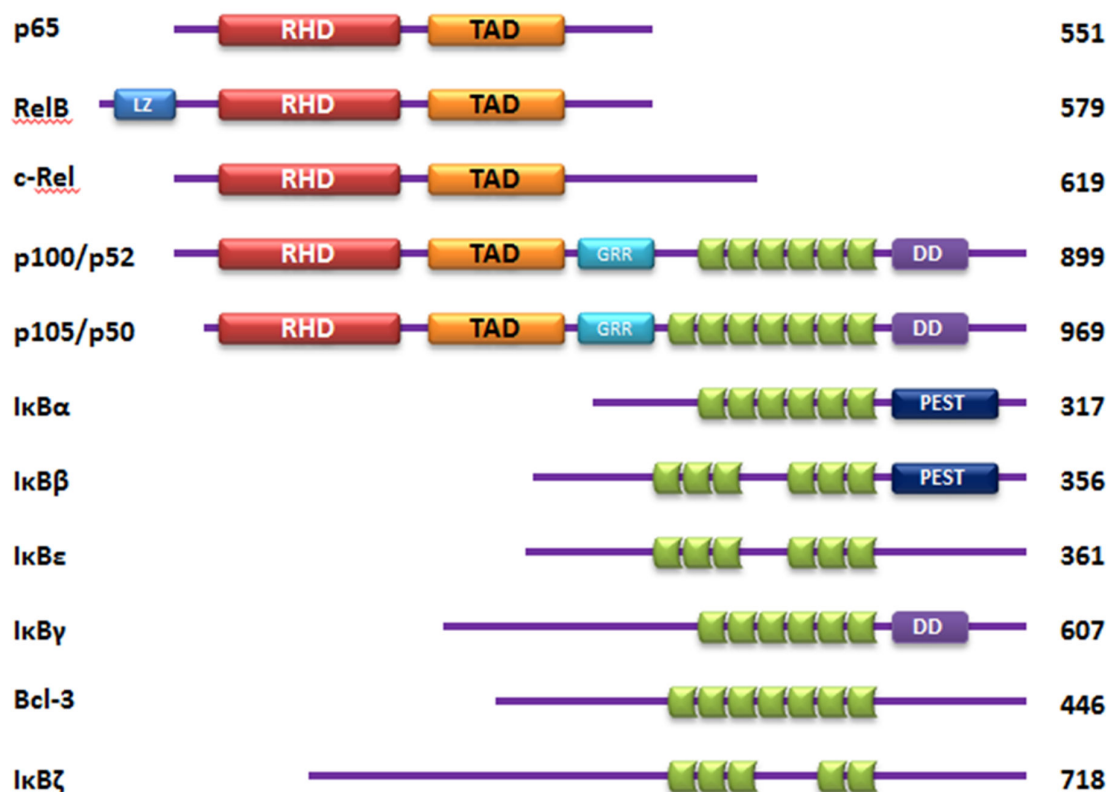


Figure A.7. Structural organisation of NF-κB and IκB proteins. Members of the NF-κB, and IκB families are shown. NF-κB family members form dimers with the most abundant being the p50/p65 heterodimer. p65 has a strong transactivation domain (TAD) and is responsible for most of NF-κB's transcriptional activity. p50/p65 and p50/c-Rel dimers are regulated by IκBs which cause their retention in the cytosol. All NF-κB proteins contain a Rel-homology domain (RHD) which mediates their dimerization and binding to the DNA. IκBs trap NF-κB proteins by masking the NLS which is within the RHD. Activation of NF-κB requires the proteolytic degradation of IκBs via their N-terminal region which contains sequences prone to ubiquitination. PEST sequences are acidic and are required for the inhibition of NF-κB from DNA binding. GRR: glycine-rich region; LZ: leucine zipper domain; DD: death domain. On the right the amino-acid sequence length is shown.



A.3.3. Activation of NF- κ B

There are two well-established pathways for NF- κ B activation and a novel third pathway, which comprises a distinct processing of the NF- κ B proteins. Although their importance is unquestionable, the analysis for the current thesis will focus on the so-called canonical NF- κ B activation pathway.

NF- κ B activation is accomplished by stimuli that degrade I κ B α . Phosphorylation of I κ B α proteins and subsequent ubiquitination, targets them to the 26s proteasome and the IKK complex is primarily involved in this phosphorylation process (Karin & Ben-Neriah, 2000). The IKK components are IKK α and IKK β and the NF- κ B essential modulator (NEMO) or IKK γ . IKK α and IKK β form homo or hetero-dimers however, IKK β is mostly involved in the canonical pathway to phosphorylate I κ B α (Delhase *et al.*, 1999). NEMO is a scaffold, sensing protein that binds IKK α or IKK β and amplifies the signal transduction pathway of kinases to phosphorylate I κ B α (Hayden & Ghosh, 2012). The distinction between the canonical and non-canonical pathways is made by the requirement of NEMO. In the non-canonical pathway an upstream inducing kinase (NIK) activates IKK α and signals towards the activation of p100 which is phosphorylated and converted into p52 (Hayden & Ghosh, 2012).

Generally, the two pathways can be activated concurrently but they have very distinct regulatory functions (Bonizzi & Karin, 2004; Silverman & Maniatis, 2001). The activation of the canonical pathway is triggered by activation of



Tumour necrosis factor receptors 1/2 (TNFR1/2), T cell receptors (TCR), B cell receptors (BCR) and Toll like receptors (TLR). Upon upregulation of transcription of genes regulating chemokines, cytokines, adhesion molecules, cell survival and inflammatory responses, the canonical pathway shuts down. On the other hand the non-canonical pathway is triggered by activation of specific TNF receptor family members like LT β R (lymphotoxin β receptor), BAFF-R (B cell activating factor), CD40 or CD30. This pathway is controlling development of lymphoid organs and adaptive immune system (Gilmore, 2006; Hayden & Ghosh, 2004, 2008, 2012).

The above mentioned third mechanism of NF- κ B activation does not necessarily result in the degradation of the NF- κ B inhibitory protein and involves NF- κ B kinases. IKK α , by itself, possesses NF- κ B kinase activity as well as many other proteins, which are part of the NF- κ B kinase family of proteins are able to phosphorylate RelA, in this way causing I κ B dissociation but not ubiquitination (Hayashi *et al.*, 1993).

A.3.4. Feedback loops and termination of NF- κ B

Active NF- κ B in the nucleus promotes the transcription of several genes involved in inflammation, such as cytokines, cytokine receptors, growth factors, and adhesion molecules. It was mentioned earlier that pro-inflammatory cytokines, e.g. IL-1 and TNF- α , can trigger NF- κ B activation; the cytokines expressed by these



genes, which make up a number of the NF- κ B target genes, result in a positive feedback cascade, further enhancing the inflammatory response (Halliwell & Gutteridge, 2007).

To counter the inflammatory cascade, NF- κ B can also promote the transcription of anti-inflammatory cytokines such as IL-10 to generate a negative feedback (Macdonald *et al.*, 2003). An important target gene of NF- κ B is I κ B α . By promoting the expression of one of its own inhibitors the signal is able to generate a negative feedback, which is normally sufficient to turn itself off. However, this mechanism does not explain how the active DNA-bound NF- κ B is diminished once signalling is terminated. Newly synthesised I κ B α can, in the nucleus, dissociate NF- κ B from the DNA to stop the transcriptional activation of target genes and also, in the cytoplasm, sequester active NF- κ B dimers to again inactivate them, as described earlier. However, p65, IKK α , suppressor of cytokine signalling (SOCS-1) are some of the molecules that can themselves affect the termination of the NF- κ B response (Hayden & Ghosh, 2008). Efforts are under way to further analyse this aspect of NF- κ B, which proves extremely important especially in diseases that have de-regulated NF- κ B (Baeuerle & Henkel, 1994; Hayden & Ghosh, 2008; Li & Verma, 2002).



A.3.5. Relation of NF- κ B to apoptosis

NF- κ B targets many genes that promote cell proliferation and survival and has often been found to be associated with an ability to negatively influence the cell's susceptibility to apoptosis (Aggarwal, 2000; Baichwal & Baeuerle, 1997; Bauerle *et al.*, 2010; Clifton *et al.*, 1998; Edderkaoui *et al.*, 2008; Van Antwerp *et al.*, 1996; Van Antwerp *et al.*, 1998).

RelA^{-/-} mice, for example, did not live to birth and showed high levels of apoptotic cell death in the developing liver, suggesting an anti-apoptotic activity for NF- κ B (Baichwal & Baeuerle, 1997). TNF- α induced apoptosis is known to be variable in its effectiveness in different cell types. This property of the cytokine has been attributed to the activity of NF- κ B, which we know it also activates (Baichwal & Baeuerle, 1997; Beg & Baltimore, 1996; Liu *et al.*, 1996b; Van Antwerp *et al.*, 1996; Van Antwerp *et al.*, 1998; Wang *et al.*, 1996). NF- κ B stimulates the transcription of TNF as was already mentioned, but also other anti-apoptotic molecules such as cellular inhibitors of apoptosis (cIAPs), caspase 8/FADD-like I β -converting enzyme inhibitory protein (cFLIP) and members of the Bcl2 family like Bcl-X_L (Karin *et al.*, 2002; Karin & Lin, 2002). Apoptosis induced by ionizing radiation and chemotherapeutics is also attenuated by NF- κ B and this ability of NF- κ B has led several groups to analyse this effect (Wang *et al.*, 1999). Many cancers in which NF- κ B subunits are highly expressed or NF- κ B constitutively activated have been shown to become more susceptible to apoptosis when



treated with compounds that inhibit NF- κ B activation (Edderkaoui *et al.*, 2008). It is understood, too that some pathogenic bacteria activate NF- κ B in their host cells in order to prevent apoptosis and promote cell survival (Clifton *et al.*, 1998).

With the aforementioned in mind, NF- κ B is regarded as an anti-apoptotic protein system. Hence I κ B α , in its cytoplasmic role as an inhibitor of NF- κ B, is widely deemed to possess a pro-apoptotic function since it inhibits the anti-apoptotic NF- κ B.

A.3.6. Mitochondrial localisation of NF- κ B and I κ B α

In recent years it has become apparent that NF- κ B subunits and I κ B α can also be found at mitochondria. Electron microscopy studies revealed that I κ B α can be identified in all the mitochondrial compartments, the inter mitochondrial membrane space (IMS), the matrix and OMM (Bottero *et al.*, 2001; Cogswell *et al.*, 2003; Lee *et al.*, 2008; Zamora *et al.*, 2004). Emphasis was given to the I κ B α localised in the IMS since it was found to be able to interact with ANT1. NF- κ B subunits were also detected in mitochondria and were also found to negatively regulate apoptosis induced by ANT1 overexpression. NF- κ B was recruited to mitochondria after ANT1 overexpression thereby abolishing its DNA binding, gene activation, and contributing to extensive apoptosis (Zamora *et al.*, 2004).

NF- κ B that has been found to be localised to mitochondria and is believed to be involved in the regulation of mitochondrial gene expression (Cogswell *et al.*,



2003; Lee *et al.*, 2008; Reuther & Baldwin, 1999). However, using mitochondriaLly localised I κ B α (I κ B α when overexpressed is found to be localised in mitochondria as well as the cytosol) to block NF- κ B activation in mitochondria during TNF- α treatment, revealed no reduction in the mRNA levels of proteins encoded by mitochondria-specific genes compared to the normal state where a reduction occurs when NF- κ B can be normally activated (Cogswell *et al.*, 2003).

The I κ B α :NF- κ B complex has been found in the IMS, where it is currently thought to interact with ANT, one of the components of the PT-Pore and the I κ B α :NF- κ B complex is supposed to have at least some influence on the apoptotic cascade (Bottero *et al.*, 2001; Zamora *et al.*, 2004) since it was found that p65 and I κ B α were released from mitochondria upon Fas-ligand induced apoptosis.

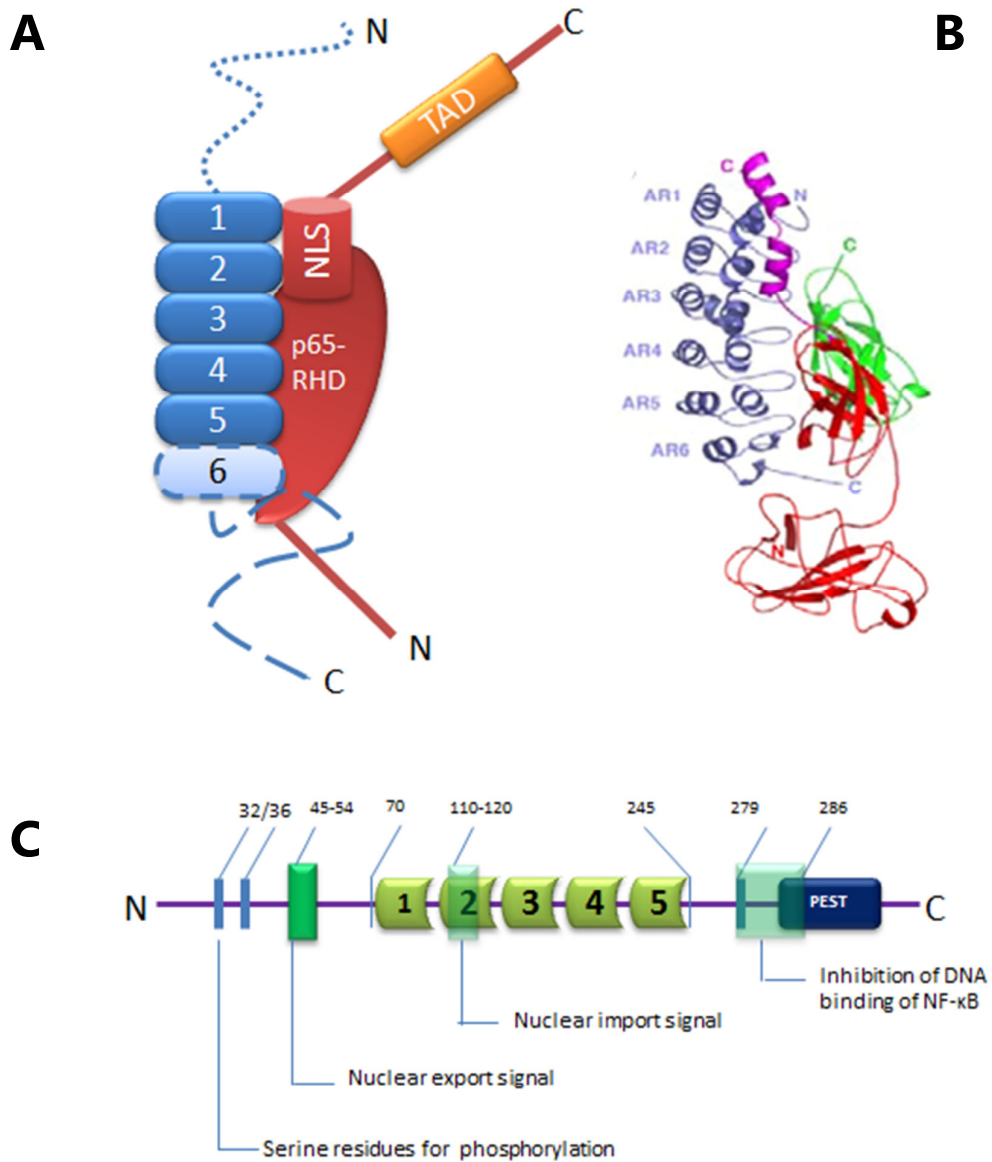


Figure A.8. Representation of I κ B α . **(A)** Cartoon representation of I κ B α domains (blue) which bind p65. Ankyrin repeats and the C-terminus are responsible of p65 binding. **(B)** Structural representation of I κ B α binding to p65 (Bergqvist S *et al.*, 2009). **(C)** Important residues and domains of I κ B α protein



A.4. Cancer

A.4.1. Cancer and apoptosis

Cancer is a disease that originates from diverse aetiologies and affects equally diverse cell targets, therefore resulting in an extremely heterogeneous appearance. Even though this complexity seems to make it daunting to define general mechanisms underlying all human cancers, defects in the programmed suicide machinery characterise all tumours. This apoptosis inhibition can contribute to cell immortality and represents one of the cancer hallmarks (Hanahan & Weinberg, 2000). It is supposed to thwart pro-apoptotic signals that arise during the transformation process. The discovery of oncogenes such as *bcl-2* or *myc* and their ability to inhibit apoptosis (Kerr et al., 1972; Strasser et al., 1990), initiated the now vast field of apoptosis investigation on cancer, at the molecular level. The emerging conclusion is that maintaining the balance of pro-apoptotic and anti-apoptotic signals within a normal cell is a fundamental prerequisite for its homeostasis. Current research so far suggests that obstructing the apoptotic machinery can then dramatically affect the dynamics of tumour progression. Moreover, a plethora of data indicate that anti-cancer compounds such as 5-fluorouracil, cis-platin, and etoposide exert their effect by inducing apoptosis in malignant cells (Mow *et al.*, 2001). So, apoptosis can be harnessed for cancer therapy. (Barry *et al.*, 1990; Kaufmann, 1989; Walker *et al.*, 1991). During treatment,



tumour cells are selected for the repression of this cell death program. In order to enhance current drug efficacy and discover more effective treatment options, it is essential to unravel how cells attain their insensitivity to cell death signals. Therefore, the molecular regulators of anti-apoptotic signalling pathways are currently the subject of intense investigation.

A.4.2. Cancer and PT-pore

As mentioned above, the molecular pathways that govern apoptosis are pivotal for controlling tumourigenesis, cancer progression, and for cancer treatment. One of the molecular complexes that play such a role is the aforementioned PT-pore. During tumourigenesis, specific modulators of the PT-pore are activated hence contributing to tumour formation. Evading hypoxia, metastasis, OMM permeabilisation, reactive oxygen species (ROS) formation or Ca^{+2} release are only some of the processes that contribute to carcinogenesis, relate to apoptosis and involve the PT-pore (Brenner & Grimm, 2006).

More specifically, hypoxia is an environmental condition of reduced oxygen levels. It is a scenario that all tumour cells experience especially at the initial stages of tumour formation and metastasis. Normally, hypoxia constitutes a pro-apoptotic signal and a stimulus for PT-pore activation. The mechanisms that cancer cells use in order to maintain survival under hypoxic conditions are largely unknown but inhibition of the PT-pore opening even in low oxygen is a



characteristic of cancer cells (Harris, 2002). Hypoxia inducible factor (HIF) and pro-apoptotic Bcl-2 family members are thought to be involved in the PT-pore activation and in normal cells the PT-pore is very sensitive to hypoxia (Kubasiak *et al.*, 2002; Vande Velde *et al.*, 2000).

A cell detached from the main tissue or tumour will, under normal conditions, undergo apoptosis in the blood stream through a form of apoptosis called anoikis. Tumour cells are able to bypass this process and survive forming metastases at a distant site. Anoikis is thought to be controlled by the pro-apoptotic proteins Bid and Bax that can directly modulate the PT-pore (Valentijn & Gilmore, 2004; Valentijn *et al.*, 2003) as described previously.

Excessive levels of ROS have been shown to induce apoptosis through the PT-pore but at normal levels ROS are favouring cell proliferation (Pelicano *et al.*, 2004; Petronilli *et al.*, 1994). Oxidative stress is caused by elevated ROS levels, which all cancer cells generate excessively putting them under permanent oxidative stress. It is logical to assume that such high levels of ROS would make cancer cells more prone to apoptosis induction but tumours are found to escape this signal as well. The PT-pore can be effectively activated by ROS but due to the upregulation of antioxidants, tumours are able to evade apoptosis and survive under conditions of stress (Brenner & Grimm, 2006; Le Bras *et al.*, 2005).



Calcium levels between cell organelles and the balance of Ca^{+2} should be maintained for cell homeostasis. It was mentioned before that the PT-pore is extremely sensitive to Ca^{+2} and under several conditions Ca^{+2} increases and extensive permeability transition occurs leading to the rupture of the OMM and subsequently apoptosis. A large number of tumours show resistance to several chemotherapeutic drugs that force the release of calcium from intracellular stores. Calcium is released from the endoplasmic reticulum (ER) under conditions of oxidative stress (Ermak & Davies, 2002) and, as it was noted before, cancer cells are under permanent oxidative stress. As a potent activator of the megapore, Ca^{+2} is expected to activate apoptotic signaling extremely efficiently, however, again cancer cells seem to manipulate intracellular signalling circuits in order to evade apoptosis (Brenner & Grimm, 2006).

The composition of the PT-pore is still under intense investigation but there is a consensus about the proteins that modulate the activation of this megachannel. Irrespective of being a component of the pore or a modulator of the pore, several proteins are found to mediate anti-apoptotic signals via PT-pore inhibition. Hence, protein-protein interactions and protein levels play a pivotal role in maintaining cell homeostasis. Cancer cells show differential expression of proteins associated with or comprising the pore, thereby contributing to cell survival and evading apoptosis. Peripheral Benzodiazepine Receptor (PBR), Cyclophilin D (Cyp-D), creatine kinase 1 (CK1) adenine nucleotide translocase 2



(ANT2) and hexokinase II are the PT-pore modulators or components that are found to be up-regulated in many cancers (Azoulay-Zohar *et al.*, 2004; Baines *et al.*, 2005; Bonod-Bidaud *et al.*, 2001; Giraud *et al.*, 1998; Hardwick *et al.*, 1999; Katz *et al.*, 1990; Nakagawa *et al.*, 2005; Schubert & Grimm, 2004).

Summarizing the above, it appears that tumourigenesis is accompanied by differential expression of PT-pore components/modulators and coincides with processes that inhibit apoptosis through opening of this megachannel. It seems that protein-protein interactions in general are absolutely pivotal for controlling apoptosis in contrast to cellular processes like the cell cycle that is mainly controlled by protein phosphorylation (Brenner & Grimm, 2006). The importance of protein levels in complexes should be also applicable to the PT-pore complex, which is so important in governing cell death via apoptosis and necrosis depending on the stimulus and the protein level of the pore constituents as described above. As it can be concluded from the above, the PT-pore appears to be a major modulator of apoptosis and since apoptosis evasion is a cancer hallmark, the PT-pore should be considered as one of the main determinants of cancer formation.

A.4.2.1. The PT-pore, the Warburg effect and cancer

Nobel prize winner Otto Warburg has made a significant contribution to the cancer field after he discovered that cancer cells prefer to conduct glycolysis



over respiration even under conditions of sufficient oxygen. This high glycolytic rate observed in cancer was named the “Warburg Effect”. This effect is so universal that cancer imaging in the clinic makes use of the glucose analogue 2-¹⁸Fluoro-2-deoxy-D-glucose in combination with positron emission tomography in order to detect primary tumours and metastases (Kroemer & Pouyssegur, 2008). The reason for this shift of cancer cells by metabolic reprogramming from oxidative phosphorylation (OXPHOS) to glycolysis relies on (1) a reduced dependence on oxygen that might be observed before angiogenesis takes place in the tumour, (2) a rearrangement of the tumour environment to recycle products of anaerobic metabolism, (3) the production of anti-oxidants through nicotinamide dinucleotide phosphate (NADPH) and (4) the usage of glycolytic pathway intermediates for anabolic reactions (Kroemer & Pouyssegur, 2008).

Hexokinase catalyses the first step of glycolysis, namely the conversion of glucose to glucose-6-phosphate and in highly glycolytic tumours hexokinase II (HXKII) is localized at the OMM anchored to VDAC1 (Azoulay-Zohar *et al.*, 2004). It is thought that the HXKII bound to the OMM prevents apoptosis via several routes, primarily by preventing the activation of the PT-pore through reducing the availability of VDAC sites that can bind BAX (Crompton *et al.*, 2002; Pastorino *et al.*, 2002). It is also thought to prevent the localisation of Bad at the OMM or change the formation of the megachannel thus altering the conformation of VDAC and ANT (Mathupala *et al.*, 2006).



The Akt protein and its signalling is widely accepted as a modulator of the VDAC-HXKII interaction through stabilisation of the complex. Akt can inhibit apoptosis via strengthening the VDAC-HXKII interaction irrespective of Bax and Bak. The VDAC-HXKII complex is now considered as an anti-apoptotic complex since it can prevent the activation of Bax and Bak by tBid (Majewski *et al.*, 2004a; Majewski *et al.*, 2004b). Another protein that showed that the stabilisation of the VDAC-HXKII complex and ultimately the inhibition of the PT-pore is crucial for cancer progression and tumour survival is the enterobacterial protein FimA. It was shown that FimA can inhibit apoptosis via strengthening the complex between VDAC1 and HXKII. It was shown to prevent Bax integration to mitochondria and inhibit cytochrome c release. In this study MOMP was inhibited by this interaction in human colon cancer cells, providing another link between cancer and the PT-pore (Sukumaran *et al.*, 2010b) since VDAC and hexokinase are modulators of the megachannel.

Cyclophilin D along with ANT was also implicated in the complex of VDAC-HXKII. It is thought that ANT, as a modulator of PT-pore, alters its conformation to a state so that permeability transition cannot occur. Also cyclophilin D, which is a core component of the megapore, can, when overexpressed, prevent pore opening and inhibit apoptosis. It is thought that this anti-apoptotic effect of Cyp-D is due to the binding of HXKII and vice versa and directly correlates with the level of association. The way how a matrix component like Cyp-D can affect an



OMM associated component of the PT pore, is not known but it is believed that through conformational changes of other PT-pore components (Azoulay-Zohar *et al.*, 2004; Chiara *et al.*, 2008; Crompton, 1999; Machida *et al.*, 2006; Majewski *et al.*, 2004b; Mathupala *et al.*, 2006; Pastorino & Hoek, 2008b; Vyssokikh & Brdiczka, 2003).

Either by abrogating Bax binding to mitochondria or by changing the conformation of pore components or modulators, HXKII binding to VDAC1 is inhibiting the formation of the PT-pore and this is a phenomenon observed in the majority of cancers. Hexokinase, which was once considered only to mediate glycolysis in highly malignant tumours, has now been shown to be one of the most important anti-apoptotic effectors upon its binding to VDAC1. Understanding the molecular mechanisms that facilitate this interaction will provide more effective treatment choices via targeting the PT-pore and cancer metabolism since this complex (VDAC-HXK) is considered as cancer's Achilles' hill (Kroemer & Pouyssegur, 2008; Mathupala *et al.*, 2006).

A.4.2.2. Cancer-associated mechanisms and NF- κ B

As already mentioned above NF- κ B is a small family of closely related proteins that share a sequence domain that is able to bind to DNA and activate transcription of a large number of genes involved in the immune response, inflammation, and cell survival (Ghosh *et al.*, 1998). A justification for analysing NF-



κ B mechanisms in the context of cancer is to obtain further insight in the already established link between NF- κ B, apoptosis, the PT-pore and cancer. This study attempts to interconnect these major fields so as to provide a basis for targeting cancer through NF- κ B and the PT-pore.

The discovery that one of the subunits of NF- κ B (p50) is a member of the reticuloendotheliosis family (REL) and the fact that v-REL is an retroviral oncoprotein, provided the first link between cancer and NF- κ B (Gilmore, 1999). Moreover, p52 was found to be structurally mutated in myelomas and lymphomas and Bcl-3 (a member of the I κ B family) was found overexpressed in leukemias. The I κ B α gene contains deletions and mutations in a set of Hodgkin's lymphomas and c-rel gene is found to be amplified in a number of such malignancies (Courtois & Gilmore, 2006).

Biochemical and translational alterations might also occur along with genetic changes. Since NF- κ B is a transcription factor controlling hundreds of genes, it was found that modification of the transcription of genes affecting apoptosis, angiogenesis, growth and proliferation are altered in cancers (Basseres & Baldwin, 2006). NF- κ B-dependent targets include cytokines, chemokines, cyclin D1, anti-apoptotic proteins of Bcl-2 family, matrix metalloproteinases, c-Myc, cIAPs, c-FLIP, A20 and many more. The most important link between this transcription factor and cancer is that NF- κ B has been shown to mediate the resistance to apoptotic stimuli (Ravi & Bedi, 2004). Hence, accumulating evidence



shows that NF- κ B plays a major role in cancer initiation, establishment and progression at later stages (metastasis) (Baldwin, 2001; Karin *et al.*, 2002).

A.4.2.3. Cancer, apoptosis and NF- κ B

NF- κ B can promote the proliferation of cells by the regulation of target genes and cancer cells use this ability of NF- κ B to proliferate and grow. Cyclin D1, which is involved in the cell cycle, is affected by the NF- κ B activity directly or indirectly by Bcl-3 that potently trans-activates NF- κ B through p52 (Karin, 2006; Kim *et al.*, 2006b; Westerheide *et al.*, 2001). IKK α is required for the expression of cyclin D1 during the development of mammary glands and HIF1 factor depends on NF- κ B for its upregulation during hypoxia providing a direct link between cancer, PT-pore and NF- κ B (Jung *et al.*, 2003). Consistent with the above are studies with mouse xenografts that use inhibitors of NF- κ B and show a decreased growth and proliferation when NF- κ B is impaired (Baldwin, 2001; Karin *et al.*, 2002).

The link between apoptosis and NF- κ B has already been discussed in this report. However, it provides the basis for a more direct link between NF- κ B and cancer since apoptosis evasion is one of the cancer hallmarks. Bcl-2 and Bcl-X_L are target genes of NF- κ B and act on the mitochondrial pathway to inhibit apoptosis. Numerous cancers have been found to up-regulate these two genes thus creating conditions of robust apoptosis inhibition (Burstein & Duckett, 2003; Dutta *et al.*,



2006). Blocking NF- κ B by I κ B α or transcriptional inhibitors of NF- κ B leads to extensive apoptosis in many tumours confirming that survival of these cells are dependent on constitutive NF- κ B activation (Baldwin, 2001; Karin *et al.*, 2002; Kim *et al.*, 2006b). Mutation or loss of expression of p53 and Foxo3 (both transcription factors) affect cell survival during cancer. Both are targets of NF- κ B and also activate this transcription factor. NF- κ B can suppress c-Jun NH2-terminal kinase activation and generation of ROS thereby providing another method by which NF- κ B is blocking cell death (Basseres & Baldwin, 2006). Figure A.9. shows a representation of NF- κ B target genes that affect different stages of oncogenesis.

A.4.2.4. NF- κ B in cancers and mechanisms of its activation

Major control points in the pathway of NF- κ B activation are the activation of the IKK complex, the degradation of I κ B α , the formation of p52 from p100, and the binding of NF- κ B to specific promoters to activate transcription of target genes. It was described previously how this pathway is stimulated under physiological conditions but the same check points are also used in cancer cells to de-regulate NF- κ B and escape apoptosis.

Several reports show that the activation of oncoproteins is affecting the NF- κ B activation pathway. H-Ras and ErbB2 impact on the IKK complex and require Rel proteins for their transformation (Arsura *et al.*, 2000). In chronic myeloid leukemias the oncoprotein Bcr-Abl is permanently activating NF- κ B. The super



repressor I κ B α -SR can inhibit this thus providing evidence for the dependence of these tumour cells on constitutive NF- κ B activation by Bcr-Abl (Cilloni *et al.*, 2006). Pim-2, Vav, B-Raf and Tax oncoproteins are only few of those drivers of cancer formation that were found to directly bind the IKK complex or the IKK scaffold protein NEMO and activate it (Basseres & Baldwin, 2006; Hiscott *et al.*, 2006).

Apart from the oncoproteins directly affecting the activation of NF- κ B, oncogene-associated pathways and growth factors can also influence the NF- κ B system. For instance the PI3/Akt-dependent signalling is stimulating NF- κ B via IKK α and its level affects the tumour progression and cell survival in lymphoid malignancies or melanomas (Gustin *et al.*, 2004). Additionally, the IKK complex can also be activated by FADD phosphorylation, CK2 and β TRCP1, which are all members of signalling pathways that can affect cancer progression and resistance in lung adenocarcinomas, breast cancer and pancreatic cancer respectively (Basseres & Baldwin, 2006). An example of a growth factor affecting NF- κ B signalling is the epidermal growth factor (EGF), which can induce the recruitment of p65 to specific promoters without subsequent canonical NF- κ B signalling and thereby affecting tumourigenesis (Sitcheran *et al.*, 2005).

Nearly all malignancies include cancer sub-types in which NF- κ B is constitutively active and the description of specific pathways or proteins that confer this would require a review of hundreds of papers and would probably be incomplete since the exact mechanisms are still not completely understood. A



basis for all those malignancies is that NF- κ B contributes to the inhibition of apoptosis and thereby to chemotherapy resistance. A few major examples are breast cancers, melanomas, pancreatic cancers, and haematological malignancies, which provide evidence that NF- κ B turned from friend to foe and hence to a major culprit for tumourigenesis (Basseres & Baldwin, 2006; Karin *et al.*, 2002; Ravi & Bedi, 2004). Cancers with constitutive NF- κ B activity are listed in Figure A.10.

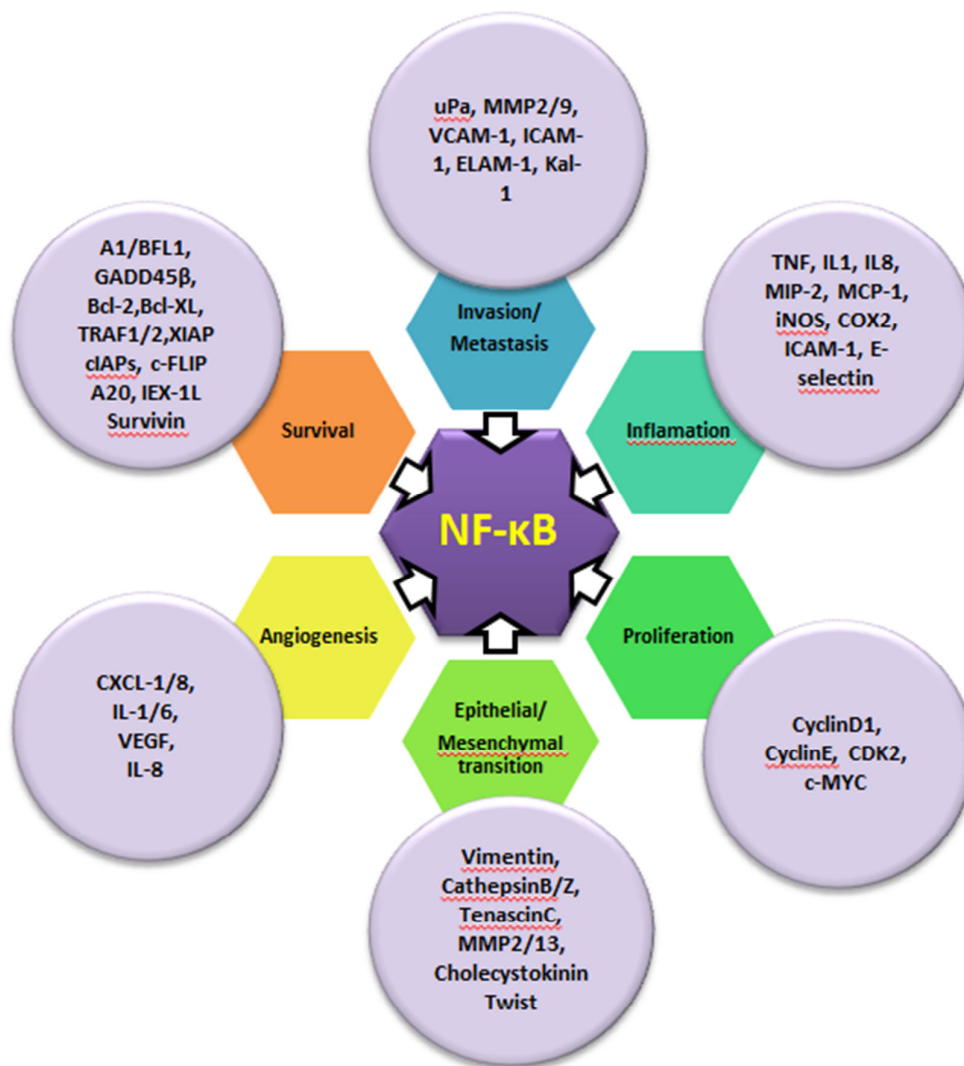


Figure A.9. Schematic representation of NF- κ B-dependent targets which are involved in different stages and aspects of tumourigenesis

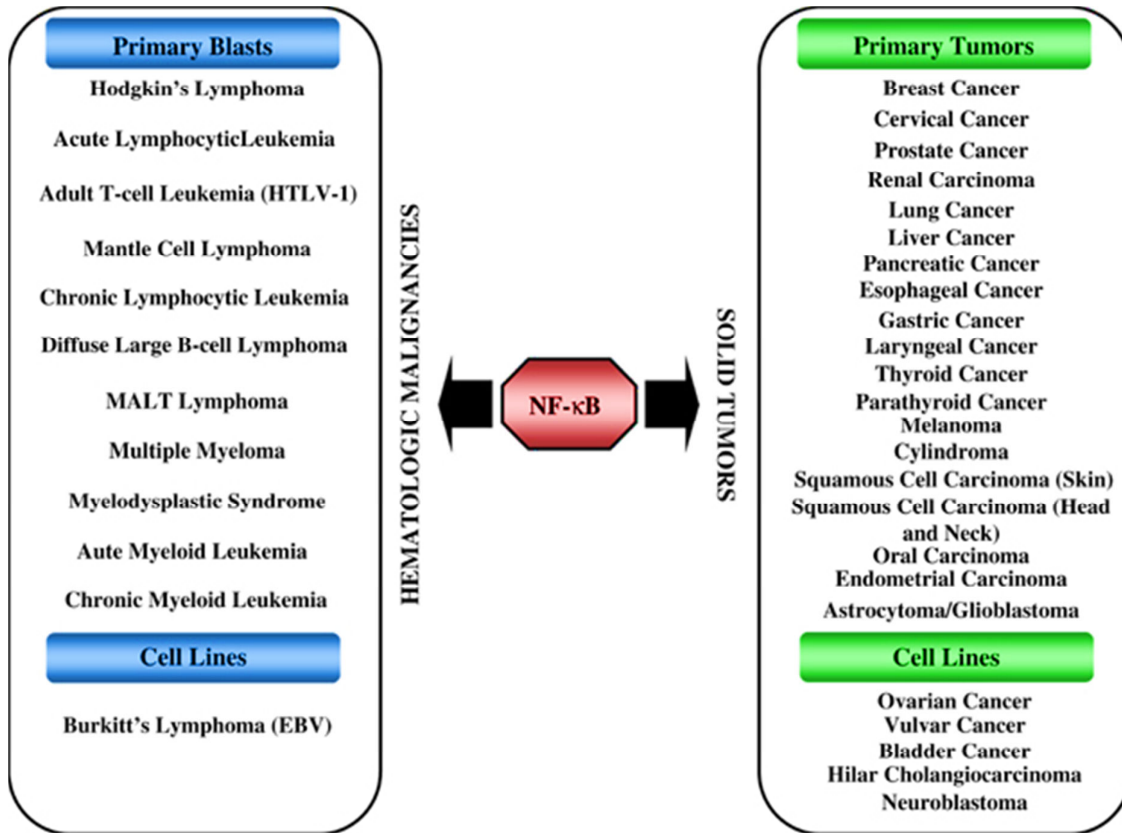


Figure A.10. Constitutive NF-κB activation in human cancers (figure from Basseres DS & Baldwin AS, 2006). Representation of several cancers showing constitutive and enhanced NF-κB activation. The list is only showing a few types since it is currently extended. Many established cell lines are now shown to have constitutive NF-κB activation.



B. Objectives and Hypothesis



B.1. Origin of the project

Determining the mechanisms that underlie the resistance of cancer cells to apoptosis induction is important to improve current treatment regimes and to reveal novel strategies against cancer. NF- κ B is a transcription factor that is constitutively active in many tumour cells and protects them from apoptosis by inducing the transcription of anti-apoptotic genes. However, because of the widespread importance of the NF- κ B factor for inflammatory responses, it has been difficult to develop NF- κ B inhibitors that act specifically in cancer cells and do not compromise the immune response in patients.

Over the years the focus of the laboratory has been signalling of apoptosis and the isolation of genetic apoptosis inducers or inhibitors. The finding that ANT-1 can dominantly induce apoptosis was a success story of our laboratory (Bauer *et al.*, 1999). The effect on ANT-1 to induce apoptosis is dramatic given that even potent apoptosis inhibitors such as Bcl-X_L are virtually unable to overcome the strong apoptosis signal of ANT-1 (Schubert & Grimm, 2004). Given that apoptosis inhibition is a hallmark of cancer cells (Hanahan & Weinberg, 2000), identification of molecules that can inhibit apoptosis are potentially targets for cancer treatment provided that the level of these proteins are altered during cancer progression.

In 2001, Virginie Bottero and colleagues (Bottero *et al.*, 2001) found that ANT-1 can interact with I κ B α , the inhibitor of NF- κ B. There was a direct implication



of I κ B α in apoptosis since NF- κ B subunits were found to be released from mitochondria upon Fas ligand binding and also it was found that ANT-1 immunoprecipitates with I κ B α in an overexpression pull-down. That finding was of specific importance to the laboratory since potential inhibitors of apoptosis and especially ANT-1 was studied. Two other studies revealed that I κ B α resides in the IMS of mitochondria and it was speculated that is involved in mitochondrial apoptosis (Cogswell *et al.*, 2003; Zamora *et al.*, 2004). However, none of the above publications could demonstrate a functional role of I κ B α and NF- κ B in mitochondria and no mechanism was identified. The effect of I κ B α on apoptosis was only indirect and was only put into a context of NF- κ B inhibition.

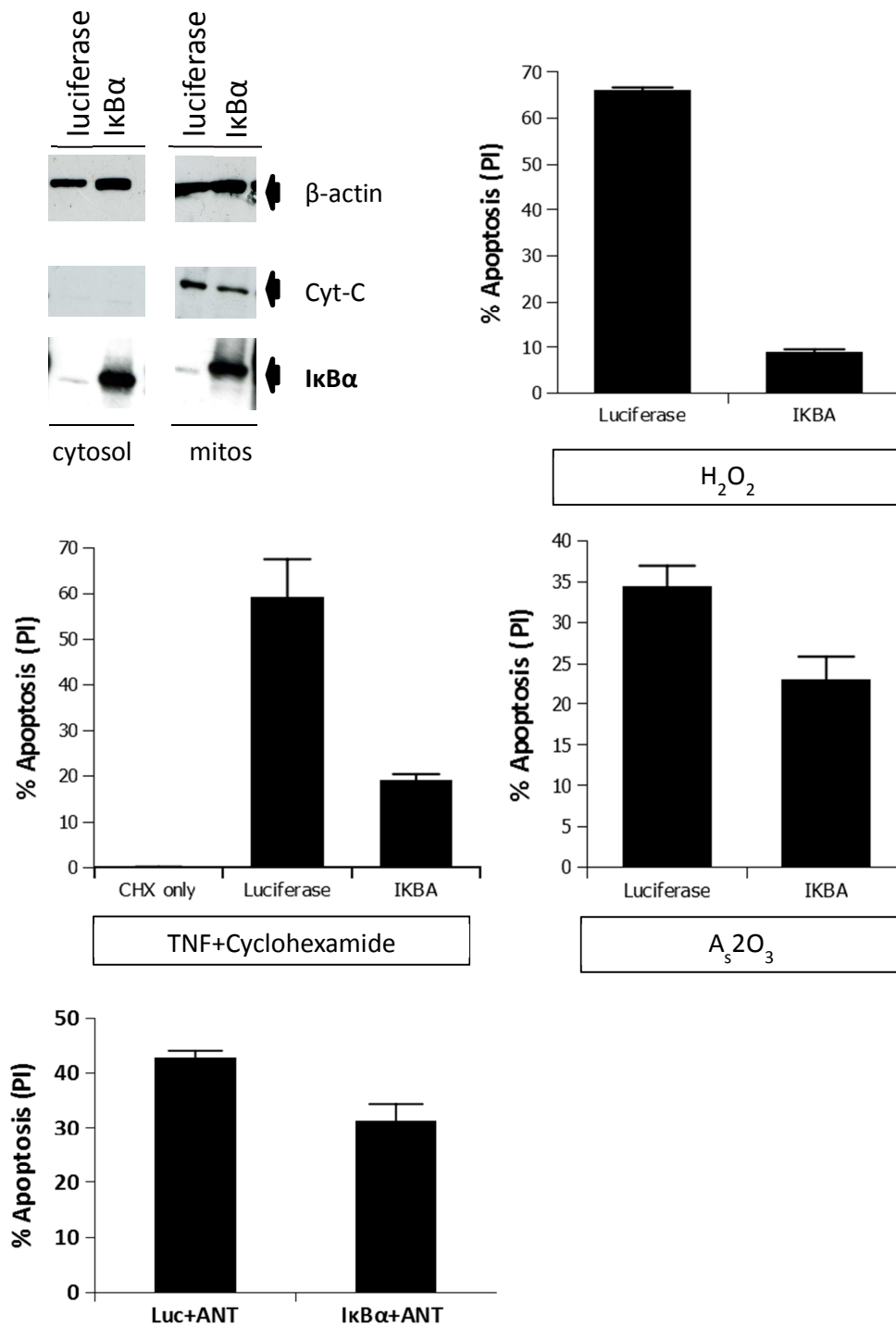
Dr Foy Osborne initiated experiments (Figure B.1.) in our laboratory that could potentially address the question of the role of I κ B α in mitochondria. She found that I κ B α -wt can reduce apoptosis induced by ANT-1 overexpression. The inhibitory potential of I κ B α was also tested with other apoptosis inducers such as arsenic trioxide and TNF and apoptosis reduction was also observed upon I κ B α overexpression. Consequently, a hypothesis was formulated that I κ B α in mitochondria can inhibit apoptosis. Overexpression of I κ B α was visualized on a western blot and fractionation experiments revealed that part of the overexpressed I κ B α is directed to mitochondria. In 2005 another paper was published on TRAIL apoptosis with one of the experiments showing that adenovirus-mediated ectopic expression of I κ B α could inhibit apoptosis of TRAIL. However,



this result was not discussed as a feature of I κ B α itself but rather as related to its inhibitor activity of NF- κ B.

The current study was initiated taking into consideration all the preliminary results and the literature that existed until then. The aim of the project was to identify the role of I κ B α in mitochondria in respect to apoptosis regulation. The identification of interaction partners was another part of the project's aim since they could provide important information about the mechanism. The results section of this thesis provides information and experiments confirming the role and the mechanism of I κ B α in apoptosis inhibition when associated with mitochondria.

