AMERICAN UNIVERSITY OF BEIRUT

NOXA AND CERAMIDE: CROSSTALK BETWEEN BCL-2 FAMILY AND P53-DEPENDENT CERAMIDE ACCUMULATION IN MEDIATING INTRINSIC APOPTOSIS IN MOLT-4 HUMAN T-CELL LEUKEMIA AND HCT116 COLON CANCER CELL LINES

by
HADILE O. KOBEISSY

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HADILE O. KOBEISSY

Approved by:

Dr. Ghassan Dbaibo, Professor — Advisor
Department of Biochemistry and Molecular Genetics

Dr. Georges Nemer, Associate Professor — Member of Committee
Department of Biochemistry and Molecular Genetics

Dr. Nadine Darwiche, Professor — Member of Committee
Department of Biochemistry and Molecular Genetics

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AN ABSTRACT OF THE THESIS OF

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Title: Noxa and ceramide: crosstalk between Bcl-2 family and p53-dependent ceramide accumulation in mediating intrinsic apoptosis in Molt-4 human T-cell leukemia and HCT116 colon cancer cell lines.

The imbalance between cell proliferation and apoptosis disturbs health and homeostasis of living systems and as a result might favor tumor development and accelerated cell growth or degeneration and cell loss. This equilibrium depends on the coordinated action of various tumor suppressors specifically p53 protein, the sphingolipid ceramide, and Bcl-2 family proteins. In fact, several tumors were shown to possess defects in ceramide biosynthesis such as overexpression of glucosylceramide synthase in metastatic breast cancer, stage III. Hence, ceramide functions as a tumor suppressor lipid by regulating apoptosis, cell cycle arrest and senescence. One of the known ceramide regulators is p53 which once activated will trigger ceramide accumulation and subsequently mitochondrial outer membrane permeabilization (MOMP) followed by apoptosis. Likewise, Noxa, a BH3-only pro-apoptotic protein, which is in turn activated by p53, regulates downstream proteins that initiate the apoptotic machinery. Here we identify a novel link between Noxa and ceramide in the apoptotic pathway that, to our knowledge, were previously thought to work independently. Specifically, we show that ceramide accumulation was triggered by γ-irradiation in the presence of both p53 and Noxa, whereas the blocking of either molecule has inhibited this increase in ceramide levels. In fact, the blocking of Noxa protein did not only affect ceramide generation but also prevented caspase activation and decreased cellular death, thus underscoring the role of Noxa in the apoptotic pathway triggered by γ-irradiation. Furthermore, mitochondrial ceramide accumulation and cytochrome c release occurred simultaneously, suggesting that ceramide's role, at least in this context, is intricately related to the execution phase of apoptosis. Although, in our model, both extrinsic and intrinsic pathways were triggered, Noxa and ceramide were exclusively functioning in intrinsic apoptosis. The data presented indicate that ceramide-induced apoptosis is dependent on Noxa activation and that they are interrelated in the intrinsic pathway. Moreover, these data reveal the mode of ceramide behavior in the Bcl-2-induced apoptotic machinery in addition to the ordering of ceramide in the orchestrated Bcl-2-induced apoptosis. We believe that these results shall contribute to the emerging line of research in the enhancement of the efficacy of chemotherapeutic agents.