Figure B.1. Preliminary data for I κ B α . HeLa cells were transfected with a luciferase vector and a vector for I κ B α -wt. Fractionation was performed according to standard methods and lysates was loaded on a 10% SDS-PAGE. WT I κ B α can inhibit apoptosis. HeLa were transfected with either luciferase or I κ B α and cells were treated with hydrogen peroxide (250 μ M), arsenic trioxide (10 μ M) and TNF 20ng/ml with cycloheximide (100ng/ml). Apoptosis was assessed 24h post-treatment using PI staining and FACS 293T cells were transfected with ANT-1 and I κ B α in a ratio 1:1 and apoptosis was quantified using PI staining





C. Materials and Methods



C.1. Reagents and materials

The following reagents were purchased from Sigma-Aldrich: 2-mercaptoethanol (M7522), acrylamide (40% solution, A7168) actinomycin D (A1410), agarose (A9539), ammonium persulfate (A3678), ampicillin sodium salt (A9518), arsenic trioxide (A1010) bovine serum albumin (BSA, A7906), calcium chloride (C7902), clotrimazole (C6019), cycloheximide (C7698), dimethyl sulfoxide (D2650), Dulbecco's modified eagles media (D6429), doxorubicin (D1515), EGTA (E4378), etoposide (E1383), faecal calf serum (F7524), glucose (G7021), HEPES (H3375), hexamethrine bromide (H9268), ionomycin (I3909), Kanamycin (K1876), Luria-Bertani broth (LB, L3022), L-glutamine (G7513), magnesium sulphate heptahydrate (63138), menadione (M5625) NP-40 (I3021), phosphate buffered saline (D8537) Polybrene® (H9268), propidium iodide solution (P4864), puromycin dihydrochloride (p8833), RPMI-1640 (R5886), sodium azide (S8032), sodium butyrate (30,341-0), sodium citrate (S1804) sodium deoxycholate (30970), sodium pyruvate (S8636), staurosporin (S4400), TEMED (T9281), Triton X-100 (T8532), Trypsin-EDTA (T4174), TWEEN® (P5927) and yeast extract (Y1625).

DAPI (D3571), DiOC6 (D273), blotting filter papers (25mm thickness, LC2010) library efficiency® DH5a™ chemocompetent bacteria (18263-012), MAX efficiency DH5α (18258-012) non-essential amino acids (11140035) OPTI-MEM (22600134), penicillin/streptomycin (100U/ml and 100µg/ml respectively, 15070-063), pLenti7.3/V5-TOPO® TA cloning kit (K5310-00) PureLink™ HiPure plasmid



filter purification kit (K2100-07), TMRE (T669), were from Invitrogen or Molecular Probes (now both under Life Technologies). DNA oligos were also purchased from Invitrogen

The reagents below were purchased from Fermentas (Part of Thermofisher): 5x protein loading buffer (R0891), 6x DNA loading dye (R0611), 50x TAE buffer (#B49), Bradford (500-0006), GeneRuler™ 1kb DNA ladder (SM0314), GeneRuler™ 1kb plus DNA ladder (SM1334), T4 DNA ligase HC (EL0013, PageRuler™ prestained protein ladder (SM1811 or SM0671), ProteoBlock™ protease inhibitor cocktail (R1321) and Phusion® polymerase (F530-L). All the restriction endonucleases from FastDigest™ series were also purchased from Fermentas.

0.5M Tris-HCl pH 6.8 solution (161-0799), 1.5M Tris-HCl pH 8.8 solution (161-0798), 10x TGS buffer (161-0772) and SDS solution (10% w/v, 161-0416) were from Bio-Rad Laboratories; Hyper film™ high performance chemoluminescent film (28906837) was from GE Healthcare; PureProteome™ Protein G Magnetic Beads (LSKMAGG10) and PVDF membrane (IPVH00010) were from Millipore.

QuickChange II site directed mutagenesis kit (#200523) and StrataClone mammalian expression vector systems (#240230) were from Agilent Technologies; potassium chloride (101984) and sodium chloride (10241) from BDH Laboratory Supplies; polyvinyl alcohol mounting medium with DABCO (10981) and saponine (47036) from Fluka; pAdVantage™ vector (E1711), Wizard SV gel PCR clean-up



system (A9282) and TNT[®] Quick Coupled Transcription/Translation System (L1170) were from Promega; Effectene (301427), Superfect (301307) from QIAGEN.

GelRed[™] nucleic acid gel stain (41002) from Biotium; trypton (0123-17-3) was from DIFCO laboratories; TNF- α (520-002-C050) and N-Oleyldopamine (BML-VR102-0025) from Enzo Lifesciences; LB-agar (852323) from OXOID Ltd.; enhanced chemoluminescent reagent (#32106) and mitochondria isolation kit for cultured cells (89874) and paraformaldehyde (28908) from PIERCE Thermo Scientific

C.2. Cell culture

HeLa (human cervical carcinoma), MCF7 (Metkar *et al.*, 2003), 293T (human embryonic kidney), 293FT (for viral production), MDA-MB-231 cells (human breast adenocarcinoma), HCT-116 (human colon carcinoma, gift from Dr Nabil Hajji), MEF WT (mouse embryonic fibroblasts), MEF *PPiF*^{-/-} (mouse embryonic fibroblasts), 3T3 (mouse embryonic fibroblasts) and 3T3 *I κ B α* ^{-/-} (mouse embryonic fibroblasts, gift from David Baltimore/Shengli Hao, California Institute of Technology) were cultivated in high glucose (4.5g/L) Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich). PC3 (human prostate cancer), were cultured in RPMI-1640 media (Roswell Park Memorial Institute) with HEPES modification (Sigma-Aldrich). Both culture media were supplemented with 10% v/v heat inactivated foetal calf serum (FCS, Sigma-Aldrich), 2mM L-glutamine (Sigma-Aldrich), 2mM sodium pyruvate (Sigma-Aldrich), penicillin (100U/ml, Invitrogen) and streptomycin (100 μ g/ml,



Invitrogen). Cells were cultured in tissue culture flasks or dishes and maintained in 5% atmospheric CO₂ at 37 °C in a humidified incubator.

The cell lines with stably down-regulated proteins by Lentiviral shRNA were cultured as above in the presence of 1µg/ml puromycin (Sigma-Aldrich).

Cells were routinely passaged to a new tissue culture dish or flask by detaching them with trypsin-EDTA solution (final concentration of 0.05% diluted in phosphate buffered saline, Sigma-Aldrich).

C.3. Plasmid vectors

All genes were cloned into mammalian expression vectors with Cytomegalovirus (CMV) promoters and U6 promoter for the expression of shRNA. The pcDNA3 (Invitrogen) vector was used for the expression of genes into cells. pLenti7.3 (Invitrogen) was used for the production of lentiviruses. pLKO.1 (Sigma-Aldrich) was used for the production of lentiviruses for the expression of shRNA. pEGFP-N1 (Clontech), pEGFP-C1 (Clontech) were used for the expression and verification of targeting sequences to mitochondria and as intermediate vectors for the sub-cloning of mammalian genes. pCMV-SC-CF (Agilent technologies) was used to tag a gene with a FLAG®. Apoptosis inducing genes were isolated from a genetic screen for apoptosis inducers (Albayrak & Grimm, 2003) and subsequently tagged C-terminally with hemagglutinin (HA-tag). Bax-β was purchased from Origene into the Myc-DDK-tagged pCMV6-Entry vector. Mammalian expression



vectors coding for DsRed-ER, CFP-mito, YFP-mito or GFP-mito were a kind gift of Dr Remy Sadoul (INSERM U836, Grenoble, France). I κ B α gene FLAG-tagged at the N-terminus was previously cloned into pCMV4 mammalian expression vector by Dr Foy Osborne. The Super-Repressor-I κ B α (SR-I κ B α) and I κ B α -110A3 were a kind gifts from L. Schmitz, Giessen, Germany. The I κ B α -110A3 protein contains alanine substitutions at leucine 115, leucine 117, and isoleucine 120 in I κ B α . The reporter plasmid pNF κ B-hrGFP was purchased from Stratagene (Agilent Technologies, UK).

C.4. Cloning

Standard molecular biology techniques were used for cloning. Genes of interest were cloned either by recombinant PCR, restriction digest cloning, INFUSION cloning or TOPO cloning. It should be made clear that wherever I κ B α gene is fused for targeting to the mitochondria, only the localization sequence (targeting) was used for fusion and not the entire gene.

C.4.1. Cloning of MCR1-I κ B α

The yeast *MCR1* (NADH-cytochrome reductase b5) gene contains a 47 aa N-terminal sequence for mitochondrial targeting (Haucke *et al.*, 1997). Recombinant PCR was used to amplify and fuse the MCR1 localization sequence with the I κ B α gene. Primer set 1a was used to amplify 141 bp with the BamHI enzyme and a Kozak sequence and the set 1b to amplify the I κ B α gene with XbaI restriction enzyme sequence. PCR products were mixed and the forward primer



from set 1a and the reverse primer for set 1b were used to amplify and fuse the sequences. Functionality of the construct was tested and will be analysed further in the results section. A C-terminal FLAG-tagged version of MCR1-I κ B α was generated using primers that contained the FLAG-tag (primer set 1a and 1b).

C.4.2. Cloning of MEET-I κ B α

MitoNEET is an outer mitochondrial membrane protein with a very distinct and clear OMM localization signal. Primer set 2 was used to amplify only the N-terminal 32 aa of MitoNEET that are sufficient to target any protein at the OMM (Wiley *et al.*, 2007b). pEGFP-N1 vector was digested at HindIII/PstI sites and MitoNEET (1-32) was inserted in frame with the following eGFP gene of the vector creating MEET-eGFP vector. Primer set 3 was used to amplify I κ B α (2-317) –full length human I κ B α – with restriction sites KpnI and BamHI and 5' and 3' end respectively. The PCR product was inserted into the MEET-GFP vector in frame with MitoNEET (1-32). HindIII and BamHI was used to excise MitoNEET (1-32) and I κ B α gene fusion and subsequently was inserted into pcDNA3 vector linearized with the same enzymes. The latter ligation generated MEET-I κ B α . This encodes an I κ B α protein targeted to OMM.

C.4.3. Cloning of S/D-I κ B α

The targeting sequence of Smac/DIABLO gene to the intermembrane space (IMS) of mitochondria was used for a fusion with eGFP and also I κ B α . Following



the same procedure as for MEET-I κ B α , the N-terminal 57 aa of Smac/DIABLO were amplified with Primer Set 4 and inserted into pEGFP-N1 (Ozawa *et al.*, 2007b). That created the S/D-eGFP vector were I κ B α was inserted afterwards. The full fusion was then inserted into pcDNA3 to create S/D-I κ B α that encodes an I κ B α protein targeted to OMM.

C.4.4. Cloning of MCR1_(AAQQ)-I κ B α

The MCR1 targeting sequence was found after its discovery that is able to direct proteins to two different sub-mitochondrial compartments (Haucke *et al.*, 1997). In order to identify a more specific pathway we introduced alanine substitutions to the localization sequences at positions 23 and 24 that virtually abolish the ability of the precursor localization sequence to insert itself into the OMM so it only targets the fused protein to the IMS. To generate the MCR1_(AAQQ)-I κ B α we used site directed mutagenesis using primer set 5 and introduced the mutations and amplified with long PCR the whole vector and transformed into super competent bacteria. The same principle is used with the Stratagene's kit QuickChangeII Site-Directed mutagenesis kit II.

C.4.5. Cloning of I κ B α / β -Actin

Primer set 6 and primer set 7 were used to amplify by PCR I κ B α and β -actin genes respectively. After amplification the constructs were mixed. A forward



primer from set 6 and reverse from set 7 were used for a recombinant PCR reaction to generate I κ B α / β -actin fusion protein.

C.4.6. Cloning of FLAG-I κ B α , MEET-I κ B α and I κ B α / β -actin into pLenti7.3

A commercial lentiviral expression system was used to generate the transfer vector plasmids for the production of lentiviral particles for the reconstitution of 3T3 I κ B α $-/-$ cells. pLenti7.3 is a TOPO cloning system and it was used according to the manufacturer's instruction to generate pLenti7.3.FLAG-I κ B α , pLenti7.3.MEET-I κ B α , pLenti7.3.I κ B α / β -actin and pLenti7.3GW.lacZ.TOPO (Control). The plasmid backbone bears a cytomegalovirus high expression promoter and an SV40 promoter in a bi-cistronic format. EM488 is a green fluorescence protein under the SV40 promoter within the LTRs of the HIV lentivirus.

C.4.7. Cloning of p65_{BD}/ β -actin and β -actin into pcDNA3

p65 bears a site where it binds with high affinity I κ B α (Ganchi *et al.*, 1992) and its binding has no effect on the ability of p65 to bind DNA κ B sites. Amino acids 151-312 from p65 (RelA) were amplified with PCR and restriction sites HindIII/KpnI and an ATG were inserted using primer set 8. The β -actin gene was amplified by PCR flanked with KpnI/BamHI sites using primer set 9. Both sequences were ligated into pcDNA3 sequentially with β -actin ligating first which



created the β -actin vector and after the ligation of p65's where I κ B α is binding to, the p65_{BD}/ β -actin was created.

C.4.8. Cloning of 326.CMV. β -actin and 326.CMV.p65_{BD}/ β -actin.

The 326.CMV is a monocistronic lentiviral expression vector that can facilitate high expression of a transgene stably and potently. β -actin and p65_{BD}/ β -actin were cloned into the 326.CMV vector with the use of the INFUSION kit from Clontech. Primer sets used were set 10 for β -actin and set 11 for p65_{BD}/ β -actin. In short the kit makes use of homologous recombination. The designing of the primers is in a way they include a 15-20bp sequence identical to the insertion site of the vector. After linearization of the vector at the appropriate site that should be identical to the one included in the primer, purified vector and PCR product are mixed together and with the help of the INFUSION enzyme, homologous recombination is taking place between the same sequence so the transgene is inserted into the vector. Virtually any gene can be cloned into any vector directionally.

C.4.9. Cloning of deletion mutants

In order to identify the necessary domain of I κ B α that is responsible to inhibit apoptosis, deletion mutants were generated using site directed mutagenesis, in the same way as the generation of mcr1_{A23/24Q}-I κ B α (see above). In



order to identify the sufficient domain of I κ B α for apoptosis inhibition, deletion mutants were generated using conventional cloning. The vector MEET-I κ B α was used as the backbone and the primer set 12a (MEET-I κ B α_{1-31}), set 12b (MEET-I κ B α_{1-70}), set 12c (MEET-I κ B α_{1-109}) and set 12d (MEET-I κ B α_{1-142}) were used to create the indicated deletions and subsequently cloned with the same strategy into pcDNA3 vector under the HindIII/BamHI sites. All the deletions contained a C-terminal FLAG tag and was introduced into the reverse primer.

Table B.1. Primer sets. Primer sets for the amplification of genes or sequences. Unless otherwise stated, Phusion high fidelity DNA polymerase was used for all normal PCR and mutagenesis reactions.

Primer Set	Sequence
Set 1a (mcr1)	(FOR)-ACTGTGGATCCCCGCCATGTTTTCCAGATT
	(REV)-ACTGTGAACACTTTATTAGA
Set 1b (I κ B α)	(FOR)-ATGTTCCAGGCGGCCGAGCGC
	(REV)-TCTAGATCATAACGTCAGACGCTG
Set 2 (mitoNEET)	(FOR)-GTAAGCTTCCACCATGAGTCTGACTTC
	(REV)-GCTGTCTGCAGTTTGTAAGCTAGATAACC
Set 3 (I κ B α)	(FOR)-ACTGTGGTACC ATTCCAGGCGGCCGAG
	(REV)-ACTGTGGATCCTCATAACGTCAGACG
Set 4 (Smac)	(FOR)-ACTGTAAGCTTCCGCCATGGCGGCTCTGAGA
	(REV)-ACTGTCTGCAGAATAGGAACCGCACACAG
Set 5 (mcr1 _{AAQQ})	CAAAAGCATTACCGATTGCTCTAGGTACAGTTGCTATACAGCAGGCTACC GCATTCTATTTTGCA
	TGCAAAATAGAATGCGGTAGCCTGCTGTATAGCAACTGTACCTAGAGCA



	ATCGGTAATGCTTTTG
Set 6 (IkB α)	(FOR)-AAGCTTCCACCATGTTCCAGGCGGCCGAG (REV)-TAACGTCAGACGCTGGCCTCCAAA
Set 7 (β -actin)	(FOR)-ATGGATGATGATATCGCCGCG (REV)-TCTAGACTAGAAGCATTGCGGTG
Set 8 (p65 _{BD})	(FOR)-GTAAGCTTCCACCATGGACTACGACCTGAATGCTGTG (REV)-GTGGTACCGCTGAAAGGACTCTTCTTCATGATGCT
Set 9 (β -actin)	(FOR)- GTGGTACCATGGATGATGATATCGCCGCGCTC (REV)- GTGGATCCCTAGAAAGCATTGCGGTGGACGATGGA
Set 10 (β -actin)	(FOR)-TAGAGGATCCACCGGTGGTACCCACCATGGATGATGATATC (REV)-GAGGTTGATTGTCGACGGATCCCTAGAAGCATTGTC
Set 11 (p65 _{BD} / β -actin)	(FOR)-TAGAGGATCCACCGGTAAGCTTCCACCATGGACTAC (REV)-GAGGTTGATTGTCGACGGATCCCTAGAAGCATTGTC
Set 12a (MEET-IkB α ₁₋₃₁)	(FOR)-GTAAGCTTCCACCATGAGTCTGACTTCCAGTTCAGCGTA (REV)-GGATCCTCACTTATCGTCGCATCCTTGAATCGTCGTGGCGGTCGTC
Set 12b (MEET-IkB α ₁₋₇₀)	(FOR)-GTAAGCTTCCACCATGAGTCTGACTTCCAGTTCAGCGTA (REV)-GGATCCTCACTTATCGTCGCATCCTTGAATCGAGCTGCTGCTTCCA
Set 12c (MEET-IkB α ₁₋₁₀₉)	(FOR)-GTAAGCTTCCACCATGAGTCTGACTTCCAGTTCAGCGTA (REV)-GGATCCTCACTTATCGTCGCATCCTTGAATCGTTGTTCTGGAAGTT
Set 12d (MEET-IkB α ₁₋₁₄₂)	(FOR)-GTAAGCTTCCACCATGAGTCTGACTTCCAGTTCAGCGTA (REV)-GGATCCTCACTTATCGTCGCATCCTTGAATCAAAGTCTCGGAGCTC

C.5. DNA electrophoresis and DNA Recovery

The DNA electrophoresis was performed using agarose (Sigma-Aldrich) gels. Briefly, agarose (usually 1% w/v) was dissolved in TAE buffer (Fermentas). The



Gel Red™ nucleic acid stain (Biotium) was then added (1/20000) dilution), cooled and poured into custom-made DNA gel electrophoresis cassette to solidify. The DNA was mixed with 6x DNA loading dye (Fermentas) and separated by gel electrophoresis in TAE buffer (Fermentas). The GeneRuler™ 1kb plus DNA ladder or GeneRuler™ 1kb DNA ladder (Fermentas) was used for the size estimation.

Upon completion of DNA electrophoresis, the piece of agarose containing DNA of interest was excised under the UV lamp and subsequently DNA was recovered using Wizard SV gel and PCR clean-up system (Promega). Briefly, a gel piece was mixed with binding buffer to dissolve agarose. The dissolved gel and the buffer were transferred to a spinning column, centrifuged, washed twice and eluted with DNAase / RNAase-free H₂O or TE buffer.

C.6. Transformation of *E. coli*

Plasmid DNA was transformed into chemo-competent, library efficient DH5α *E. coli* bacteria or MAX efficient DH5α *E. coli* bacteria (Invitrogen). Aliquots stored in -80°C were thawed on ice, the corresponding plasmid DNA was added to the cells. After 30 minutes of incubation on ice, the cells were treated by heat shock (45 seconds at 42°C) with a subsequent quick chill on ice (2 minutes). Then 1ml of SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 10mM MgSO₄, 2.5mM KCl, 20mM glucose) was added to the transformed cells and incubated for 1 hour at 37 °C in a shaker to allow bacteria to express resistance



gene. Afterwards, bacteria were either given LB medium containing appropriate antibiotics (usually 100µg/ml of Ampicillin or 50µg/ml Kanamycin) or were plated onto LB agar containing the appropriate antibiotics (usually 100µg/ml of Ampicillin) and cultured over night at 37°C incubator.

C.7. Extraction of DNA from bacteria

For the extraction of plasmid DNA from transformed bacteria, the bacteria were grown (3ml for miniprep and 250ml for maxiprep) in a Lauria-Bertani broth supplemented with yeast extract (10g/L, Sigma-Aldrich) in the presence of appropriate antibiotics overnight in a shaking incubator at 37°C. The plasmid DNA was extracted from the bacteria using the Plasmid Maxi Prep kit (QIAGEN) or the PureLink™ HiPure plasmid filter purification kit (Invitrogen). The resulting DNA pellet was re-suspended in 500-1000µl of DNase, RNase free water or TE buffer and the concentration measured using NanoDrop (ThermoScientific) or with PicoGreen (Molecular Probes). Briefly, DNA was sequentially diluted to make concentration of approximately 0.1µg/µl. 1µl of diluted DNA was added to a well of a 96-well plate. 1µl of standard DNA (0.1µg/µl) was also included as a reference. Then 50µl of TE buffer was added to each well followed by 50µl of diluted PicoGreen (1:200 in TE buffer). Plate was incubated 5 minutes. The fluorescence was measured with FLUOstar OPTIMA (BMG Labtech) with excitation at 480nm and emission at 520nm.



C.8. Plasmid vector transfections

HeLa cells were plated onto either 24-well ($2-2.4 \times 10^4$ cells/well) or 6-well ($1-1.2 \times 10^5$ cells/well) plates approximately 15 hours prior to the transfections. HEK293T cells were plated onto either 24-well ($10-12 \times 10^4$ cells/well) or 6-well ($5-6 \times 10^5$ cells/well) plates approximately 15 hours prior to the transfections. Effectene (QIAGEN) was used for HeLa cells and either SuperFect (QIAGEN) or Xfect (Clontech), was used for HEK293T cells for the DNA transfection. Briefly, plasmid DNA ($0.2 \mu\text{g}$ per 24-well plate or $0.8 \mu\text{g}$ per 6-well plate) was mixed in EC buffer with Enhancer, incubated for 5 minutes, Effectene reagent was added ($2.5 \mu\text{l}$ or $5 \mu\text{l}$ for 24- or 6-well plate, respectively), incubated for 10 minutes, and the DNA-lipid mix was added to the cells for Effectene transfection. For SuperFect transfections, DNA was diluted in DMEM ($1 \mu\text{g}$ or $2 \mu\text{g}$ for 24- or 6-well plate, respectively), SuperFect was added ($5 \mu\text{l}$ or $10 \mu\text{l}$ for 24- or 6-well plate, respectively), incubated for 10 minutes and the DNA-lipid mix was added to the cells. For Xfect transfections, DNA was diluted in the Xfect Reaction buffer ($1 \mu\text{g}$ or $7.5 \mu\text{g}$ for 24- or 6-well plate, respectively) and was mixed with the same volume of reaction buffer with the Xfect polymer. After vortexing and incubating for 10 minutes, complexes were transferred dropwise onto the HEK293T cells. Transfection mixes were removed from the plate after 5-6 hours, fresh medium added and incubated.



C.9. Mitochondria isolation and treatment

Mitochondria were isolated with an established protocol (Gogvadze *et al.*, 2003), re-suspended in the protocol's mitochondria buffer and kept at -80°C . Briefly, after trypsinisation, cells were harvested, washed with PBS and centrifuged for 5 minutes at 2100 rpm. 400 μl to 600 μl of mitochondria lysis buffer [Buffer A: (20mM HEPES pH 7.5, 1.5mM MgCl_2 ($6\text{H}_2\text{O}$), 10mM KCl, 1mM EDTA, pH 7.4), Lysis Buffer: 12ml Buffer A with 3ml sucrose 1.25M)] was added to the pellet and left for 2 minutes on ice. Meanwhile a dounce homogenizer (Wheaton 357542) was washed with cold lysis buffer and homogenized by 60-80 strokes. 200 μl of lysis buffer were added and lysed cells were left on ice for 20 minutes. A centrifugation step followed (3000rpm) for 10 minutes in which step the nuclei and debris were pelleted. The supernatant which contained the mitochondria was re-centrifuged at 13000rpm for 20 minutes. The pellet contained the isolated mitochondria and the supernatant constituted the cytosolic fraction. For greater mitochondria purity but smaller yield a commercial kit from Pierce (ThermoFisher, UK) was also used. 25ng/ml of Proteinase K (Sigma, UK) was applied to isolated mitochondria for 30 min at 4°C and PMSF was used to stop the reaction. Cleavage of outer mitochondria membrane (OMM) proteins was observed on western blots. Recombinant Bax was purchased from ProSpec-Tany TechnoGene LTD (Israel), diluted with mitochondria buffer and incubated with isolated mitochondria for 30min at 25°C . Transfected mitochondria with I κ B α were used or in vitro translated



(IVT) proteins using the TNT Quick Coupled Transcription-Translation kit (Promega). IVT proteins were run on an SDS page for their integrity determination and equal amounts were applied on isolated mitochondria for 30min at 25°C prior to addition of recombinant Bax. Association of IVT proteins to isolated mitochondria and the cytochrome c release induced by recombinant Bax protein from isolated mitochondria were analysed as described previously (Kim *et al.*, 2006a)

C.10. Nuclear protein extraction

Cells were pelleted at 1200 rpm and were re-suspended in 300µl of L-Buffer (0,1 % Triton X-100, 0,1% NP-40 in PBS with the addition of protease inhibitors). Lysates were left on ice for 15 min and subsequently centrifuged at 1000g for 10 min at 4°C. The supernatant which is the cytosol was removed and kept and the pellet was re-suspended in 1ml of L-buffer and centrifuged 7min at 1000g. This step was repeated 3 times and the pellet was lysed in RIPA buffer. It was left on ice for 40 min while vortexing every 10 min. The samples were finally centrifuged at 15000rpm for 20 min at 4°C.



C.11. Apoptosis assays

C.11.1. Quantification of cell death

Mitochondria dyes TMRE (tetramethylrhodamine, ethyl ester) and DiOC₆ (3,3'-dihexyloxycarbocyanine iodide) were used in conjunction with propidium iodide to quantify apoptosis (Chiara *et al.*, 2008; Majewski *et al.*, 2004a). In short, floating and adherent cells were harvested and centrifuged at 2100 RPM. Supernatant was discarded and cells were re-suspended in 150µl of PBS were DiOC₆/PI mix was added. Cells were incubated in total for 90 minutes and were then analysed using FACS (BD Biosciences) with the CellQuest programme (BD Biosciences, UK). FlowJo (TreeStar Inc.) was used for data analysis.

C.11.2. Propidium Iodide staining

The supernatant and the adherent cells were harvested, centrifuged (1500rpm, 5 minutes) and re-suspended in lysis buffer (0.1% sodium citrate and 0.1% Triton X-100 in PBS) containing propidium iodide (PI, 20µg/ml). Upon the lysis of cells, resulting nuclei were acquired with flow cytometer (FACSCalibur™, BD Biosciences) using FL-2 channel to quantify the percentage of cell population with subG1/G0 DNA content. The pan-caspase inhibitor, zVAD-fmk (MP Biomedicals), was used at a concentration of 20µM.



C.11.3. DiOC₆ / TMRE staining

To quantify the percentage of cells undergoing mitochondrial outer membrane depolarization, supernatant and the adherent cells were harvested, centrifuged (1500rpm, 5 minutes) and re-suspended in 100-200µl of PBS containing 40nM DiOC₆ or 40nM TMRE for 40 minutes in the incubator and further incubated for 40 minutes at room temperature in the dark. Cells were then analysed using a flow cytometer (BD Biosciences) using FL-1 (for DiOC₆ staining) or FL-3 (for TMRE staining) channel. Bongkreikic acid (Sigma-Aldrich), a blocker of the permeability transition pore, was used at a concentration of 75µM.

C.11.4. Caspase-3 and Bax activity assay

The supernatant and the adherent cells were harvested and centrifuged (1500rpm, 5 minutes) and fixed in 4% paraformaldehyde in PBS (PFA, Thermo Scientific) for 20 minutes at 4°C, then blocked in 3% bovine serum albumin (BSA) for 30 minutes in Tris-buffered saline (TBS, 50mM Tris-HCl, 155mM NaCl, pH 7.6) and permeabilized with saponine (0.02% w/v) in TBS (TBSS) for 30 minutes in the incubator. Cells were then centrifuged (1500rpm, 5 minutes) and the primary antibody, diluted in TBSS, that recognizes either active caspase-3 (polyclonal rabbit anti-cleaved Caspase-3 Asp175, Cell Signaling, #9661) or Bax (monoclonal mouse anti-Bax, BD Pharmingen, 556467, clone 6A7) was added (50µl) at a dilution of 1/200 or 1/100, respectively, and incubated for 1 hour in the incubator. Cells were then washed with 2ml of TBSS, centrifuged and the isotype-matched



secondary antibody conjugated with Alexa Fluor 488 (Molecular probes), diluted in TBSS at a dilution of 1/500, was added to the tube (50µl) and incubated for 1 hour in the incubator. TBSS (2ml) was then added to wash the secondary antibody; the cells were centrifuged, re-suspended in TBSS (300µl), and analysed with flow cytometer (BD Biosciences) using the FL-1 channel.

C.11.5. Cleavage of poly ADP ribosome polymerase (PARP)

The supernatant and the adherent cells were harvested and the pellet was lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 50mM Tris-HCl pH 8) containing protease inhibitors (Fermentas). The total cell lysate was separated and immunoblotted with PARP antibody to determine the procession of procaspase-3 or full length PARP (see section "SDS-PAGE and Immunoblotting").

C.12. Immunoprecipitation

C.12.1. Immunoprecipitation of endogenous proteins

Cells were washed once with PBS and lysed in 500µl of RIPA buffer containing protease inhibitors (Fermentas). Approximately 7mg of total cell lysate per condition was used. The cell lysates were placed on ice for 30min, centrifugated (13x10³ rpm) for 30 minutes at 4°C. The supernatant was pre-cleared twice with 50µl of protein G sepharoseTM 4 fast flow (GE Healthcare) or with



PureProteome magnetic protein G beads (Millipore) for 30 minutes each with gentle rotation at 4°C. After the pre-clearing, appropriate amount of antibody (usually what was suggested in the antibodies' data sheets) was added to the supernatant and incubated overnight with rotation at 4°C. For VDAC1-HXKII interaction experiments, VDAC1 antibodies (D-16 or N-18) (dilution 1:50) were used to immunoprecipitate complexes from isolated mitochondria (~100µg of protein) following the protocol above. No antibody was added to the control immunoprecipitations. The antibody-protein complexes were then precipitated with 80µl or 20µl (for the VDAC1-HXKII experiments) of beads and incubated for at least 2 hours at 4°C. Beads were washed five times with the lysis buffer and re-suspended in gel loading dye (Fermentas) supplemented with reducing agent (usually 2-mercaptoethanol). Each tube was immediately boiled for 10 minutes and immunoprecipitates were subjected to protein separation by SDS-PAGE (see section "SDS-PAGE and Immunoblotting"). For ANT-1 immunoprecipitation 10 mg of protein was used (5xT175) from HeLa or MCF7 and lysed in a buffer containing mitochondria lysis buffer and 1% (final) n-dodecyl-D-maltoside. The lysates were incubated on ice for 35 minutes and subsequently centrifuged at 32000g for 30 min. The rest of the procedure was the same as above.

C.12.2. Immunoprecipitation of overexpressed proteins

HEK293T cells were transfected with the appropriate plasmids as indicated and cell lysates were prepared in RIPA buffer. Protein concentration was measured



(see section "SDS-PAGE and Immunoblotting") and equal amounts of protein in an equal volume (adjusted with RIPA buffer) were pre-cleared once with 50 μ l of PureProteome magnetic protein G beads (Millipore) for 30 minutes with gentle rotation at 4°C. The supernatant was then immunoprecipitated with the indicated antibodies overnight. No antibody was added for the control tubes. The antibody-protein complexes were then precipitated with 80 μ l of beads and incubated for 1 hour at 4°C. Beads were washed five times with lysis buffer and re-suspended in gel loading dye (Fermentas) supplemented with reducing agent. Each tube was immediately boiled for 10 minutes and immunoprecipitates were subjected to protein separation by SDS-PAGE.

Antibodies used were polyclonal rabbit α -Flag (Sigma-Aldrich, F7425, used at 1/50 dilution), monoclonal mouse α -HA (Sigma-Aldrich, H3663, used at 1/50 dilution) and the protein raised antibodies where no tag was available.

C.13. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

C.13.1. Preparation of whole cell lysate

Cells were trypsinized, centrifuged (1500rpm, 5 minutes) and lysed, or were directly lysed on the plate by adding RIPA buffer. Total cell lysates were then incubated on ice for 30 min and subsequently centrifuged for at least 45 minutes



(13×10^3 rpm) at 4°C. The resulting supernatant, which contained soluble proteins, was transferred to a new tube and used immediately or stored at -20°C until use.

C.13.2. Protein concentration measurement

Protein concentration was determined using the Bradford assay (BioRad). To obtain a standard curve, BSA of known concentration was sequentially diluted (from 0-6µg/µl) and placed on a flat-bottom 96-well plate. To each well, 199µl of Bradford (diluted 5 times in water) was added and the absorbance measured at 595nm using FLUOstar OPTIMA (BMG Labtech). The resulting absorbance values were plotted against BSA concentration and used as a standard curve to measure the protein concentration.

C.13.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Following the protein concentration measurement, 20-100µg of the total cell lysate was separated by SDS-PAGE (stacking gel: 4.8% polyacrylamide, 125mM Tris-HCl pH6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED; resolving gel: 10-15% polyacrylamide, 375mM Tris-HCl pH8.8, 0.1% SDS, 0.1% APS, 0.05% TEMED) in TGS-running buffer (25mM Tris-HCl, 192mM Glycin, 0.1% w/v SDS). PageRuler™ pre-stained protein ladder (Fermentas) was used for the protein size estimation. Proteins were then transferred to nitrocellulose or PVDF membrane (Millipore) in transfer buffer (20% methanol in TGS buffer). Conditions used for the transfer were



as follows: limited to 500 mA and 20 volts. Proteins were transferred for 90 minutes (if two gels are transferred together) or 60 minutes (if only one gel is transferred) in a semi-dry transfer system from Bio-Rad .

C.13.4. Immunoblotting

The membranes were blocked with 3% BSA (Sigma-Aldrich) or 5% FCS in TBS containing 0.1% Tween (TBS-T) for minimum of 30 minutes, incubated with the primary antibody, washed three times with TBS-T (10 minutes each), and incubated with horseradish peroxidase-conjugated isotype-matched secondary antibody. The membrane was then washed as before, treated with enhanced chemoluminescent reagent (Pierce) and subjected to autoradiography using high performance chemoluminescence film (GE Healthcare).

Antibodies used for immunoblotting and immunoprecipitation are listed below (Table B.2).

Table B.2. Antibodies used for immunoblotting and immunoprecipitation

Antigen	Source	Clonality	Dilution	Company	Cat. no
I κ B α	Rabbit	polyclonal	1:1000 (WB) 1:100 (IP)	Santa-Cruz	sc-371
I κ B α	Goat	polyclonal	1:750 (WB) 1:100 (IP)	Santa-Cruz	sc-371-G
I κ B α	Rabbit	monoclonal	1:1000	Abcam	ab-32518



IκBα	Mouse	monoclonal	1:500(WB)	Santa-Cruz	sc-1643
β-actin	Mouse	monoclonal	1:2000	Sigma-Aldrich	A2228
Bax	Mouse	monoclonal	1:2000	BD Pharmingen	556467
FLAG	Rabbit	polyclonal	1:2000	Sigma-Aldrich	F7425
FLAG	Mouse	monoclonal	1:100 (IP)	Origene	TA50011-1
GAPDH	Mouse	monoclonal	1:2000	Santa-Cruz	sc-32233
HA	Rabbit	polyclonal	1:2000	Sigma-Aldrich	H6908
PARP	Rabbit	polyclonal	1:1000	Cell Signaling	#9542
ANT-1	Mouse	monoclonal	1:100	Mitosciences	MSA02
ANT-1	Rabbit	monoclonal	1:500	Proteintech	15997-1
VDAC1	Rabbit	polyclonal	1:1000	Abcam	15895
VDAC1	Mouse	monoclonal	1:500	Santa-Cruz	sc-58649
VDAC1	Goat	polyclonal	1:1000	Santa-Cruz	sc-32063
VDAC1/2/3	Rabbit	polyclonal	1:500	Santa-Cruz	sc-98708
GRP75	Rabbit	polyclonal	1:500	Santa-Cruz	sc-13967
HSP60	Goat	polyclonal	1:500	Santa-Cruz	sc-1722
Tom20	Rabbit	polyclonal	1:1000	Santa-Cruz	sc-11415
Cytochrome -C	Mouse	monoclonal	1:1000	BD-Pharmigen	556433



p65	Rabbit	polyclonal	1:300	Santa-Cruz	sc-372
p50	mouse	monoclonal	1:300	Santa-Cruz	sc-8414
Hexokinase 2	Mouse	monoclonal	1:500	Sigma-Aldrich	SAB140222 1
Bax	Rabiit	polyclonal	1:300	Santa-Cruz	sc-493
Creatine Kinase 1	Rabbit	polyclonal	1:750	Proteintech	15346-1AP
Cyclophilin D (ppiF)	Mouse	monoclonal	1:500	Calbiochem	AP1035
Mouse HRP	Goat		1:5000	Molecular Probes	G21040
Rabbit HRP	Goat		1:20000	Sigma-Aldrich	
Goat HRP	Rabbit		1:20000	Sigma-Aldrich	
Biotin HRP	Mouse		1:20000	Sigma-Aldrich	A0185

C.14. Immunofluorescence

C.14.1. Immunofluorescence assay

Cells, seeded on coverslips (CS), were transfected by mitoCFP, mitoGFP or mitoYFP with Effectene (Qiagen, UK) and grown for 24 hours, then fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. After a blocking step with 3% BSA in Tris-HCl 50 mM, NaCl 155 mM, pH 7.6 and 0.02% saponine (TBSS) for 30 min at room temperature (RT), cells were incubated with the primary antibody (Table B.3.)



for 2 hours at RT. Cells were rinsed five times in TBSS and then incubated with the secondary anti-rabbit Alexa Fluor 594 antibody at a dilution of 1/500 in TBSS. Cells were washed five times in TBSS and incubated 30 minutes in DAPI (2 μ g/ml, Invitrogen, UK) at RT, before being mounted in polyvinyl alcohol mounting medium with DABCO (Sigma). Cells were examined with confocal laser-scanning microscope (Leica) with a 63x objective and analysed with LAS AF software.

Plasmid vectors conjugated with fluorescent protein used are: mitoCFP, mitoGFP or mitoYFP are all fluorescent proteins conjugated at the N-terminus with a mitochondria localization signal from the precursor protein of human cytochrome c oxidase subunit VIII. Those plasmids were a kind gift of Remy Sadoul (Inserm U836, Grenoble, France). S/D-GFP and MEET-GFP were fused and cloned and verified by immunofluorescence (see above).

Table B.3. Primary antibodies used for Immunofluorescence

Antigen	Source	Clonality	Dilution	Company	Cat. no
I κ B α	Rabbit	polyclonal	1:50	Santa-Cruz	sc-371
Cyt.-C	mouse	monoclonal	1:50	Santa-Cruz	sc-13560
FLAG	Rabbit	polyclonal	1:50	Sigma-Aldrich	F7425
p65	Rabbit	polyclonal	1:50	Santa-Cruz	sc-372



C.14.2. Mitochondrial staining

Mito Tracker® Red CM-H2XRos (MitoTracker) and tetramethylrhodamine ethyl ester (TMRE) were used at a concentration of 50nM (Molecular Probes) for the visualization of mitochondria. Both probes were diluted in a culture medium and added to the cells for 15-20 minutes at 37°C. When specific mitochondrial staining was visible, dyes were removed, washed three times with DMEM and fresh medium (minimum quantity, ~250µl per a well of 24-well plate) was added.

C.15. Production of lentiviral particles

Virulent media was produced in HEK293FT cells (Invitrogen, UK) using the following optimised protocol for lentiviral production: Plasmids encoding Vesicular Stomatitis Virus Glycoprotein (pVSV-G), group antigen and reverse transcriptase (pGag.Pol) and mRNA export helper protein (pRev) were isolated from ViraPower™ lentiviral packaging mix (Invitrogen). Expression vector for the pAdVantage was from Promega. The cell line used was 293FT cells from Invitrogen.

The day before the transfection, 293FT cells were plated onto 10cm² dishes so that the plate would be approximately 50-60% confluent on the day of transfection. Two hours prior to transfection, the medium was replaced with fresh DMEM. 5.4µg of the pVSV-G, 3.8µg of the pRev, 7.8µg of the pGag.Pol, 9µg of the pAdVantage and 20µg of the lentiviral transgene plasmid were mixed in a 2ml eppendorff tube and made up to 437.5µl with H₂O. Then 62.5µl of 2M CaCl₂ was



added, mixed thoroughly and incubated for 5 minutes. Meanwhile, 500 μ l of 2xHBS (50mM HEPES, 281mM NaCl, 1.5mM Na₂HPO₄ pH 7.08-7.1, filter sterilized) was prepared in a 15ml conical tube. After 5 minutes incubation, the DNA/CaCl₂ mixture was added drop by drop into 15ml conical tube while vigorously shaking the tube on a vortex machine. The mix was immediately added to the dish drop by drop.

The culture medium was replaced with fresh warm medium containing sodium butyrate (10mM, Sigma-Aldrich), dissolved in PBS, on the following morning (16 hours post transfection). Note that sodium butyrate was freshly prepared every time. After 2 to 3 days post transfection, the supernatant was harvested and centrifuged (500g, 10 minutes, RT) to remove cells and large debris, then passed through a 0.45 μ m sterile filter. Note that 0.2 μ m filter should not be used as it can damage viral outer glycoproteins. 3T3-I κ B α -/- cells were transduced for 6 h in the presence of polybrene (6 μ g/ml, Sigma) for the reconstitution with I κ B α variants (pLenti7.3-G.O.I.). After infection, cells were sorted through a BD FACSAriaIIu fluorescent activated cell sorter for eGFP since the pLenti7.3 vector allows the expression of a fluorescent marker along with the gene of interest through a bi-cistronic vector system.



C.15.1. Calculation of viral titre

In order to measure the viral titre, the cells of interest (HEK293T or HeLa cells) were seeded on the 12-well plate to make a plate ~30% confluent on the day of transduction. On the day of the transduction the viral stock was diluted (three different dilution to achieve accurate measurements) in 400µl of DMEM containing polybrene® (hexadimethrine bromide, 6µg/ml, Sigma-Aldrich). The diluted viruses were added to the cells and incubated for 48 hours to allow integration of genome and the expression. The number of cells present in a well was counted. The cells were then selected with puromycin (2-6µg/ml) for 24 hours and the number of surviving cells was recorded.

The viral titre was calculated using the following equation:

$$TU/ml = \frac{F \times N \times D}{V}$$

Where TU is the transforming unit (=number of virions), F

is the percentage of surviving cells, N is the number of cells at the time of transduction, D is the dilution factor, and V is the volume of diluted viral sample added into each well in ml. TU can also be referred as CFU which is the Colony Forming Unit (number of virions) and is calculated on the same way. In order to avoid under-estimation of the virus titre, only the dilution that was showing less than 20% transduced cells was used. In the case of pLenti7.3 based virions puromycin and selection was not used since the backbone contains a GFP variant for an easy estimation of viral titer.



C.15.2. Production of stable cell lines

The cell line of interest was seeded on a well of 6-well plate 15 hours prior to the transduction. On the following day, cells were transduced with the viral supernatant supplemented with 1µg/ml of Polybrene® (Sigma-Aldrich) for 6 hours or overnight. The viral supernatant was then removed; fresh medium added and incubated for a further two to three days. The medium was replaced with DMEM containing puromycin (varying concentration, 5µg/ml for HeLa cells) after 72-96 post-infection and further cultivated for three days for the selection. Upon completion of the selection, cells were maintained in DMEM containing 1µg/ml of puromycin to retain the resistance.

C.16. IκBα, VDAC and hexokinase II down-regulation by shRNA

Commercial shRNA constructs for IκBα, VDAC1 and Hexokinase II from Sigma-Aldrich were tested with western blots for sufficient down-regulation of IκBα and two constructs were validated. The appropriate controls were also purchased from Sigma. The vectors were packaged into lentiviruses using the same approach as described above. Transductions of cells with constitutively active NFκB activity (MDA-231, PC3) and non-constitutively active (Hela, 293T), were performed with the help of polybrene and stable cell lines were established.



The sequences of the synthetic oligonucleotides (Invitrogen, UK) used for IκBα shRNA constructs and VDAC1 were the following:

Table B.4. Sequences of short hairpin DNA used to knock-down the proteins of the indicated genes

IκBα (sh42) 3'UTR targeting	5'→3' CCGGGACGAGAAAGATCATTGAAATCTCGAGATTTCAATGATCTT TCTCGTCTTTTT
IκBα (sh88) CDS targeting	5'→3' CCGGAGAGTCAGAGTTCACGGAGTTCTCGAGAACTCCGTGAACTC TGACTCTTTTTT
VDAC1 CDS targeting	5'→3' CCGGGCTTGGTCTAGGACTGGAATTCTCGAGAATTCCAGTCCTAGA CCAAGCTTTTT
mVDAC1 CDS targeting	5'→3' CCGGACCAGGTATCAAACCTGACGTTCTCGAGAACGTCAGTTTGAT ACCTGGTTTTTT
Hexokinase2 CDS targeting	5'→3' CCGGCCAAAGACATCTCAGACATTGCTCGAGCAATGTCTGAGATG TCTTTGGTTTTTT
Scramble Non-targeting	5'→3' CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAA CCTTAGGTTTTT



C.17. NF- κ B activity assay in 293T and 3T3 I κ B α re-constituted cells

293T cells were transfected with the reporter plasmid pNF κ B-hrGFP along with expression constructs for ANT-1, Bax and Tax (Munoz & Israel, 1995). The pan-caspase inhibitor zVAD (MPBio, UK) was applied at 20 μ M to avoid late degradation of proteins through apoptosis by Bax and ANT-1. 24 hours post-transfection, 293T cells were collected for FACS analysis. For the rest of the cell lines a non-radioactive biotinylated-oligo-pull-down assay was used to quantify the level of NF κ B activity. In short, sense and antisense oligonucleotides encoding tandem κ B sites (sense, AGCTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGG) were annealed after denaturing and slow cooling in a buffered solution. The sense strand was biotinylated at the 5' position, while a non-biotinylated oligonucleotide was used as control. Nuclear extracts were prepared as described above and incubated with annealed oligonucleotides for 60 minutes at 4°C. The oligonucleotides were subsequently precipitated by streptavidin agarose beads (Invitrogen, UK) and rinsed 3 times before proceeding to Western blot analysis.

C.18. *In vivo* experiments

MDA-MB-231 cells were obtained from ATCC and were transduced using previously prepared lentivirus for scramble of shI κ B α (42) at M.O.I. of 100. Stable clone pools were generated and cells were expanded 2 weeks post infection.



Down-regulation of I κ B α was checked with western blots. Cells were maintained in puromycin at concentration 1 μ g/ml.

Eight 4 week old, female, Balb/c^{nu/nu} athymic mice were purchased under license 70/7173. Animals were always treated under the rules and conditions of the Animals Act 1986.

A 20 μ l 1:1 mixture of matrigel and cells containing 2x10⁶ cells were injected using an insulin needle into the mammary fat pad. Both MDA-231/sc and MDA-231/shI κ B α were injected in each mouse on different sides. Preparation of cells for injection was done as following:

- Cells in T175 flasks were washed 3 times with PBS
- After final wash, 5mL trypsin was added
- When the majority of cells were detached 20mL of media (RPMI + 10% FCS) was added
- Cells were thoroughly mixed, transferred to 50mL conical tube and subsequently centrifuged.
- The supernatant was discarded, pellet re-suspended with 5mL fresh media
- Re-suspended cells from multiple conical tubes were combined into one conical tube where the cells were counted
- Cells were centrifuged and supernatant was removed
- Cells were re-suspended in a volume of PBS to give 4x10⁶ cells in 20 μ L



- Equal volumes of Matrigel and cell suspension were gently but thoroughly mixed together which provided 2×10^6 cells in 20 μ l (50% PBS/50% Matrigel).

Mice were observed on daily basis for tumour formation. This type of cells was shown before to produce xenografts in nude mice but since they have been in culture for longer they were tested for tumour formation potential. Positively engrafted mice were separated in groups and treated in weekly intervals with PBS or Doxorubicin (6mg/kg). Administration of drug or vehicle was done through the intravenous (i.v.) route. An electronic calliper was used to measure the three dimensions of tumour and the following formula was used to calculate the volume

$$V = \frac{\pi}{6 * length * width * height} .$$

C.19. Densitometry analysis

A scanned image of a western blot was uploaded onto ImageJ programme and converted to 8-bit image (image \rightarrow type \rightarrow 8-bit). The image was then inverted (Edit \rightarrow invert) and appropriate fields selected using “rectangular selection” tool. The plot profile (analyse \rightarrow plot profile) was created and the background intensities were removed by drawing a straight line using “straight line selection.” Then distinctive peaks were separated with “straight line selection” and the intensities of bands were analysed upon selection of the appropriate area with “wand tool” followed by the measurement (analyse \rightarrow measure). Note that



pressing the “Shift” key during line drawing allowed me to draw a straight line and “Ctrl+D” allowed me to approve my line draw.

C.20. Statistical analysis

Statistical analysis was performed using unpaired student’s t-test. Two samples were regarded to have an equal variance when the f-test between two sample sets gave $p > 0.05$. Data were regarded as statistically significant if $p < 0.05$ based on the t-test.



C.21. Common buffers used

Solution/Media	Composition
2M CaCl ₂	11.1 gr in 50ml and sterilize by filtering
2X HBS	50mM HEPES (Sigma, UK), 280 mM NaCl, 1.5mM Na ₂ HPO ₄ . Bring pH to 7.06-7.08 and sterilize
Blocking Buffer	Wash Buffer + 3% BSA (Sigma,UK) or Wash buffer+5% FCS
Lactacystin	40ng/ml in DMSO (Cayman Europe, Estonia)
Loading Buffer 3X	4% SDS, 10% 2-Mercaptoethanol, 20% Glycerol, 0.125M Tris-HCl pH 6.8 (Fermentas, UK)
Luria-Bertani (LB) Broth	1% Bacto tryptone, 0.5% Bacto-yeast extract, 1% NaCl Bring pH 7.5 by adding NaOH
Mitochondria Lysis Buffer	Buffer A: 20mM HEPES pH 7.5, 1.5mM MgCl ₂ (6H ₂ O), 10mM KCl, 1mM EDTA, 1mM EGTA, H ₂ O up to 1L, pH to 7.4 Lysis Buffer: 12ml Buffer A + 3ml Sucrose 1.25M
Paraformaldehyde 4% (60ml)	4g of paraformaldehyde dissolved in 50 ml of ddH ₂ O, add 100µl of NaOH, allow to dissolve completely, add 10ml of PBS and cool down to RT. pH: 7.4 via HCl
Phosphate Buffered Saline (PBS)	8g/L NaCl, 0.2g/L KCl, 0.2g/LKH ₂ PO ₄ , 1.15g/L Na ₂ HPO ₄ , pH 7.4 (Invitrogen, UK)
Polyacrylamide	40% (Bio-Rad, UK)
Propidium Iodide staining lysis buffer	0.1% sodium citrate, 0.1% Triton X-100 in PBS + propidium iodide 20µg/ml (final)
Resolving gel	10-15% polyacrylamide, 375mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS and 0.05% TEMED
RIPA Lysis Buffer	150 mM Sodium Chloride (NaCl), 1% NP-40 or Triton X-



	100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris. pH 8.0, add before using PMSF and the protease inhibitor Cocktail (Roche, UK)
Running Buffer 1X	25mM Tris base, 190mM Glycine, 0.1% SDS (Bio-Rad,UK)
S.O.C. media	2% tryptone, 0.5% yeast extract, 10mM NaCl, 10mM MgSO ₄ , 2.5mM KCl and 20mM glucose
TBS 20X (2L)	400ml 1M Tris pH7.4, 175.22gr NaCl, 100ml 0.5M EDTA, pH8.0, 40ml Tween-20, Water up to 2 litres
Stacking gel	4.8% polyacrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% APS and 0.1%TEMED
TAE buffer	40 mM Tris-acetate and 1 mM EDTA at pH 8.3
TE buffer	100 mM Tris-HCl (pH 7.5) and 10 mM EDTA (pH 8.0)
Transfer Buffer before use	400 ml Methanol, 200 ml Running Buffer (10X), 1.4 L Water
Wash Buffer (TBS-T)	TBS + 0.1% Tween 20 (Sigma, UK)



D. Results



D.1. Localisation of I κ B α

For more than a quarter of a century I κ B α has been thought to only shuttle between the cytosol and the nucleus of every mammalian cell type in order to regulate NF- κ B. While its function as a NF- κ B inhibitor in these different locales has been extensively investigated, it was recently found by two groups to also reside in mitochondria of cells with an unknown function in this organelle. Since this study set out to give further insight into the function of I κ B α associated with mitochondria we attempted to reproduce the finding of the I κ B α localisation at mitochondria and subsequently explore its biochemical function in terms of apoptosis regulation.

Here I show that I κ B α resides both in the cytosol and the mitochondria. The mitochondrial I κ B α undergoes normal degradation upon TNF treatment following similar kinetics than its cytosolic counterpart but in contrast to the cytosolic I κ B α , its degradation is proteasome independent. Furthermore, I κ B α resides not only at the IMS as the previous studies reported but also at the OMM of all the cell lines that we tested and we are the first to show that in tumour cell lines with constitutive NF- κ B activity, the majority of I κ B α protein is actually found associated with the OMM of these cell types.

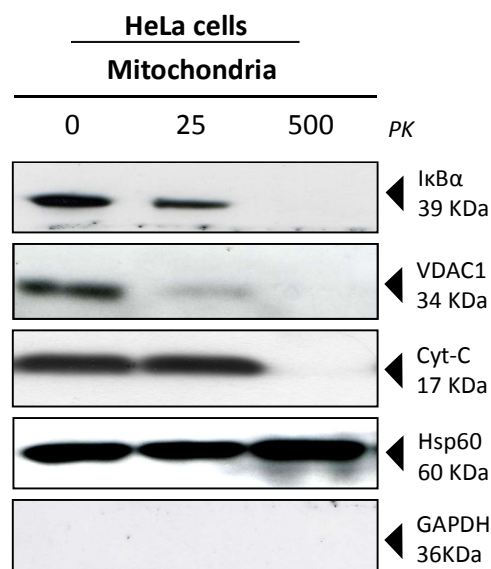
D.1.1. Defining the correct experimental setting

In order to confirm previous findings of Cogswell et al (2002) that I κ B α resides at mitochondria of some cell types I initiated the study by determining a suitable experimental setting. Mitochondria were isolated from HeLa cells (cervical carcinoma) and separated from the cytosol. Proteinase K, which has the ability to cleave any protein was utilised to assess the localisation of proteins.

To determine the correct setting, several conditions were investigated to identify the buffer, the temperature and the concentration needed to be able to cleave non-specifically all the OMM proteins without destroying the OMM and being able to access the IMS. The suitable concentration was identified to be 25ng/ml in PBS on ice for 30 minutes. As figure D.1 shows, 25ng/ml of PK was able to cleave most of VDAC1, but it did not alter the level of Cyt-c in samples that were free from any cytosolic contaminants as the GAPDH probe shows.

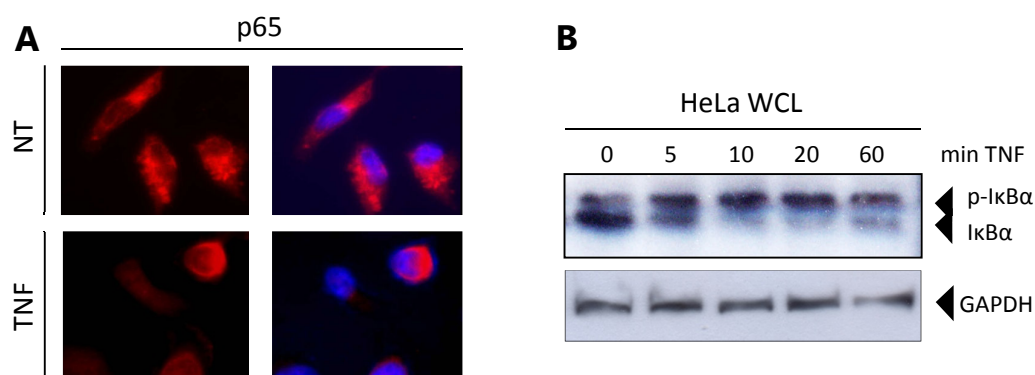
Figure D.1 25ng/ml of Proteinase K (PK) is sufficient to distinguish proteins within mitochondria from those at OMM and in the cytosol.

Several concentrations of PK were used on isolated mitochondria. Samples were treated for 30 min on ice, mitochondria were washed again in mitochondria buffer and lysed with RIPA buffer. A 12% SDS-gel was used to separate the proteins and suitable antibodies to detect for the specific antigens.



D.1.2. The kinetics of I κ B α degradation by TNF in mitochondria are similar to those in the cytosol

It is known that upon binding of TNF, I κ B α gets phosphorylated, ubiquitinated with K48 ubiquitin chains and then targeted to the proteasome for its degradation. Since I κ B α was found in the mitochondria we wanted to investigate its degradation kinetics since the proteasome is unlikely to have access to the mitochondria. We hypothesized that I κ B α would be protected from proteasomal degradation and show no or slower degradation since it resides in the IMS where it is protected. We first showed by immunofluorescence that the TNF concentration used was sufficient to induce the translocation of RelA (p65) subunit into the nucleus (Figure D.2A) confirming the activation of the NF- κ B pathway. We show in figure D.2C that mitochondrial I κ B α follows the same kinetics as the cytosolic I κ B α .



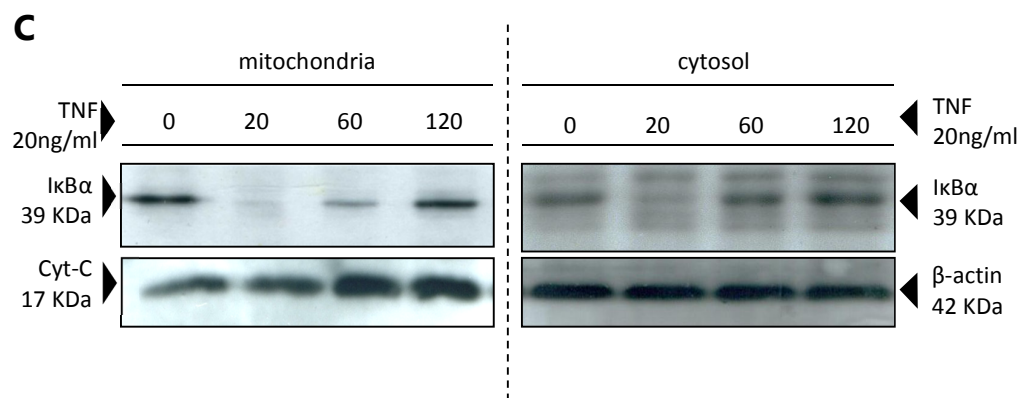


Figure D.2. Upon TNF treatment, I κ B α is degraded with the same kinetics in the mitochondria as in the cytosol. (A) HeLa cells were treated with 20ng/ml TNF to activate NF- κ B. The activation is confirmed by the translocation of p65 and p50 from the cytosol into the nucleus. (B) HeLa cells were treated with TNF and the I κ B α and subsequently the p-I κ B α antibody were used to probe for activated I κ B α . (C) WB of cytosolic and mitochondrial HeLa lysates indicate that upon TNF application I κ B α is degraded with the same kinetics in both cell fractions. Mitochondria were treated with Proteinase K to ensure mitochondria purity. (WCL: Whole Cell Lysate)

D.1.3. I κ B α degradation in mitochondria is proteasome-independent

The results of the degradation kinetics of mitochondrial I κ B α upon TNF treatment served as a reference for an experiment in which we checked the I κ B α degradation when the proteasome was inhibited. We used the cell permeable and irreversible proteasome inhibitor lactacystin, which specifically inhibits the 26s proteasome (MCP; multicatalytic proteinase complex). It blocks the proteasome activity by targeting its catalytic β -subunit and has no effect on other serine or cysteine proteases. Blocking the proteasome inhibited I κ B α degradation in the

cytosol upon TNF treatment but had no effect on mitochondrial I κ B α degradation. This result suggests that TNF also activates a separate protease that degrades the mitochondrial I κ B α (Figure D.3).

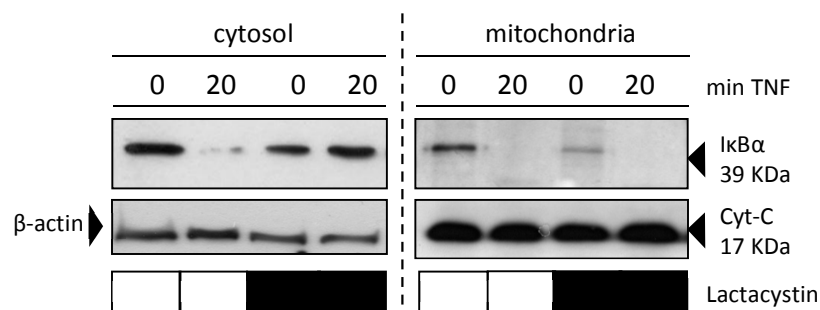


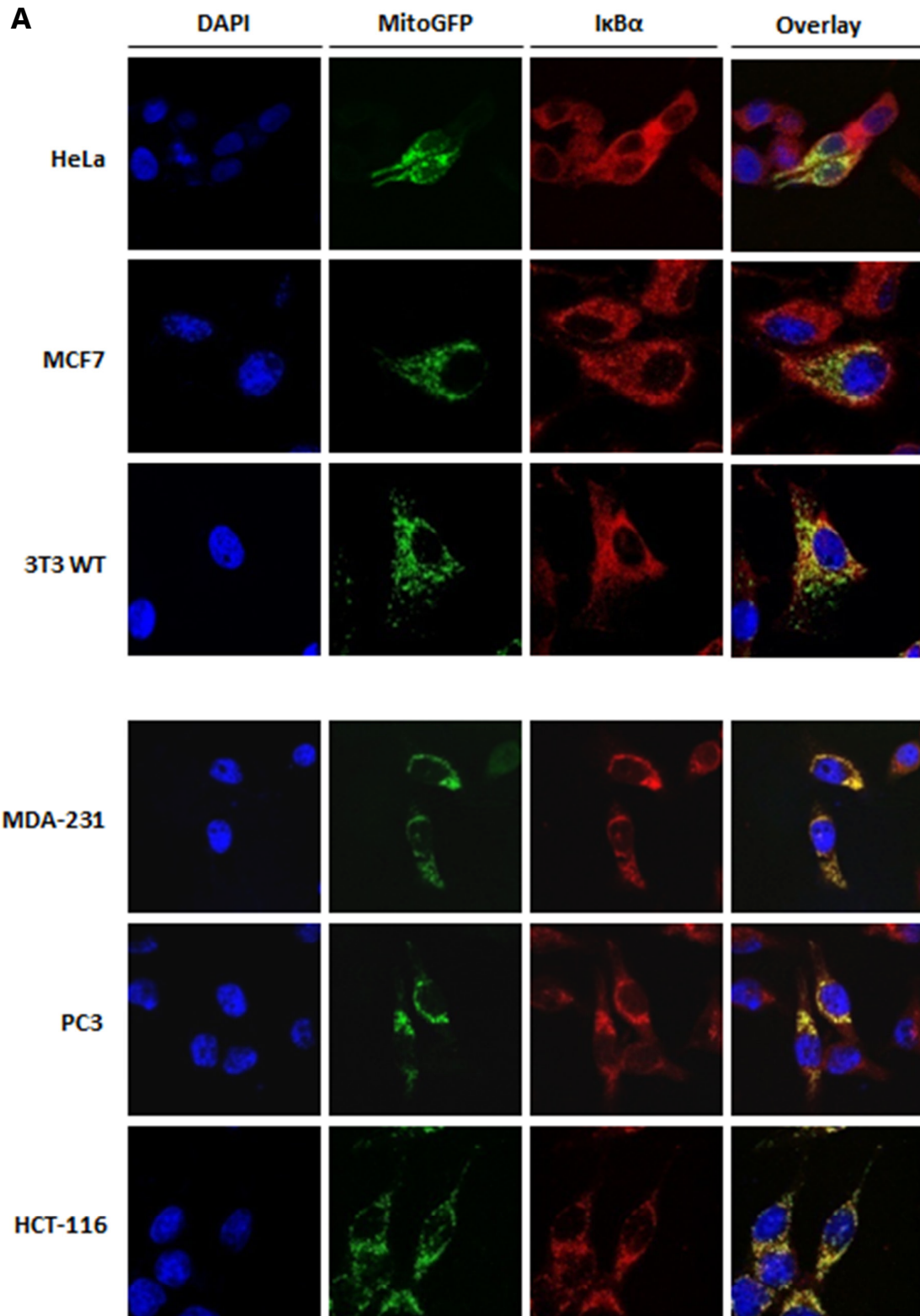
Figure D.3. Mitochondrial I κ B α degradation upon TNF treatment is proteasome independent. HeLa cells were treated with DMSO or lactacystin (40ng/ml) for one hour and subsequently with TNF (20ng/ml) for the indicated times. Mitochondrial and cytosolic fractions were separated with standard methods and loaded on a 12% SDS gel, which was probed with the indicated antibodies.

D.1.4. I κ B α associates with the OMM of several cell lines

Following our hypothesis that I κ B α at mitochondria plays a role on apoptosis, we wanted to examine the level of its protein at the mitochondria and also the sub-cellular residence of I κ B α within the organelle. We used immunofluorescence and determined that in HeLa, MCF7, and 3T3 cells I κ B α partly co-localised with mitochondria. In MDA-231, PC3, and HCT-116 cells that display constitutive NF- κ B activity (Dejardin *et al.*, 1999; Nakshatri *et al.*, 1997b; Sakamoto *et al.*, 2009; Suh *et al.*, 2002), the co-localisation of I κ B α with mitochondria was



even more pronounced (Figure D.4A). This distribution was substantiated in cell fractionation experiments which revealed that MDA-MB-231 cells show high expression levels of I κ B α at mitochondria whereas its cytosolic levels were diminished. In contrast MCF7 cells, which are also a carcinoma cell line from the breast tissue, revealed only partial localisation of I κ B α to mitochondria and the cytosolic levels were much higher (Figure D.4B). These results implied that there is a preference for I κ B α localisation to mitochondria in cell lines with constitutive NF- κ B activity. In order to test the exact localisation of I κ B α in several cell lines we utilized the conditions that we optimised earlier for the proteolytic cleavage of proteins at the OMM. Treatment of intact mitochondria with proteinase K isolated from Hela cells revealed that the enzyme gained access to I κ B α as it could substantially reduce the I κ B α signal in a protein blot and likewise that of the OMM protein Tom20 as a control. In contrast, the inner mitochondrial protein cytochrome c was protected (Figure D.4C). With mitochondria from the remaining cell lines we likewise found that I κ B α could be cleaved by proteinase K suggesting that it was also associated with the OMM (Figure D.4D).



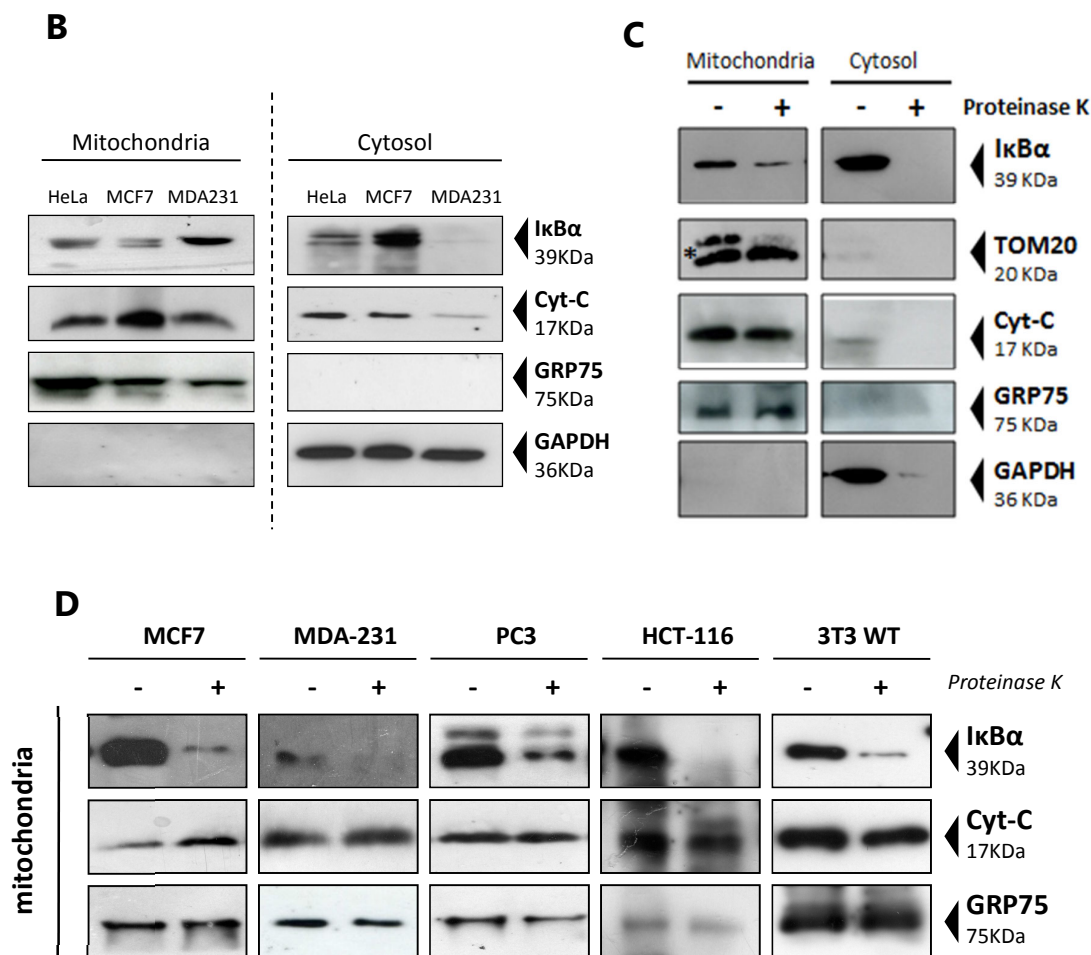


Figure D.4. IkBα is found at the outer mitochondrial membrane. (A) Confocal photomicrographs of IkBα distribution in normal cells (upper panels) and cells with constitutively active NF-κB (lower panels) **(B)** Mitochondrial and cytosolic fractions from HeLa, MCF7 and MDA-231 cells were loaded on a 12% SDS gel. Cytosolic fractions were 10X more concentrated than the mitochondrial fraction since mitochondrial proteins represent ~10% of the total protein content. **(C)** Mitochondrial and cytosolic fractions were isolated from HeLa cells and treated with proteinase K to identify the localisation of IkBα. IkBα was found to associate with the OMM. **(D)** Mitochondrial fractions of the indicated cell lines were treated with proteinase K to validate the localisation of IkBα within the organelle. The same conditions and mitochondria purity were used as in (C). (* indicates an unspecific band).



D.2. I κ B α is an apoptosis inhibitor

Having identified that I κ B α resides at mitochondria of several cell lines and with some of them its expression level being high, we employed transient expression of I κ B α to check its effect on apoptosis. Various genes, implicated in apoptosis induction or inhibition, have the dominant capacity to exert their effect upon overexpression. This feature is even conserved across species (McCarthy & Dixit, 1998). Several constructs directing I κ B α to mitochondria (OMM and IMS) or sequestering it in the cytosol were employed to address its apoptosis regulation from different subcellular locales.

Using specific N-terminal fusions of I κ B α to the mitochondrial localisation sequences of mitoNEET (Wiley *et al.*, 2007a) and Smac/DIABLO (S/D) (Ozawa *et al.*, 2007a), we aimed to target I κ B α to the OMM and to the mitochondrial intermembrane space, respectively. Immunofluorescence confirmed the localisation of the fusion constructs to mitochondria and proteinase K digest on isolated mitochondria in combination with Western blotting supported their residence in the expected compartments. We then tested the activity of the fusion constructs in an apoptosis assay. MitoNEET-I κ B α (MEET-I κ B α) completely abolished apoptosis induced by ANT1 transfection. WT I κ B α led to an intermediate apoptosis reduction, while the S/D-I κ B α fusion had no effect on apoptosis. I κ B β was likewise ineffective for cell death inhibition. A fusion of I κ B α to the mitochondria localisation sequence of yeast mcr1, which among other membranes



also associated with the OMM (Hahne *et al.*, 1994b), led to inhibition of ANT1-induced apoptosis in 293T, MCF7, and HeLa cells (Figure D.6B). To determine how general the inhibitory effects on apoptosis were, we tested additional apoptosis-inducing genes and observed a strong apoptosis reduction when the pro-apoptotic genes Bax, VDAC1, and tBid were co-transfected with MEET-I κ B α , while apoptosis induced by caspase-8 was only weakly affected.

D.2.1. Generation and validation of constructs allowing targeting of I κ B α to the mitochondria

For the yeast *mcr1* gene we used the first 141 bp in order to direct I κ B α to the mitochondria. According to the model that Haucke *et al.* (1997) have published, the first 12 amino acids of MCR1 are inserted into the inner mitochondrial membrane and amino acids 13 to 47 reside in the intermembrane space where they are cleaved in order to release the fused protein. The constructs for I κ B α , *mcr1*-I κ B α and *mcr1*-I κ B α -FLAG were inserted into a mammalian expression vector and were verified by sequencing and western blotting. To confirm the localization of the MCR1-I κ B α protein, HeLa cells were transfected with the fusion gene *mcr1*-I κ B α and cell fractionation for mitochondria and cytosol was performed. The results show the correct localization of *mcr1*-I κ B α at mitochondria with minimal or no contamination of overexpressed protein in the cytosol (Figure D.5).

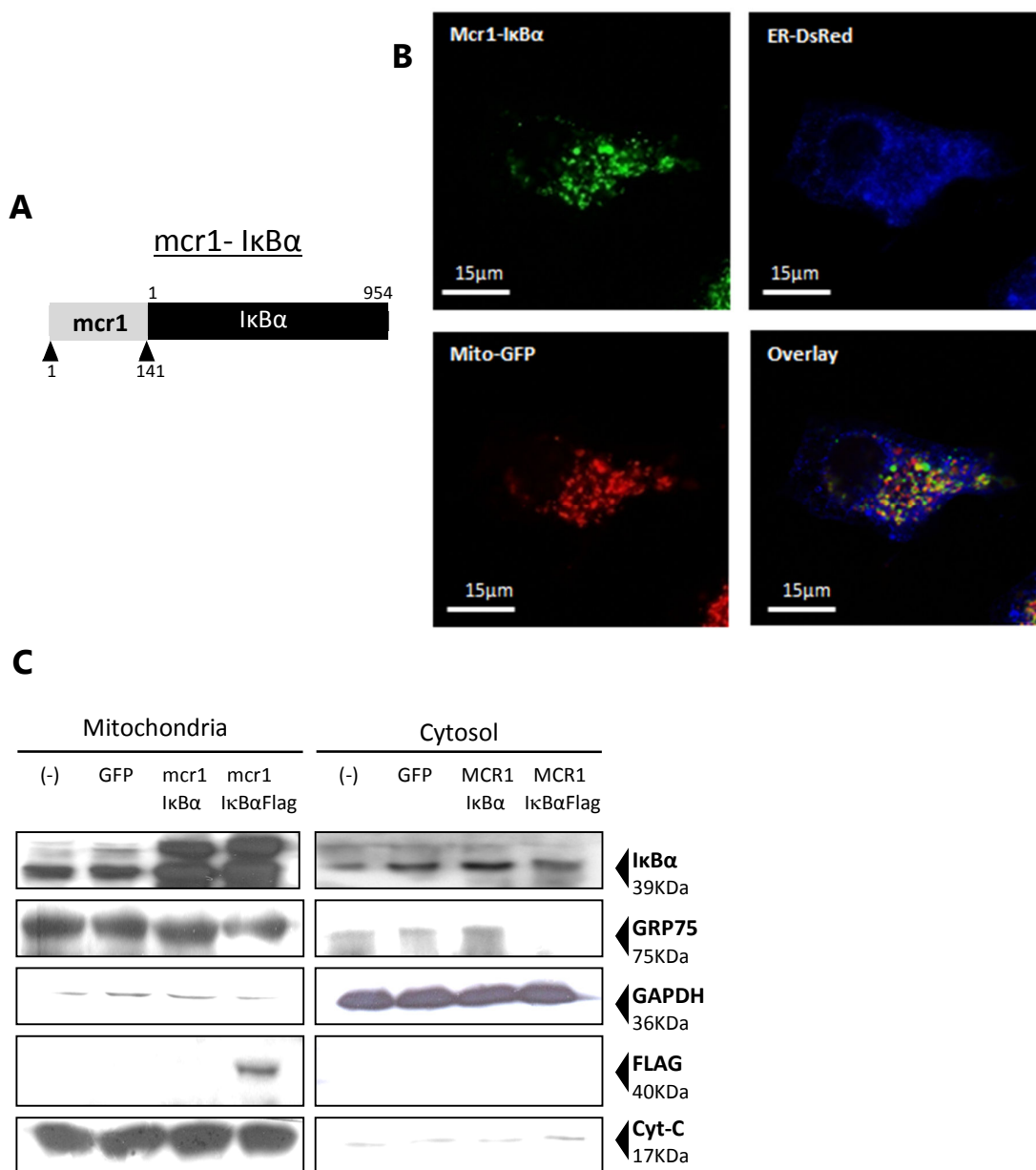


Figure D.5. Mcr1-IκBα is targeted to mitochondria. (A) Cartoon representation of IκBα fused with the localization signal of yeast mcr1. (B) Confocal photomicrographs of HeLa cells transfected with mcr1-IκBα-FLAG, mitoGFP and ER-DsRed. (C) HeLa cells were transfected with the indicated constructs and mitochondrial extracts were separated from cytosol with standard procedures.



D.2.2. I κ B α is a strong apoptosis inhibitor

The investigation of I κ B α at the mitochondrial level required the establishment of an experimental setting relevant for the localisation of the endogenous protein. Our lab has identified that the Adenine Nucleotide Translocase 1 (ANT-1) which resides at the IMM is a strong apoptosis inducer when overexpressed (Bauer *et al.*, 1999). We initially applied this finding to induce apoptosis in 293T, HeLa and MCF7 cells and investigated if I κ B α can have any effect on this apoptosis. I κ B α could indeed completely abrogate the strong apoptosis inducer ANT-1 and this effect was even more pronounced when it was targeted to mitochondria (Figure D.6A). A titration experiment revealed that even wt-I κ B α could completely inhibit ANT-1 apoptosis. The effect was not cell-specific since similar results were obtained in HeLa and MCF7 cells (Figure D.6B). I κ B α and mcr1-I κ B α could also inhibit the apoptosis observed in HeLa cells after administration of ionomycin. This drug initiates an influx of calcium to mitochondria, which leads to mitochondrial membrane potential dissipation and collapse of the OMM integrity. I κ B α targeted to mitochondria could potentially inhibit this apoptosis signal (Figure D.6C)

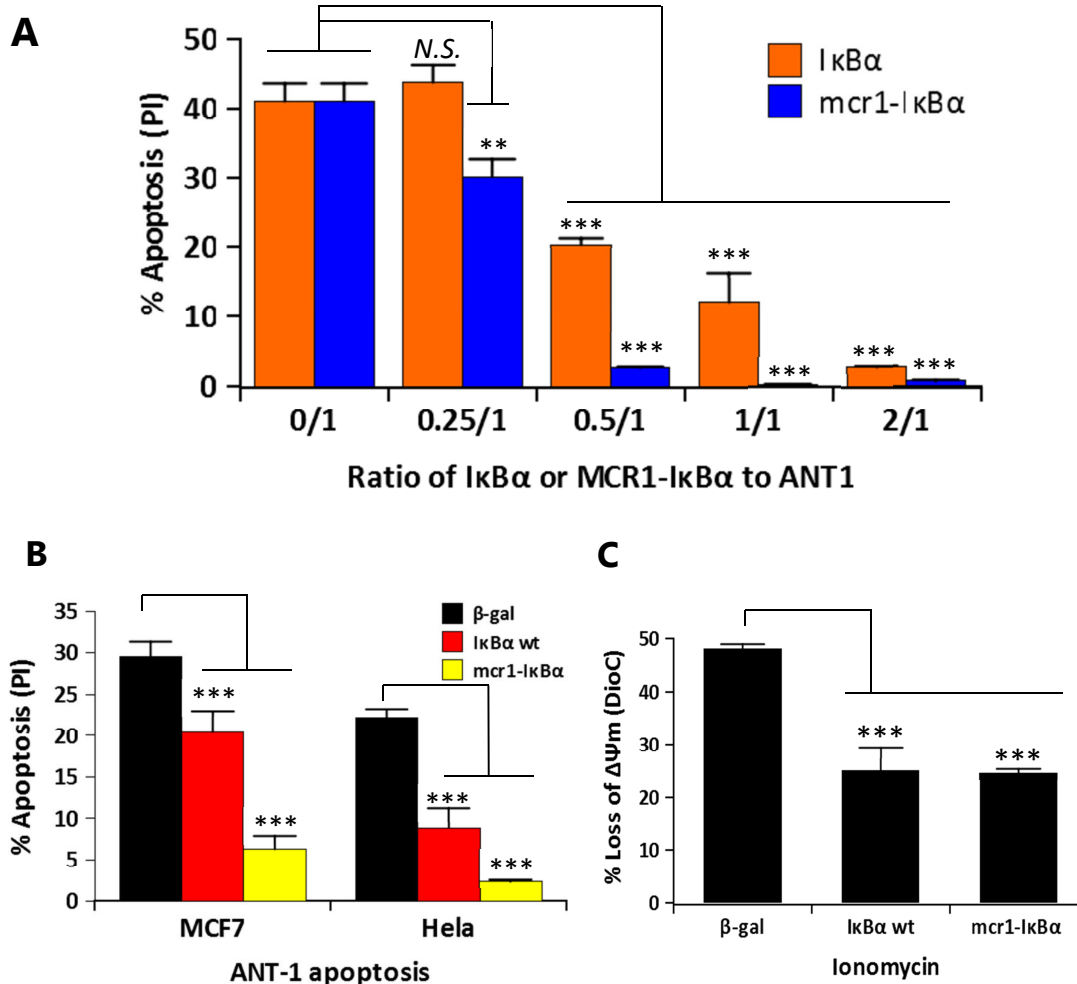


Figure D.6. $\text{IkB}\alpha$ is a strong apoptosis inhibitor at mitochondria. (A) $\text{IkB}\alpha$ wt and mcr1- $\text{IkB}\alpha$ inhibit ANT-1 apoptosis in a dose-dependent manner. 293T cells were transfected using Superfect with the indicated constructs and after 24h, apoptosis was quantified by PI staining with cell lysis. **(B)** MCF7 and HeLa cells were transfected using Effectene and 24h post transfection cell death was quantified by PI staining with lysis. **(C)** $\text{IkB}\alpha$ can inhibit the loss of $\Delta\Psi_m$. HeLa cells were transfected with Effectene and 24 post transfection Ionomycin was used at 20 μM . 2 hours later the mitochondria membrane potential was quantified using DioC staining. (***) $p < 0.0001$)



D.2.3. Generation and validation of constructs allowing targeting of I κ B α to specific sub-mitochondrial localisation

The mitochondrial NADH-cytochrome b5 reductase gene (*mcr1*) is an example of a gene encoding a protein that is directed to two sub-mitochondrial compartments (Haucke *et al.*, 1997). The N-terminal 47aa of the yeast *mcr1* gene are able to target the same protein to the OMM and IMS. Likewise, any protein fused to the first 47 amino acids of the *mcr1* gene would be targeted both to the OMM and the IMS. On the contrary it has been shown in the past that alanine substitution for glutamine at positions 23 and 24 can direct any protein almost exclusively to the IMS (Haucke *et al.*, 1997).

In order to identify the locale of I κ B α that is required for its anti-apoptotic effect, *mcr1*_{AAQQ}-I κ B α was generated in order to determine if the IMS localisation is responsible. Moreover, the constructs S/D-I κ B α and MitoNEET-I κ B α were generated that can potently and exclusively direct I κ B α to the IMS and OMM, respectively. Figure D.7 shows that the constructs were validated by immunofluorescence and western-blotting for their expected sub-organelle localisation.

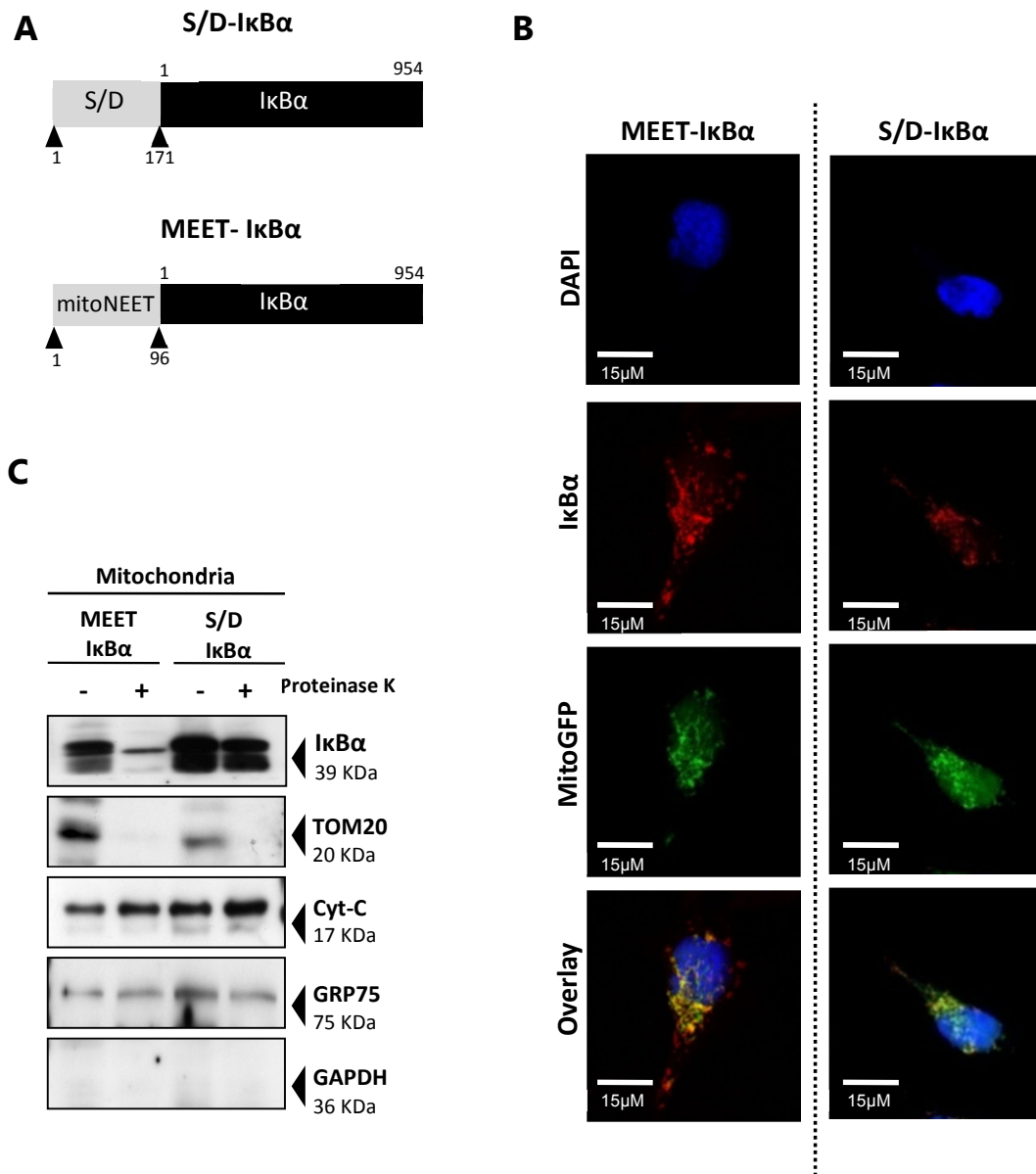


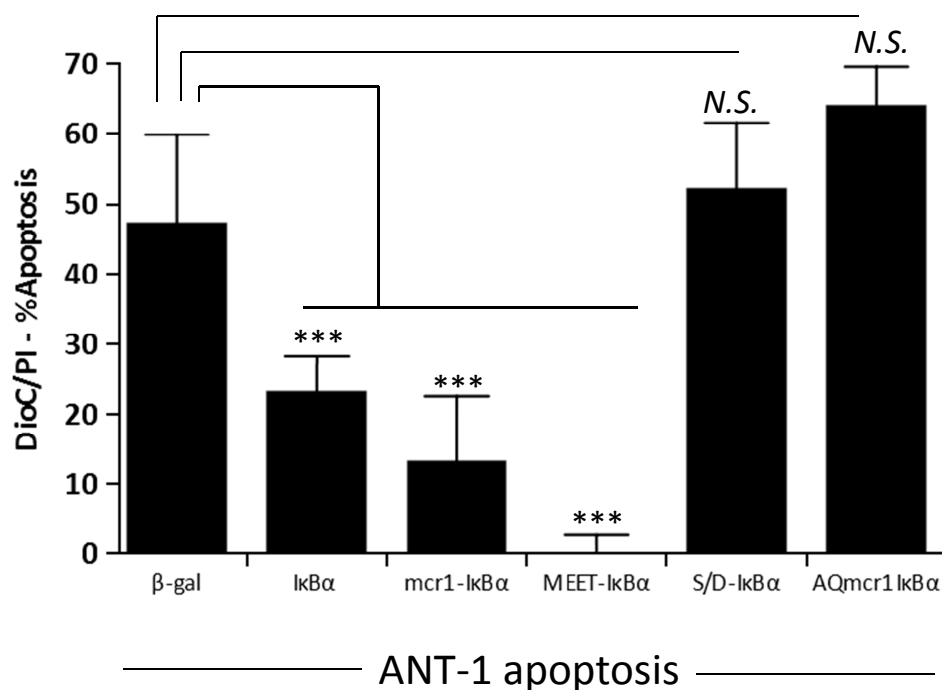
Figure D.7. MEET-IκBα and S/D-IκBα are targeted to the OMM and IMS respectively. (A) Cartoon representation of MEET-IκBα and S/D-IκBα. (B) Confocal photomicrographs showing co-localization of the indicated constructs at mitochondria. HeLa cells were transfected with mito-GFP and the indicated fusions of IκBα and 24h post transfection immunofluorescence was performed. (C) Fusion constructs reside in the correct sub-mitochondrial fractions. Proteinase at 25ng/ml was utilized to cleave OMM proteins leaving the IMS unaffected.



D.2.4. I κ B α inhibits apoptosis at the OMM

After the validation of the constructs we proceeded with the same experimental setting as previously to assess the locale at which I κ B α exerts its anti-apoptotic effect. It was found that I κ B α inhibits apoptosis at the outer mitochondrial membrane and is ineffective when targeted to the IMS (Figure D.8A-B). ANT-1 was not the only apoptosis inducer that I κ B α could inhibit. Transfection of other pro-apoptotic genes such as Bax, VDAC1, or tBid led to apoptosis initiation and I κ B α could inhibit this effect as well. When it was targeted to the OMM the effect was even more pronounced (Figure D.8C).