CONTENTS

ILLUSTRATIONS

TABLES

ABBREVIATIONS

AIF: Apoptosis-Inducing Factor

Alk-SMase: Alkaline Sphingomyelinase

APL: Acute Promyelocytic Leukemia

ASMase: Acid Sphingomyelinase

ATF: Activating Transcription Factor

BAD: Bcl-2 Antagonist of Cell death

BAK: Bcl-2 Antagonist Killer

BAX: Bcl-2 Associated X

BCL-xL: B Cell Lymphoma-Long Isoform

BCL-2: B cell lymphoma 2

BH: Bcl-2 Homology Domain

BID: Bcl-2 Interacting Protein

BIM: Bcl-2 Interacting Mediator of Cell death

CAPP: Ceramide Activated Protein Phosphatase

CDC25C: Cell Division Cycle 25C

CDK: Cyclin-Dependent Kinase

CerS: Ceramide Synthase

CERT: Ceramide Transfer Protein

CHK: Checkpoint Kinase

CHOP: C/EBP-Homologous Protein

CML: Chronic Myeloid Leukemia

DD: Death Domain

DGK: Di-acyl Glycerol Kinase

DISC: Death-Inducing Signaling Complex

DRP-1: Dynamin Related Protein-1

EGR1: Early Growth Response 1

ER: Endoplasmic Reticulum

ERK: Extracellular Signal-Regulated Kinase

FADD: FAS-Associated Death Domain

LASS: Longevity Assurance gene

MAM: Mitochondrial Associated Membrane

MCL1: Myeloid Cell Leukemia 1

MOM: Mitochondrial Outer Membrane

MOMP: Mitochondrial Outer Membrane Permeabilization

MTT: Microculture Tetrazolium Assay

NEAA: Non-Essential Amino Acid

NFLD: Nonalcoholic Fatty Liver Disease

NMR: Nuclear Magnetic Resonance

NSMase: Neutral Sphingomyelinase

PARP: Poly (ADP-Ribose) Polymerase

PERK: Protein kinase RNA-like ER Kinase

PI: Propidium Iodide

PKC: Protein Kinase C

PMAIP1: Phorbol-12-Myristate-13-Acetate Inducing Protein 1

PTEN: Phosphatase and Tensin homolog

PUMA: P53 Up regulated Modulator of Apoptosis

RB: Retinoblastoma

ROS: Reactive Oxygen Species

RPE: Retinal Pigment Epithelial

SM: Sphingomyelin

SMase: Sphingomyelinase

SPT: Serine-Palmitoyl Transferase

S1P: Sphingosine 1 Phosphate

S15: Serine 15

S46: Serine 46

TNF: Tumor Necrotic Factor

TNFR: Tumor Necrotic Factor Receptor

TRAIL: TNF-Related Apoptosis-Inducing Ligand

TRAILR: TNF-Related Apoptosis-Inducing Ligand Receptor

UPR: Unfolded Protein Response

UV: Ultra Violet

CHAPTER I

LITERATURE REVIEW

A. Ceramide in Cancer

In targeted therapy we aim to execute cancer cells while spare normal ones along with averting cancer recurrence. Triggering apoptosis as well as preventing proliferation of cancer cells while leaving normal cells unaffected is the role of targeted therapy; here comes the role of ceramide, a sphingolipid molecule. Only recently, was the role of ceramide appreciated in this mode of therapy. For instance, introducing C2 ceramide in retinal pigment epithelial (RPE) cells has induced selective apoptosis in non-polarized RPE while sparing normal RPE monolayer [1]. Today, ceramide garnered major interest to cancer biologists and clinical oncologists as a foremost regulator of cancer development. In fact, several sphingolipids, and not only ceramide, had demonstrated their important functions in cancer development irrespective of whether anti- or pro-. Ceramide is one of the biologically active molecules that negatively regulates cell growth by initiating apoptosis, senescence or cell cycle arrest, its significance was recognized since 1995 by Supriya Jayadev and his colleagues [2] . Alternatively, is sphingosine-1-phosphate (S1P), generated from sphingosine the precursor of ceramide, promotes cancer cell survival and migration by triggering anti-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins and extracellular signal-regulated kinases (ERK) activation, respectively [3-6]. In fact, several tumors possess imbalanced expression of sphingolipid enzymes therefore pointing out the significance of ceramide molecule in cancer development (see Table 1). For example, overexpressed glucosylceramide synthase, an enzyme mediating ceramide reduction by converting it into anti-apoptotic