A



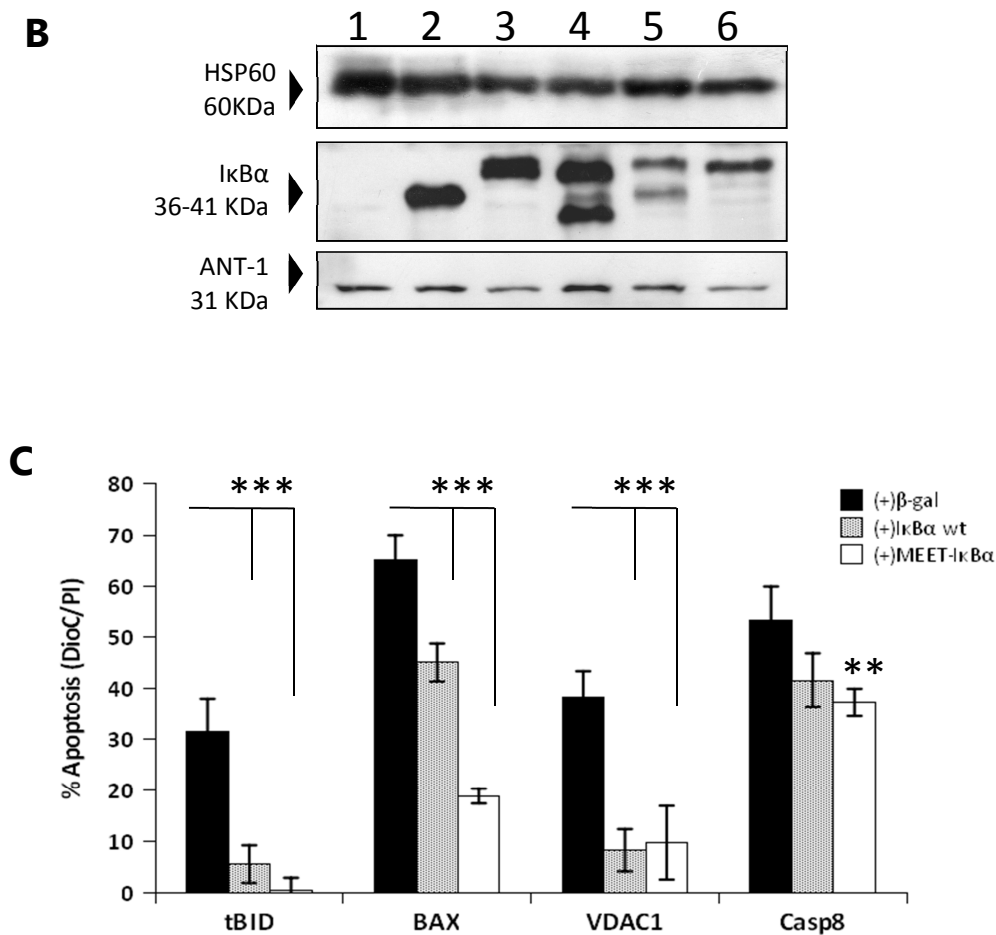
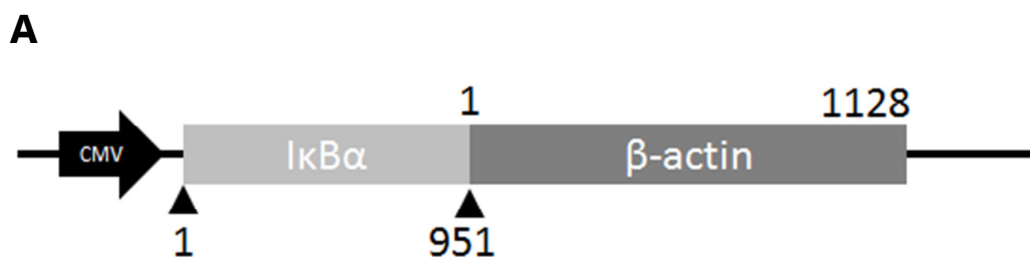


Figure D.8. IκBα is a strong apoptosis inhibitor at the OMM. (A) 293T cells were co-transfected with ANT-1 and the indicated constructs at a ratio 1:2 using calcium phosphate. 24 hours post-transfection cells were collected and DioC/PI staining was used to quantify apoptosis. Data represent six independent experiments with triplicates. (B) Expression pattern of IκBα-fusions for the indicated experiment. Numbers represent the sequence of the upper panel (1:β-gal, 2:IκBα wt, 3:mcr1-IκBα, 4:MEET-IκBα, 5:S/D-IκBα, 6:AA_{23/24}QQmcr1-IκBα). (C) tBid, Bax-β, VDAC1, and Caspase 8 were co-transfected with the IκBα-wt and MEET-IκBα to assess the inhibition profile of IκBα. Four independent experiments with triplicates were combined in the graph. (**p<0.001, ***p<0.0001)

D.2.4.1. I κ B α but not I κ B β can inhibit apoptosis and its effect is specific at mitochondria

After the discovery that I κ B α can inhibit apoptosis especially when targeted to mitochondria, the investigation of its specificity was the next step. I κ B α shares extensive sequence similarity with I κ B β and its effect on apoptosis was tested in a similar assay. As figure D.9C shows, I κ B β was completely incapable to inhibit apoptosis compared to I κ B α -wt. Furthermore, the specificity of apoptosis inhibition at mitochondria was tested in order to assess if the locale was specifically required or if the inhibitory ability was due to non-specific effects of over-expression. In order to investigate that possibility we generated a fusion of I κ B α with the β -actin gene (Figure D.9A) to sequester I κ B α in the cytosol and avoid its mitochondrial localisation. Figure D.9B shows that the actin fusion of I κ B α was not co-localised at mitochondria in contrast to MEET-I κ B α and was unable to inhibit apoptosis induced by ANT-1 (Figure D.9C).



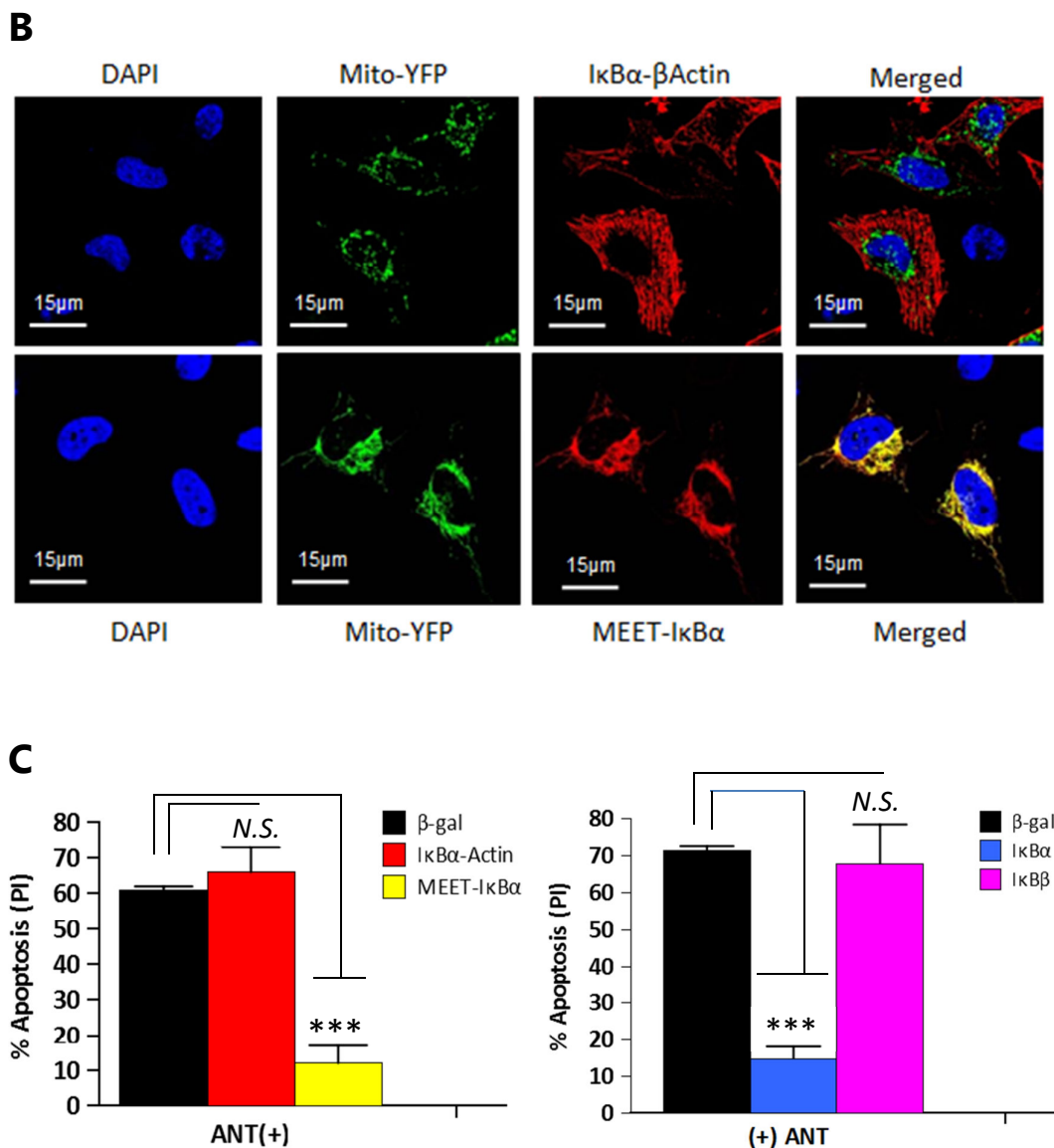


Figure D.9. IκBα but not IκBβ inhibits apoptosis exclusively at the OMM.
(A) Cartoon representation of IκBα/βActin in pcDNA3 expression vector. **(B)** Confocal images of HeLa cells transfected with mito-YFP, MEET-IκBα or IκBα/βActin. Cells were stained for IκBα antibody. **(C)** 293T cells were transfected using Xfect with ANT-1 and the indicated plasmids and apoptosis was quantified 24h post transfection using PI staining. IκBα sequestered in the cytosol is unable to inhibit apoptosis in contrast to IκBα targeted to mitochondrial OMM (left panel). *** $p < 0.001$, ** $p < 0.01$



D.2.5. Reconstitution of I κ B α in 3T3 I κ B α -/- cells renders them resistant to apoptosis

Genetic knockouts have provided an experimental tool for the identification of possible consequences of the absence of the respective protein under investigation. I κ B α -/- mice show constitutive NF- κ B activation, and neonatal lethality. We tried to investigate the effect on apoptosis using a re-constitution model for I κ B α at different subcellular compartments and in parallel to confirm the results from the ectopic expression experiments described earlier. We utilized a lentiviral expression strategy in order to reconstitute I κ B α only at the outer mitochondrial membrane or only in the cytosol. MEET-I κ B α and I κ B α / β Actin proved to be the suitable fusions for this purpose. We used a lentiviral vector which contains a CMV promoter for the transgene expression, followed by a SV40 promoter expressing a GFP mutant so that the positive colonies would be visually identified.

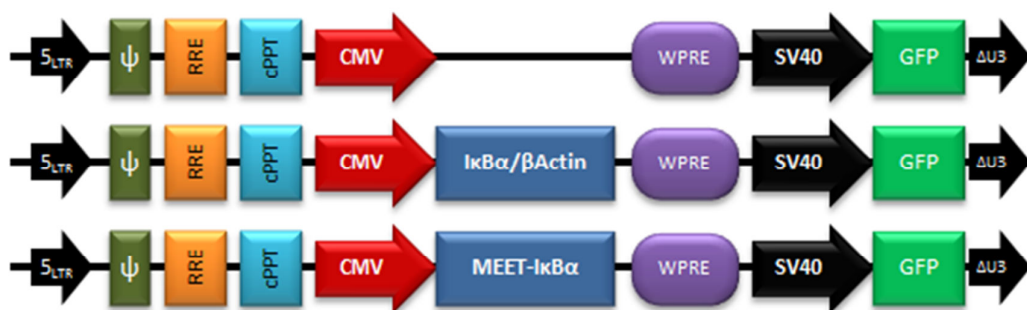
We reconstituted I κ B α at the OMM at a level similar or lower than the one observed in wild type fibroblasts (Figure D.10B) by modifying the multiplicity of infection of the virus. Reconstitution of I κ B α at mitochondria of I κ B α -/- fibroblasts significantly reduced the sensitivity of these cells to several apoptotic stimuli whereas I κ B α sequestered in the cytosol had the opposite effect (Figure D.10C). We checked the level of active NF- κ B in order to have a baseline for comparison and interpretation of the results. A non-radioactive assay with a biotinylated-

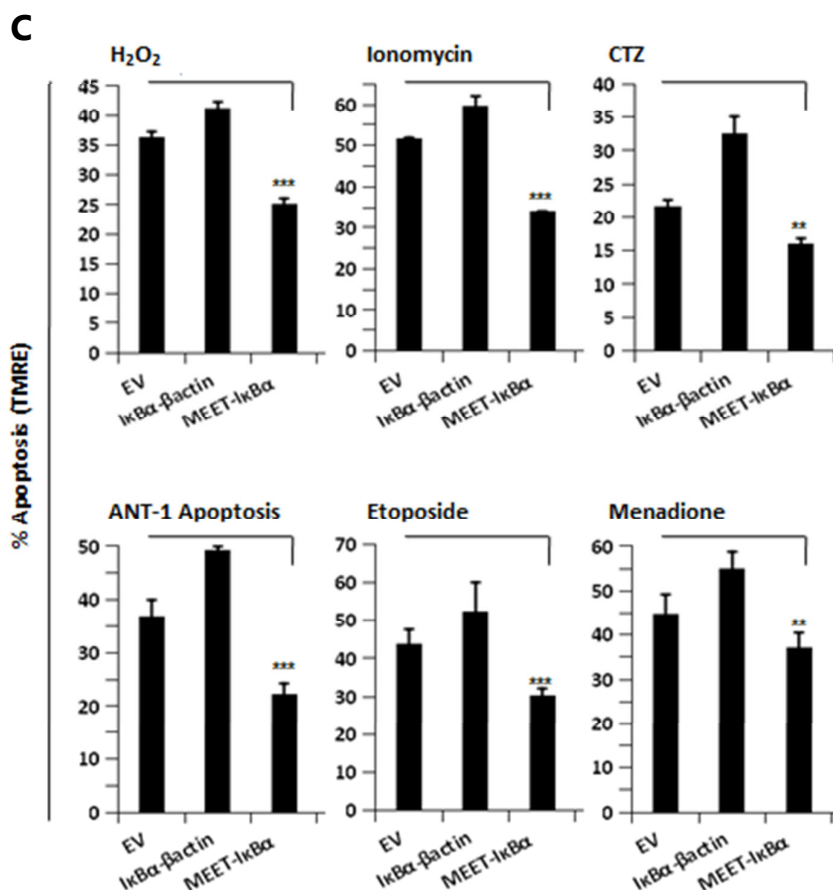
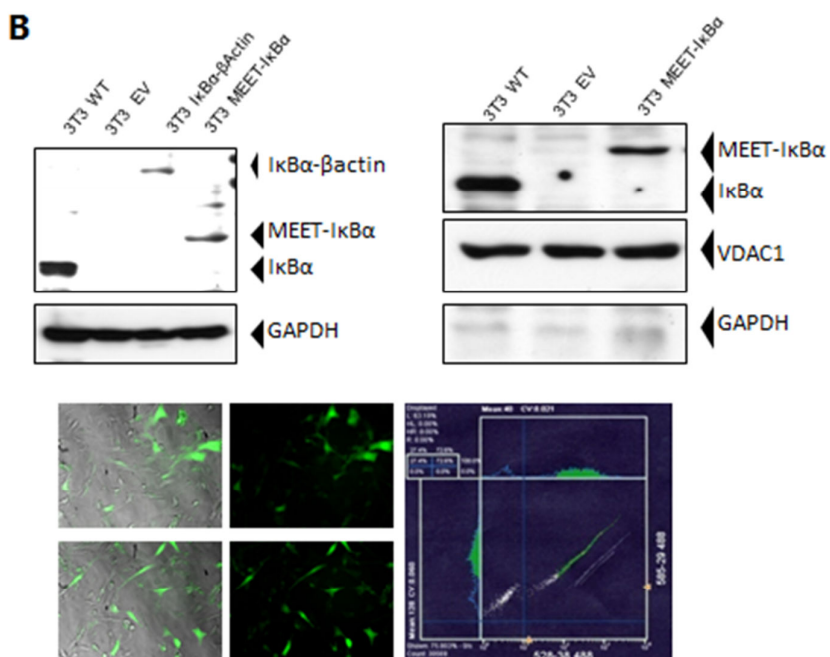


tandem- κ B-oligo-pulldown, showed that both MEET- $\text{I}\kappa\text{B}\alpha$ and $\text{I}\kappa\text{B}\alpha/\beta$ Actin inhibited the NF- κ B activation to the same extent, which made the experimental setting more accurate since the comparison could be made (Figure D.10E)

NF- κ B activity and especially constitutive NF- κ B activation has been observed in several aggressive tumours as discussed in the introduction chapter. Since NF- κ B has an anti-apoptotic activity in these tumours we investigated if NF- κ B has an anti-apoptotic activity on these cells so we can take that under consideration when interpreting the reconstitution results. Using a specific NF- κ B inhibitor (N-Oleoyldopamine) and two signals for apoptosis we inhibited the NF- κ B system in $\text{I}\kappa\text{B}\alpha$ $-/-$ cells (reconstituted with an empty vector) and we found that these cells are more sensitive when NF- κ B is inhibited showing that in those cells constitutive NF- κ B activation confers an anti-apoptotic signal (Figure D.10D).

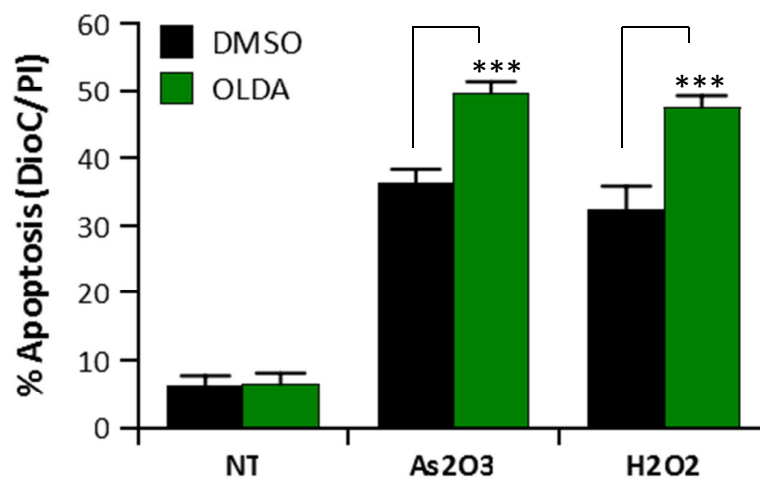
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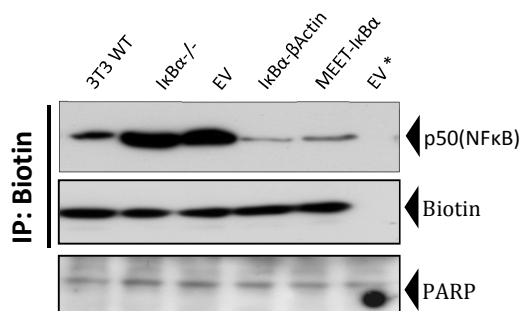




Figure D.10. Effect of I κ B α reconstitution at the OMM and the cytosol in I κ B α $-/-$ cells. **(A)** Cartoon representation of the lentiviral constructs used for the reconstitution of the indicated genes in 3T3 I κ B α $-/-$ cells. A GFP variant was also expressed in a bi-cistronic format for easier sorting of the cells. **(B)** Two weeks post-transduction, cells were selected in a FACS sorter for GFP and WB from total lysates (left panel) was used to identify the level of expression after reconstitution. Both I κ B α / β Actin and MEET-I κ B α are expressed at a lower level than 3T3-WT cells. Mitochondrial I κ B α after reconstitution was examined after fractionation and separation from the cytosol. (EV: Empty Vector, WT: Wild type) **(C)** Effect on apoptosis of mitochondria-specific I κ B α reconstitution in I κ B α $-/-$ (constitutive NF- κ B positive) cells. I κ B α $-/-$ cells were reconstituted and treated with the indicated signals for apoptosis. Apoptosis was assessed by FACS analysis. **(D)** 3T3 I κ B α $-/-$ reconstituted cells with an empty vector were assessed for their dependence on constitutive NF- κ B activation. N-Oleyoldopamine (OLDA) was used to inhibit NF- κ B and cells were subsequently treated with the indicated drugs. Apoptosis was quantified 24h post treatment. (NT: Non-treated) **(E)** Reconstitution of mitochondrial or cytosolic I κ B α equally inhibits the constitutive NF- κ B activation observed in I κ B α $-/-$ cells. Biotin conjugated κ B tandem repeats was immunoprecipitated from nuclear extracts of the indicated reconstituted cells and SDS-PAGE was used to assess the level of active NF- κ B.



D.2.6. Knock down of I κ B α in cancer cell lines with constitutive NF- κ B activity sensitises them for apoptosis

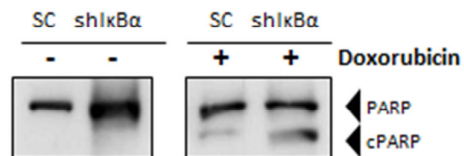
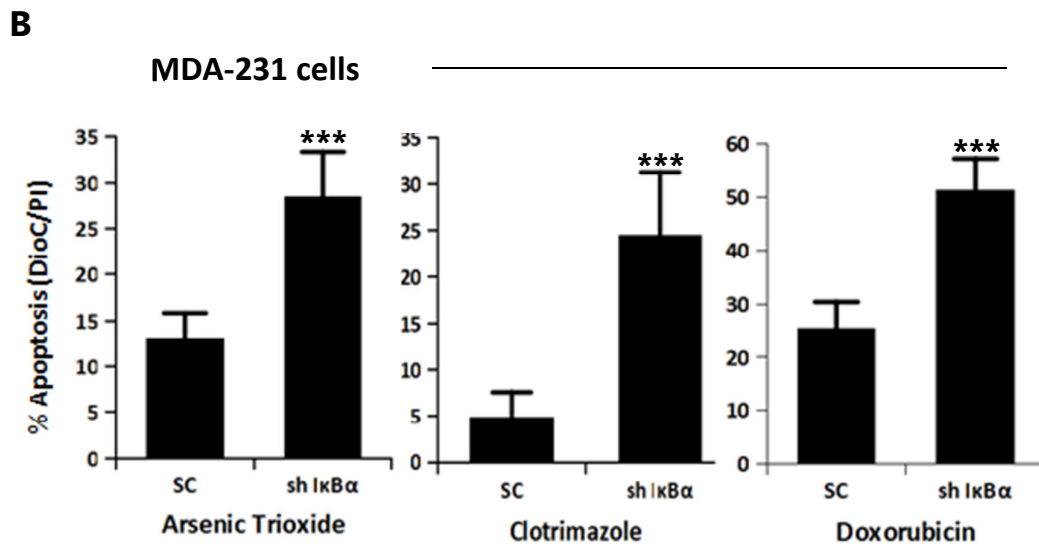
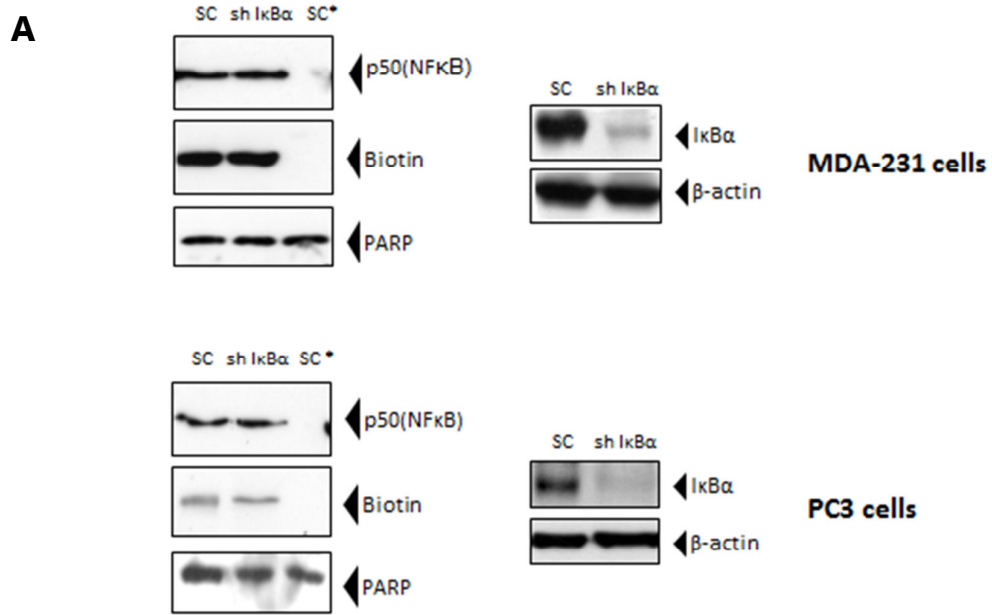
Having established that I κ B α can potently inhibit apoptosis at the OMM and possibly contributes to the apoptosis resistance observed in many cancers, we progressed to investigate its function in those cancer cells. We established the experimental setting in a way so that the cells we used had I κ B α at mitochondria, had constitutively active NF- κ B, and were very resistant to apoptosis. PC3 and MDA-MB-231 cells were found to fulfil these criteria. It was shown earlier that MDA-231 cells accumulate I κ B α at mitochondria with minimal expression in the cytosol (Figure D.4B). These cells have constitutively active NF- κ B that is not dependent on I κ B α degradation and are extremely resistant to several chemotherapeutics.

Since I κ B α has an anti-apoptotic role when expressed at mitochondria and since in MDA-231 cells I κ B α is found mainly at mitochondria, we reduced the expression of I κ B α by lentiviral shRNA so that the *mitochondrial* I κ B α is significantly down-regulated. We observed, that when I κ B α was knocked-down, MDA-231 cells were sensitized to several apoptotic signals, which were ineffective previously (Figure D.11C-E). Likewise, PC3 cells in which immunofluorescence likewise showed enhanced localization of I κ B α at mitochondria and proteinase K treatment revealed that I κ B α is located at their OMM, were sensitized to apoptotic signals (Figures D.4A, D.4D). HCT-116 cells show the same trend for apoptosis



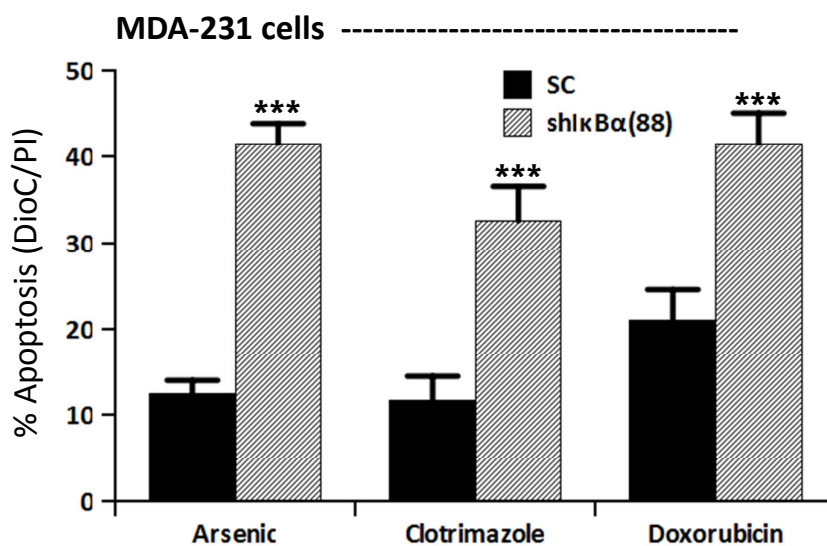
sensitization after I κ B α knock-down (Figure D.11F). In order to exclude off-target effects of the shRNA sequence that we used (sh I κ B α -42) we generated an additional stable cell line with an alternative targeting sequence for I κ B α knock-down. Figure D.11C shows that the effects were specific and shI κ B α (88) likewise sensitized MDA-231 cells for apoptosis induction. A cell viability assay (MTT) revealed that MDA-231 cells with reduced I κ B α expression showed reduced viability compared to normal cells (scramble) (Figure D.11D) confirming the sensitisation to apoptosis observed previously (Figure D.11B)

Since NF- κ B activation is conferring an anti-apoptotic signal to several cancers we investigated the effect of I κ B α knock-down to MDA-231 and PC3 cells. If the NF- κ B system was affected the results would have been interpreted accordingly. We show in figure D.11A that shRNA-mediated knock-down of I κ B α in MDA-231 and PC3 cells did not affect the level of endogenous constitutively active NF- κ B.

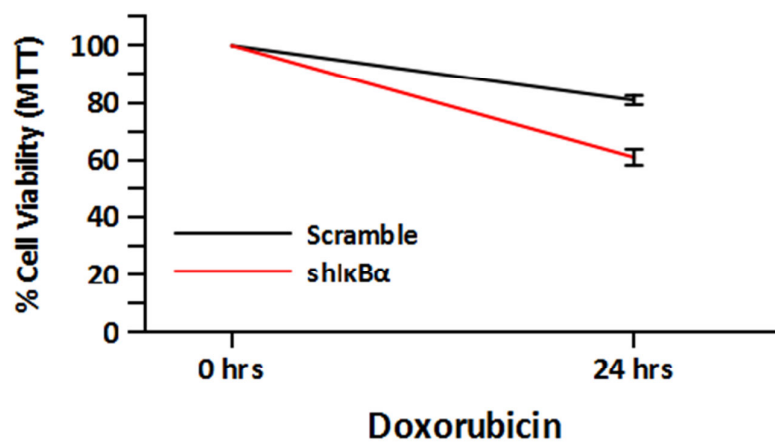




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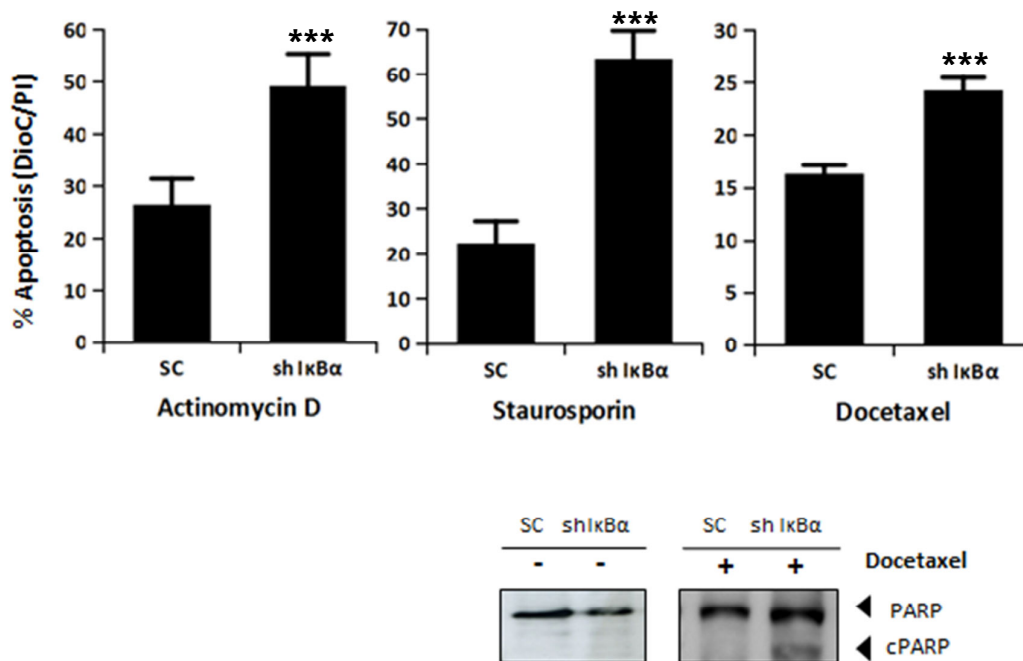


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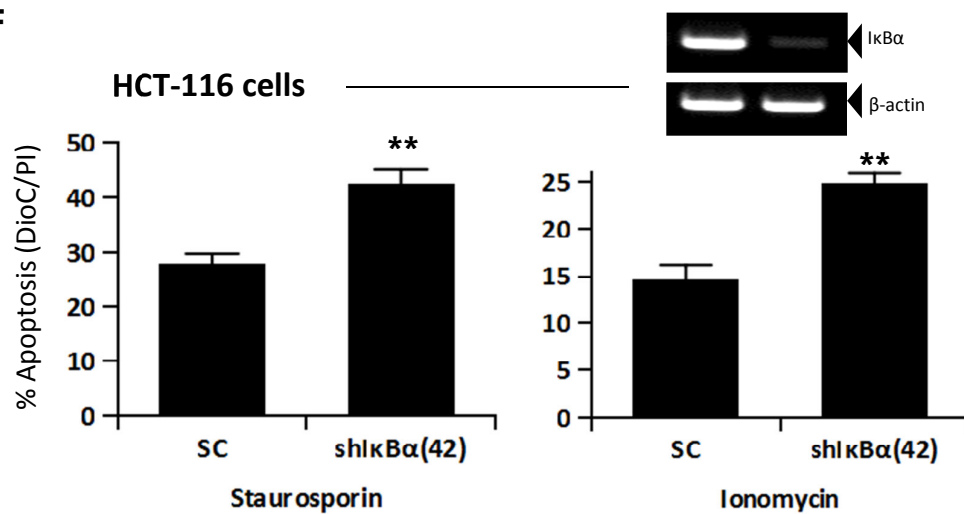




Figure D.11. Knock-down of I κ B α in cells with constitutively active NF- κ B sensitizes them to apoptosis. (A) Lentiviral-based knock-down of I κ B α by shRNA diminished the expression of its protein in MDA-MB-231 cells (upper panel) and in PC3 cells (lower panel). NF- κ B was not affected by the down-regulation as indicated by a biotinylated-oligo-pull-down assay. (SC*: Non-labelled oligo in 1000X excess) (B) MDA-231 cells were treated with the indicated drugs (arsenic 10 μ M, clotrimazole 40 μ M-4 hours- and doxorubicin 1 μ M for 24h) and apoptosis was assessed by FACS and PARP cleavage. (C) An alternative shRNA construct for I κ B α was also used to exclude possible off-target effects, showed similar results with (B) after treatment under the same conditions. (D) MDA-231 cells were treated with 1 μ M doxorubicin and subjected to an MTT assay for cell viability. (E) Identical approach with (B) was used for PC3 cells which were treated with 1,4 μ M actinomycin D, 1 μ M Staurosporine (STS) and 5nM Docetaxel for 24h. Apoptosis was assessed with FACS and PARP cleavage. (F) Knock-down of I κ B α in HCT-116 cells was confirmed by semi-quantitative RT-PCR. Ionomycin 10 μ M and STS 1 μ M were used to induce apoptosis in those cells.*** $p < 0.001$, ** $p < 0.01$



D.2.7. *In vivo* MDA-231 tumour shrinkage with doxorubicin after knock-down of I κ B α

Following the above observations *in vitro* for the role of I κ B α on the resistance to chemotherapeutics that is observed in cells lines with increased levels of I κ B α at mitochondria, we sought to examine the role of I κ B α *in vivo*. MDA-231 as the *in vitro* study shows are extremely resistant to chemotherapeutics such as doxorubicin. We transduced MDA-231 cells with lentiviruses encoding for scramble or for shRNA targeting I κ B α . We used these cells to generate xenografts by injecting 2×10^6 cells of each cell line in the mammary fat pad of 8 Balb/c nu/nu mice. After four weeks the mice were examined for tumour formation. Tumour growth was observed only in the MDA-231/shI κ B α cells but we decided to proceed to the treatment in order to determine relevant concentrations of doxorubicin.

Two out of eight mice did not develop palpable tumours. We randomly split the 6 mice into two groups for PBS or doxorubicin treatment. Mice were weighted and PBS or doxorubicin (6mg/kg) was administered intravenously (i.v.). Figure D.12A shows the days when the tumours were measured and the drug was administered. 14 days after the initiation of the treatment PBS treated mice showed tumour formation in both sides which grew substantially to the same level. 21 days post treatment initiation MDA-231/scramble also started growing and eventually continued growing even when they were under doxorubicin



treatment. MDA-231/shIkB α tumours despite being substantially bigger and the only ones growing at the initial treatment stages, were re-sensitized and shrank even stronger than the MDA-231/scramble cells. Figure D.12B shows the average tumour size on the various days of measurement for PBS- and doxorubicin-treated mice. However, statistical significance was not achieved due to the small mice number. Results shown were derived from a small pilot study to determine the ability of MDA-231 cells to develop tumours and determine an effective doxorubicin dose for a later, large scale study. All in vivo experiments were conducted following the 1986 Act for Animals (Scientific Procedures) and under PPL : 70/7173

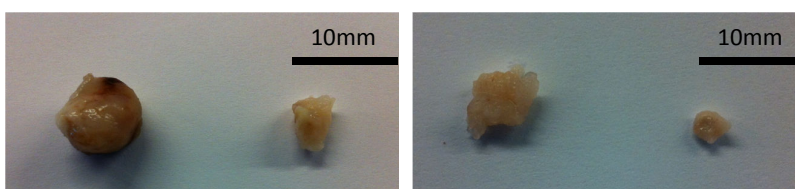
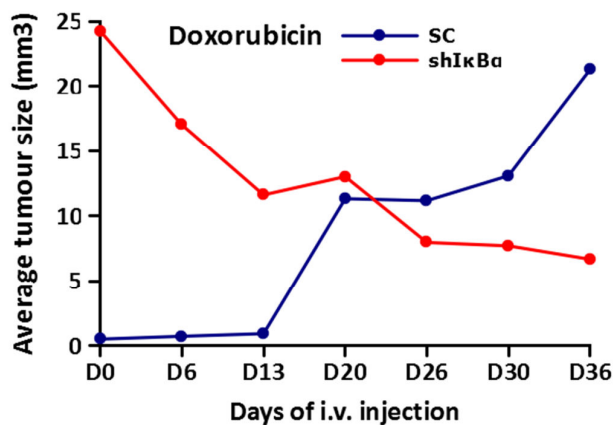
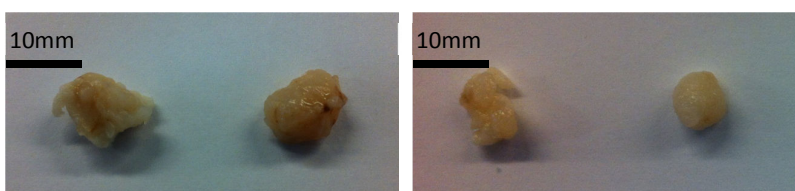
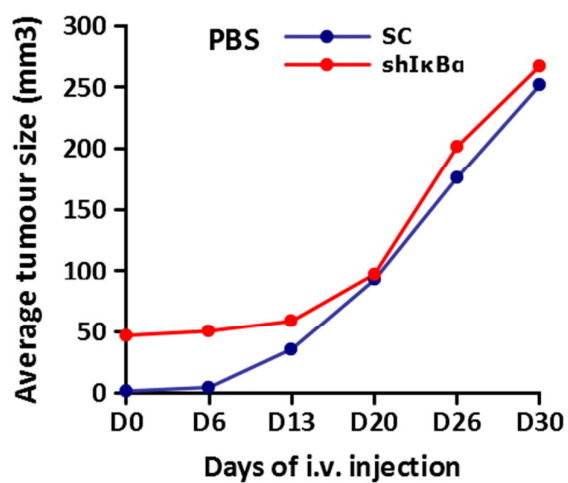




Figure D.12. Mouse xenografts from chemotherapy-resistant MDA-231 cells are re-sensitized after IκBα knockdown. Random grouping of previously engrafted mice in the mammary fat pad (mfp) with MDA-231/scramble and MDA-231/shIκBα cells were injected i.v. with PBS (upper graph and upper panel) or doxorubicin (6mg/kg)(lower graph and lower panel) for the days indicated in the graphs. Calliper measurements were taken on the same days as treatment. Graphs are showing average tumour sizes from 3 Balb/c nu/nu mice in each group. Tumour size was calculated following the formula:

$$V \text{ (mm}^3\text{)} = \frac{\pi}{6 * \text{length} * \text{width} * \text{height}}$$
. PBS mice were sacrificed at D30 to comply with the conditions of the Act since tumour sizes reached the limit of the project licence.



D.3. I κ B α is a PT-pore modulator

In an effort to explore the function of I κ B α at the OMM we tried to identify the interaction partners of I κ B α at this locale. Using immunoprecipitations we discovered a strong signal with the OMM protein VDAC1 and Hexokinase II, but not with cytochrome-c. I κ B α was also able to co-immunoprecipitate with CK1 and ANT1, which are localised in the intermembrane space and the inner mitochondrial membrane, respectively (Figure D.13). This suggested that I κ B α can associate with molecules that are part of the permeability transition (PT) pore. Accordingly, deletion of cyclophilin D, which inhibits the PT pore (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005), also completely inhibited the activity of MEET-I κ B α to reduce apoptosis (Figure D.14). In addition, ANT1-induced apoptosis was efficiently inhibited by I κ B α and is mediated by the PT pore as it is dependent on cyclophilin D (Figure D.14). Moreover, the shRNA-mediated knockdown of VDAC1 or Hexokinase II also reduced the potential of MEET-I κ B α to inhibit apoptosis (Figure D.15). These experiments suggested that I κ B α targets VDAC1 and/or HKII to inhibit apoptosis. In an effort to determine the behaviour of I κ B α under apoptotic conditions, it was found that I κ B α loses its interaction with VDAC1 while maintaining its interaction with hexokinase II (Figure D.15).



D.3.1. Endogenous interaction of I κ B α and components of the PT-pore

Having identified the locale of I κ B α that is required for apoptosis inhibition I sought to identify those molecules that are necessary for I κ B α to interact and exert its anti-apoptotic effect. I used isolated mitochondria from HeLa cells and immunoprecipitated I κ B α . I found that I κ B α can interact with VDAC1, ANT-1, CK1 and Hexokinase II but was un-able to interact with cytochrome-c (Figure D.13A-B) establishing a physical interaction with members of the PT-pore.

In the previous section I κ B α was found to exert its anti-apoptotic effect at the OMM. One prominent molecule that resides at the OMM is VDAC1 and I κ B β was not found able to interact with VDAC1 as I κ B α (Figure D.13C). The latter was performed after the finding that I κ B α , but not I κ B β , can inhibit apoptosis (see chapter D.2.4.1).

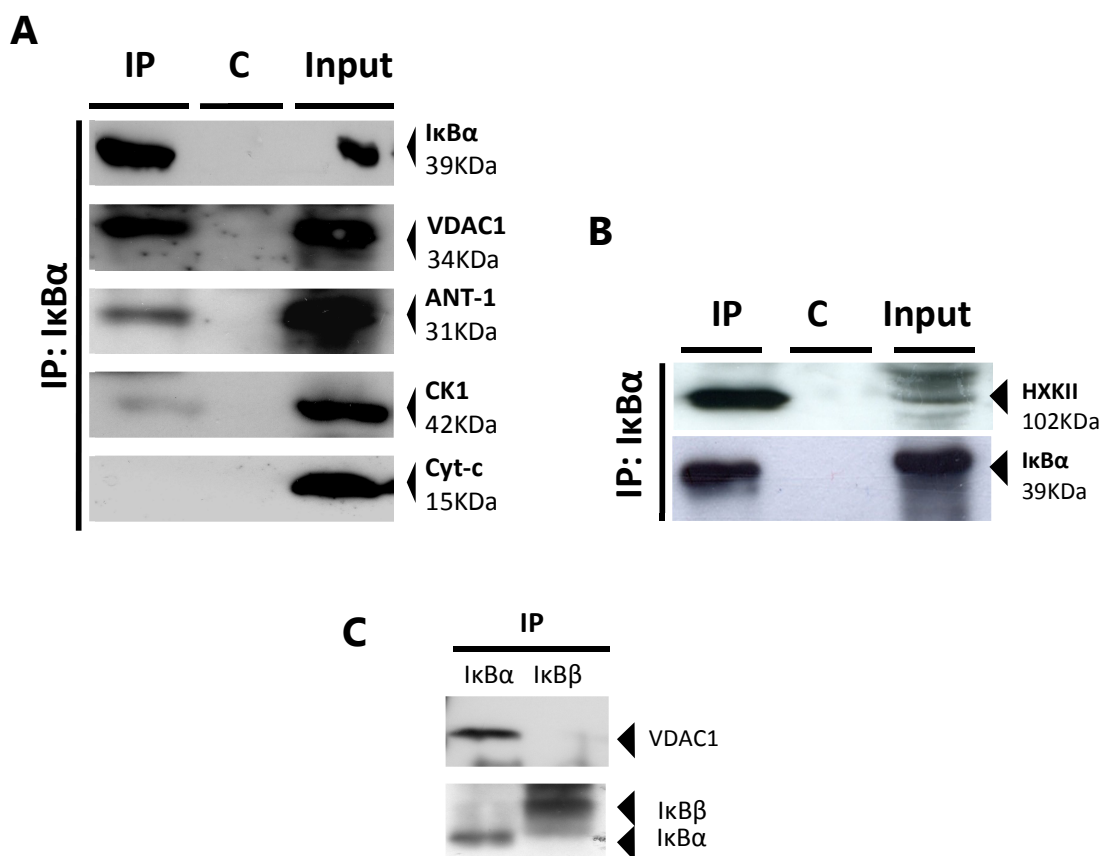


Figure D.13. IκBα interacts with subunits of the PT-pore. (A) HeLa cells were lysed with RIPA buffer and 5% n-dodecyl-D-maltoside added and the lysates incubated on ice for 30 min. After high speed centrifugation the supernatant was recovered and IκBα was immunoprecipitated using a monoclonal antibody. The pulled-down proteins were separated by SDS-PAGE (IP: Immunoprecipitation, C: Control). The blot was probed with several antibodies against subunits of the PT pore or proteins of the IMS. **(B)** MCF7 cells, which express high levels of HXKII, were used to pull-down IκBα and identify the IκBα-HXKII interaction **(C)** Whole cell lysates were used to pull down IκBα or IκBβ and a sequential staining for IκBα and IκBβ was performed for the pull-down/input while a monoclonal VDAC1 antibody was used to identify interaction.



D.3.2. I κ B α requires the PT-pore to exert its anti-apoptotic effect

The PT-pore is acknowledged to play an important role in apoptosis as a protein complex, but individual members such as ANT-1, VDAC1 and cyclophilin D have also independently been implicated in apoptosis regulation. We sought to identify the importance of the PT-pore for I κ B α to exert its anti-apoptotic effect. We used cells in which cyclophilin-D is absent (*ppif*^{-/-}) and which, as a consequence, show increased resistance to apoptotic stimuli since the PT-pore cannot be activated.

As described in previous sections we used ANT-1 to induce apoptosis and I κ B α as an apoptosis inhibitor. Previous findings from our lab show that ANT-1 apoptosis can be inhibited by bongkreikic acid (Bauer *et al.*, 1999), a specific inhibitor of the PT-pore. We initially tested ANT-1 and found that its apoptosis induction is mediated by the PT-pore since transfection of ANT-1 in *ppif*^{-/-} cells rendered ANT-1 incapable of inducing apoptosis (Figure D.14A). Likewise, we tested the ability of I κ B α to inhibit apoptosis when the PT-pore cannot be activated (using *ppif*^{-/-} cells). We found that I κ B α could inhibit apoptosis induced by several drugs in normal mouse embryonic fibroblasts but was not able to further inhibit apoptosis in cells with a defective PT-pore (Figure D.14B).