glucosylceramide, was found to be associated with cancer resistance to ordinary chemotherapeutics such as vincristine, adriamycin, doxorubicin and imatinib-resistant chronic myeloid leukemia (CML) [7-11]. The inhibition of glucosylceramide synthase activity or by adding-on ceramide has averted the latter phenomenon. Today's research have shown that the mode of action of several chemotherapeutic agents do bring about ceramide accumulation in one way or another. For instance, Arsenic trioxide used for treating acute promyelocytic leukemia (APL) was shown to stimulate ceramide synthesis prior to cell death [12]. Ceramide-mediated therapy is advancing rapidly, with most recently the integration of C6-ceramide into pegylated nanoliposomes to enhance its intracellular delivery, owing to ceramide alone is difficult to be delivered intravenously due to its hydrophobic nature [13].

Table 1. Cancers with altered sphingolipid enzymes [14].

Enzyme	Expression	Cancer model
Glucosylceramide synthase	Overexpressed	Human cell lines of glioblastoma, leukaemia and neuroblastoma; and of colon, ovarian and breast cancer. Mouse cell line of melanoma and metastatic breast cancer, stage III
Acid ceramidase	Overexpressed	Human cell lines of prostate, breast and liver cancer; and of leukaemia and melanoma. Overexpressed in 70% of head and neck squamous cell tumours
Ceramide kinase	Overexpressed	Human tumour samples and human cell lines of breast and lung cancer and of leukaemia. Correlated with ERBB2 status in breast cancer
Sphingosine kinase 1	Overexpressed	Prostate cancer cells and prostate cancer specimens with Gleason score of ≥4+3
Sphingomyelinase	Downregulated	Human cell lines of colon and liver cancer
Serine palmitoyltransferase	Downregulated	Human cell lines of colon cancer

B. Ceramide Structure and Biosynthesis

Sphingolipids were named by J.L.W Thudichum in 1884 after the shinx of Greek mythology, attributable to their enigmatic structure. A large body of work had exerted in understanding sphingolipids structure, biosynthesis and function. Ceramide in particular show simple structure with complex biophysical properties. Ceramide, also called *N*-acylsphingosine, consist of fatty acid chain bound to an amino group at the sphingoid base, called sphingosine (Figure 1), which in turn has two hydroxyl groups present at Carbon 1 and 3 [15]. Moreover, the fatty acyl chain may be saturated or monounsaturated and can exhibit hydroxyl group at C2 position or at the very end carbon of the acyl chain (α- or ω-carbon, respectively). In general, ceramide molecules possess little polarity with high hydrophobic structure. Therefore, they are insoluble in biological fluids or in cytoplasm instead they are mainly located on membranous structures such as cell membranes, mitochondrial membranes and endoplasmic reticulum (ER).

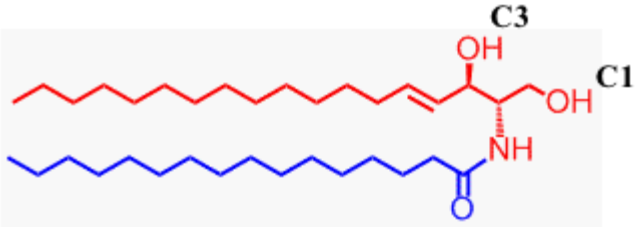


Figure 1. Ceramide structure. The diagram represents the structure of the common ceramide species, *N*-palmitoyl-sphingosine. The red structure represents the sphingoid base whereas the blue is the added acyl chain (C_{16}).

In normal conditions ceramides are scarcely found in a cell, but with excessive signaling, its production will drastically increase. The endogenous ceramide generation is mediated by complex and interrelated pathways. One of these pathways is the *de novo* pathway which is the building block of ceramide biosynthesis. In this pathway, Serine-palmitoyltransferase (SPT) condenses serine and palmitoyl-CoA which will form