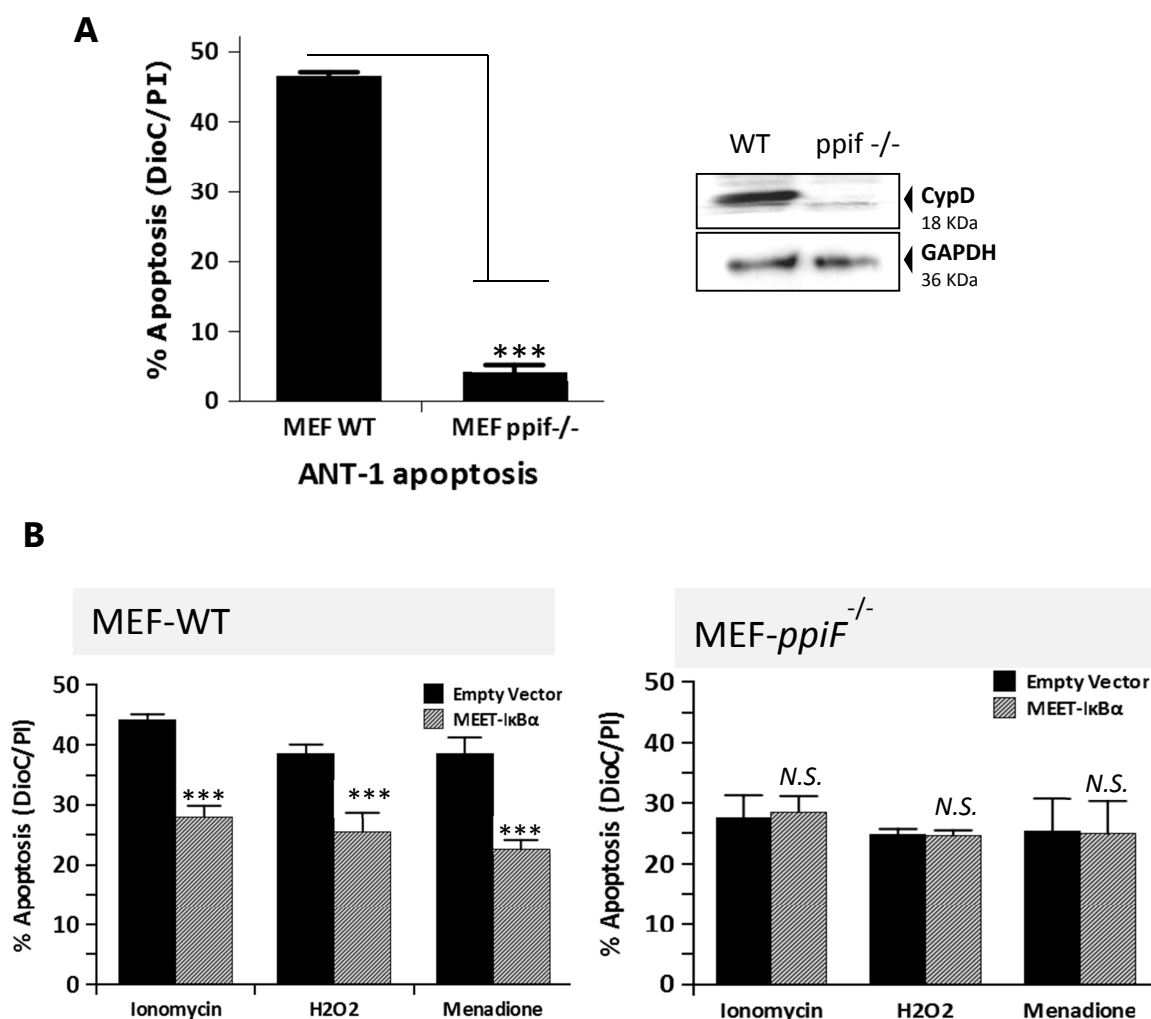


Figure D.14. IκBα exerts an anti-apoptotic effect through the PT-pore. (A)

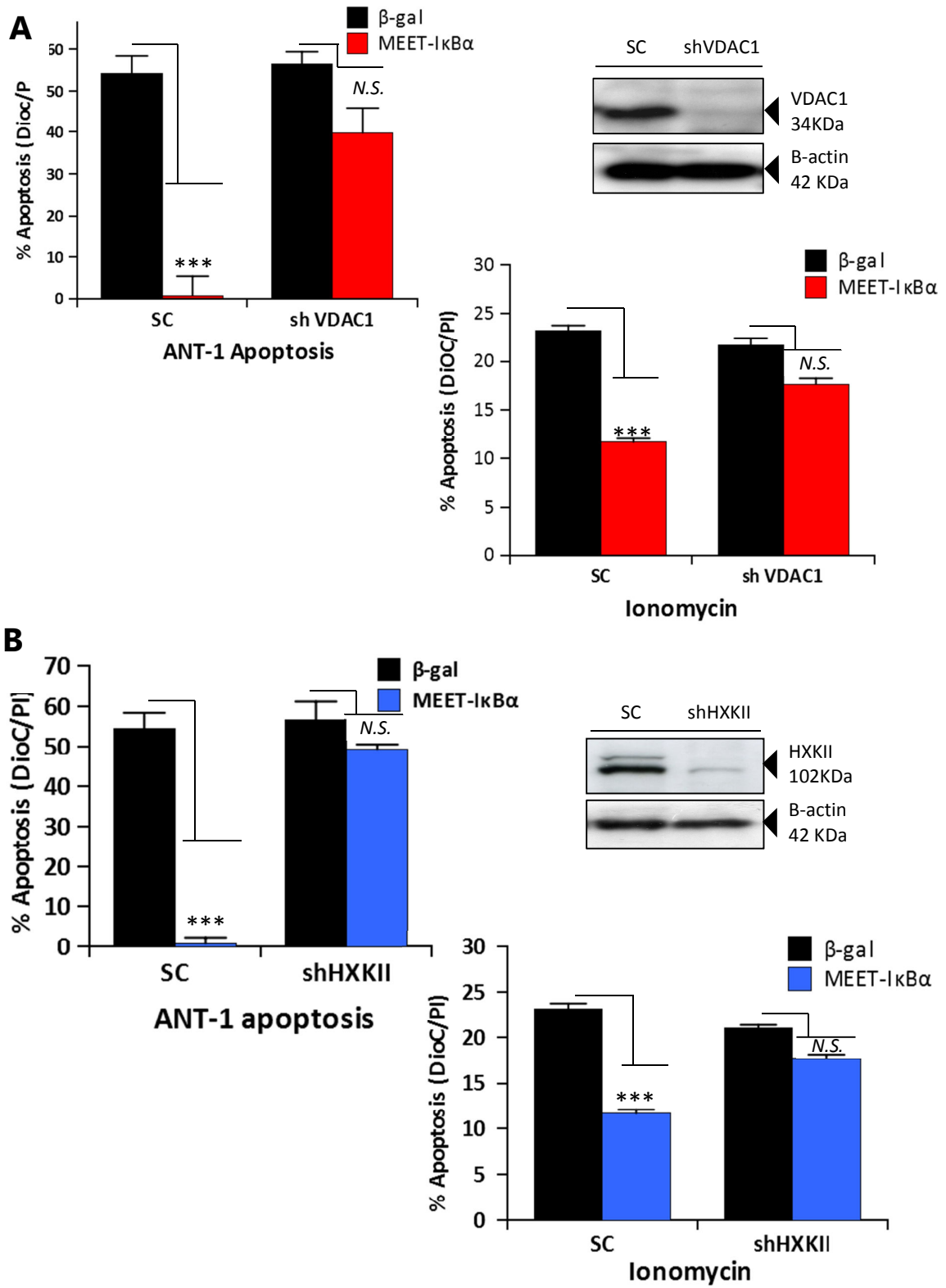
MEF-WT and MEF-*ppiF*^{-/-} were tested with a WB for the validation of the gene knock-out (right panel). MEF-WT and MEF-*ppiF*^{-/-} were transfected with X-fect with ANT-1 and 24h post transfection DiOC/PI was measured to quantify apoptosis. **(B)** MEF-WT and MEF-*ppiF*^{-/-} were transfected with an empty vector and with MEET-IκBα. 24 hours post-transfection cells were treated with ionomycin (10μM, 3h), menadione (15μM, 20h), and H₂O₂ (500μM, 24h). Apoptosis was quantified by DiOC/PI staining and FACS. (***: p<0.0001, N.S.: Non-significant)



D.3.3. I κ B α targets VDAC1 to exert its anti-apoptotic effect

An important modulator of the PT-pore that induces apoptosis when over-expressed is VDAC1 as mentioned in the introduction. We established that I κ B α has a very strong anti-apoptotic effect when targeted to the outer mitochondrial membrane and that the PT-pore is necessary for this inhibitory effect. Bearing in mind the above we attempted to identify the interaction partner that I κ B α is targeting to exert its effect. At the OMM two important proteins that form an essential anti-apoptotic complex were tested for this purpose. VDAC1 and Hexokinase II (HXKII) were found to be required for I κ B α 's anti-apoptotic effect.

Using knock-downs of VDAC1 and HXKII in 293T cells and in 3T3 I κ B α -/- reconstituted cells we determined that both VDAC1 and HXKII are necessary for apoptosis inhibition via I κ B α . Knock-down of VDAC1 completely abolished the ability of I κ B α to inhibit apoptosis (Figure D.15A, D.15C) and the effect was observed both with overexpressed I κ B α and reconstituted I κ B α . Likewise, HXKII reduction abrogated the inhibitory effect of I κ B α (Figure D.15B). Immunoprecipitation of I κ B α from cells with stably reduced VDAC1 left the I κ B α -HXKII interaction unaffected as similar to when HXKII was knocked-down and the interaction between VDAC1 and I κ B α was examined (Figure D.15D).



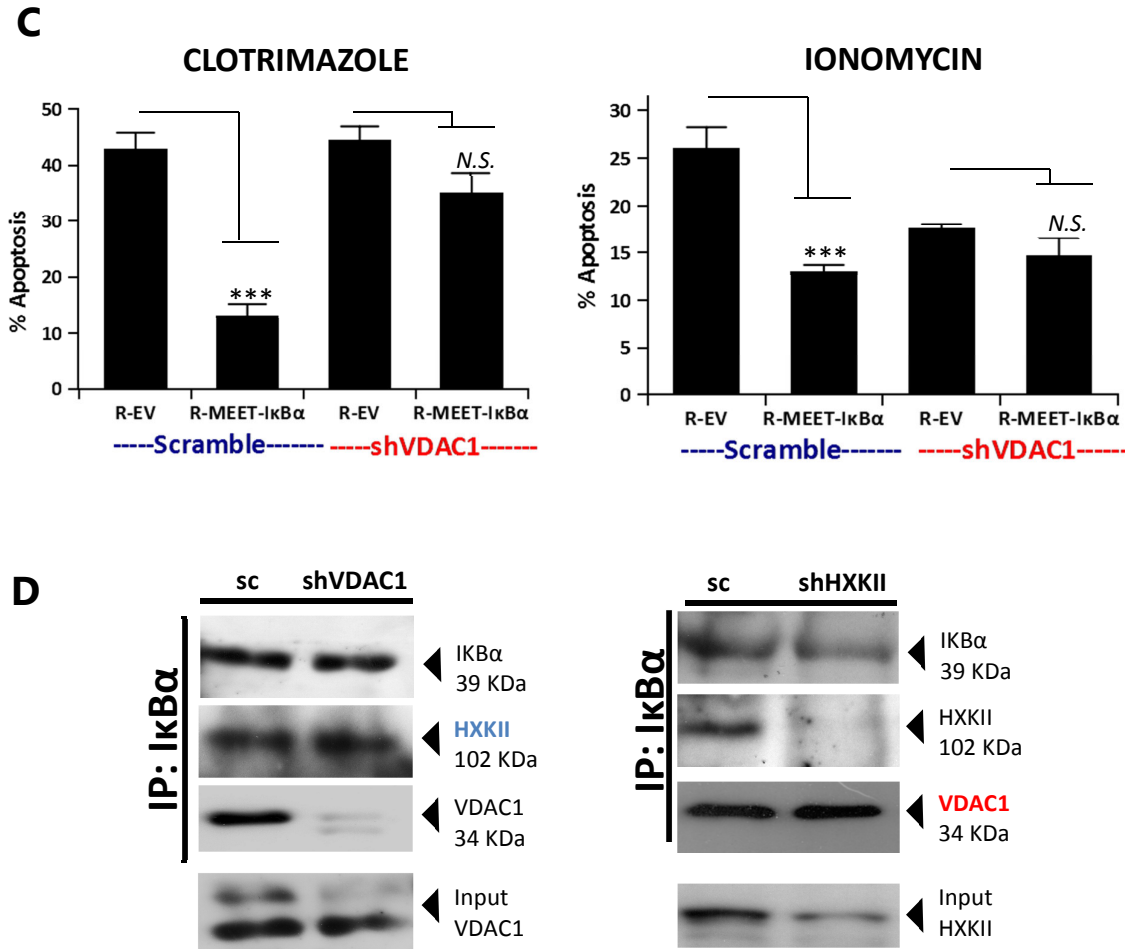


Figure D.15. IκBα targets VDAC1 to exert its anti-apoptotic effect. (A) VDAC1 was stably knocked-down in 293T cells using lentiviral particles. Its expression was checked in a WB after 7 days of puromycin selection. ANT-1 and 20μM ionomycin were used for apoptosis induction and transfection of empty vector or MEET-IκBα. Apoptosis was quantified by FACS. **(B)** The same procedure as (A) was used to knock-down HXKII and likewise the ability of IκBα to inhibit apoptosis was examined. **(C)** 3T3 IκBα^{-/-} cells reconstituted with an empty vector or with MEET-IκBα were transduced with lentiviral particles to reduce VDAC1. Apoptosis was induced with Ionomycin (20μM) or clotrimazole (40μM). **(D)** Stable knock-down 293T cells for the indicated proteins were used to immunoprecipitate IκBα and its HXKII interaction or VDAC1 interaction was assessed.



D.3.4. I κ B α dissociates from VDAC1 upon apoptosis

I κ B α was found to interact endogenously with members of the PT-pore under physiological conditions. Since I κ B α is able to inhibit apoptosis via its interaction with VDAC1 and HXKII, we anticipated that upon apoptosis these interactions might change. We used HeLa and HCT-116 cells and identified the conditions, which induce apoptosis after 24h but without any morphological or biochemical sign of cell death after 10 hours of induction. We chose 2 different cell lines to avoid cell type-specific effects and used the 10h time point to avoid degradation of proteins due to apoptosis initiation.

Using immunoprecipitations of I κ B α we found that upon induction of apoptosis with arsenic trioxide I κ B α lost its ability to interact with VDAC1 but maintained the HXKII interaction (Figure D.16B). In parallel the lack of overt apoptosis was assessed biochemically using DioC/PI staining 10 hours post treatment with As₂O₃ (Figure D.16A).

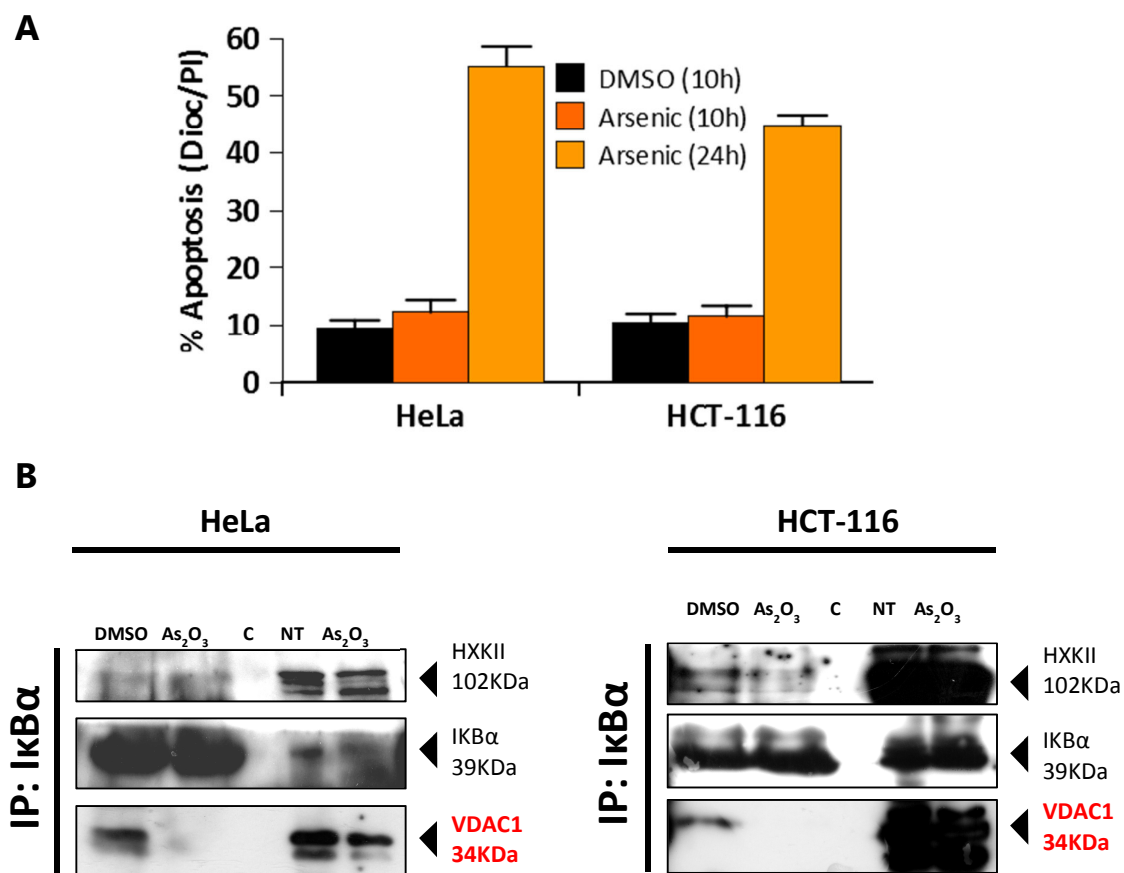


Figure D.16. IκBα dissociates from VDAC1 upon apoptosis. (A) HeLa and HCT-116 cells were treated with arsenic trioxide (As₂O₃) for 10 and 24 hours and apoptosis was assessed with DioC/PI double staining. **(B)** DMSO- and arsenic-treated HeLa and HCT-116 cells were collected 10 hours post-treatment and IκBα was immunoprecipitated from total cell lysates. Hexokinase II and VDAC1 protein levels were examined.



D.4. The anti-apoptotic effect of I κ B α is structurally and functionally independent from its function on NF- κ B inhibition

The discovery of I κ B α as a mitochondrial apoptosis inhibitor prompted questions about this role and the exact mechanism of action. The well-established role of I κ B α as the inhibitor of NF- κ B gives it characteristics of an pro-apoptotic molecule but at the same time I κ B α can very potently inhibit several forms of mitochondrial apoptosis signals. We took the initial step to determine if the NF- κ B activity played any role in the anti-apoptotic activity of I κ B α . Using functional NF- κ B inhibitor mutants of I κ B α we discovered that the anti-apoptotic activity was not altered. A non-degradable form of I κ B α (I κ B α -S) and an I κ B α mutant impaired in NF- κ B inhibition (I κ B α -110A3) (Sachdev *et al.*, 1998a) were as effective as WT-I κ B α for inhibition of apoptosis by Bax and ANT1 (Figure D.17). A reporter assay confirmed that under these conditions the endogenous NF- κ B system was not activated (Figure D.17). In order to explore whether the effect on apoptosis could structurally be separated from the known activity of I κ B α to inhibit NF- κ B, we generated MEET-I κ B α deletion mutants including one of the I κ B α N-terminus and one of its C-terminus that are dispensable and required for NF- κ B inhibition, respectively (Hatada *et al.*, 1993a). An inverse structural requirement was found for apoptosis inhibition: The N-terminus of I κ B α was necessary for apoptosis



inhibition, while the C-terminus could be deleted without compromising apoptosis repression by I κ B α (Figure D.18). The N-terminus of I κ B α interacts with VDAC1, which is the required domain for apoptosis inhibition (Figure D.18B-C). Furthermore, The N-terminus and the first ankyrin repeat constitute a domain sufficient for apoptosis inhibition as identified by sequential deletions of the I κ B α 's C-terminus (Figure D.19). All together these experiments strongly suggested that the effects on apoptosis exerted by I κ B α were independent of NF- κ B inhibition.



D.4.1. Functional mutants of I κ B α can still inhibit apoptosis

Several I κ B α mutants have been established, which alter its function on NF- κ B inhibition. The I κ B α super-repressor (I κ B α -SR) is a mutant of I κ B α with alterations of the amino acids 32 and 36, which render I κ B α resistant to degradation by the proteasome. The protein product of this construct is able to permanently inhibit NF- κ B. Mutations at the ankyrin repeat 2 of I κ B α have been shown to neutralise the ability of I κ B α of inhibiting NF- κ B. The product of the construct I κ B α -110A3 is permanently incapable of NF- κ B inhibition. We used these constructs along with I κ B α -wt in order to determine if alteration of I κ B α for NF- κ B inhibition activity had also any effect on its ability to inhibit apoptosis. We tested if the NF- κ B system was induced by ectopic expression of ANT-1 or Bax in order to establish a system that allows making comparisons. It was found that I κ B α mutants maintained their ability to inhibit ANT-1 or Bax apoptosis (Figure D.17A). NF- κ B activation initiates an anti-apoptotic signal so we sought to ensure that such conditions were not realised. Under our conditions the endogenous NF- κ B system was not induced as confirmed by an assay using a reporter plasmid for NF- κ B activation while the TAX gene expression potently activated the system (Figure D.17B). That further indicated that I κ B α inhibits apoptosis independently of its function on NF- κ B.

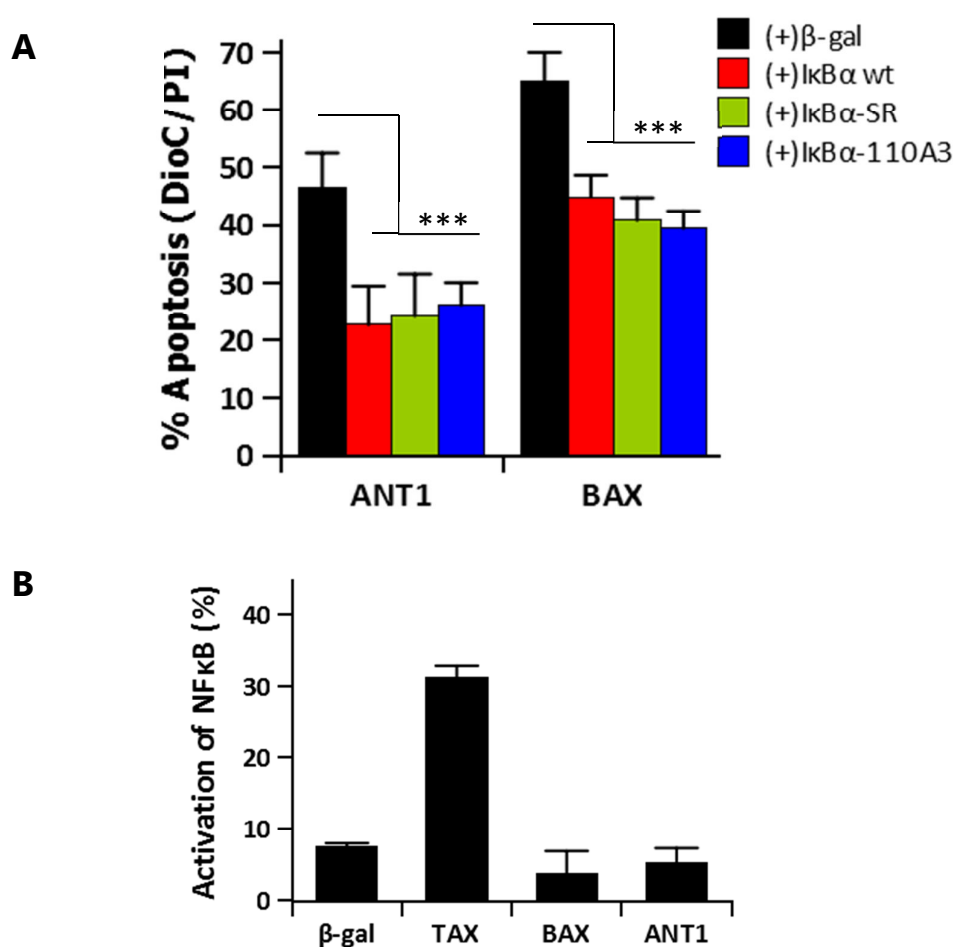


Figure D.17. Mutants of IκBα for NF-κB inhibition function can diminish apoptosis. (A) ANT-1 or BAX were co-transfected with β-gal, IκBα-wt, IκBα-SR, and IκBα-110A3 at a ratio 1:2 in 293T cells. X-fect was used to ensure a high transfection efficiency and apoptosis was quantified 24 hours post-transfection. Means from 4 independent experiments are shown. **(B)** 293T cells were transfected with pcis-hNF-κB and β-gal, TAX, BAX or ANT-1. EGFP was measured 24 hours post transfection to quantify the activation of NF-κB using FACS-FL1 channel. zVAD was used in all cases to inhibit apoptosis and potential degradation of proteins. (***) $p < 0.0001$



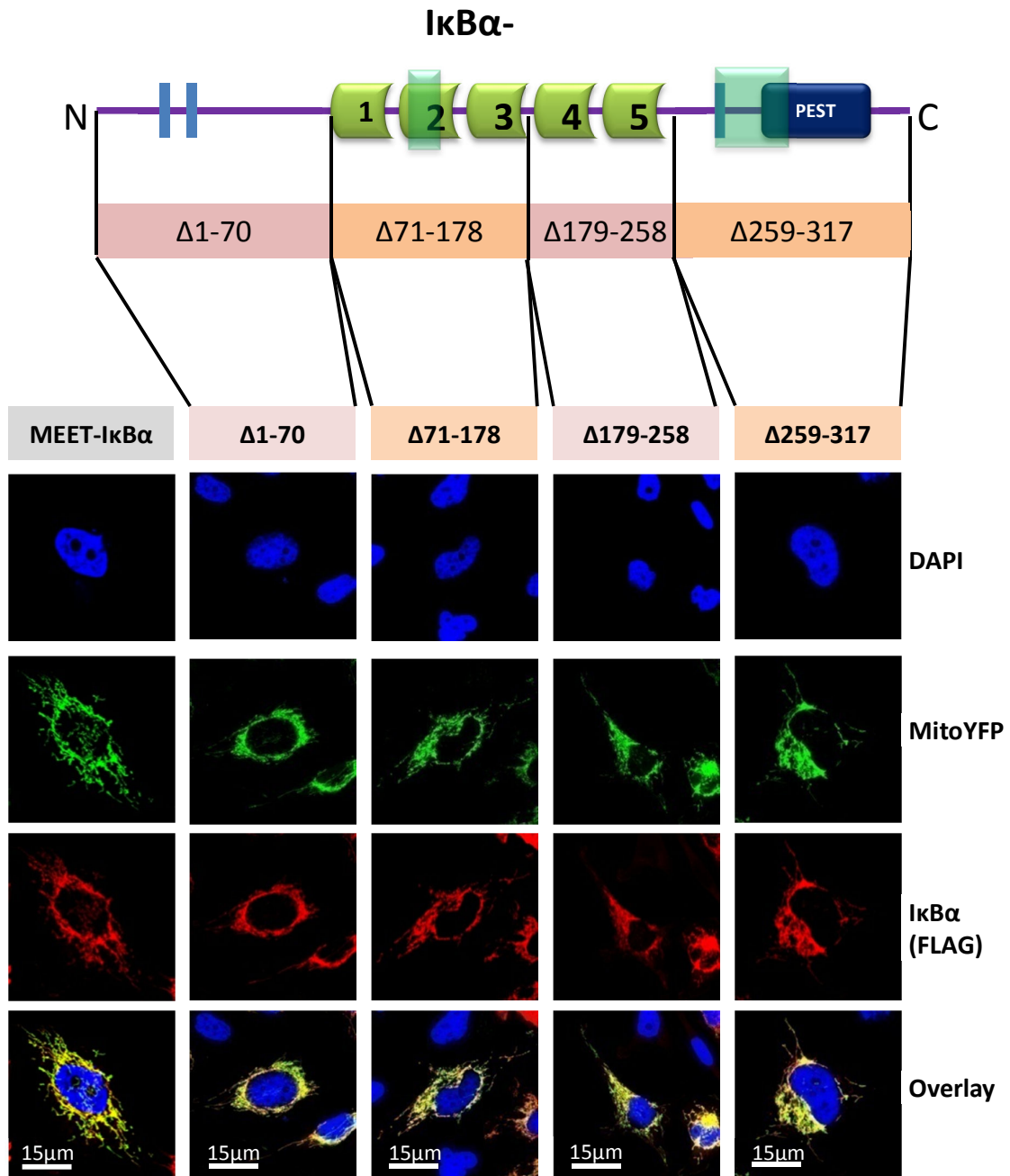
D.4.2. The N-terminal domain of I κ B α is necessary for apoptosis inhibition

Using site-directed mutagenesis and recombinant PCR we generated deletion mutants of I κ B α to identify the domain necessary for apoptosis inhibition. We used the MEET-I κ B α fusion in order to ensure validity of the effect since MEET-I κ B α expression completely abrogates even strong apoptosis signals and allows to separate domains responsible for apoptosis inhibition from those responsible for mitochondrial targeting. We deleted the N-terminus, the ankyrin repeats 1-3, the ankyrin repeat 4-5 and the C-terminus. We examined the correct localisation and expression of these constructs (-FLAG-tagged) with immunofluorescence (Figure D.18A)

In order to identify the necessary domain for apoptosis inhibition we tested these deletions in an apoptosis assay and expressed them together with ANT-1. We found that the N-terminal domain of I κ B α (1-70) was not able to inhibit apoptosis as the other deletions did (Figure D.18B). Having established VDAC1 as the primary target of I κ B α at mitochondria we used immunoprecipitations to test the ability of mutants to interact with this protein. It was found that deletion of the N-terminus renders I κ B α incapable to interact with VDAC1 (Figure D.18C) explaining why this construct cannot inhibit apoptosis.



A



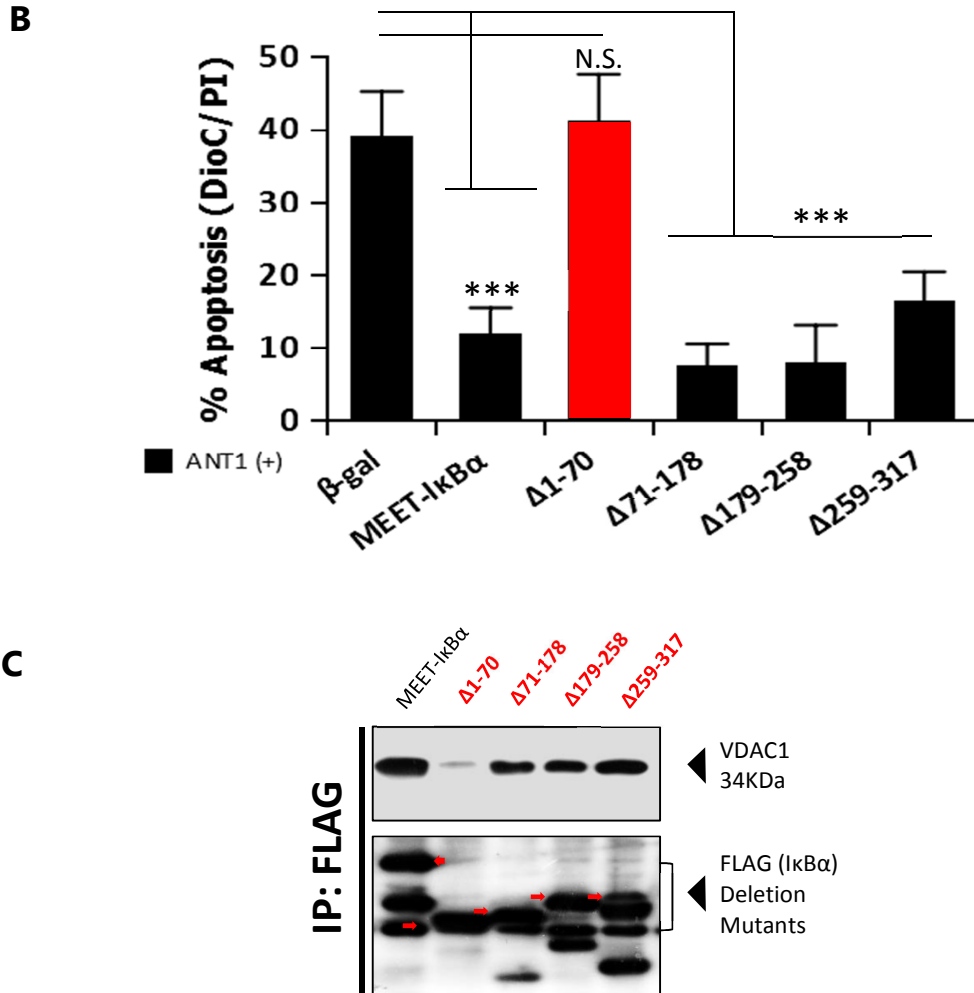


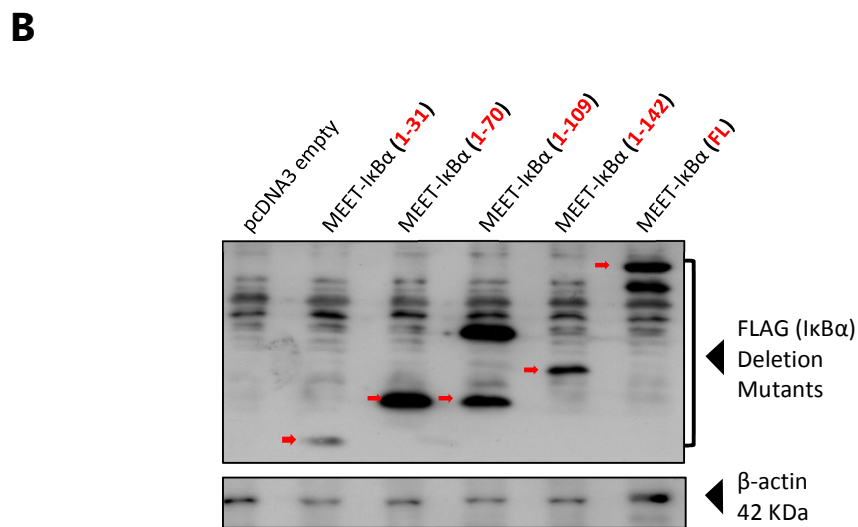
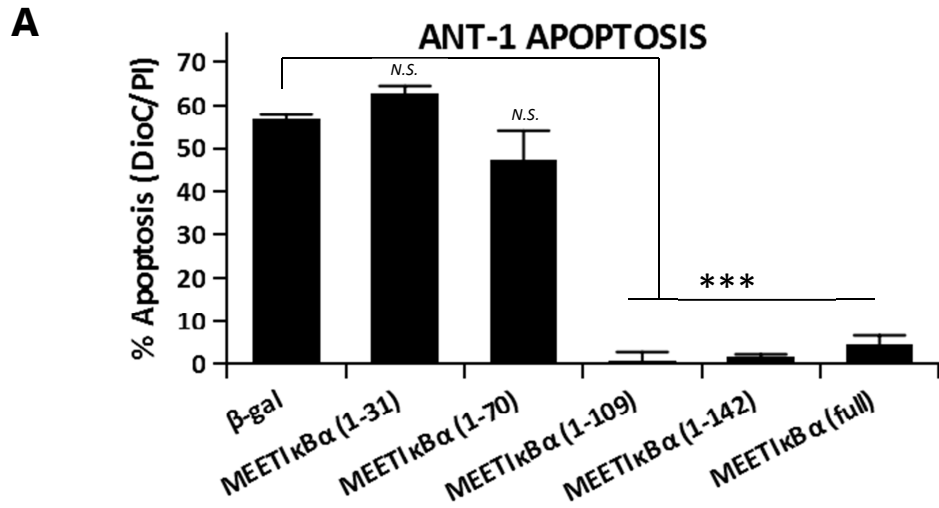
Figure D.18. The N-terminal domain of IκBα interacts with VDAC1 and is necessary for apoptosis inhibition. (A) Schematic representation of the deleted domains of IκBα (upper panel). Confocal microphotographs of HeLa cells transfected with the indicated deletion mutants of MEET-IκBα, which were co-transfected with mitoYFP and stained for FLAG (fused at the C-terminus of IκBα) 24h post transfection. (B) ANT-1 was co-transfected with the indicated deletion mutants of MEET-IκBα and 24h post-transfection apoptosis was assessed by FACS. (C) Deletions of MEET-IκBα were transfected into 293T cells and a FLAG antibody was used to pull-down. A VDAC1 antibody was used to determine interaction.



D.4.3. The I κ B α domain sufficient for apoptosis inhibition consist of the N-terminus and the first ankyrin repeat

As it was found in the previous chapter that the N-terminus of I κ B α is necessary for apoptosis inhibition, the next question we asked was if that N-terminus represents a functional moiety of I κ B α , which is also sufficient to inhibit apoptosis. We used the MEET-I κ B α fusion protein to generate deletion mutants by starting with the expression of only the first 31 amino acids (immediately before the phosphorylation site) of I κ B α , the first 70 amino acids, the amino acids 1-109 (N-terminus and 1st ankyrin repeat) and amino acids 1-142 (N-terminus and ankyrin repeats 1 and 2). A schematic representation of all the deletions for determining the necessary and sufficient domain can be found in figure D.19C

Expression of the above constructs was confirmed by western blotting and they were tested in an apoptosis assay using ANT-1 transfection. The N-terminus only, irrespective of its stability and expression level, was unable to inhibit apoptosis. When the first ankyrin repeat was expressed along with the N-terminus, ANT-1 apoptosis could completely be inhibited as figure D.19A shows. Immunoblotting of cells transfected with the above plasmids confirmed the expression of the genes and the results of the apoptotic assay evaluated with DioC/PI (Figure D19A).





C

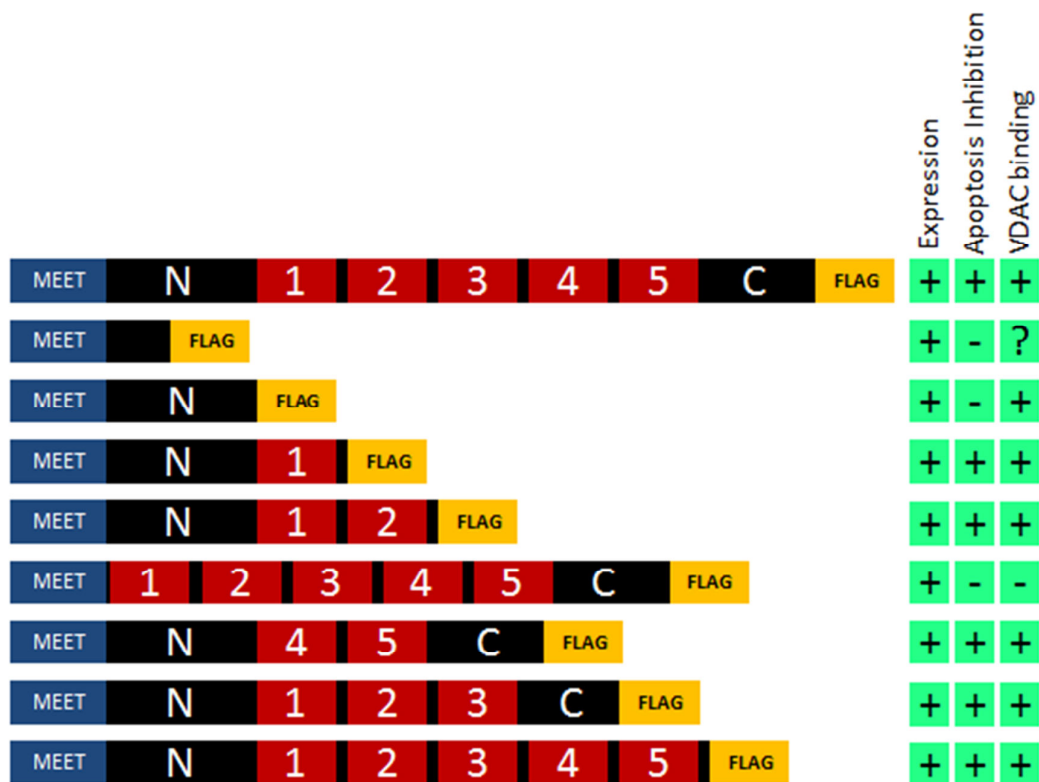


Figure D.19. $\text{I}\kappa\text{B}\alpha$'s N-terminal domains with the first ankyrin repeat are sufficient to inhibit apoptosis. (A) Deletion mutants of MEET- $\text{I}\kappa\text{B}\alpha$ were generated with recombinant PCR and tagged with FLAG[®] (in parentheses are the amino-acids of $\text{I}\kappa\text{B}\alpha$ that are present in each construct). Their potency to inhibit apoptosis was tested in an ectopic expression assay with ANT-1 and quantification was done with FACS and DioC/PI staining. (B) Cells from (A) were lysed with RIPA buffer and lysates were blotted on a 15% SDS-PAGE for each deletion's expression level. (C) Schematic representation of MEET- $\text{I}\kappa\text{B}\alpha$ deletion mutants that were used for identification of the domains necessary and sufficient for apoptosis inhibition. On the right the characteristics of each construct are summarized. Ankyrin repeats are shown in red and N- or C-termini are represented by letters N or C.



D.5. Mechanism of apoptosis inhibition by I κ B α

In this report we unravelled a novel pathway of apoptosis inhibition by I κ B α at mitochondria. We observed a strong inhibitory effect on several signals for apoptosis and complete abrogation on apoptosis induced by ANT-1. The observation that OMM-targeted I κ B α is a stronger inhibitor of apoptosis led us to its target at the outer mitochondria membrane which is VDAC1. Re-constitution, over-expression and knockdown studies, all reveal that I κ B α acts on VDAC1 to inhibit apoptosis and this inhibition is mediated by a domain on I κ B α , which is separable from domains affecting its ability to inhibit NF- κ B. Although the effect was very clear, the mechanism by which I κ B α exerts its effect was, till then, only based on observations and possible interaction partners of I κ B α at the mitochondria level. Further analysis and experiments helped to identify the mechanism involved. I κ B α inhibits apoptosis by inhibiting Bax integration by its association with VDAC1, thereby stabilizing the VDAC1-HXKII complex.



D.5.1. I κ B α inhibits Bax-induced cytochrome-C release

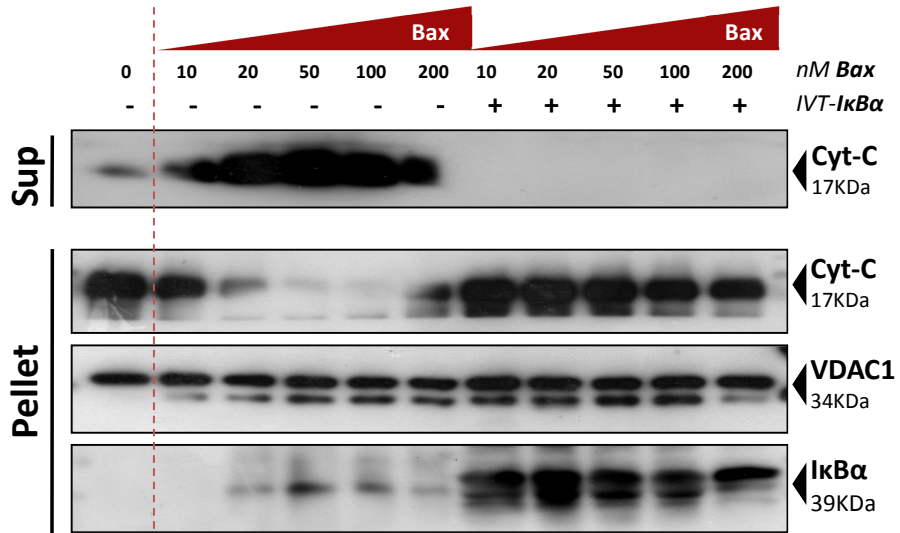
VDAC1 was found to be the target of I κ B α at the mitochondrial level as shown in the previous sections. As a member or effector of the PT-pore VDAC1 is a key molecule involved in apoptosis since upon apoptosis induction it allows the docking of Bax and eventually the permeabilisation and rapture of the OMM leading to cytochrome-C release. The release of apoptogenic factors is the “point of no return” for apoptosis execution and so far anti-apoptotic Bcl-2 family members were the sole guards of the OMM integrity acting directly on Bax (a mechanism that is extensively described in the introduction). We investigated if I κ B α can have any effect on cytochrome-C release since VDAC1 is its target and is involved in this function. We used Bax- β to induce apoptosis in 293T cells and I κ B α targeted to the OMM was able to nearly completely inhibit Bax- β apoptosis (Figure D.20B). Using in vitro-translated (IVT) I κ B α and recombinant Bax we induced cytochrome-C release from isolated mitochondria. When IVT-I κ B α was present cytochrome-C release was completely abrogated (Figure D.20A). We used the same setting but instead of IVT-I κ B α we transfected these cells with MEET-I κ B α . This had the same effect on cytochrome-C release (Figure D.20C). We showed previously that PC3 cells are resistant to apoptotic stimuli but when I κ B α is reduced, these cells are re-sensitized to apoptosis. I κ B α levels at mitochondria are found elevated in those cells, so knockdown would be a physiologically relevant system to explore its function on BAX and VDAC1. We used staurosporin



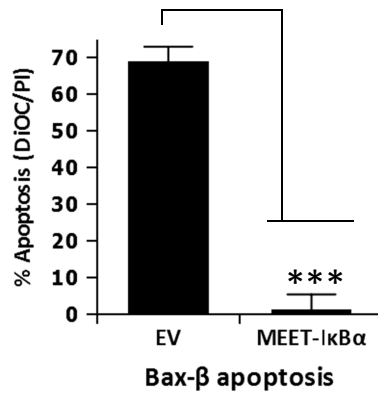
that induces Bax translocation to mitochondria and sought to investigate the effect of I κ B α on cytochrome-C release. When I κ B α (PC3/scramble) was present and after staurosporin administration, cytochrome-C was released after 6-12 hours and Bax was moderately integrated in the OMM. When PC3/shI κ B α cells were used and I κ B α was found absent in mitochondria fractions, cytochrome-C could be released after only 2 hours of STS administration. Bax was found to be strongly integrated in the OMM when compared with the PC3/scramble cells on the same membrane (Figure D.20D). All the above results indicated a clear effect of I κ B α on Bax-induced cytochrome-C release.