sphinganine that will be then acylated and dihydroceramide is formed by dihydroceramide synthase (or ceramide synthase, CerS) (Figure 2) [16]. Dihydroceramide is then desaturated generating ceramide by dihydroceramide desaturase. The first reaction of this pathway is the rate-limiting step. Ceramide is generated on the ER membrane that will be ultimately transported to the Golgi apparatus where more complex sphingolipids are then synthesized [17]. The cell mobilizes ceramide from ER to Golgi by two major mechanisms either through ceramide transfer protein (CERT) or by vesicles formation, termed fission, which will be discussed in the following section. Alternative to the *de novo* pathway is the sphingomyelinase (SMase) or hydrolysis pathway that involves the hydrolysis of sphingomyeline (SM) molecule into ceramide (Figure 2). Sphingomyelinases are mainly found on cell membranes, vesicles and lysosomes/endosomes and they are responsible for recruiting diverse signaling molecules and consequently receptor activation. Three different SMases have been characterized based on their optimal working PH, these are acid (ASMase) alkaline (Alk-SMase) and neutral (NSMase) sphingomyelinases [18]. Although Alk-SMases are not long ago appreciated in cancer field, ASMase and NSMase are well recognized [18-19]. Last, is the salvage pathway, or sphingolipid recycling, is another pathway that initiates complex molecule catabolism such as ceramide-1-phosphate, glucosylceramide and glucocerebroside by number of specialized enzymes allocated mainly on lysosomes [20]. These complex molecules will be broke down into sphingosine that will be acylated to re-generate ceramide (Figure 2).

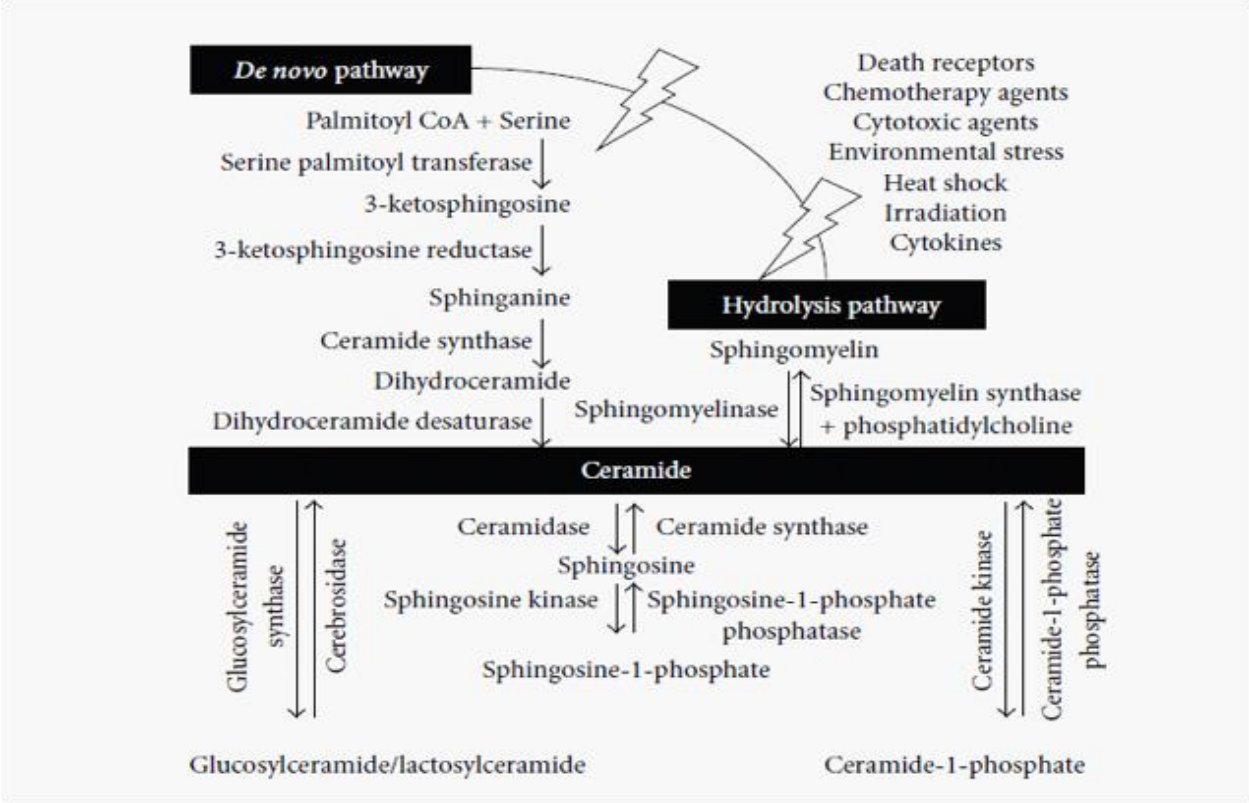


Figure 2. Ceramide Biosynthesis pathways. Under diverse stress signaling, ceramide can be synthesized primarily by *de novo* synthesis, through SPT and ceramide synthase enzymes. The sphingomyelinase or the hydrolysis pathway involves the hydrolysis of sphingomyelin into ceramide by sphingomyelinase enzymes. Finally, the salvage or sphingolipid recycling pathway requires the depicted enzymes for breaking down complex sphingolipids into ceramide. Adapted from Huang *et al*. [21].

C. Ceramide Structure Dictates its Function

To understand ceramide function in cell biology we first need to discuss how ceramide physico-chemical properties affect cellular activities. Sphingolipids are amphiphile molecules where they possess both hydrophobic and hydrophilic moieties. Most ceramide molecules contain only two hydroxyl groups, therefore compromising its hydrophilic nature compared to other more complex sphingolipids such as, ceramide-1-phosphate, sphingosine-1-phosphate and phosphorylcholine where their phosphate groups contribute to their high hydrophilic properties. The physical structure of ceramide indicates its biological properties, such as hydrophobicity, hydrophilicity,

saturation or unsaturation and the fatty acid chain length, all these properties dictates ceramide function in metabolism, cell biology and signaling [15, 22].

1. Fusion and fission

Ceramides have their fatty acid chain length varying starting from short with only 2 carbons to long with 24 carbons. Increasing the acyl chain length greatly contribute to increase in the hydrophobicity of ceramide [23], and consequently its ability to fuse to cell membranes [24]. For instance, short ceramides, or detergents, are capable of micelle formation and solubilizing phospholipid layers. However, long-chain ceramide can form high gel-to-liquid crystalline phase due to their hydrophobic nature, but then again they can transform into non-lamellar phase owing to their minimal hydrophilic nature [24-27]. This was demonstrated by nuclear magnetic resonance (NMR), calorometric and X-ray scattering studies. The transient configuration of these phases at different locations on the membrane induces morphological changes with negative curvatures and consequently vesicle formation, called fission [28-29]. Moreover, their hydroxyl functional group allows hydrogen bonding between ceramide molecules contributing to their high lamellar to non-lamellar transition temperature and thus their stability [30-31]. These features link ceramides to each other compelling ceramide domain formation called “rafts” [15, 32-33]. Movement of ceramide molecules unrestricted on plasma membranes also commences a “flip-flop” mechanism (for a review see [15]). Fission and fusion of cell membranes were speculated in several pathophysiological mechanisms. For instance, host cells infected with engulfed pathogens or the release of cytokines from vesicles, all are mediated by raft membrane spatial and temporal organization of ceramide-enriched membrane platforms [34].

2. Membrane permeability

Ceramide channel formation on mitochondrial membrane followed by protein leakage was first studied by Siskind and Colombini in 2000 using electrophysiological methods [35]. From there on, series of experiments were done on ceramide structural features to identify the physico-chemical properties behind these channels. These channels are found attributable to ceramide domain formation and phase transition as was explained in the previous section. It has been found that synthetic or naturally produced ceramide forms channels composed of six antiparallel monomers having a barrel structure on membranes, thus permitting protein exchange of ~25kDa size [36-37]. These monomers are held together by hydrogen bonds between the amides and the carbonyl groups in addition to, proposed hydrogen bonding between the C1-hydroxyl group and the polar molecules surrounding the inner pore of the channel, thus permitting strong structural stability as well as release of polar molecules from the mitochondria, respectively (Figure 3) [38]. The increase in acyl length contributes to ceramide solubility thus affecting their anchoring properties rather than channel assembly. In fact, short chain ceramide channels may dissociate and anchor on other organelles' membrane causing their leakage. Ceramide properties and their metabolism directs mitochondrial [39] and lysosomal permeability [40], ER stress [41] and extracellular signaling [42] contributing to apoptosis, cell cycle arrest, autophagy, senescence or differentiation.