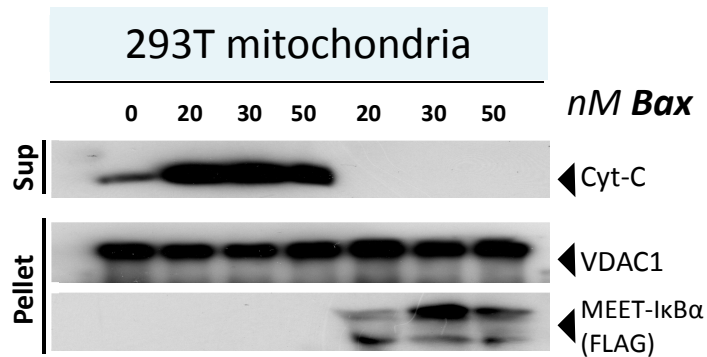
A



B



C



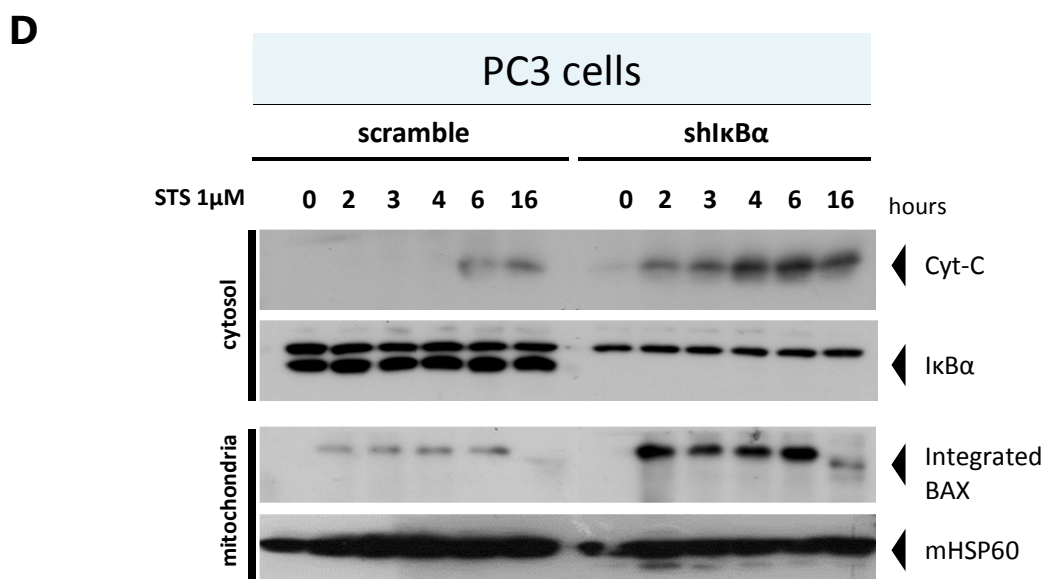


Figure D.20. IkB α inhibits Bax-induced cytochrome-C release. (A)

Mitochondria from 293T cells were isolated and kept on ice. Reticulocyte lysate from IVT- β -gal and reticulocyte lysate from IVT-IkB α were incubated with mitochondria at 25°C for 30min. Increasing concentration of recombinant-Bax were added afterwards and cytochrome release assessed. **(B)** 293T cells were transfected with an empty vector (EV) and MEET-IkB α and 24h later they were transfected with Bax- β . Apoptosis was quantified by DioC/PI staining and FACS. **(C)** 293T transfected either with an empty vector or MEET-IkB α were used and their mitochondria were isolated. Increasing concentrations of recombinant Bax were added and cytochrome-C release was assessed. **(D)** PC3/scramble and PC3/shIkB α cells were treated with 1 μ M staurosporine (STS) for the indicated times. Subsequently, alkali wash was used on isolated mitochondria according to standard methods and cytochrome-C release or Bax integration was qualitatively measured by a 15% SDS-PAGE. IkB α levels were measured before the alkali wash. An unspecific band was observed showing equal loading. 16 hours after STS administration integrated Bax appears cleaved in both cell lines possibly due to general protein degradation from excessive STS apoptosis.



D.5.2. I κ B α stabilizes the VDAC1-HXKII complex

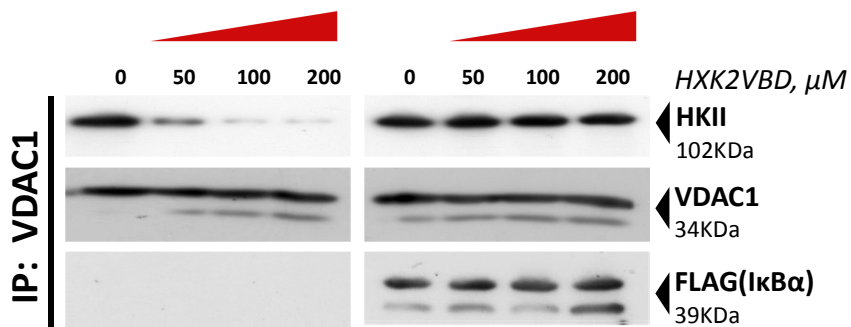
The ability of I κ B α to inhibit Bax-induced cytochrome-C gave further insight into the mechanism that I κ B α uses to inhibit apoptosis. Since Bax integration was delayed and cytochrome-C was inhibited, VDAC1 was the most obvious molecule to be investigated for a postulated mechanism. VDAC1 forms a complex with Hexokinase II (HXKII) and this complex is anti-apoptotic since HXKII, which is found bound to VDAC1 especially in cancer cells, makes the VDAC1 docking sites for Bax inaccessible. I speculated that I κ B α could act on this complex and possibly stabilise it so that apoptosis is inhibited. We described in the introduction that the enterobacterial *E. coli* protein FimA was found to stabilise this complex and to confer resistance to colon cancer cells. We followed the same strategy to explore if I κ B α had similar characteristics comparable to the bacterial protein. We designed a peptide that resembles the initial 15 amino acids of HXKII and when applied to isolated mitochondria, dissociates HXKII from them. The antibiotic clotrimazole has the same properties as the peptide but its effects are more unspecific.

We obtained indications that I κ B α is probably involved in this complex, initially when we knocked-down VDAC1 or HXKII (see figure D.15). The finding that I κ B α was able to endogenously interact with HXKII (Figure D.13) was an additional step towards the characterization of the mechanism. I κ B α was found to stabilise the VDAC1-HXKII complex in mitochondria from HeLa cells transfected with I κ B α -wt since even maximal amounts of HXK2VBD peptide were unable to dissociate

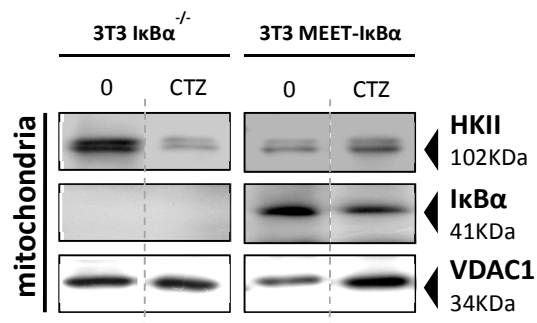


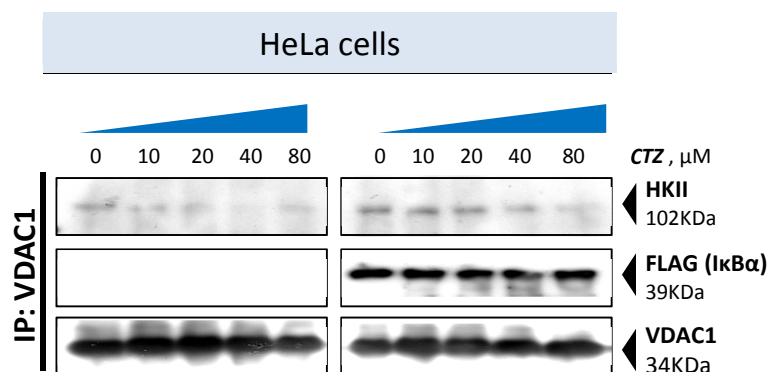
HXKII when I κ B α was present (Figure D.21A). Moreover, mitochondria from HeLa cells transfected with I κ B α -wt became more resistant to dissociation caused by clotrimazole since greater amounts of the drug were required for the dissociation (Figure D.21C). Similar results were observed in a genetically more defined system when we used I κ B α ^{-/-} and MEET-I κ B α reconstituted cells (Figure D.21B).

A



B



**C****Figure D.21. IkB α stabilizes the VDAC1-HXKII complex. (A)**

Mitochondria from control- or IkB α -transfected HeLa cells were treated with the HKII-release peptide HXK2VBD and the interaction between HKII and VDAC1 was monitored with immunoprecipitations. **(B)** Mitochondria isolated from IkB α ^{-/-} or MEET-IkB α -reconstituted 3T3 cells were treated with clotrimazole (CTZ, 80 μ M, 30 min). Subsequently, mitochondria were washed extensively, pelleted and then the indicated proteins were detected associated with mitochondria. (Note that all lysates were loaded on the same gel and blotted onto the same membrane but between 0 and 80 μ M CTZ the lanes used for controls and the drug were not added. Only the necessary parts of the blots are shown.) **(C)** Mitochondria from control- or IkB α -transfected HeLa cells were treated with increasing amounts of clotrimazole. The stability of the VDAC1-HXKII interaction was monitored with immunoprecipitations of VDAC1.



E. Discussion



E.1. Summary of the study

In most cell types the activation of the transcription factor NF- κ B leads to an anti-apoptotic response through the transcriptional upregulation of cell death inhibitors. I have demonstrated here that, unexpectedly, the NF- κ B target gene *I κ B α* , besides its inhibitory effect on NF- κ B and its pro-apoptotic consequence, also guards the integrity of the OMM and thereby represses the intrinsic pathway for apoptosis. *I κ B α* can exert its anti-apoptotic effect due to its stable interaction with VDAC1 and HKII through its N-terminal domain as mapping experiments revealed. *I κ B α* stabilises the VDAC1-HXKII complex and inhibits Bax recruitment and cytochrome-C release. The PT-pore, which spans the mitochondrial membranes, is actively involved in this process since I could demonstrate that *I κ B α* uses the PT-pore to exert its anti-apoptotic effect. Re-constitution and mapping studies reveal that the domains of *I κ B α* involved in apoptosis inhibition and in NF- κ B inhibition are separate. Overall, I show here that *I κ B α* constitutes a target for cancer therapeutics since specific down-regulation sensitised previously non-sensitive tumour cells to chemotherapeutics: tumours in nude mice were considerably more sensitive to chemotherapy when *I κ B α* was knocked-down in comparison with control tumours that showed no sensitivity to small doses of the drug. Further research will be required for the design of molecules specifically targeting the VDAC1/HKII-*I κ B α* interaction.



E.2. Mitochondrial localisation of I κ B α and apoptosis inhibition

Consistent with the identification of I κ B α in mitochondria (Bottero *et al.*, 2001; Cogswell *et al.*, 2003) we confirm in this study that I κ B α is indeed found associated with mitochondria of several cancer cell lines (Figure D.4). Of note is the fact that in cell lines with constitutive NF- κ B activity the mitochondrial localisation of I κ B α is almost complete (Figure D.4A). The kinetics of I κ B α degradation within mitochondria upon TNF α treatment were almost identical with the cytosolic effect (Figure D.2). That was an interesting finding since the proteasome does not have access to the inner compartments of mitochondria (Cogswell *et al.*, 2003) and a possible transport of I κ B α out of mitochondria is unlikely unless it is very fast. We tested the kinetics of mitochondrial and cytosolic I κ B α degradation upon specific blockage of the proteasome using the specific and irreversible inhibitor lactacystin. The results indicate that the mechanism of I κ B α degradation in mitochondrial differs from the one in the cytosol (Figure D.3). Under these conditions I κ B α in mitochondria is still degraded upon TNF α treatment but at the same time its degradation is blocked in the cytosol. Consequently, a different mechanism for I κ B α degradation is suggested by these results. This could be another protease that is recruited into the mitochondria where it degrades I κ B α . Calpain has been shown to cleave I κ B α by interacting with residues 50-51 but the degradation effect was so far not tested on mitochondrial I κ B α (Schaecher *et al.*, 2004). Although the



finding that mitochondrial I κ B α is possibly degraded by another mechanism is interesting, it is not providing additional information about the OMM-associated I κ B α that we show here is anti-apoptotic. The lactacystin experiments were looking at the I κ B α localised at the IMS.

As a predominantly cytosolic protein with a pro-apoptotic function (since it inhibits the anti-apoptotic NF- κ B transcription factor) mutational changes in I κ B α were so far mainly connected with constitutive NF- κ B activity (Biswas *et al.*, 2004; Gasparian *et al.*, 2002a; Robe *et al.*, 2004; Romieu-Mourez *et al.*, 2001). Trying to identify possible differences between cells with normal NF- κ B activity or cells with constitutively active NF- κ B, we separated mitochondria and cytosol from HeLa, MCF and MDA-231 cells. There are so far no data showing the expression levels of the I κ B α protein in mitochondria and our results compared for the first time three different cell lines, one with constitutively active NF- κ B (MDA-MB-231) and two with normal NF- κ B activity (Hela, MCF7). When analysing the results shown in figure D.4B we observed that in the MDA-MB-231 cell line, I κ B α levels in mitochondria are comparatively higher than in MCF7 cells (from the same tissue) or in HeLa cells (another carcinoma). On the other hand, the levels of the protein in the cytosol are diminished in MDA-MB-231 cells, probably because of the constitutive degradation by the proteasome as observed in other tumours (Biswas *et al.*, 2004; Gasparian *et al.*, 2002a; Robe *et al.*, 2004; Romieu-Mourez *et al.*, 2001).



Using a proteinase K assay, I κ B α was found within the mitochondria and at the OMM of several cell lines including tumour or normal cell lines (Figure D.4D). Various genes implicated in apoptosis induction or inhibition have the dominant capacity to exert their effect upon overexpression. This feature is even conserved across species (McCarthy & Dixit, 1998). For this reason and since I κ B α is found to accumulate at mitochondria in cells with constitutive NF- κ B activity, it was interesting to investigate the effect of I κ B α when over-expressed. We generated a fusion protein with the first 47 amino acids of the MCR1 yeast protein to target the I κ B α to the mitochondria intermembrane space (IMS) and the outer mitochondrial membrane (Figure D.5). The localisation of this protein to mitochondria is of particular interest since they are pivotal organelles for apoptosis control that contain numerous cell death regulators. Ultimately, they govern the disruption of the outer mitochondrial membrane and the release of pro-apoptotic factors such as cytochrome c and AIF, which cause cell destruction. We show here that when I κ B α is specifically targeted to mitochondria it can inhibit even strong apoptosis signals such as ANT-1 (Figure D.6). One recent report suggests that ANT-1 induces apoptosis by titrating out nuclear NF- κ B. However, the Hela cells used in this study do not contain constitutively active NF- κ B (Grimm *et al.*, 1996; Zamora *et al.*, 2004). Rather, we believe that ANT-1 is a specific inducer of the PT pore for apoptosis, which is supported by its dependence on Cyp-D (Figure D.14). Upon I κ B α expression we have also observed a reduced break-down of the mitochondrial



membrane potential $\Delta\Psi_m$, an early sign of apoptosis induction, when cells were treated with an ionophore that increases the mitochondrial Ca^{2+} level (Figure D.6C). This, together with ANT-1 apoptosis inhibition, supports the notion of I κ B α at mitochondria as an apoptosis inhibitor. A dose response was also tested by transfecting different amounts of I κ B α or mcr1-I κ B α plasmids while maintaining the ANT-1 levels. At a 2:1 ratio with an ANT-1 expression construct I κ B α -wt and mitochondria-targeted I κ B α (mcr1-I κ B α) could completely abrogate the ANT-1 apoptosis signal (Figure D.6A). The effect was not cell-specific since MCF7 and HeLa cells responded in the same manner upon I κ B α and ANT-1 co-transfection (Figure D.6B).

The localisation signal of the yeast *MCR1* gene is not specific for any sub-organelle compartment, however specific mutagenesis on two amino acids targeted mcr1-I κ B α almost completely to the IMS. (Hahne *et al.*, 1994a; Haucke *et al.*, 1997; Meineke *et al.*, 2008). For this reason I generated fusion constructs for this specific targeting of I κ B α . Localisation sequences from Smac/DIABLO for the IMS and mitoNEET for the OMM were fused with I κ B α thereby generating S/D-I κ B α and MEET-I κ B α (Figure D.7). Upon ANT-1 transfection only MEET-I κ B α and I κ B α -wt were able to inhibit the apoptotic signal suggesting that only when I κ B α associates with the OMM, it can inhibit apoptosis (Figure D.8). Other apoptosis inducers were likewise tested such as t-BID, VDAC1, Bax or Caspase-8. In all cases I κ B α targeted to the OMM could inhibit apoptosis. For caspase-8 the effect of



I κ B α -wt or of MEET-I κ B α was only moderate suggesting a specificity of the signals that mitochondrial I κ B α can inhibit. Caspase-8 activation can be the end result of apoptosis induced by the extrinsic pathway but a communication exists between the plasma membrane receptor and the mitochondria-mediated pathways through BID. Caspase-8 can cleave and activate BID and this is possibly underlying the effect on apoptosis inhibition that we observe (Figure D.8C).

I κ B β was not found at mitochondria in the study that discovered I κ B α in this organelle (Cogswell *et al.*, 2003). However, I κ B β shares extensive homology with I κ B α , consequently our intention was to evaluate the capacity of I κ B β to inhibit apoptosis. Figure D.9C shows that I κ B β does not inhibit apoptosis. The specificity of the apoptosis inhibition was further highlighted by an experimental setting that had I κ B α sequestered in the cytosol, which rendered it unable to inhibit apoptosis: the I κ B α / β -actin fusion protein was generated in order to retain the I κ B α protein in the cytosol (Figure D.9A-9B). ANT-1 apoptosis could not be inhibited by this fusion construct (Figure D.9C). Hence, the sole site for apoptosis inhibition by I κ B α remained the outer membrane of mitochondria.

Re-constitution studies have long served as an option to evaluate the function of a protein. We obtained I κ B α ^{-/-} fibroblasts in order to examine the effects of I κ B α on apoptosis since such an investigation was not performed when the I κ B α ^{-/-} mice were generated (Beg *et al.*, 1995a). These mice show constitutive NF- κ B activation, which we confirmed confers apoptosis resistance to those cells



(Figure D.10D). In order to avoid overexpression of proteins we used a lentiviral system that allowed us to transduce the knock-out fibroblasts with different M.O.I.s so that the reconstitution of the proteins would be at the same or lower level than the 3T3 WT fibroblasts. We used $I\kappa B\alpha/\beta$ -actin, and empty vector (EV) and MEET- $I\kappa B\alpha$ to target $I\kappa B\alpha$ only to the cytosol or to the OMM. Since these cells show constitutive NF- κB activity we used a non-radioactive- κB -oligo-pull-down assay using nuclear extracts to determine the status of NF- κB before and after reconstitution. Figure D.10E show that upon reconstitution of $I\kappa B\alpha$ at physiological levels (Figure D.10B) NF- κB activity was diminished in both cases making our results comparable and independent of any apoptosis resistance conferred by NF- κB activation (Figure D.10E). After setting up our cellular system, I used apoptosis assays in order to evaluate the effect of $I\kappa B\alpha$ variants in specific cellular compartments after apoptosis induction. OMM-targeted $I\kappa B\alpha$ conferred resistance to all apoptotic stimuli used in re-constituted cells, despite the in-activation of the anti-apoptotic NF- κB (Figure D.10C). Cytosol-sequestered $I\kappa B\alpha$ was unable to inhibit apoptosis and in most cases even sensitised the cells for apoptosis possibly due to the inhibition of NF- κB (Figure D.10E).



E.3. Evidence for resistance to apoptosis through mitochondrial I κ B α : proof of concept

In order to investigate the role of I κ B α at the OMM for apoptosis regulation and its function as a NF- κ B target gene we made use of cells with constitutive NF- κ B activity that express high level of I κ B α at the OMM (Figure D.4A-B). The selection of this cell type was particularly important since I previously identified that these cells (MDA-MB-231), apart from the high levels of I κ B α at mitochondria, have diminished levels of I κ B α in the cytosol. Hence, RNAi against I κ B α would primarily down-regulate the mitochondria-associated I κ B α . Using lentiviruses we stably knocked down *I κ B α* in MDA-MB-231 cells. NF- κ B remained unaffected indicating that it is activated by a process other than I κ B α degradation in these cells (Prasad *et al.*, 2009) (Figure D.11A). The treatment with various apoptosis inducers revealed that *I κ B α* reduction rendered these cells significantly more sensitive to apoptosis (Figure D.11). A similar effect was observed when *I κ B α* was targeted in PC3 cells, which likewise display constitutively active NF- κ B and a high amount of I κ B α at mitochondria (Figure D.11).

Treatment-resistance of tumour cells with constitutively active NF- κ B has been reported extensively (Lee *et al.*, 1999; Nakshatri *et al.*, 1997a; Sovak *et al.*, 1997; Wang *et al.*, 1996) emphasising the importance of our experimental findings. Indeed MDA-MB-231 cells are extremely resistant to apoptosis (Ciucci *et al.*, 2006)



and several studies report that inhibition of NF- κ B made these cells more sensitive to apoptosis signals. However, we have emphasised in the introductory sections of this thesis the importance of NF- κ B in scenarios other than apoptosis. Targeting NF- κ B would have detrimental effects on the immune response, something that needs to be avoided. MDA-MB-231 cells have constitutively active NF- κ B and the knockdown of I κ B α , which resides primarily at mitochondria, did not affect its activation and I κ B α . Targeting I κ B α proved to be an effective method to sensitise these cells to the well-established chemotherapeutic doxorubicin. A small *in vivo* pilot study using mice engrafted with MDA-MB-231 cells confirmed my *in vitro* results and doxorubicin-treated tumours with I κ B α shrank substantially, whereas normal tumours were unresponsive to doxorubicin and continued to grow (Figure D.12).

PC3 cells display the same characteristics as the MDA-MB-231 cells in terms of resistance to apoptosis and chemotherapy agents due to constitutive NF- κ B activation (Gasparian *et al.*, 2002a; Gasparian *et al.*, 2002b; Lu *et al.*, 2004; Muenchen *et al.*, 2000). The underlying mechanism for constitutive NF- κ B activation is not known but I κ B α was also found associating with the OMM in those cells (Figure D.4) and knock-down of I κ B α had no effect on further activating NF- κ B (Figure D.11A). Docetaxel has been identified as an efficient chemotherapeutic to target breast, lung and prostate cancer and has extensively been used in the clinic (Morse *et al.*, 2005). However, PC3 cells display resistance to



this drug (Figure D.11E). I κ B α knockdown sensitised these cells to docetaxel and efficiently induced apoptosis (Figure D.11E).

MDA-MB-231 and PC3 are two cell lines that exhibit the enhanced accumulation of I κ B α at mitochondria and both of them were sensitised upon I κ B α RNAi-mediated reduction. In order to explore how universal is the effect on apoptosis resistance we also tested the human colon cancer cell line (HCT-116) which likewise is extremely resistant to apoptosis and displays constitutive NF- κ B activity. An effect on apoptosis sensitisation was also observed with those cells when I κ B α was reduced. We used staurosporin and ionomycin, which initiate apoptosis through Bax translocation to mitochondria and activation of the PT-pore through calcium influx, respectively (Schubert & Grimm, 2004; Zhang *et al.*, 2004). In both cases I κ B α was found to be the gatekeeper of mitochondria protecting their integrity through a mechanism that we will discuss below in more detail. Our findings supported our initial concept that I κ B α accumulates at mitochondria, is protected from constitutive degradation and protects cells from apoptosis. We demonstrated that I κ B α is, possibly among others, a molecule that confers resistance to apoptosis and chemotherapeutics through its interaction with mitochondria.



E.4. Mechanism of apoptosis inhibition by I κ B α

The outer mitochondria membrane is a very important site for apoptosis regulation since it is there where several protein-protein interactions take place to protect or permeabilise this organelle in order to induce apoptosis. The localisation of NF- κ B subunits to mitochondria was revealed previously (Bottero *et al.*, 2001; Cogswell *et al.*, 2003), but this was limited to the matrix and the intermembrane space of this organelle and its role for apoptosis remained unknown. In those studies overexpressed ANT-1 and I κ B α were found to interact. We proved a more direct involvement of I κ B α in apoptosis. The mechanism that underlies the inhibition effect of mitochondrial I κ B α was the subject of this study. Testing a set of I κ B α interactors (Figure D.13) indicated many potential mechanisms. As endogenous I κ B α is able to physically interact with endogenous VDAC1, ANT-1, HXKII and CK1, all of which are members of the classical PT-pore, this protein complex became the prime candidate for the target of I κ B α (Figure D.13). Indeed, my work revealed that I κ B α specifically associates with the OMM and inhibits apoptosis via its most prominent partners VDAC and HXKII. I κ B α stabilises the complex of HKII and VDAC1 since a peptide able to specifically dissociate HXKII from VDAC1 was incapable to do so when I κ B α was present (Figure D.21). Clotrimazole, which also has the ability of HXKII dissociation from



mitochondria, was also diminished by the presence of I κ B α since greater amounts of the drug were required for dissociation (Figure D.21C).

Hexokinase II was also found to be necessary for the inhibitory effect of I κ B α . shRNA-mediated reduction of the protein levels rendered I κ B α incapable of inhibiting apoptosis even when overexpressed (Figure D.15B). However, since HXKII is itself an apoptosis inhibitor (Pastorino *et al.*, 2002), silencing could have contributed to the enhanced apoptosis that was observed. Therefore, HXKII might also be an important component of the mechanism of I κ B α inhibition but with our data we cannot claim its necessity for apoptosis inhibition through I κ B α .

HKII is known to bind to VDAC1, the most abundant VDAC isoform, via its N-terminus and its putative BH4 domain (Pastorino & Hoek, 2008a). This seems to favour the oligomeric association of VDAC proteins and keeps these proteins in a state, which renders them unable to interact with Bax for apoptosis induction. With this novel activity at the OMM, I κ B α exerts the same effect as the enterobacterial fimA protein, which likewise stabilises the VDAC-HKII interaction and inhibits apoptosis (Sukumaran *et al.*, 2010a). I κ B α can therefore potentially represent the human equivalent of this bacterial protein and contribute to apoptosis inhibition observed in cancer cells, in particular since we have data showing increased levels of I κ B α at mitochondria of several cancer cell lines (see chapter D.1.4 above). The importance of our findings is striking for cancer cells but one can extrapolate the same level of significance to normal cells. The overall expression of I κ B α in a



cancer cell with constitutive NF- κ B is either not altered or reduced (Nakshatri *et al.*, 1997a). However, our results indicate that there is a preferential shift of the localisation of I κ B α from the cytosol to the mitochondria altering the apoptosis sensitivity of the cell. The best binding partner of I κ B α , p65 (relA), resides in the nucleus of cells with constitutive NF- κ B activity and that disturbs the interaction of I κ B α and p65. Newly synthesized I κ B α or I κ B α resistant to degradation would therefore complex with the VDAC1 as its alternative interactor. On the other hand, we have also observed I κ B α at mitochondria of 3T3 WT cells. I κ B α appears to be able to bind both p65 and VDAC1 simultaneously but since the affinity of p65 with I κ B α is much greater the ability of VDAC1 binding, despite being present, appears reduced (Figure E.1) .

Using recombinant and IVT proteins we were able to show that Bax-mediated cytochrome-C release was blocked by I κ B α (Figure D.20A). Using PC3 cells, which have previously been shown to be resistant to apoptosis by chemotherapeutics but became sensitive upon I κ B α knockdown, we observed that mitochondrial I κ B α delays the release of cytochrome-C via its ability to inhibit enhanced Bax integration into the OMM (Figure D.20D). This effect is possibly due to the stabilisation of the VDAC1-HXKII complex since VDAC sites are occupied by HXKII and are not accessible for Bax.

Cyp-D knockout fibroblasts (*ppif*^{-/-}) show increased resistance to cell death (Baines *et al.*, 2005) and are unable to undergo permeability transition since they



cannot open the PT-pore. With the help of these cells ANT-1 was found to induce apoptosis through the PT-pore confirming the observation of Bauer *et al* (1999) with bongkreikic acid, a compound that specifically inhibits the PT-pore (Bauer *et al.*, 1999). The requirement for cyp-D in the mitochondrial matrix to observe I κ B α 's apoptosis repression (Figure D.14B) suggests that mitochondrial I κ B α is a component or an effector of the PT pore. This protein complex connects the inner with the outer mitochondrial membrane and is converted into an unspecific channel for apoptosis induction (Zamzami *et al.*, 2005). Its structure consists of cypD, ANT and VDAC proteins as the core constituents with probably many more associated regulatory factors (Verrier *et al.*, 2004). The role of the latter two components has been questioned, though (Baines *et al.*, 2007; Kokoszka *et al.*, 2004) but an extensive collection of publications in the scientific literature exists arguing for their function in mitochondrial apoptosis (Shoshan-Barmatz *et al.*, 2010; Tsujimoto & Shimizu, 2002).

One of the publications critical of the contribution of VDAC molecules to apoptosis induction via the PT pore observed no effect on cell death when VDAC1 and VDAC3 were genetically removed and VDAC2 was reduced by knock-down with RNAi (Baines *et al.*, 2007). Our results could contribute to an explanation as I κ B α 's apoptosis inhibition ability depends on VDAC1 (Figure D.15A). Firstly, above study used VDAC1/3^(-/-/-) cells with only a knockdown of the VDAC2 isoform since VDAC2-null mice are not viable. One cannot exclude a compensatory effect of



VDAC2 for apoptosis since complete knockdown would have killed the cells. Secondly, while VDAC silencing would make the cells more resistant for cell death it would also mask the anti-apoptotic activity of I κ B α and – on balance - could thereby abrogate any net effect on cell death. Analysis of the I κ B α -re-constituted MEF cells with shRNA against VDAC1 indicated similar effects as observed in human cell lines (Figure D.15C), which contributed to the identification of VDAC1 as a target of I κ B α . The PT pore has been shown to mediate necrosis but a growing body of evidence also implicates it in apoptosis regulation (Kinnally et al., 2010) as extensively described in the introductory sections of this study. Possibly, the concentration of the cell death inducers in those studies claiming sole involvement in necrosis could have been too high to detect apoptosis via the PT pore (Baines et al., 2005; Nakagawa et al., 2005). As was previously analysed the intensity and the quality of the signal are the crucial modulators of apoptosis versus necrosis execution through the PT-pore.

We are now able to explain the effects that we see on apoptosis sensitisation upon down-regulation of I κ B α . PC3 cells that are responding poorly to staurosporin became extremely sensitised to apoptosis when I κ B α was not present to protect them from Bax integration and cytochrome-C release (Figure D.20D). I κ B α guards the integrity of the OMM by stabilising the VDAC1-HXKII complex and thereby inhibiting Bax translocation to mitochondria and cytochrome-C release. Since I κ B α is a gatekeeper of the PT-pore opening we



sought to investigate the initial stages of apoptosis when only biochemical changes upstream of effector pathways occur. We induced apoptosis with As_2O_3 which was previously shown to be executed through VDAC1 (Yu *et al.*, 2007; Zheng *et al.*, 2004) and observed the VDAC1-I κ B α interaction ten hours after drug administration when no morphological changes to cells were observed and mitochondria and plasma membrane integrity were not affected (Figure D.16A). Upon apoptosis induction and early in the apoptotic phase, I κ B α was released from VDAC1 (Figure D.16B). This result confirms our hypothesis that I κ B α is a gatekeeper of the mitochondrial permeability transition. Cells exhibited extensive apoptosis after 24 hours when I κ B α was absent and could not stabilise the VDAC1-HXKII complex to inhibit Bax translocation and binding to VDACS for subsequently cytochrome-C release.

Whether VDAC1 contributes to apoptosis or not and whether it is a member of the PT-megapore, along with ANT-1, was not the main subject of this study. However, all our data indicate that VDAC1 constitutes the target of I κ B α at the OMM and this transporter therefore does regulate apoptosis. Through this interaction I κ B α is able to stabilise the anti-apoptotic VDAC1-HXKII complex and inhibit apoptosis. If *ppif*^{-/-} cells are regarded as a suitable tool to show the role of the PT-pore in apoptosis, then I κ B α is most probably a member of the PT-pore complex, since I could show the requirement of Cyp-D for apoptosis inhibition by I κ B α (Figure D.14). Nevertheless, the mechanism that we propose in this study is



based on mitochondrial apoptosis effectors that are not part of the scientific debate on the identity of the PT pore components. The VDAC1/HXKII complex has been established as an anti-apoptotic complex at the OMM, which $\text{I}\kappa\text{B}\alpha$ can stabilise thereby inhibiting cytochrome-C release and Bax integration.

The current status of the debate on the composition of the PT-pore is that no clear conclusions can be derived on the dispensability of any of its components. The classical PT-pore seems to be the key complex for the interpretation of our results since we can see in all cases the requirement of cyclophilin-D, VDAC1 and hexokinase from $\text{I}\kappa\text{B}\alpha$ to inhibit apoptosis. $\text{I}\kappa\text{B}\alpha$ requires all three and possibly other proteins in this complex to exert its anti-apoptotic effect. Whether any of the three components is or is not at the same time a member of the PT pore, is unresolved. Still, our data indicate that $\text{I}\kappa\text{B}\alpha$ is most likely an effector of a protein complex that includes all the aforementioned factors and is possibly by the same token a component of the PT- pore since the associations were detected in resting cells.



E.5. I κ B α domain mapping and its dual activity

As the inhibitor of the anti-apoptotic transcription factor NF- κ B, I κ B α exerts a pro-apoptotic function, which is counteracted by its novel activity to repress apoptosis at the OMM as revealed in this work. Therefore, during its degradation in the activation cycle of NF- κ B, while it is re-synthesised as a consequence of its being a target gene of the transcriptional activity of NF- κ B, I κ B α contributes to the balance of survival and cell death signals.. This is especially important as many of the signals that activate NF- κ B constitute cell stress.

Our mapping experiments indicated that, in contrast to the inhibition of NF- κ B, which is exerted through the C-terminus and the ankyrin repeats, the N-terminus of I κ B α is responsible for its repression of apoptosis (Figure D.18). The N-terminus is a regulatory domain for degradation of I κ B α (Traenckner *et al.*, 1995) in the canonical NF- κ B signalling but it does not display domain interactions with p65/p50 heterodimers (Baeuerle, 1998; Van Antwerp & Verma, 1996). Immunoprecipitations with MEET-I κ B α lacking the N-terminus displayed a minimal ability to interact with VDAC1 explaining the inability of this deletion mutant to inhibit apoptosis (Figure D.18). Moreover, specific I κ B α mutants with either reduced or constitutive NF- κ B inhibition (Sachdev *et al.*, 1998b; Traenckner *et al.*, 1995) showed an identical ability to inhibit Bax- or ANT1-induced apoptosis



(Figure D.17A). I κ B α -SR, for example, is able to constitutively retain NF- κ B in the cytosol making its canonical activation impossible. The I κ B α 110-A3 mutant responds normally to degradation signals but is not able to dissociate NF- κ B from the nucleus making the NF- κ B inactivation impossible. This construct was likewise active for apoptosis inhibition. These experiments gave credence to the hypothesis that I κ B α has a dual activity. The N-terminus is responsible for apoptosis inhibition at mitochondria and the rest of I κ B α is able to respond and act on NF- κ B signalling.

The N-terminus is the domain necessary for apoptosis inhibition but further mapping experiments showed that is not sufficient. I κ B α requires the N-terminus and the first ankyrin repeat for apoptosis inhibition (Figure D.19). On the other hand, deletion of nearly the total I κ B α domain, which is required for NF- κ B retention and inhibition, did not have any effect on the ability of I κ B α to inhibit apoptosis (Figure D.19).

Our assays, on which we based the discovery that I κ B α inhibits apoptosis, involved overexpression of ANT-1 and Bax. In order to define if the experimental settings were clean in terms of other effects on apoptosis we tested the activation of NF- κ B. NF- κ B activation confers, for the majority of times, an anti-apoptotic signal, so a possible activation during overexpression of ANT-1 or Bax would have masked any net effect of apoptosis inhibition by I κ B α . We used a fluorescence based NF- κ B activation assay to see if ANT-1- or Bax-induced apoptosis involves



NF- κ B activation. As a positive control we used the viral TAX gene which was previously reported to induce NF- κ B activation (Arima *et al.*, 1991; Sun *et al.*, 1994). Under conditions of ectopic expression with several transfection reagents ANT-1 or Bax could not activate the NF- κ B system in contrast to TAX (Figure D.17B). This result supported the mapping results and contributed to the hypothesis that there are distinct domains on I κ B α which allow apoptosis inhibition irrespective of its ability to inhibit/retain NF- κ B.

I κ B α and I κ B β share extensive homology and some of their domains are identical. Analysis with the protein database Uniprot revealed this homology. However, the N-terminal part of I κ B α seems to have the least homology with any other part of I κ B β . Only the regulatory domain responsible for I κ B α degradation (DSGLDS-31/36) is identical with a domain on I κ B β . However, we have shown that mutations in this domain (I κ B α -SR) did not affect the ability of I κ B α to inhibit apoptosis. There is also a domain in the I κ B α -N-terminus that is completely unique compared to I κ B β since it does not match with any sequence in I κ B β . Amino-acids 13-30 and 50-54 showed no similarity and since the N-terminus (1-70) is the necessary domain for apoptosis inhibition and since I κ B β cannot inhibit apoptosis we can hypothesize that those domains are possibly the domains relevant for the effect of I κ B α on apoptosis. The sequence alignment of the two proteins can be found in the Appendix of this thesis and further experimentation will shed new light on this hypothesis.



Differences in the N-terminus of I κ B α and I κ B β can potentially explain the functional differences of these two molecules on apoptosis. However, the two proteins share extensive functional similarity for NF- κ B signalling. They are both degraded upon cytokine administration, they can both sequester NF- κ B in the cytosol and inhibit NF- κ B activation (Rao *et al.*, 2010). Mice lacking I κ B α and with the gene of I κ B β knocked-in under the I κ B α promoter, show a normal phenotype compared to the severe effects of I κ B α knock-out (Cheng *et al.*, 1998). One would expect that since they share so many functions they should also have the same effect on apoptosis. Our experiments, however, show the opposite for apoptosis regulation. I κ B β cannot inhibit apoptosis and the reason might be that only I κ B α is a target gene of NF- κ B. Indeed, I κ B α is rapidly degraded and re-synthesized within 30 minutes of cell stimulation. I κ B β shows much slower kinetics. I κ B β is claimed to be a stronger cytoplasmic inhibitor of NF- κ B since it is able to mask both NLS on NF- κ B in resting cells. On the contrary, I κ B α is only masking one NLS and show a more dynamic localization pattern shifting between cytosol and the nucleus (Malek *et al.*, 2001). The latter finding might explain the dual role of I κ B α . A molecule with two activities should be able to preferentially function on both systems that can affect in order to maintain homeostasis. I κ B β would not have been able to do so since it is a very good sequestering molecule but with poor NF- κ B dissociation ability. However, since I κ B α has a dual function its degradation creates a window in which cells should appear more sensitive to apoptosis since



I κ B α is not present in the cytosol but also not in the mitochondria. Since I κ B α is a target gene, cells up-regulate it rapidly so the equilibrium between pro- and anti-apoptotic proteins is restored.

I κ B α variants with differential localisations (Figure D.9) allowed the separation of the pro- and the anti-apoptotic activity of I κ B α . Moreover, experiments under conditions with (Figures D.10-D.11) or without (Figure D.6-D.8) constitutive NF- κ B activity, indicated that the novel activity of I κ B α to repress apoptosis at the OMM is distinct from its known ability to inhibit NF- κ B. Of note, the C-terminus of I κ B α is mutated in Hodgkin's lymphoma, while its N-terminus remains intact (Cabannes et al. 1999). This would disturb only its effect on retaining NF- κ B and preserves its anti-apoptotic effect at the OMM.

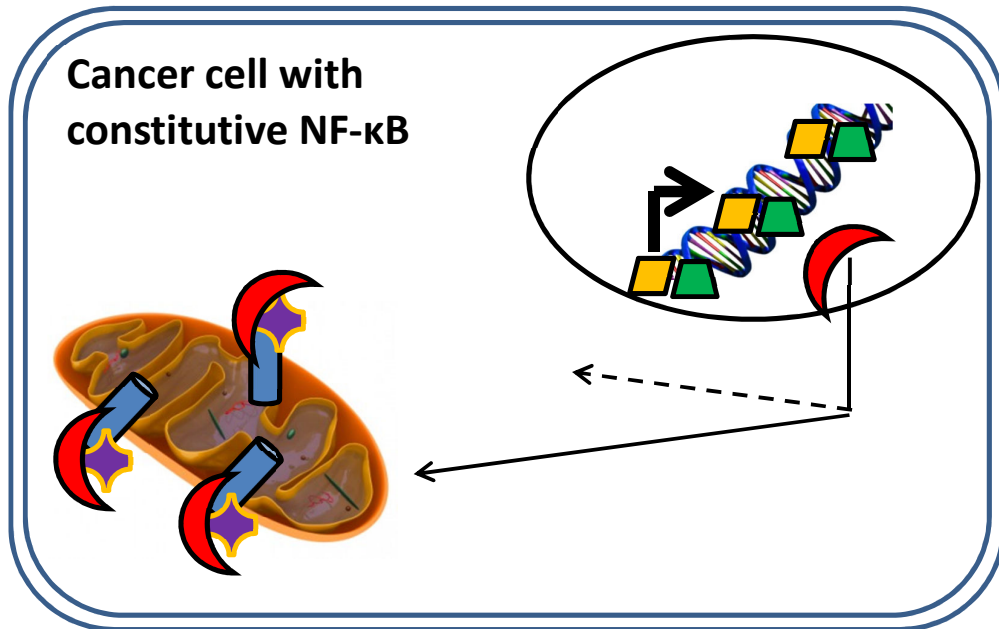
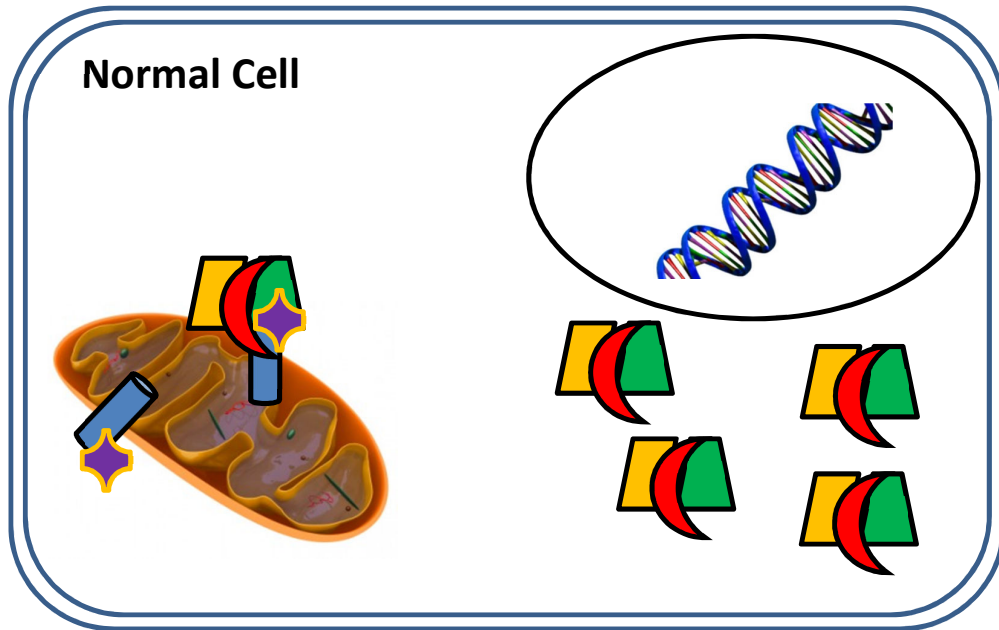
If the equilibrium of NF- κ B-activating and -inhibiting factors is disturbed this inducible transcription factor can contribute to various diseases. In a number of tumour models NF- κ B is found to be constitutively active and this seems to be required for the survival of the malignant cells (Prasad et al., 2009). Permanently activated NF- κ B also contributes to chronic inflammation in a variety of disease models (Gordon *et al.*, 2011; Nichols *et al.*, 2008; Rangan *et al.*, 2009; Roman-Blas & Jimenez, 2008; Yao & Rahman, 2009). As a consequence, NF- κ B is targeted by pharmacological interference in inflammatory and autoimmune diseases (Karin et al., 2004) and in cancer therapy (Baud & Karin, 2009). These efforts could benefit from our findings on the novel mitochondrial activity of I κ B α as it reveals more







specific interference options. A successful compound could bind to I κ B α and either degrade it or block the N-terminus in order to prevent association with VDAC1. Since NF- κ B is the key modulator of the immune response, compounds that target its subunits would have a great effect on the inflammatory and immune responses. Targeting the I κ B α -VDAC1 interaction could prove a more relevant option since one could achieve an apoptosis sensitising effect by the dissociation of I κ B α from mitochondria without affecting the ability of I κ B α to function properly on NF- κ B. In the context of cancer therapy I κ B α dissociation from mitochondria could be therapeutically relevant but in the physiological context of normal cells such a dissociation would have no or minimal effects on the normal functioning of NF- κ B.