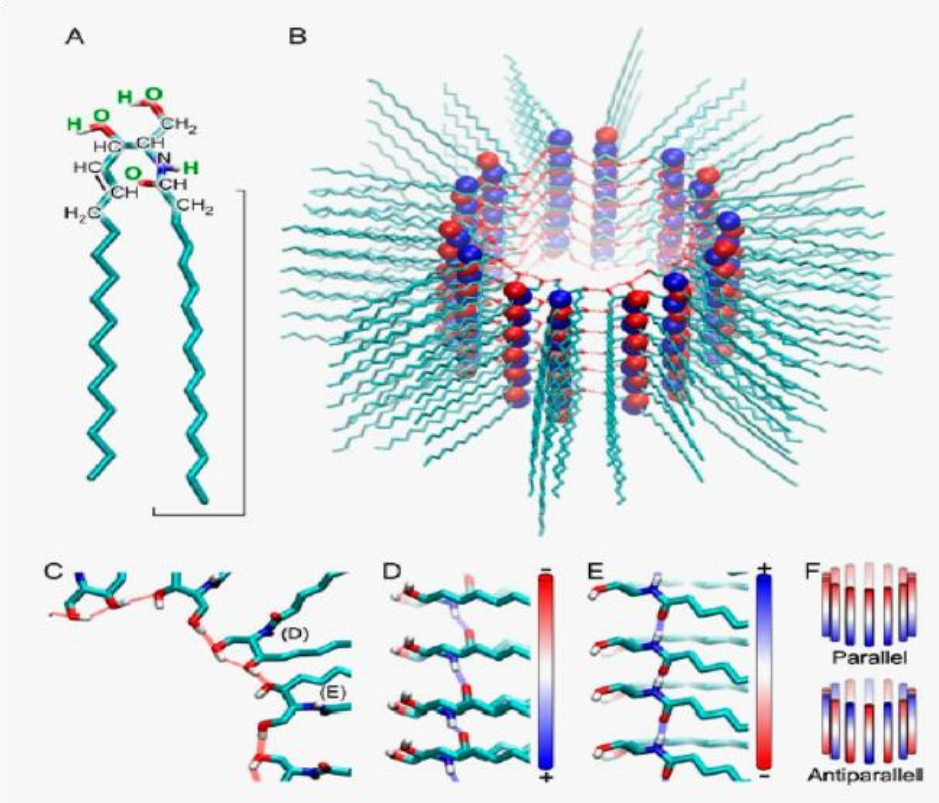


Figure 3. A model of ceramide channel. **(A)** The above structure is C_{16} ceramide showing the polar head group atoms nitrogen (red), oxygen (blue), hydroxyl group that are capable of hydrogen bonding (green) and the non-polar carbon tail(cyan). **(B)** The structure of ceramide assembly in pore formation. Ceramide channel is barrel shaped made up of 6 layers and 14 columns of C_{16} ceramide monomers. Notice that the head groups are facing the pore side whereas the tails splay out in the membrane. The hydroxyl groups in the head group will hydrogen bond with polar molecules surrounding. **(C)** Hydrogen bonding between the amide group and the carbonyl group of ceramide monomers shown in red lines which are forming circular structure. **(D-E)** Each represents a column of 6 layered ceramides in antiparallel orientation. As noticed, the nitrogen atom (red) in D are in opposite direction of that in E. **(F)** Two types of ceramide channel arrangements (parallel and antiparallel). Adapted from Anishkin *et al*. [36].