A



 p50/p65
 IκBα

 PT-pore
 Hexokinase



B

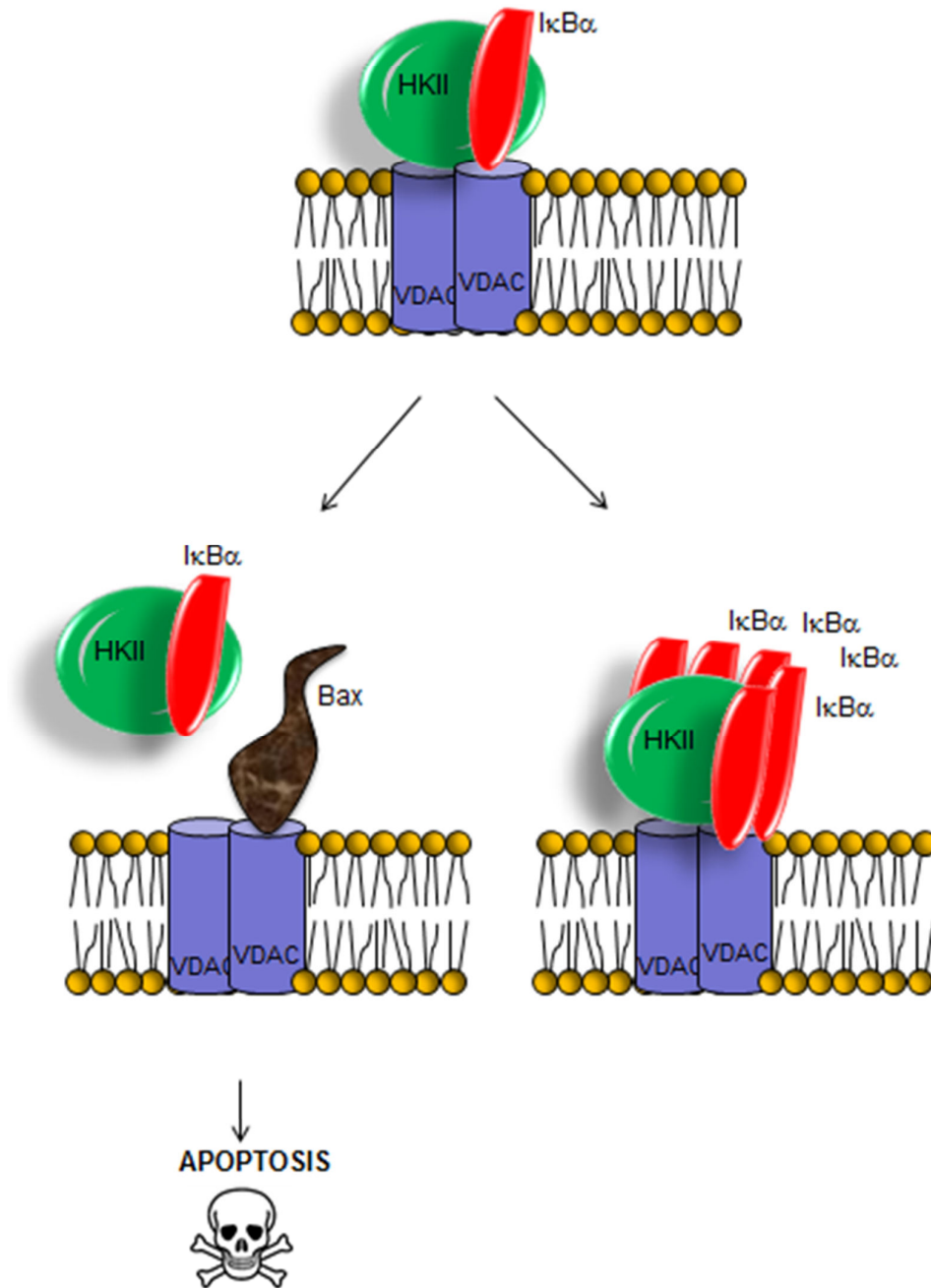




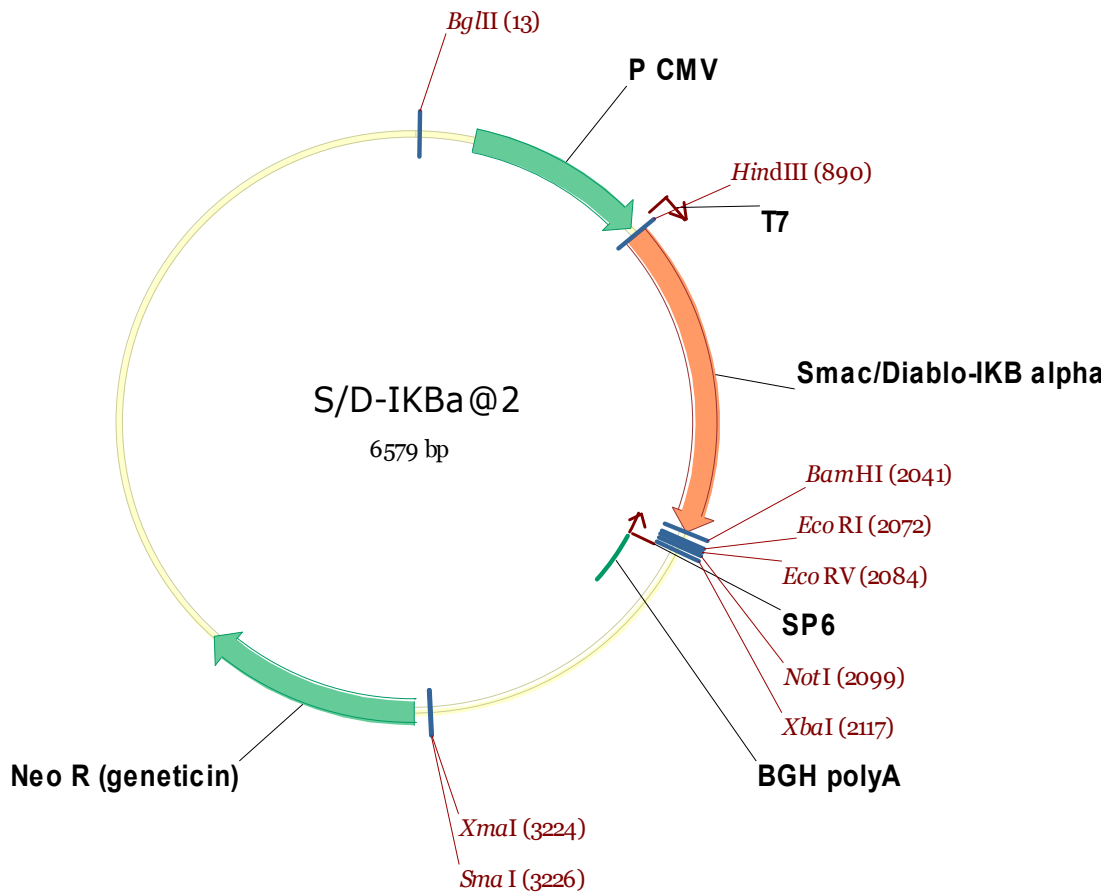
Figure E.1. Model of I κ B α localisation and function. (A) In normal cell I κ B α is sequestering the NF- κ B heterodimers in the cytosol. The N-terminus of I κ B α allows the association with VDAC1 in parallel with the NF- κ B association. I κ B α contributes to NF- κ B signalling and also inhibits apoptosis at mitochondria maintaining the equilibrium for homeostasis. When a cell shifts to a cancerous state NF- κ B is constitutively active by mechanisms independent of I κ B α degradation. This allows the translocation of the NF- κ B dimer to enter the nucleus and transcribe target genes. Such translocation creates an imbalance for the association of I κ B α with p65 and VDAC1. I κ B α then preferentially shifts to a mitochondrial localisation. This, in turn, allows I κ B α to exert its second function, namely to inhibit apoptosis through the PT-pore. NF- κ B activation and I κ B α -VDAC1 association occur concurrently with enhanced I κ B α localisation at mitochondria. The overall net effect is apoptosis resistance. **(B)** In normal and tumour cells that lack constitutive active NF- κ B some I κ B α proteins are associated with VDAC1 and HXKII. Upon apoptosis induction, they dissociate from VDAC and Bax is recruited for apoptosis induction (Lower left hand panel). In those tumour cells that suffer mutations that render NF- κ B constitutively active and in which I κ B α accumulates at the OMM, the VDAC1-HXKII interaction is stabilized and inhibits Bax recruitment, PT-pore opening, cytochrome-c release and apoptosis induction (lower right hand panel).



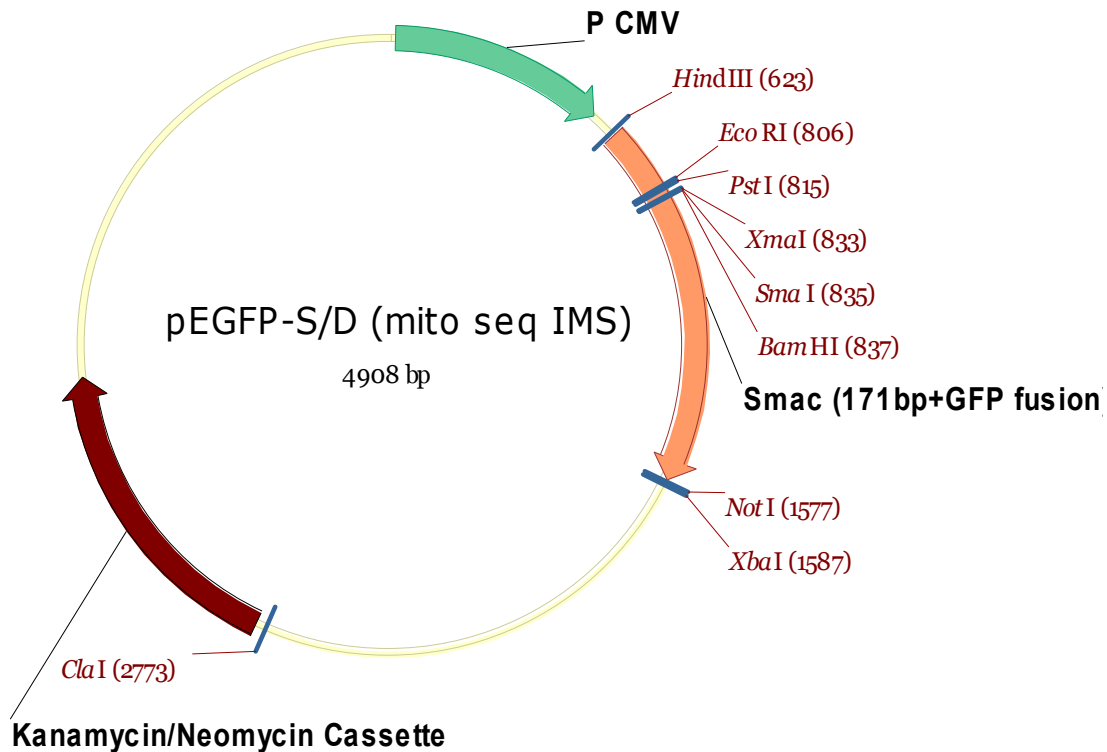
F. Appendix



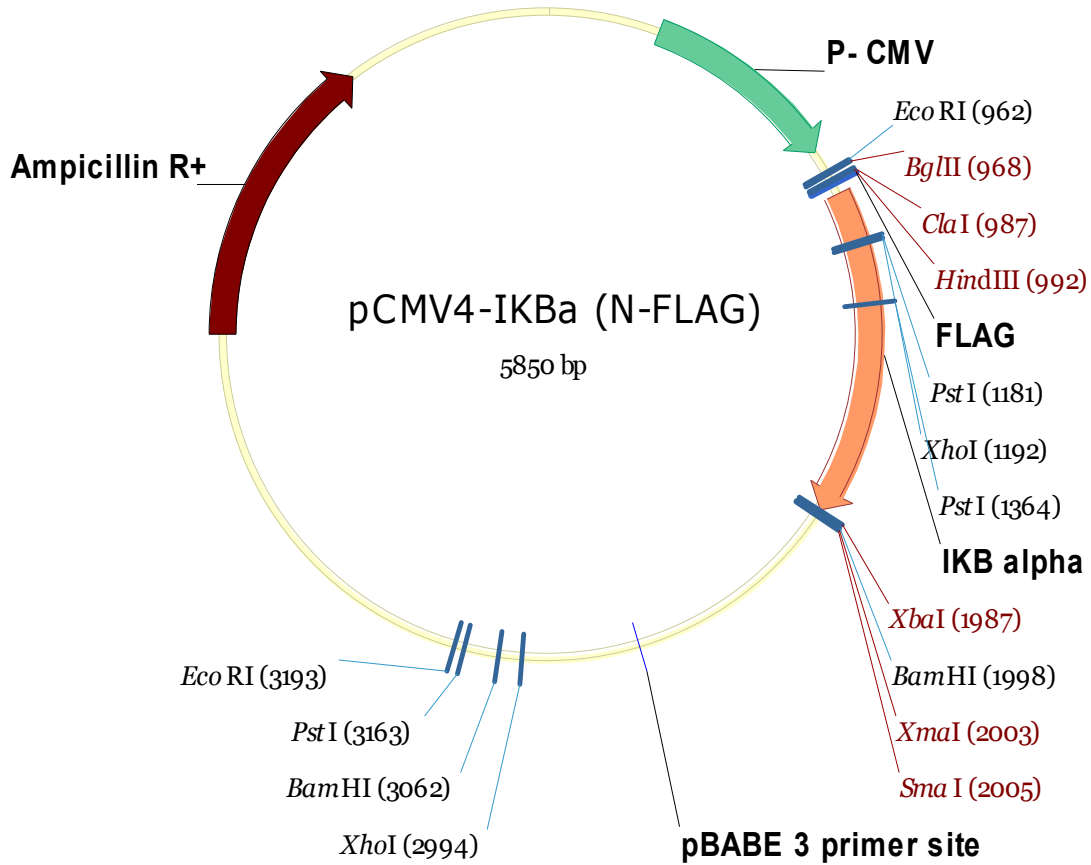
F.1. Plasmids



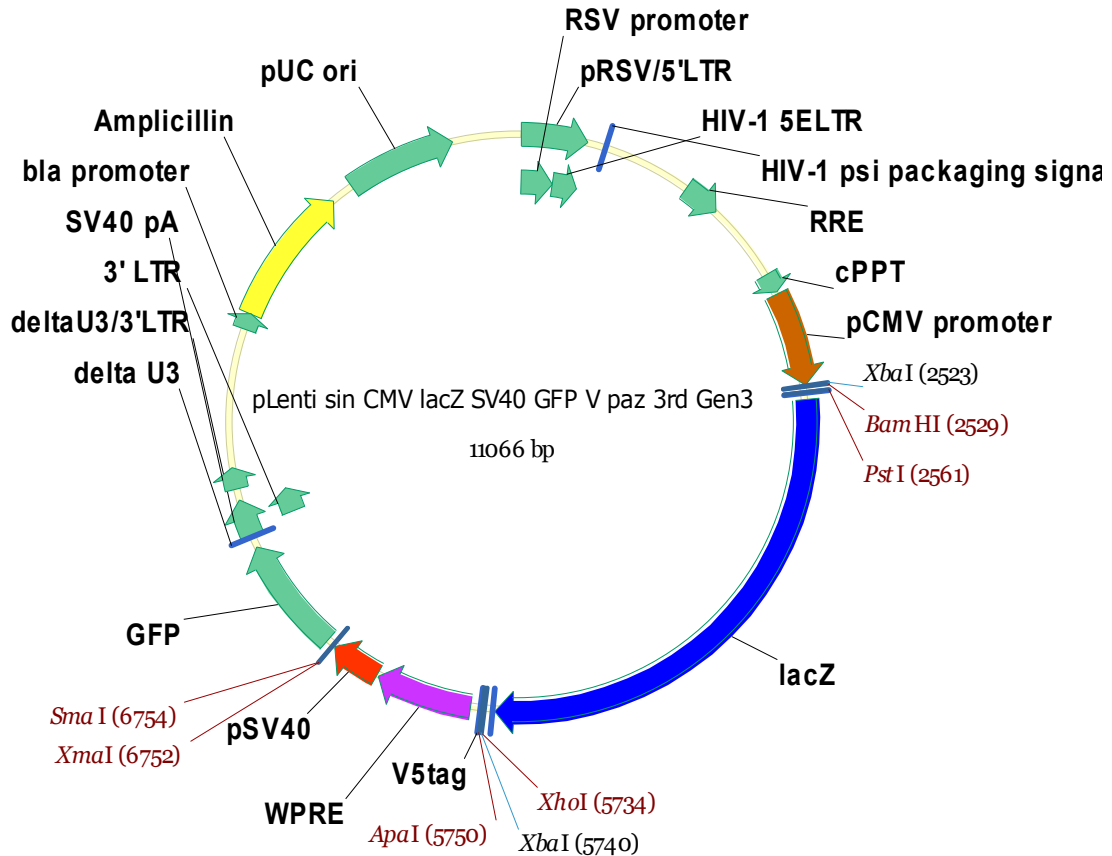
IκBα gene without ATG fused with the localization sequence for mitochondria from the Smac/Diablo gene. S/D first 171 bp were first inserted into pEGFP-N1 and IκBα followed into KpnI/BamHI sites. The whole fusion from HindIII/BamHI was then inserted into pcDNA3. The plasmid was tested for correct localization of the protein with immunofluorescence. Between Smac/Diablo and IκBα there is a linker sequence CTG CAG TCG ACG GTA CC in frame to produce the fusion. All the above apply for the generation of MEET-IκBα vector.



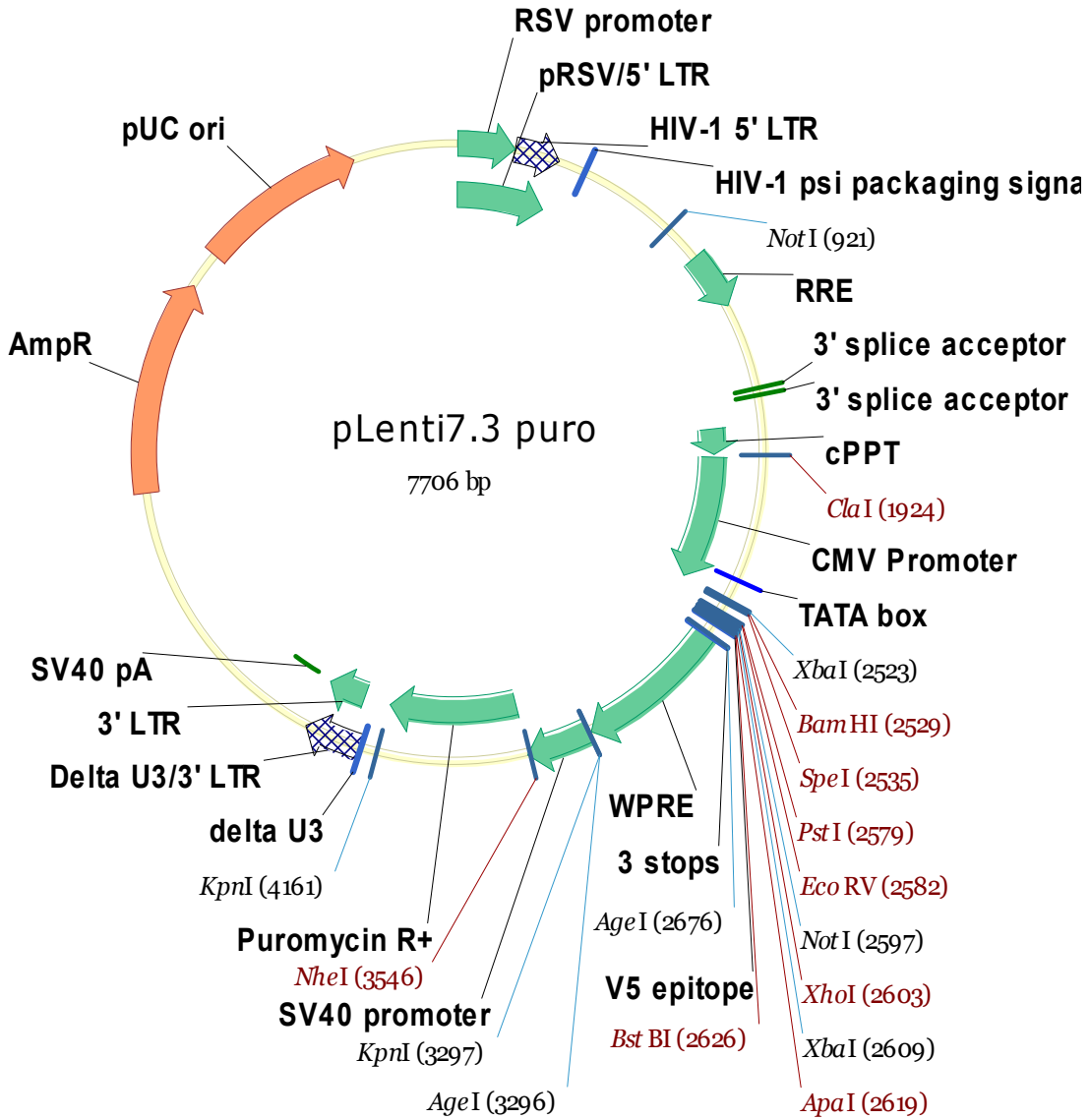
171bp from the Smac/Diablo gene of mouse origin and a Kozak consensus were into the HindIII and PstI sites of pEGFP-N1 vector to create a fusion protein with EGFP gene. The same strategy was followed for the generation of MEET-GFP plasmid. The plasmids can be used as fluorescent markers for the Intermembrane space of mitochondria (IMS) or the OMM respectively. The constructs were sequenced–verified and validated with Immunofluorescence. It can be used to visualize mitochondria.



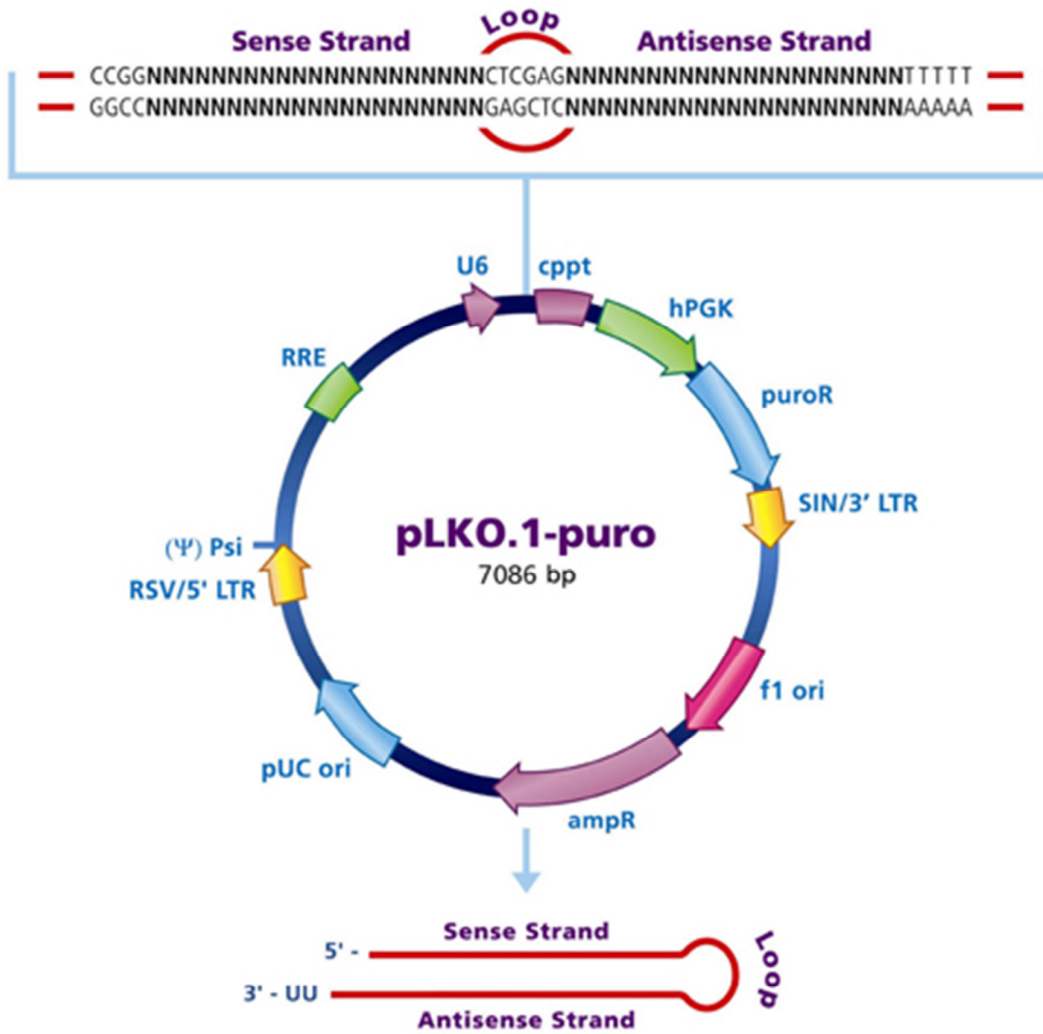
I κ B α gene human was inserted into pCMV4 plasmid and a FLAG tag was inserted with recombinant PCR technology in the N-terminal part of I κ B α . The plasmid was sequence-verified and validated using transfections. Restrictions sites *Bgl*III, *Hind*III and *Cla*I with *Xba*I, *Xma*I can be used to excise the I κ B α TAGGED gene. pCEP forward and pBABE Reverse primers can be used with relevant safety to sequence the gene



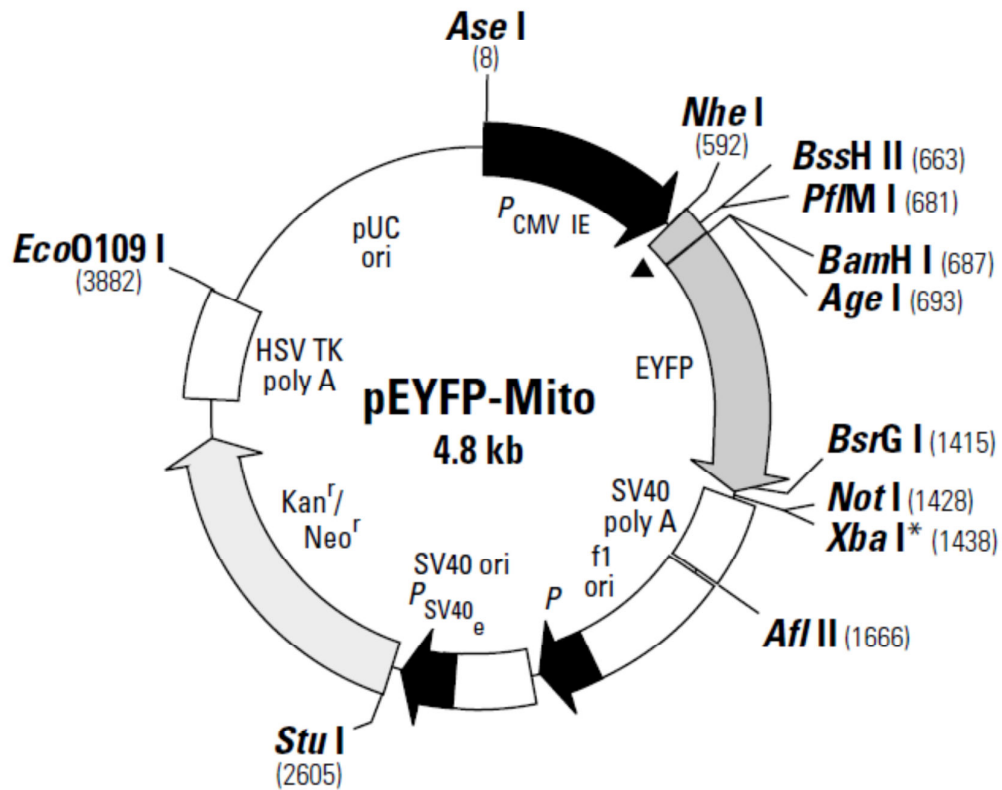
lacZ control gene in the commercial pLenti7.3 vector that also contains eGFP under the SV40 promoter. The lacZ is under a strong mammalian expression pCMV. The plasmid is to be used for production of control lentiviral particles with a 3rd generation lentiviral production system. MEET-IκBα and IκBα/βactin were also cloned into this vector.



Lentiviral transfer vector plasmid with Puromycin gene for selection in mammalian cells



pLKO.1 vector for the expression of shRNA. The vector contains a puromycin resistance cassette for mammalian selection in a bi-cistronic format with U6 promoter. PvuII restriction digest can be used for pattern checking



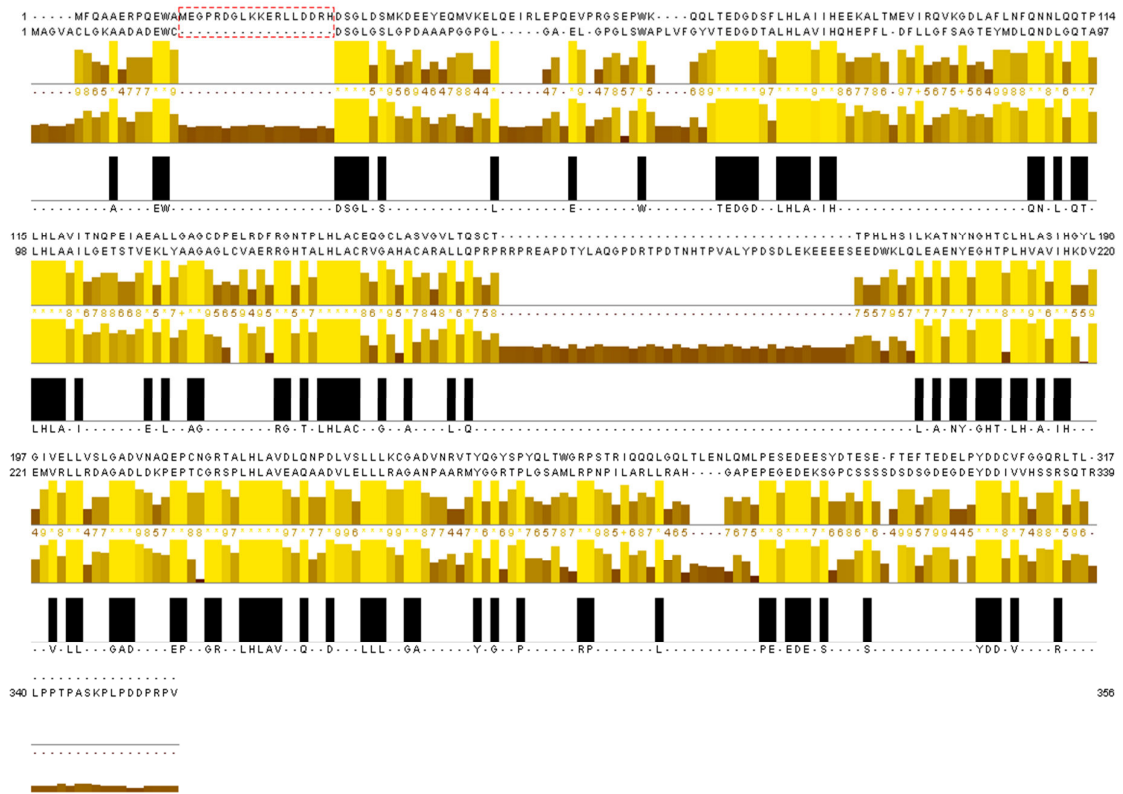
▲ = mitochondrial targeting sequence (Mito)

pEYFP-mito encodes a fusion of EYFP and the mitochondrial targeting sequence from the subunit VIII of human cytochrome c oxidase which targets the EYFP gene to the mitochondrial matrix. Substitutions of the EYFP gene with the CFP or DsRed gene created alternatives for fluorescent labelling of mitochondria. The mitochondrial targeting sequence finishes at the 683th bp and before BamHI site.



F.2. IκBα and IκBβ

1	-----MFQAAERPQEWAMEGPRDGLKKERLLDDRHD	55	P25963	IKBA_HUMAN
1	MAGVACLGKAADADEWC-----DSGLGSLGPDAAAPGGPGL-----G	37	Q15653	IKBB_HUMAN
	: * : ** . : * * * * * * : *			
56	PQEVPRGSEPWK---QQLTEDGDSFLHLAIHHEEKALTEVIQVKGDALFLNFQNNLQ	111	P25963	IKBA_HUMAN
38	A-EL-GPGLSWAPLVFGYVTEGDGDTALHLAVIHQHEPFL-DFLLGFSAGTEYMDLQNDLG	94	Q15653	IKBB_HUMAN
	* : . * : * * * * * : * * * * * : : : : : : : : : : : : * * * * *			
112	QTPLHLAVITNQPEIAEALLGAGCDPELRFDRNTPLHLACEQGCLASVGLTQSCT---	168	P25963	IKBA_HUMAN
95	QTALHAAIIGETSTVEKLYAAGAGLCVAERRGHTALHLACRVGAHACARALLQPRPRRP	154	Q15653	IKBB_HUMAN
	** * * * * * * : . . * * * * * . : : * * * * * * * * * * . * . . . * *			
169	-----TPHLHSILKATNYNGHTCLHLA	190	P25963	IKBA_HUMAN
155	REAPDTYLAQGPDRTPDTNHTPVALYPDSOLEKEEESEEDWKLQLEAENYEGHTPLHVA	214	Q15653	IKBB_HUMAN
	. : * : * * * * * * * * *			
191	SIHGYLGIVELLVSLGADVNAQEPNCRGTALHLAVDLQNPDLVSLLLKCGADVNRVTYQG	250	P25963	IKBA_HUMAN
215	VIHKDVMVRLLRDAGADLDKPEPTCGRSPLHLAVEAQADVLELLLKAGANPAARMYGG	274	Q15653	IKBB_HUMAN
	** : : * * * * . * * * : * * * * * : * * : : * * * * : * * *			
251	YSPYQLTWGRPSTRIQQQLGQLTLENLQMLPESEDEESYDTESE-FTEFTEDELPHYDDCV	309	P25963	IKBA_HUMAN
275	RTPLGSAMLRPNPILARLLRAH----GAPEPEGEDEKSGPCSSSSSDSDGDEGDEYDDIV	330	Q15653	IKBB_HUMAN
	: * : * * : : * * : * * * * * * * : : : * * * *			
310	FGGQRLTL-----	317	P25963	IKBA_HUMAN
331	VHSSRSQTRLPPPTASKPLPDDPRPV	356	Q15653	IKBB_HUMAN
	. . . *			





G. Bibliography



Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X. & Akey, C.W. (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell*, **9**, 423-432.

Adrain, C., Creagh, E.M. & Martin, S.J. (2001) Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *EMBO J*, **20**, 6627-6636.

Aggarwal, B.B. (2000) Apoptosis and nuclear factor-kappa B: a tale of association and dissociation. *Biochem Pharmacol*, **60**, 1033-1039.

Ahmad, M., Srinivasula, S.M., Wang, L., Talanian, R.V., Litwack, G., Fernandes-Alnemri, T. & Alnemri, E.S. (1997) CRADD, a novel human apoptotic adaptor molecule for caspase-2, and FasL/tumor necrosis factor receptor-interacting protein RIP. *Cancer Res*, **57**, 615-619.

Albayrak, T. & Grimm, S. (2003) A high-throughput screen for single gene activities: isolation of apoptosis inducers. *Biochem Biophys Res Commun*, **304**, 772-776.

Alimonti, J.B., Shi, L., Baijal, P.K. & Greenberg, A.H. (2001) Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J Biol Chem*, **276**, 6974-6982.

Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. & Martinou, J.C. (2000) Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochem J*, **345 Pt 2**, 271-278.

Arima, N., Molitor, J.A., Smith, M.R., Kim, J.H., Daitoku, Y. & Greene, W.C. (1991) Human T-cell leukemia virus type I Tax induces expression of the Rel-related family of kappa B enhancer-binding proteins: evidence for a pretranslational component of regulation. *J Virol*, **65**, 6892-6899.

Arsura, M., Mercurio, F., Oliver, A.L., Thorgeirsson, S.S. & Sonenshein, G.E. (2000) Role of the IkappaB kinase complex in oncogenic Ras- and Raf-mediated transformation of rat liver epithelial cells. *Mol Cell Biol*, **20**, 5381-5391.

Ashkenazi, A. (2008) Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Rev*, **19**, 325-331.



Ashkenazi, A. & Dixit, V.M. (1998) Death receptors: signaling and modulation. *Science*, **281**, 1305-1308.

Azoulay-Zohar, H., Israelson, A., Abu-Hamad, S. & Shoshan-Barmatz, V. (2004) In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *Biochem J*, **377**, 347-355.

Baeuerle, P.A. (1998) I κ B-NF- κ B structures: at the interface of inflammation control. *Cell*, **95**, 729-731.

Baeuerle, P.A. & Henkel, T. (1994) Function and activation of NF- κ B in the immune system. *Annu Rev Immunol*, **12**, 141-179.

Baichwal, V.R. & Baeuerle, P.A. (1997) Activate NF- κ B or die? *Curr Biol*, **7**, R94-96.

Baines, C.P., Kaiser, R.A., Purcell, N.H., Blair, N.S., Osinska, H., Hambleton, M.A., Brunskill, E.W., Sayen, M.R., Gottlieb, R.A., Dorn, G.W., Robbins, J. & Molkentin, J.D. (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature*, **434**, 658-662.

Baines, C.P., Kaiser, R.A., Sheiko, T., Craigen, W.J. & Molkentin, J.D. (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol*, **9**, 550-555. Epub 2007 Apr 2008.

Baldwin, A.S. (2001) Control of oncogenesis and cancer therapy resistance by the transcription factor NF- κ B. *J Clin Invest*, **107**, 241-246.

Barry, M.A., Behnke, C.A. & Eastman, A. (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem Pharmacol*, **40**, 2353-2362.

Basanez, G., Sharpe, J.C., Galanis, J., Brandt, T.B., Hardwick, J.M. & Zimmerberg, J. (2002) Bax-type apoptotic proteins porate pure lipid bilayers through a mechanism sensitive to intrinsic monolayer curvature. *J Biol Chem*, **277**, 49360-49365.

Basseres, D.S. & Baldwin, A.S. (2006) Nuclear factor- κ B and inhibitor of κ B kinase pathways in oncogenic initiation and progression. *Oncogene*, **25**, 6817-6830.



Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M.A. & Bernardi, P. (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem*, **280**, 18558-18561. Epub 12005 Mar 18525.

Baud, V. & Karin, M. (2001) Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol*, **11**, 372-377.

Baud, V. & Karin, M. (2009) Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat Rev Drug Discov.*, **8**, 33-40.

Bauer, M.K., Schubert, A., Rocks, O. & Grimm, S. (1999) Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis. *J Cell Biol*, **147**, 1493-1502.

Bauerle, K.T., Schweppe, R.E. & Haugen, B.R. (2010) Inhibition of nuclear factor-kappa B differentially affects thyroid cancer cell growth, apoptosis, and invasion. *Mol Cancer*, **9**, 117.

Beg, A.A. & Baltimore, D. (1996) An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*, **274**, 782-784.

Beg, A.A., Sha, W.C., Bronson, R.T. & Baltimore, D. (1995a) Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev*, **9**, 2736-2746.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S. & Baltimore, D. (1995b) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature*, **376**, 167-170.

Bernardi, P. (1999) Mitochondria in muscle cell death. *Ital J Neurol Sci*, **20**, 395-400.

Beurel, E. & Jope, R.S. (2006) The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog Neurobiol*, **79**, 173-189.

Beutner, G., Ruck, A., Riede, B., Welte, W. & Brdiczka, D. (1996) Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. *FEBS Lett*, **396**, 189-195.



Biswas, D.K., Shi, Q., Baily, S., Strickland, I., Ghosh, S., Pardee, A.B. & Iglehart, J.D. (2004) NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc Natl Acad Sci U S A*, **101**, 10137-10142.

Boatright, K.M., Renatus, M., Scott, F.L., Sperandio, S., Shin, H., Pedersen, I.M., Ricci, J.E., Edris, W.A., Sutherlin, D.P., Green, D.R. & Salvesen, G.S. (2003) A unified model for apical caspase activation. *Mol Cell*, **11**, 529-541.

Bohm, I. & Schild, H. (2003) Apoptosis: the complex scenario for a silent cell death. *Mol Imaging Biol*, **5**, 2-14.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G. & Thompson, C.B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597-608.

Boldin, M.P., Mett, I.L., Varfolomeev, E.E., Chumakov, I., Shemer-Avni, Y., Camonis, J.H. & Wallach, D. (1995) Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *J Biol Chem*, **270**, 387-391.

Bonizzi, G. & Karin, M. (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol*, **25**, 280-288.

Bonod-Bidaud, C., Chevrollier, A., Bourasseau, I., Lachaux, A., Mousson de Camaret, B. & Stepien, G. (2001) Induction of ANT2 gene expression in liver of patients with mitochondrial DNA depletion. *Mitochondrion*, **1**, 217-224.

Bottero, V., Rossi, F., Samson, M., Mari, M., Hofman, P. & Peyron, J.F. (2001) Ikappa b-alpha, the NF-kappa B inhibitory subunit, interacts with ANT, the mitochondrial ATP/ADP translocator. *J Biol Chem*, **276**, 21317-21324.

Brdiczka, D. (1991) Contact sites between mitochondrial envelope membranes. Structure and function in energy- and protein-transfer. *Biochim Biophys Acta*, **1071**, 291-312.

Brenner, C. & Grimm, S. (2006) The permeability transition pore complex in cancer cell death. *Oncogene*, **25**, 4744-4756.

Broekemeier, K.M., Dempsey, M.E. & Pfeiffer, D.R. (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem*, **264**, 7826-7830.



Brown, G.C. & Borutaite, V. (2008) Regulation of apoptosis by the redox state of cytochrome c. *Biochim Biophys Acta*, **1777**, 877-881.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X. & Wang, X. (1999) Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol*, **15**, 269-290.

Burstein, E. & Duckett, C.S. (2003) Dying for NF-kappaB? Control of cell death by transcriptional regulation of the apoptotic machinery. *Curr Opin Cell Biol*, **15**, 732-737.

Capano, M. & Crompton, M. (2002) Biphasic translocation of Bax to mitochondria. *Biochem J*, **367**, 169-178.

Castedo, M., Ferri, K., Roumier, T., Metivier, D., Zamzami, N. & Kroemer, G. (2002) Quantitation of mitochondrial alterations associated with apoptosis. *J Immunol Methods*, **265**, 39-47.

Cecconi, F., Alvarez-Bolado, G., Meyer, B.I., Roth, K.A. & Gruss, P. (1998) Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell*, **94**, 727-737.

Chen, F.E. & Ghosh, G. (1999) Regulation of DNA binding by Rel/NF-kappaB transcription factors: structural views. *Oncogene*, **18**, 6845-6852.

Chen, G. & Goeddel, D.V. (2002) TNF-R1 signaling: a beautiful pathway. *Science*, **296**, 1634-1635.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T. & Korsmeyer, S.J. (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell*, **8**, 705-711.

Cheng, J.D., Ryseck, R.P., Attar, R.M., Dambach, D. & Bravo, R. (1998) Functional redundancy of the nuclear factor kappa B inhibitors I kappa B alpha and I kappa B beta. *J Exp Med*, **188**, 1055-1062.

Chiara, F., Castellaro, D., Marin, O., Petronilli, V., Brusilow, W.S., Juhaszova, M., Sollott, S.J., Forte, M., Bernardi, P. & Rasola, A. (2008) Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels. *PLoS One*, **3**, e1852.



Chinnaiyan, A.M., O'Rourke, K., Tewari, M. & Dixit, V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 505-512.

Chipuk, J.E., Kuwana, T., Bouchier-Hayes, L., Droin, N.M., Newmeyer, D.D., Schuler, M. & Green, D.R. (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*, **303**, 1010-1014.

Chittenden, T., Harrington, E.A., O'Connor, R., Flemington, C., Lutz, R.J., Evan, G.I. & Guild, B.C. (1995) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature*, **374**, 733-736.

Cilloni, D., Messa, F., Arruga, F., Defilippi, I., Morotti, A., Messa, E., Carturan, S., Giugliano, E., Pautasso, M., Bracco, E., Rosso, V., Sen, A., Martinelli, G., Baccarani, M. & Saglio, G. (2006) The NF-kappaB pathway blockade by the IKK inhibitor PS1145 can overcome imatinib resistance. *Leukemia*, **20**, 61-67.

Ciucci, A., Gianferretti, P., Piva, R., Guyot, T., Snape, T.J., Roberts, S.M. & Santoro, M.G. (2006) Induction of apoptosis in estrogen receptor-negative breast cancer cells by natural and synthetic cyclopentenones: role of the IkappaB kinase/nuclear factor-kappaB pathway. *Mol Pharmacol*, **70**, 1812-1821.

Clifton, D.R., Goss, R.A., Sahni, S.K., van Antwerp, D., Baggs, R.B., Marder, V.J., Silverman, D.J. & Sporn, L.A. (1998) NF-kappa B-dependent inhibition of apoptosis is essential for host cell survival during *Rickettsia rickettsii* infection. *Proc Natl Acad Sci U S A*, **95**, 4646-4651.

Cogswell, P.C., Kashatus, D.F., Keifer, J.A., Guttridge, D.C., Reuther, J.Y., Bristow, C., Roy, S., Nicholson, D.W. & Baldwin, A.S., Jr. (2003) NF-kappa B and I kappa B alpha are found in the mitochondria. Evidence for regulation of mitochondrial gene expression by NF-kappa B. *J Biol Chem*, **278**, 2963-2968.

Colombini, M. (1983) Purification of VDAC (voltage-dependent anion-selective channel) from rat liver mitochondria. *J Membr Biol*, **74**, 115-121.

Courtois, G. & Gilmore, T.D. (2006) Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene*, **25**, 6831-6843.

Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem J*, **341 (Pt 2)**, 233-249.



Crompton, M. (2000) Mitochondrial intermembrane junctional complexes and their role in cell death. *J Physiol*, **529 Pt 1**, 11-21.

Crompton, M., Barksby, E., Johnson, N. & Capano, M. (2002) Mitochondrial intermembrane junctional complexes and their involvement in cell death. *Biochimie*, **84**, 143-152.

Crompton, M. & Costi, A. (1990) A heart mitochondrial Ca²⁺-dependent pore of possible relevance to re-perfusion-induced injury. Evidence that ADP facilitates pore interconversion between the closed and open states. *Biochem J*, **266**, 33-39.

Crompton, M., Virji, S. & Ward, J.M. (1998) Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. *Eur J Biochem*, **258**, 729-735.

Danial, N.N. & Korsmeyer, S.J. (2004) Cell death: critical control points. *Cell*, **116**, 205-219.

de Jong, S., Timmer, T., Heijnenbrok, F.J. & de Vries, E.G. (2001) Death receptor ligands, in particular TRAIL, to overcome drug resistance. *Cancer Metastasis Rev*, **20**, 51-56.

Dejardin, E., Deregowski, V., Chapelier, M., Jacobs, N., Gielen, J., Merville, M.P. & Bours, V. (1999) Regulation of NF-kappaB activity by I kappaB-related proteins in adenocarcinoma cells. *Oncogene*, **18**, 2567-2577.

Delhase, M., Hayakawa, M., Chen, Y. & Karin, M. (1999) Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science*, **284**, 309-313.

Desagher, S. & Martinou, J.C. (2000) Mitochondria as the central control point of apoptosis. *Trends Cell Biol*, **10**, 369-377.

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. & Martinou, J.C. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol*, **144**, 891-901.

Devin, A., Lin, Y. & Liu, Z.G. (2003) The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. *EMBO Rep*, **4**, 623-627.



Dewson, G. & Kluck, R.M. (2009) Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *J Cell Sci*, **122**, 2801-2808.

DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S. & Karin, M. (1996) Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol*, **16**, 1295-1304.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E. & Karin, M. (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature*, **388**, 548-554.

Donepudi, M., Mac Sweeney, A., Briand, C. & Grutter, M.G. (2003) Insights into the regulatory mechanism for caspase-8 activation. *Mol Cell*, **11**, 543-549.

Duan, H. & Dixit, V.M. (1997) RAIDD is a new 'death' adaptor molecule. *Nature*, **385**, 86-89.

Duchen, M.R. (2000) Mitochondria and calcium: from cell signalling to cell death. *J Physiol*, **529 Pt 1**, 57-68.

Dutta, J., Fan, Y., Gupta, N., Fan, G. & Gelinas, C. (2006) Current insights into the regulation of programmed cell death by NF-kappaB. *Oncogene*, **25**, 6800-6816.

Edderkaoui, M., Odinokova, I., Ohno, I., Gukovsky, I., Go, V.L., Pandol, S.J. & Gukovskaya, A.S. (2008) Ellagic acid induces apoptosis through inhibition of nuclear factor kappa B in pancreatic cancer cells. *World J Gastroenterol*, **14**, 3672-3680.

Edinger, A.L. & Thompson, C.B. (2004) Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol*, **16**, 663-669.

Eldering, E., Mackus, W.J., Derks, I.A., Evers, L.M., Beuling, E., Teeling, P., Lens, S.M., van Oers, M.H. & van Lier, R.A. (2004) Apoptosis via the B cell antigen receptor requires Bax translocation and involves mitochondrial depolarization, cytochrome C release, and caspase-9 activation. *Eur J Immunol*, **34**, 1950-1960.

Elliott, W.H. & Elliott, D.C. (2009) *Biochemistry and molecular biology*. Oxford University Press, Oxford.



Ernak, G. & Davies, K.J. (2002) Calcium and oxidative stress: from cell signaling to cell death. *Mol Immunol*, **38**, 713-721.

Ernst, M.K., Dunn, L.L. & Rice, N.R. (1995) The PEST-like sequence of I kappa B alpha is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers. *Mol Cell Biol*, **15**, 872-882.

Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A. & Martinou, J.C. (1998) Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg²⁺ ions. *J Cell Biol*, **143**, 217-224.

Eskes, R., Desagher, S., Antonsson, B. & Martinou, J.C. (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol*, **20**, 929-935.

Farrow, S.N., White, J.H., Martinou, I., Raven, T., Pun, K.T., Grinham, C.J., Martinou, J.C. & Brown, R. (1995) Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. *Nature*, **374**, 731-733.

Faustin, B., Rossignol, R., Rocher, C., Benard, G., Malgat, M. & Letellier, T. (2004) Mobilization of adenine nucleotide translocators as molecular bases of the biochemical threshold effect observed in mitochondrial diseases. *J Biol Chem*, **279**, 20411-20421.

Fiek, C., Benz, R., Roos, N. & Brdiczka, D. (1982) Evidence for identity between the hexokinase-binding protein and the mitochondrial porin in the outer membrane of rat liver mitochondria. *Biochim Biophys Acta*, **688**, 429-440.

Ganchi, P.A., Sun, S.C., Greene, W.C. & Ballard, D.W. (1992) I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. *Mol Biol Cell*, **3**, 1339-1352.

Gasparian, A.V., Yao, Y.J., Kowalczyk, D., Lyakh, L.A., Karseladze, A., Slaga, T.J. & Budunova, I.V. (2002a) The role of IKK in constitutive activation of NF-kappaB transcription factor in prostate carcinoma cells. *J Cell Sci*, **115**, 141-151.

Gasparian, A.V., Yao, Y.J., Lu, J., Yemelyanov, A.Y., Lyakh, L.A., Slaga, T.J. & Budunova, I.V. (2002b) Selenium compounds inhibit I kappa B kinase (IKK) and nuclear factor-kappa B (NF-kappa B) in prostate cancer cells. *Mol Cancer Ther*, **1**, 1079-1087.



Ghavami, S., Mutawe, M.M., Hauff, K., Stelmack, G.L., Schaafsma, D., Sharma, P., McNeill, K.D., Hynes, T.S., Kung, S.K., Unruh, H., Klönisch, T., Hatch, G.M., Los, M. & Halayko, A.J. (2010) Statin-triggered cell death in primary human lung mesenchymal cells involves p53-PUMA and release of Smac and Omi but not cytochrome c. *Biochim Biophys Acta*, **1803**, 452-467.

Ghosh, S., May, M.J. & Kopp, E.B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*, **16**, 225-260.

Gilmore, T.D. (1999) Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. *Oncogene*, **18**, 6925-6937.

Gilmore, T.D. (2006) Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene*, **25**, 6680-6684.

Giraud, S., Bonod-Bidaud, C., Wesolowski-Louvel, M. & Stepien, G. (1998) Expression of human ANT2 gene in highly proliferative cells: GRBOX, a new transcriptional element, is involved in the regulation of glycolytic ATP import into mitochondria. *J Mol Biol*, **281**, 409-418.

Gogvadze, V., Orrenius, S. & Zhivotovsky, B. (2003) Analysis of mitochondrial dysfunction during cell death. *Curr Protoc Cell Biol*, **Chapter 18**, Unit 18 15.

Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I. & Green, D.R. (2000) The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat Cell Biol*, **2**, 156-162.

Gordon, J.W., Shaw, J.A. & Kirshenbaum, L.A. (2011) Multiple facets of NF-kappaB in the heart: to be or not to NF-kappaB. *Circ Res*, **108**, 1122-1132.

Green, D.R. (2005) Apoptotic pathways: ten minutes to dead. *Cell*, **121**, 671-674.

Green, D.R., Knight, R.A., Melino, G., Finazzi-Agro, A. & Orrenius, S. (2004) Ten years of publication in cell death. *Cell Death Differ*, **11**, 2-3.

Green, D.R. & Kroemer, G. (2004) The pathophysiology of mitochondrial cell death. *Science*, **305**, 626-629.



Green, D.R. & Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309-1312.

Griffiths, G.J., Dubrez, L., Morgan, C.P., Jones, N.A., Whitehouse, J., Corfe, B.M., Dive, C. & Hickman, J.A. (1999) Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. *J Cell Biol*, **144**, 903-914.

Grimm, S., Bauer, M.K., Baeuerle, P.A. & Schulze-Osthoff, K. (1996) Bcl-2 down-regulates the activity of transcription factor NF-kappaB induced upon apoptosis. *J Cell Biol*, **134**, 13-23.

Grimm, S. & Brdiczka, D. (2007) The permeability transition pore in cell death. *Apoptosis*, **12**, 841-855.

Gross, A., Jockel, J., Wei, M.C. & Korsmeyer, S.J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J*, **17**, 3878-3885.

Gross, A., Yin, X.M., Wang, K., Wei, M.C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P. & Korsmeyer, S.J. (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem*, **274**, 1156-1163.

Gustin, J.A., Ozes, O.N., Akca, H., Pincheira, R., Mayo, L.D., Li, Q., Guzman, J.R., Korgaonkar, C.K. & Donner, D.B. (2004) Cell type-specific expression of the IkkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. *J Biol Chem*, **279**, 1615-1620.

Haanen, C. & Vermes, I. (1995) Apoptosis and inflammation. *Mediators Inflamm*, **4**, 5-15.

Hahne, K., Haucke, V., Ramage, L. & Schatz, G. (1994a) Incomplete arrest in the outer membrane sorts NADH-cytochrome b5 reductase to two different submitochondrial compartments. *Cell*, **79**, 829-839.

Hahne, K., Haucke, V., Ramage, L. & Schatz, G. (1994b) Incomplete arrest in the outer membrane sorts NADH-cytochrome b5 reductase to two different submitochondrial compartments. *Cell*, **79**, 829-839.



Hakem, R., Hakem, A., Duncan, G.S., Henderson, J.T., Woo, M., Soengas, M.S., Elia, A., de la Pompa, J.L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S.A., Lowe, S.W., Penninger, J.M. & Mak, T.W. (1998) Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell*, **94**, 339-352.

Halestrap, A.P., Woodfield, K.Y. & Connern, C.P. (1997) Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. *J Biol Chem*, **272**, 3346-3354.

Halliwell, B. & Gutteridge, J.M.C. (2007) *Free radicals in biology and medicine*. Oxford University Press, Oxford.

Hanahan, D. & Weinberg, R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57-70.

Hardwick, M., Fertikh, D., Culty, M., Li, H., Vidic, B. & Papadopoulos, V. (1999) Peripheral-type benzodiazepine receptor (PBR) in human breast cancer: correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization, and PBR-mediated cell proliferation and nuclear transport of cholesterol. *Cancer Res*, **59**, 831-842.

Harris, A.L. (2002) Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer*, **2**, 38-47.

Harwood, S.M., Yaqoob, M.M. & Allen, D.A. (2005) Caspase and calpain function in cell death: bridging the gap between apoptosis and necrosis. *Ann Clin Biochem*, **42**, 415-431.

Haskill, S., Beg, A.A., Tompkins, S.M., Morris, J.S., Yurochko, A.D., Sampson-Johannes, A., Mondal, K., Ralph, P. & Baldwin, A.S., Jr. (1991) Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. *Cell*, **65**, 1281-1289.

Hatada, E.N., Naumann, M. & Scheidereit, C. (1993a) Common structural constituents confer I kappa B activity to NF-kappa B p105 and I kappa B/MAD-3. *EMBO J*, **12**, 2781-2788.

Hatada, E.N., Naumann, M. & Scheidereit, C. (1993b) Common structural constituents confer I kappa B activity to NF-kappa B p105 and I kappa B/MAD-3. *EMBO J*, **12**, 2781-2788.



- Haucke, V., Ocana, C.S., Honlinger, A., Tokatlidis, K., Pfanner, N. & Schatz, G. (1997) Analysis of the sorting signals directing NADH-cytochrome b5 reductase to two locations within yeast mitochondria. *Mol Cell Biol*, **17**, 4024-4032.
- Hayashi, T., Sekine, T. & Okamoto, T. (1993) Identification of a new serine kinase that activates NF kappa B by direct phosphorylation. *J Biol Chem*, **268**, 26790-26795.
- Hayden, M.S. & Ghosh, S. (2004) Signaling to NF-kappaB. *Genes Dev*, **18**, 2195-2224.
- Hayden, M.S. & Ghosh, S. (2008) Shared principles in NF-kappaB signaling. *Cell*, **132**, 344-362.
- Hayden, M.S. & Ghosh, S. (2012) NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev*, **26**, 203-234.
- Hiscott, J., Nguyen, T.L., Arguello, M., Nakhaei, P. & Paz, S. (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. *Oncogene*, **25**, 6844-6867.
- Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. & Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348**, 334-336.
- Hsu, H., Shu, H.B., Pan, M.G. & Goeddel, D.V. (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*, **84**, 299-308.
- Hsu, H., Xiong, J. & Goeddel, D.V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*, **81**, 495-504.
- Hsu, Y.T., Wolter, K.G. & Youle, R.J. (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci U S A*, **94**, 3668-3672.
- Hu, S., Vincenz, C., Ni, J., Gentz, R. & Dixit, V.M. (1997) I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem*, **272**, 17255-17257.



Hunter, D.R., Haworth, R.A. & Southard, J.H. (1976) Relationship between configuration, function, and permeability in calcium-treated mitochondria. *J Biol Chem*, **251**, 5069-5077.

Jacobs, M.D. & Harrison, S.C. (1998) Structure of an IkappaBalpha/NF-kappaB complex. *Cell*, **95**, 749-758.

Janssen, O., Sanzenbacher, R. & Kabelitz, D. (2000) Regulation of activation-induced cell death of mature T-lymphocyte populations. *Cell Tissue Res*, **301**, 85-99.

Jeffers, J.R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K.H., Han, J., Chittenden, T., Ihle, J.N., McKinnon, P.J., Cleveland, J.L. & Zambetti, G.P. (2003) Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell*, **4**, 321-328.

Jiang, Y., Woronicz, J.D., Liu, W. & Goeddel, D.V. (1999) Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. *Science*, **283**, 543-546.

Johnson, D.E. (2000) Noncaspase proteases in apoptosis. *Leukemia*, **14**, 1695-1703.

Joza, N., Susin, S.A., Daugas, E., Stanford, W.L., Cho, S.K., Li, C.Y., Sasaki, T., Elia, A.J., Cheng, H.Y., Ravagnan, L., Ferri, K.F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y.Y., Mak, T.W., Zuniga-Pflucker, J.C., Kroemer, G. & Penninger, J.M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature*, **410**, 549-554.

Jung, Y.J., Isaacs, J.S., Lee, S., Trepel, J. & Neckers, L. (2003) IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J*, **17**, 2115-2117.

Jurgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D. & Reed, J.C. (1998) Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A*, **95**, 4997-5002.

Kang, N., Zhang, J.H., Qiu, F., Tashiro, S., Onodera, S. & Ikejima, T. (2010) Inhibition of EGFR signaling augments oridonin-induced apoptosis in human laryngeal cancer cells via enhancing oxidative stress coincident with activation of both the intrinsic and extrinsic apoptotic pathways. *Cancer Lett*, **294**, 147-158.



Karin, M. (2006) Nuclear factor-kappaB in cancer development and progression. *Nature*, **441**, 431-436.

Karin, M. & Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol*, **18**, 621-663.

Karin, M., Cao, Y., Greten, F.R. & Li, Z.W. (2002) NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer*, **2**, 301-310.

Karin, M. & Lin, A. (2002) NF-kappaB at the crossroads of life and death. *Nat Immunol*, **3**, 221-227.

Karin, M., Yamamoto, Y. & Wang, Q.M. (2004) The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov.*, **3**, 17-26.

Katz, Y., Ben-Baruch, G., Kloog, Y., Menczer, J. & Gavish, M. (1990) Increased density of peripheral benzodiazepine-binding sites in ovarian carcinomas as compared with benign ovarian tumours and normal ovaries. *Clin Sci (Lond)*, **78**, 155-158.

Kaufmann, S.H. (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res*, **49**, 5870-5878.

Kerr, J.F., Wyllie, A.H. & Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, **26**, 239-257.

Kiechle, F.L. & Zhang, X. (2002) Apoptosis: biochemical aspects and clinical implications. *Clin Chim Acta*, **326**, 27-45.

Kiefer, M.C., Brauer, M.J., Powers, V.C., Wu, J.J., Umansky, S.R., Tomei, L.D. & Barr, P.J. (1995) Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature*, **374**, 736-739.

Kim, H., Rafiuddin-Shah, M., Tu, H.C., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J. & Cheng, E.H. (2006a) Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol*, **8**, 1348-1358.



Kim, H.J., Hawke, N. & Baldwin, A.S. (2006b) NF-kappaB and IKK as therapeutic targets in cancer. *Cell Death Differ*, **13**, 738-747.

Kim, J.H., Kim, B., Cai, L., Choi, H.J., Ohgi, K.A., Tran, C., Chen, C., Chung, C.H., Huber, O., Rose, D.W., Sawyers, C.L., Rosenfeld, M.G. & Baek, S.H. (2005) Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes. *Nature*, **434**, 921-926.

Kim, J.W., Choi, E.J. & Joe, C.O. (2000) Activation of death-inducing signaling complex (DISC) by pro-apoptotic C-terminal fragment of RIP. *Oncogene*, **19**, 4491-4499.

Kinloch, R.A., Treherne, J.M., Furness, L.M. & Hajimohamadreza, I. (1999) The pharmacology of apoptosis. *Trends Pharmacol Sci*, **20**, 35-42.

Kinnally, K.W., Peixoto, P.M., Ryu, S.Y. & Dejean, L.M. (2010) Is mPTP the gatekeeper for necrosis, apoptosis, or both? *Biochim Biophys Acta*, **1813**, 616-622. Epub 2010 Oct 2011.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H. & Peter, M.E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J*, **14**, 5579-5588.

Klement, J.F., Rice, N.R., Car, B.D., Abbondanzo, S.J., Powers, G.D., Bhatt, P.H., Chen, C.H., Rosen, C.A. & Stewart, C.L. (1996) IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. *Mol Cell Biol*, **16**, 2341-2349.

Kluck, R.M., Bossy-Wetzell, E., Green, D.R. & Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275**, 1132-1136.

Kokoszka, J.E., Waymire, K.G., Levy, S.E., Sligh, J.E., Cai, J., Jones, D.P., MacGregor, G.R. & Wallace, D.C. (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature*, **427**, 461-465.

Konishi, A., Shimizu, S., Hirota, J., Takao, T., Fan, Y., Matsuoka, Y., Zhang, L., Yoneda, Y., Fujii, Y., Skoultchi, A.I. & Tsujimoto, Y. (2003) Involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks. *Cell*, **114**, 673-688.



- Korsmeyer, S.J. (1995) Regulators of cell death. *Trends Genet*, **11**, 101-105.
- Kroemer, G. (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat Med*, **3**, 614-620.
- Kroemer, G., Dallaporta, B. & Resche-Rigon, M. (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol*, **60**, 619-642.
- Kroemer, G., Galluzzi, L. & Brenner, C. (2007) Mitochondrial membrane permeabilization in cell death. *Physiol Rev*, **87**, 99-163.
- Kroemer, G. & Pouyssegur, J. (2008) Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell*, **13**, 472-482.
- Kroemer, G. & Reed, J.C. (2000) Mitochondrial control of cell death. *Nat Med*, **6**, 513-519.
- Kubasiak, L.A., Hernandez, O.M., Bishopric, N.H. & Webster, K.A. (2002) Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. *Proc Natl Acad Sci U S A*, **99**, 12825-12830.
- Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P. & Flavell, R.A. (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell*, **94**, 325-337.
- Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R. & Newmeyer, D.D. (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell*, **111**, 331-342.
- Le Bras, M., Clement, M.V., Pervaiz, S. & Brenner, C. (2005) Reactive oxygen species and the mitochondrial signaling pathway of cell death. *Histol Histopathol*, **20**, 205-219.
- Lee, F.S., Hagler, J., Chen, Z.J. & Maniatis, T. (1997) Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell*, **88**, 213-222.
- Lee, F.S., Peters, R.T., Dang, L.C. & Maniatis, T. (1998) MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta. *Proc Natl Acad Sci U S A*, **95**, 9319-9324.



Lee, H.H., Dadgostar, H., Cheng, Q., Shu, J. & Cheng, G. (1999) NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc Natl Acad Sci U S A*, **96**, 9136-9141.

Lee, J., Sharma, S., Kim, J., Ferrante, R.J. & Ryu, H. (2008) Mitochondrial nuclear receptors and transcription factors: who's minding the cell? *J Neurosci Res*, **86**, 961-971.

Leist, M. & Jaattela, M. (2001) Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol*, **2**, 589-598.

Leist, M., Single, B., Castoldi, A.F., Kuhnle, S. & Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med*, **185**, 1481-1486.

Lemarie, A. & Grimm, S. (2011) Mitochondrial respiratory chain complexes: apoptosis sensors mutated in cancer? *Oncogene*, **30**, 3985-4003.

Lemarie, A., Huc, L., Pazarentzos, E., Mahul-Mellier, A.L. & Grimm, S. (2011) Specific disintegration of complex II succinate:ubiquinone oxidoreductase links pH changes to oxidative stress for apoptosis induction. *Cell Death Differ*, **18**, 338-349.

Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S. & Korsmeyer, S.J. (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*, **2**, 183-192.

Leu, J.I., Dumont, P., Hafey, M., Murphy, M.E. & George, D.L. (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol*, **6**, 443-450.

Leung, A.W. & Halestrap, A.P. (2008) Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore. *Biochim Biophys Acta*, **1777**, 946-952.

Lewis, K. (2000) Programmed death in bacteria. *Microbiol Mol Biol Rev*, **64**, 503-514.

Li, H., Zhu, H., Xu, C.J. & Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, **94**, 491-501.



Li, L.Y., Luo, X. & Wang, X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*, **412**, 95-99.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. & Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479-489.

Li, Q. & Verma, I.M. (2002) NF-kappaB regulation in the immune system. *Nat Rev Immunol*, **2**, 725-734.

Lin, Y., Devin, A., Rodriguez, Y. & Liu, Z.G. (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev*, **13**, 2514-2526.

Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V.M., Adelman, D.M., Simon, M.C., Ma, A., Golden, J.A., Evan, G., Korsmeyer, S.J., MacGregor, G.R. & Thompson, C.B. (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell*, **6**, 1389-1399.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R. & Wang, X. (1996a) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, **86**, 147-157.

Liu, Z.G., Hsu, H., Goeddel, D.V. & Karin, M. (1996b) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell*, **87**, 565-576.

Lu, T., Burdelya, L.G., Swiatkowski, S.M., Boiko, A.D., Howe, P.H., Stark, G.R. & Gudkov, A.V. (2004) Secreted transforming growth factor beta2 activates NF-kappaB, blocks apoptosis, and is essential for the survival of some tumor cells. *Proc Natl Acad Sci U S A*, **101**, 7112-7117.

Lucken-Ardjomande, S. & Martinou, J.C. (2005) Newcomers in the process of mitochondrial permeabilization. *J Cell Sci*, **118**, 473-483.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*, **94**, 481-490.



Luque, I. & Gelinas, C. (1998) Distinct domains of IkappaBalpha regulate c-Rel in the cytoplasm and in the nucleus. *Mol Cell Biol*, **18**, 1213-1224.

Macdonald, J., Galley, H.F. & Webster, N.R. (2003) Oxidative stress and gene expression in sepsis. *Br J Anaesth*, **90**, 221-232.

Machida, K., Ohta, Y. & Osada, H. (2006) Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells. *J Biol Chem*, **281**, 14314-14320.

Mahul-Mellier, A.L., Pazarentzos, E., Datler, C., Iwasawa, R., Abuali, G., Lin, B. & Grimm, S. (2011) De-ubiquitinating protease USP2a targets RIP1 and TRAF2 to mediate cell death by TNF. *Cell Death Differ*.

Majewski, N., Nogueira, V., Bhaskar, P., Coy, P.E., Skeen, J.E., Gottlob, K., Chandel, N.S., Thompson, C.B., Robey, R.B. & Hay, N. (2004a) Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell*, **16**, 819-830.

Majewski, N., Nogueira, V., Robey, R.B. & Hay, N. (2004b) Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases. *Mol Cell Biol*, **24**, 730-740.

Malek, S., Chen, Y., Huxford, T. & Ghosh, G. (2001) IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *J Biol Chem*, **276**, 45225-45235.

Mancini, M., Nicholson, D.W., Roy, S., Thornberry, N.A., Peterson, E.P., Casciola-Rosen, L.A. & Rosen, A. (1998) The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. *J Cell Biol*, **140**, 1485-1495.

Mathupala, S.P., Ko, Y.H. & Pedersen, P.L. (2006) Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. *Oncogene*, **25**, 4777-4786.

McCarthy, J.V. & Dixit, V.M. (1998) Apoptosis induced by Drosophila reaper and grim in a human system. Attenuation by inhibitor of apoptosis proteins (cIAPs). *J Biol Chem*, **273**, 24009-24015.



McConkey, D.J., Zhivotovsky, B. & Orrenius, S. (1996) Apoptosis--molecular mechanisms and biomedical implications. *Mol Aspects Med*, **17**, 1-110.

McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., McKearn, J.P. & Korsmeyer, S.J. (1989) bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell*, **57**, 79-88.

McDonnell, T.J. & Korsmeyer, S.J. (1991) Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature*, **349**, 254-256.

Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H. & Peter, M.E. (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J*, **16**, 2794-2804.

Meineke, B., Engl, G., Kemper, C., Vasiljev-Neumeyer, A., Paulitschke, H. & Rapaport, D. (2008) The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway. *FEBS Lett*, **582**, 855-860.

Metkar, S.S., Wang, B., Ebbs, M.L., Kim, J.H., Lee, Y.J., Raja, S.M. & Froelich, C.J. (2003) Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis. *J Cell Biol*, **160**, 875-885.

Mikhailov, V., Mikhailova, M., Degenhardt, K., Venkatachalam, M.A., White, E. & Saikumar, P. (2003) Association of Bax and Bak homo-oligomers in mitochondria. Bax requirement for Bak reorganization and cytochrome c release. *J Biol Chem*, **278**, 5367-5376.

Mitchell, P. & Moyle, J. (1965a) Evidence discriminating between the chemical and the chemiosmotic mechanisms of electron transport phosphorylation. *Nature*, **208**, 1205-1206.

Mitchell, P. & Moyle, J. (1965b) Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. *Nature*, **208**, 147-151.

Morse, D.L., Gray, H., Payne, C.M. & Gillies, R.J. (2005) Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. *Mol Cancer Ther*, **4**, 1495-1504.



Mow, B.M., Blajeski, A.L., Chandra, J. & Kaufmann, S.H. (2001) Apoptosis and the response to anticancer therapy. *Curr Opin Oncol*, **13**, 453-462.

Muenchen, H.J., Lin, D.L., Walsh, M.A., Keller, E.T. & Pienta, K.J. (2000) Tumor necrosis factor-alpha-induced apoptosis in prostate cancer cells through inhibition of nuclear factor-kappaB by an IkappaBalpha "super-repressor". *Clin Cancer Res*, **6**, 1969-1977.

Munoz, E. & Israel, A. (1995) Activation of NF-kappa B by the Tax protein of HTLV-1. *Immunobiology*, **193**, 128-136.

Nagata, S. (1997) Apoptosis by death factor. *Cell*, **88**, 355-365.

Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., Inohara, H., Kubo, T. & Tsujimoto, Y. (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature*, **434**, 652-658.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D.A., Goulet, R.J., Jr. & Sledge, G.W., Jr. (1997a) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol*, **17**, 3629-3639.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D.A., Goulet, R.J., Jr. & Sledge, G.W., Jr. (1997b) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol*, **17**, 3629-3639.

Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R.J., Matsuda, H. & Tsujimoto, Y. (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc Natl Acad Sci U S A*, **95**, 14681-14686.

Newmeyer, D.D. & Ferguson-Miller, S. (2003) Mitochondria: releasing power for life and unleashing the machineries of death. *Cell*, **112**, 481-490.

Nichols, D., Chmiel, J. & Berger, M. (2008) Chronic inflammation in the cystic fibrosis lung: alterations in inter- and intracellular signaling. *Clin Rev Allergy Immunol*, **34**, 146-162.

Nicholson, D.W. & Thornberry, N.A. (1997) Caspases: killer proteases. *Trends Biochem Sci*, **22**, 299-306.



Oltvai, Z.N., Milliman, C.L. & Korsmeyer, S.J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609-619.

Ozawa, T., Natori, Y., Sako, Y., Kuroiwa, H., Kuroiwa, T. & Umezawa, Y. (2007a) A minimal peptide sequence that targets fluorescent and functional proteins into the mitochondrial intermembrane space. *ACS Chem Biol*, **2**, 176-186. Epub 2007 Mar 2009.

Ozawa, T., Natori, Y., Sako, Y., Kuroiwa, H., Kuroiwa, T. & Umezawa, Y. (2007b) A minimal peptide sequence that targets fluorescent and functional proteins into the mitochondrial intermembrane space. *ACS Chem Biol*, **2**, 176-186.

Palombella, V.J., Rando, O.J., Goldberg, A.L. & Maniatis, T. (1994) The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell*, **78**, 773-785.

Pastorino, J.G. & Hoek, J.B. (2003) Hexokinase II: the integration of energy metabolism and control of apoptosis. *Curr Med Chem*, **10**, 1535-1551.

Pastorino, J.G. & Hoek, J.B. (2008a) Regulation of hexokinase binding to VDAC. *J Bioenerg Biomembr*, **40**, 171-182.

Pastorino, J.G. & Hoek, J.B. (2008b) Regulation of hexokinase binding to VDAC. *J Bioenerg Biomembr*, **40**, 171-182.

Pastorino, J.G., Shulga, N. & Hoek, J.B. (2002) Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. *J Biol Chem*, **277**, 7610-7618.

Pedersen, P.L., Mathupala, S., Rempel, A., Geschwind, J.F. & Ko, Y.H. (2002) Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochim Biophys Acta*, **1555**, 14-20.

Pelicano, H., Carney, D. & Huang, P. (2004) ROS stress in cancer cells and therapeutic implications. *Drug Resist Updat*, **7**, 97-110.

Pereira, C., Camougrand, N., Manon, S., Sousa, M.J. & Corte-Real, M. (2007) ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome c release in yeast apoptosis. *Mol Microbiol*, **66**, 571-582.



Pereira, C., Silva, R.D., Saraiva, L., Johansson, B., Sousa, M.J. & Corte-Real, M. (2008) Mitochondria-dependent apoptosis in yeast. *Biochim Biophys Acta*, **1783**, 1286-1302.

Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S. & Bernardi, P. (1994) The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. *J Biol Chem*, **269**, 16638-16642.

Petronilli, V., Miotto, G., Canton, M., Brini, M., Colonna, R., Bernardi, P. & Di Lisa, F. (1999) Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys J*, **76**, 725-734.

Petronilli, V., Szabo, I. & Zoratti, M. (1989) The inner mitochondrial membrane contains ion-conducting channels similar to those found in bacteria. *FEBS Lett*, **259**, 137-143.

Piret, J.P., Arnould, T., Fuks, B., Chatelain, P., Remacle, J. & Michiels, C. (2004) Caspase activation precedes PTP opening in TNF-alpha-induced apoptosis in L929 cells. *Mitochondrion*, **3**, 261-278.

Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A. & Ashkenazi, A. (1996) Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem*, **271**, 12687-12690.

Prasad, S., Ravindran, J. & Aggarwal, B.B. (2009) NF-kappaB and cancer: how intimate is this relationship. *Mol Cell Biochem*, **336**, 25-37. Epub 2009 Oct 2008.

Precht, T.A., Phelps, R.A., Linseman, D.A., Butts, B.D., Le, S.S., Laessig, T.A., Bouchard, R.J. & Heidenreich, K.A. (2005) The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis. *Cell Death Differ*, **12**, 255-265.

Raffray, M. & Cohen, G.M. (1997) Apoptosis and necrosis in toxicology: a continuum or distinct modes of cell death? *Pharmacol Ther*, **75**, 153-177.

Rangan, G., Wang, Y. & Harris, D. (2009) NF-kappaB signalling in chronic kidney disease. *Front Biosci*, **14**, 3496-3522.



Rao, P., Hayden, M.S., Long, M., Scott, M.L., West, A.P., Zhang, D., Oeckinghaus, A., Lynch, C., Hoffmann, A., Baltimore, D. & Ghosh, S. (2010) IkappaBbeta acts to inhibit and activate gene expression during the inflammatory response. *Nature*, **466**, 1115-1119.

Ravi, R. & Bedi, A. (2004) NF-kappaB in cancer--a friend turned foe. *Drug Resist Updat*, **7**, 53-67.

Reuther, J.Y. & Baldwin, A.S., Jr. (1999) Apoptosis promotes a caspase-induced amino-terminal truncation of IkappaBalpha that functions as a stable inhibitor of NF-kappaB. *J Biol Chem*, **274**, 20664-20670.

Robe, P.A., Bentires-Alj, M., Bonif, M., Rogister, B., Deprez, M., Haddada, H., Khac, M.T., Jolois, O., Erkmen, K., Merville, M.P., Black, P.M. & Bours, V. (2004) In vitro and in vivo activity of the nuclear factor-kappaB inhibitor sulfasalazine in human glioblastomas. *Clin Cancer Res*, **10**, 5595-5603.

Rodriguez, M.S., Wright, J., Thompson, J., Thomas, D., Baleux, F., Virelizier, J.L., Hay, R.T. & Arenzana-Seisdedos, F. (1996) Identification of lysine residues required for signal-induced ubiquitination and degradation of I kappa B-alpha in vivo. *Oncogene*, **12**, 2425-2435.

Roff, M., Thompson, J., Rodriguez, M.S., Jacque, J.M., Baleux, F., Arenzana-Seisdedos, F. & Hay, R.T. (1996) Role of IkappaBalpha ubiquitination in signal-induced activation of NFkappaB in vivo. *J Biol Chem*, **271**, 7844-7850.

Roje, Z., Matic, D., Librenjak, D., Dokuzovic, S. & Varvodic, J. (2011) Necrotizing fasciitis: literature review of contemporary strategies for diagnosing and management with three case reports: torso, abdominal wall, upper and lower limbs. *World J Emerg Surg*, **6**, 46.

Roman-Blas, J.A. & Jimenez, S.A. (2008) Targeting NF-kappaB: a promising molecular therapy in inflammatory arthritis. *Int Rev Immunol*, **27**, 351-374.

Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D.C., Traish, A.M., Mercurio, F. & Sonenshein, G.E. (2001) Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer. *Cancer Res*, **61**, 3810-3818.

Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B. & Borner, C. (1998) Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature*, **391**, 496-499.



Sachdev, S., Hoffmann, A. & Hannink, M. (1998a) Nuclear localization of IkappaB alpha is mediated by the second ankyrin repeat: the IkappaB alpha ankyrin repeats define a novel class of cis-acting nuclear import sequences. *Mol Cell Biol*, **18**, 2524-2534.

Sachdev, S., Hoffmann, A. & Hannink, M. (1998b) Nuclear localization of IkappaB alpha is mediated by the second ankyrin repeat: the IkappaB alpha ankyrin repeats define a novel class of cis-acting nuclear import sequences. *Mol Cell Biol*, **18**, 2524-2534.

Sakamoto, K., Maeda, S., Hikiba, Y., Nakagawa, H., Hayakawa, Y., Shibata, W., Yanai, A., Ogura, K. & Omata, M. (2009) Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. *Clin Cancer Res*, **15**, 2248-2258. Epub 2009 Mar 2210.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. & Peter, M.E. (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*, **17**, 1675-1687.

Schaecher, K., Goust, J.M. & Banik, N.L. (2004) The effects of calpain inhibition on Ikb alpha degradation after activation of PBMCs: identification of the calpain cleavage sites. *Neurochem Res*, **29**, 1443-1451.

Scherer, D.C., Brockman, J.A., Chen, Z., Maniatis, T. & Ballard, D.W. (1995) Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. *Proc Natl Acad Sci U S A*, **92**, 11259-11263.

Schinzl, A.C., Takeuchi, O., Huang, Z., Fisher, J.K., Zhou, Z., Rubens, J., Hetz, C., Danial, N.N., Moskowitz, M.A. & Korsmeyer, S.J. (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc Natl Acad Sci U S A*, **102**, 12005-12010.

Schubert, A. & Grimm, S. (2004) Cyclophilin D, a component of the permeability transition-pore, is an apoptosis repressor. *Cancer Res*, **64**, 85-93.

Shearwin-Whyatt, L.M., Harvey, N.L. & Kumar, S. (2000) Subcellular localization and CARD-dependent oligomerization of the death adaptor RAIDD. *Cell Death Differ*, **7**, 155-165.



Shoshan-Barmatz, V., De Pinto, V., Zweckstetter, M., Raviv, Z., Keinan, N. & Arbel, N. (2010) VDAC, a multi-functional mitochondrial protein regulating cell life and death. *Mol*, **31**, 227-285. Epub 2010 Mar 2023.

Silverman, N. & Maniatis, T. (2001) NF-kappaB signaling pathways in mammalian and insect innate immunity. *Genes Dev*, **15**, 2321-2342.

Sitcheran, R., Gupta, P., Fisher, P.B. & Baldwin, A.S. (2005) Positive and negative regulation of EAAT2 by NF-kappaB: a role for N-myc in TNFalpha-controlled repression. *EMBO J*, **24**, 510-520.

Sloviter, R.S. (2002) Apoptosis: a guide for the perplexed. *Trends Pharmacol Sci*, **23**, 19-24.

Smith, C.A., Farrah, T. & Goodwin, R.G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell*, **76**, 959-962.

Sovak, M.A., Bellas, R.E., Kim, D.W., Zanieski, G.J., Rogers, A.E., Traish, A.M. & Sonenshein, G.E. (1997) Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. *J Clin Invest*, **100**, 2952-2960.

Sperandio, S., de Belle, I. & Bredesen, D.E. (2000) An alternative, nonapoptotic form of programmed cell death. *Proc Natl Acad Sci U S A*, **97**, 14376-14381.

Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T. & Alnemri, E.S. (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell*, **1**, 949-957.

Srinivasula, S.M., Ahmad, M., Otilie, S., Bullrich, F., Banks, S., Wang, Y., Fernandes-Alnemri, T., Croce, C.M., Litwack, G., Tomaselli, K.J., Armstrong, R.C. & Alnemri, E.S. (1997) FLAME-1, a novel FADD-like anti-apoptotic molecule that regulates Fas/TNFR1-induced apoptosis. *J Biol Chem*, **272**, 18542-18545.

Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J.A., Binder, B.R. & Lipp, J. (1998) Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med*, **188**, 211-216.

Stennicke, H.R., Jurgensmeier, J.M., Shin, H., Deveraux, Q., Wolf, B.B., Yang, X., Zhou, Q., Ellerby, H.M., Ellerby, L.M., Bredesen, D., Green, D.R., Reed, J.C., Froelich,



C.J. & Salvesen, G.S. (1998) Pro-caspase-3 is a major physiologic target of caspase-8. *J Biol Chem*, **273**, 27084-27090.

Stoica, B.A. & Faden, A.I. (2010) Cell death mechanisms and modulation in traumatic brain injury. *Neurotherapeutics*, **7**, 3-12.

Strasser, A., Harris, A.W., Bath, M.L. & Cory, S. (1990) Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature*, **348**, 331-333.

Suh, J., Payvandi, F., Edelstein, L.C., Amenta, P.S., Zong, W.X., Gelinas, C. & Rabson, A.B. (2002) Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. *Prostate*, **52**, 183-200.

Sukumaran, S.K., Fu, N.Y., Tin, C.B., Wan, K.F., Lee, S.S. & Yu, V.C. (2010a) A soluble form of the pilus protein FimA targets the VDAC-hexokinase complex at mitochondria to suppress host cell apoptosis. *Mol*, **37**, 768-783.

Sukumaran, S.K., Fu, N.Y., Tin, C.B., Wan, K.F., Lee, S.S. & Yu, V.C. (2010b) A soluble form of the pilus protein FimA targets the VDAC-hexokinase complex at mitochondria to suppress host cell apoptosis. *Mol Cell*, **37**, 768-783.

Sun, S.C., Elwood, J., Beraud, C. & Greene, W.C. (1994) Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. *Mol Cell Biol*, **14**, 7377-7384.

Susin, S.A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K.F., Irinopoulou, T., Prevost, M.C., Brothers, G., Mak, T.W., Penninger, J., Earnshaw, W.C. & Kroemer, G. (2000) Two distinct pathways leading to nuclear apoptosis. *J Exp Med*, **192**, 571-580.

Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M. & Kroemer, G. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med*, **184**, 1331-1341.

Susin, S.A., Zamzami, N. & Kroemer, G. (1998) Mitochondria as regulators of apoptosis: doubt no more. *Biochim Biophys Acta*, **1366**, 151-165.

Suzuki, M., Youle, R.J. & Tjandra, N. (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell*, **103**, 645-654.



Syntichaki, P. & Tavernarakis, N. (2003) The biochemistry of neuronal necrosis: rogue biology? *Nat Rev Neurosci*, **4**, 672-684.

Szabo, I. & Zoratti, M. (1991) The giant channel of the inner mitochondrial membrane is inhibited by cyclosporin A. *J Biol Chem*, **266**, 3376-3379.

Szabo, I. & Zoratti, M. (1992) The mitochondrial megachannel is the permeability transition pore. *J Bioenerg Biomembr*, **24**, 111-117.

Tergaonkar, V., Correa, R.G., Ikawa, M. & Verma, I.M. (2005) Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. *Nat Cell Biol*, **7**, 921-923.

Thornberry, N.A., Peterson, E.P., Zhao, J.J., Howard, A.D., Griffin, P.R. & Chapman, K.T. (1994) Inactivation of interleukin-1 beta converting enzyme by peptide (acyloxy)methyl ketones. *Biochemistry*, **33**, 3934-3940.

Traenckner, E.B., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S. & Baeuerle, P.A. (1995) Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *EMBO J*, **14**, 2876-2883.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. & Croce, C.M. (1985) The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science*, **229**, 1390-1393.

Tsujimoto, Y. & Shimizu, S. (2002) The voltage-dependent anion channel: an essential player in apoptosis. *Biochimie.*, **84**, 187-193.

Turpin, P., Hay, R.T. & Dargemont, C. (1999) Characterization of IkappaBalpha nuclear import pathway. *J Biol Chem*, **274**, 6804-6812.

Valentijn, A.J. & Gilmore, A.P. (2004) Translocation of full-length Bid to mitochondria during anoikis. *J Biol Chem*, **279**, 32848-32857.

Valentijn, A.J., Metcalfe, A.D., Kott, J., Streuli, C.H. & Gilmore, A.P. (2003) Spatial and temporal changes in Bax subcellular localization during anoikis. *J Cell Biol*, **162**, 599-612.



- Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R. & Verma, I.M. (1996) Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science*, **274**, 787-789.
- Van Antwerp, D.J., Martin, S.J., Verma, I.M. & Green, D.R. (1998) Inhibition of TNF-induced apoptosis by NF-kappa B. *Trends Cell Biol*, **8**, 107-111.
- Van Antwerp, D.J. & Verma, I.M. (1996) Signal-induced degradation of I(kappa)B(alpha): association with NF-kappaB and the PEST sequence in I(kappa)B(alpha) are not required. *Mol Cell Biol*, **16**, 6037-6045.
- Vande Velde, C., Cizeau, J., Dubik, D., Alimonti, J., Brown, T., Israels, S., Hakem, R. & Greenberg, A.H. (2000) BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol*, **20**, 5454-5468.
- Varfolomeev, E.E., Schuchmann, M., Luria, V., Chiannikulchai, N., Beckmann, J.S., Mett, I.L., Rebrikov, D., Brodianski, V.M., Kemper, O.C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K.B., Goncharov, T., Holtmann, H., Lonai, P. & Wallach, D. (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity*, **9**, 267-276.
- Vaux, D.L., Cory, S. & Adams, J.M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*, **335**, 440-442.
- Vaux, D.L. & Korsmeyer, S.J. (1999) Cell death in development. *Cell*, **96**, 245-254.
- Vermeulen, K., Strnad, M., Havlicek, L., Van Onckelen, H., Lenjou, M., Nijs, G., Van Bockstaele, D.R. & Berneman, Z.N. (2002) Plant cytokinin analogues with inhibitory activity on cyclin-dependent kinases exert their antiproliferative effect through induction of apoptosis initiated by the mitochondrial pathway: determination by a multiparametric flow cytometric analysis. *Exp Hematol*, **30**, 1107-1114.
- Verrier, F., Deniaud, A., Lebras, M., Metivier, D., Kroemer, G., Mignotte, B., Jan, G. & Brenner, C. (2004) Dynamic evolution of the adenine nucleotide translocase interactome during chemotherapy-induced apoptosis. *Oncogene*, **23**, 8049-8064.
- Vousden, K.H. & Lu, X. (2002) Live or let die: the cell's response to p53. *Nat Rev Cancer*, **2**, 594-604.



Vyssokikh, M. & Brdiczka, D. (2004) VDAC and peripheral channelling complexes in health and disease. *Mol Cell Biochem*, **256-257**, 117-126.

Vyssokikh, M.Y. & Brdiczka, D. (2003) The function of complexes between the outer mitochondrial membrane pore (VDAC) and the adenine nucleotide translocase in regulation of energy metabolism and apoptosis. *Acta Biochim Pol*, **50**, 389-404.

Wajant, H., Pfizenmaier, K. & Scheurich, P. (2003) Tumor necrosis factor signaling. *Cell Death Differ*, **10**, 45-65.

Walczak, H. & Krammer, P.H. (2000) The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res*, **256**, 58-66.

Waldmeier, P.C., Feldtrauer, J.J., Qian, T. & Lemasters, J.J. (2002) Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporin derivative NIM811. *Mol Pharmacol*, **62**, 22-29.

Walker, P.R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J.F. & Sikorska, M. (1991) Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res*, **51**, 1078-1085.

Wang, C.Y., Cusack, J.C., Jr., Liu, R. & Baldwin, A.S., Jr. (1999) Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. *Nat Med*, **5**, 412-417.

Wang, C.Y., Mayo, M.W. & Baldwin, A.S., Jr. (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science*, **274**, 784-787.

Weaver, J.G., Tarze, A., Moffat, T.C., Lebras, M., Deniaud, A., Brenner, C., Bren, G.D., Morin, M.Y., Phenix, B.N., Dong, L., Jiang, S.X., Sim, V.L., Zurakowski, B., Lallier, J., Hardin, H., Wettstein, P., van Heeswijk, R.P., Douen, A., Kroemer, R.T., Hou, S.T., Bennett, S.A., Lynch, D.H., Kroemer, G. & Badley, A.D. (2005) Inhibition of adenine nucleotide translocator pore function and protection against apoptosis in vivo by an HIV protease inhibitor. *J Clin Invest*, **115**, 1828-1838.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. & Korsmeyer, S.J. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, **292**, 727-730.



Westerheide, S.D., Mayo, M.W., Anest, V., Hanson, J.L. & Baldwin, A.S., Jr. (2001) The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. *Mol Cell Biol*, **21**, 8428-8436.

Wiley, S.E., Murphy, A.N., Ross, S.A., van der Geer, P. & Dixon, J.E. (2007a) MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity. *Proc Natl Acad Sci U S A*, **104**, 5318-5323. Epub 2007 Mar 5321.

Wiley, S.E., Murphy, A.N., Ross, S.A., van der Geer, P. & Dixon, J.E. (2007b) MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity. *Proc Natl Acad Sci U S A*, **104**, 5318-5323.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. & Youle, R.J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol*, **139**, 1281-1292.

Xiang, J., Chao, D.T. & Korsmeyer, S.J. (1996) BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. *Proc Natl Acad Sci U S A*, **93**, 14559-14563.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P. & Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*, **275**, 1129-1132.

Yao, H. & Rahman, I. (2009) Current concepts on the role of inflammation in COPD and lung cancer. *Curr Opin Pharmacol.*, **9**, 375-383. Epub 2009 Jul 2015.

Yeh, W.C., Pompa, J.L., McCurrach, M.E., Shu, H.B., Elia, A.J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Deiry, W.S., Lowe, S.W., Goeddel, D.V. & Mak, T.W. (1998) FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science*, **279**, 1954-1958.

Yoshida, H., Kong, Y.Y., Yoshida, R., Elia, A.J., Hakem, A., Hakem, R., Penninger, J.M. & Mak, T.W. (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell*, **94**, 739-750.

Yu, J., Qian, H., Li, Y., Wang, Y., Zhang, X., Liang, X., Fu, M. & Lin, C. (2007) Therapeutic effect of arsenic trioxide (As₂O₃) on cervical cancer in vitro and in vivo through apoptosis induction. *Cancer Biol Ther*, **6**, 580-586.



Zaid, H., Abu-Hamad, S., Israelson, A., Nathan, I. & Shoshan-Barmatz, V. (2005) The voltage-dependent anion channel-1 modulates apoptotic cell death. *Cell Death Differ*, **12**, 751-760.

Zamora, M., Merono, C., Vinas, O. & Mampel, T. (2004) Recruitment of NF-kappaB into mitochondria is involved in adenine nucleotide translocase 1 (ANT1)-induced apoptosis. *J Biol Chem*, **279**, 38415-38423.

Zamzami, N., El Hamel, C., Maise, C., Brenner, C., Munoz-Pinedo, C., Belzacq, A.S., Costantini, P., Vieira, H., Loeffler, M., Molle, G. & Kroemer, G. (2000) Bid acts on the permeability transition pore complex to induce apoptosis. *Oncogene*, **19**, 6342-6350.

Zamzami, N. & Kroemer, G. (2001) The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol*, **2**, 67-71.

Zamzami, N. & Kroemer, G. (2003) Apoptosis: mitochondrial membrane permeabilization--the (w)hole story? *Curr Biol*, **13**, R71-73.

Zamzami, N., Larochette, N. & Kroemer, G. (2005) Mitochondrial permeability transition in apoptosis and necrosis. *Cell Death Differ.*, **12**, 1478-1480.

Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.A., Petit, P.X., Mignotte, B. & Kroemer, G. (1995a) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med*, **182**, 367-377.

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X. & Kroemer, G. (1995b) Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med*, **181**, 1661-1672.

Zhang, X.D., Gillespie, S.K. & Hersey, P. (2004) Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways. *Mol Cancer Ther*, **3**, 187-197.

Zheng, Y., Shi, Y., Tian, C., Jiang, C., Jin, H., Chen, J., Almasan, A., Tang, H. & Chen, Q. (2004) Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome c release induced by arsenic trioxide. *Oncogene*, **23**, 1239-1247.



Zoratti, M. & Szabo, I. (1995) The mitochondrial permeability transition. *Biochim Biophys Acta*, **1241**, 139-176.

Zoratti, M., Szabo, I. & De Marchi, U. (2005) Mitochondrial permeability transitions: how many doors to the house? *Biochim Biophys Acta*, **1706**, 40-52.