D. Ceramide in Function

Sphingolipids do not only serve as structural component on membranes but they can also have functional roles. Ceramide is considered the hub of sphingolipids in mediating biological functions. Ceramides have several intriguing functions that correspond to different cellular responses, however here we will focus specifically on

their role in apoptosis and cell cycle arrest in addition to the signaling molecules conscientious for their accumulation.

1. Apoptosis

Apoptosis, also termed programmed cell death, is triggered when something is awry within the cell. The term apoptosis was first used by Kerr *et al* in 1972, in attempt to describe a morphologically distinct form of cell death [43]. These morphological hallmarks include cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and phosphatidylserine that "flip-flop" from inner side of plasma membrane to outside [44]. It is initiated by cascade of executioners that ultimately orchestrate self-destruction mechanism [45]. Ceramide, a tumor-suppressor lipid, serve in this cascade of events. Numerous apoptotic stimuli arouse drastic increase of endogenous ceramide levels prior to apoptosis [46]. These stressors include hypoxia/hyperoxia [47-48], irradiation [49], ultra-violet (UV) light [50], tumor necrotic factor (TNF) and TNF-related apoptosis inducing-ligand (TRAIL) [51]. However, in most cases these signals lead to caspase activation prior to apoptosis and in pursuit of ceramide accumulation. Based on the death signal and type of activated caspase, apoptosis is classified into three major pathways 1) extrinsic apoptosis 2) intrinsic apoptosis and 3) others such as ER stress and mitotic catastrophe. At the end they all merge into one execution pathway characterized by activation of terminal caspase 3. The activated caspase 3 will in turn cleave Poly (ADP-Ribose) Polymerase (PARP) that will translocate to the nucleus and fragments DNA leading to cell death.

a. Extrinsic apoptosis

Extrinsic apoptosis or death receptor-mediated pathway, propagate series of signaling cascade that starts by binding of death ligands such as FasL (or CD-95) and TRAIL to their corresponding receptors in the TNF receptor (TNFR) super family which includes TRAIL receptors (TRAILR), Fas/Apo1 and TNFR1 [14, 52]. Once bound, the intracellular death domain (DD) of the activated receptor will activate FAS-associated death domain (FADD) adaptor protein which in turn will interact with pro-caspase 8 rendering it active (Figure 4) [21]. This formed complex is called death-inducing signaling complex (DISC) and it is required for further activation of caspase 8. Next, cleaved caspase 8 activates and translocates ASMase found in lysosomes to the plasma membrane where sphingomyelin is hydrolysed into ceramide [53]. Ceramides will form rafts on plasma membrane and potentiates receptor oligomerization and DISC formation. Thereby ceramide role in extrinsic apoptosis is to facilitate and amplify apoptotic signaling. Further on, caspase 8 can either directly activate caspase 3 or truncate Bcl-2 interacting protein (tBid), a pro-apoptotic Bcl-2 member, allowing for cross communication with the intrinsic apoptosis pathway and meets in initiating mitochondrial perforation, releasing mitochondrial proteins and consequently caspase 3 activation and apoptosis [54]. This is only a general view (for a review refer to [55]) of extrinsic apoptosis. For instance, ASMase can also initiate intrinsic apoptosis[56] as well as other cellular mechanisms although mainly found to play an important role in extrinsic apoptosis. Moreover, chemotherapeutics [57] and irradiation [58-59] are genotoxic stressors known to stimulate intrinsic apoptosis but can also induce extrinsic apoptosis this depends on cell type, yet the precise mechanism of non-receptor ligand in initiating extrinsic apoptosis still remains unclear.

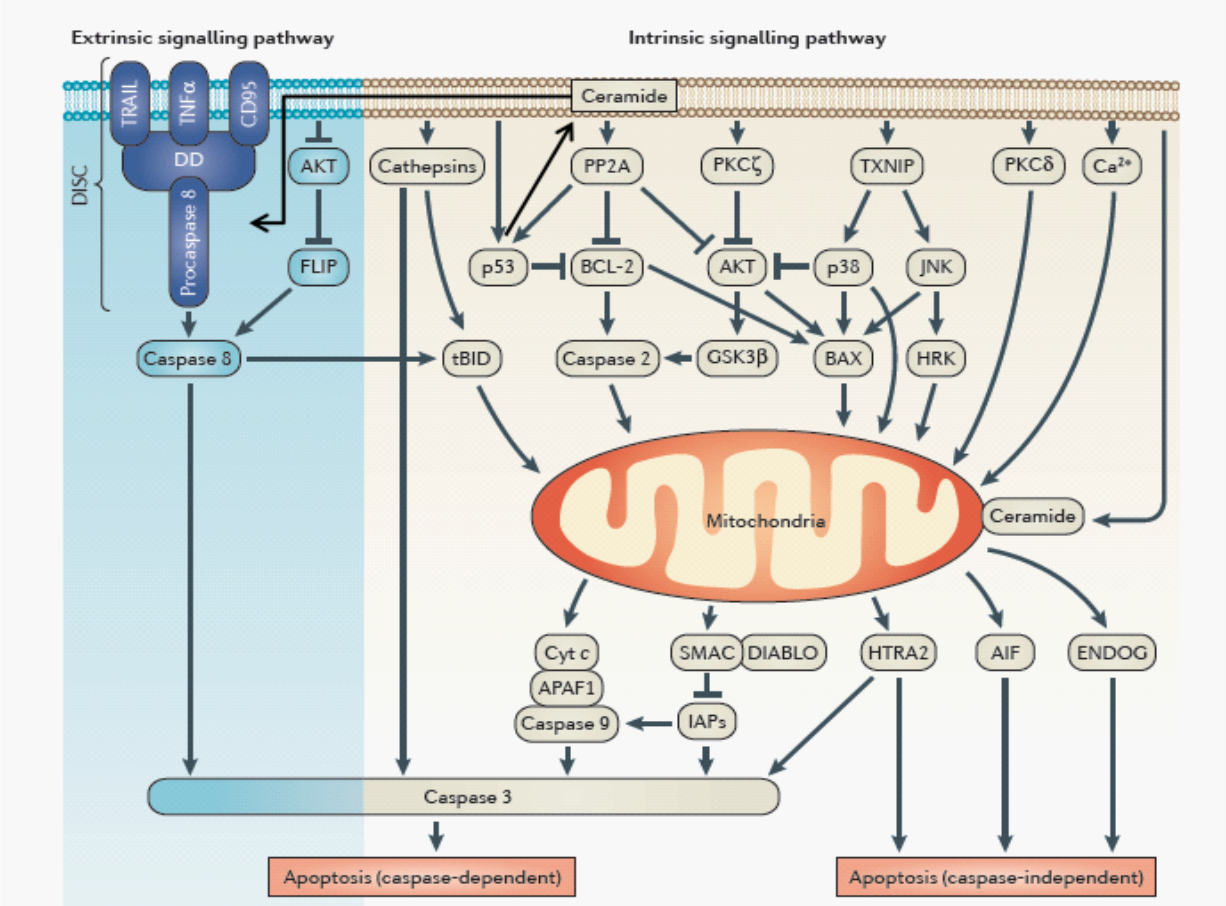


Figure 4. Ceramide in extrinsic and intrinsic apoptosis. In the extrinsic pathway, the binding of the ligand to the receptor will initiate the death domain (DD) to activate the adaptor protein FAS-associated death domain (FADD) which will sequentially activates caspase 8 to form a death-inducing signaling complex (DISC). Ceramide will enhance DISC formation that is required for caspase 8 cleavage. Subsequently, caspase 8 will either directly induce caspase 3 cleavage or indirectly through truncating Bid which will lead to mitochondrial permeability and subsequently caspase 3activation. Ceramide can also induce intrinsic apoptosis through activating p53 or p53 can induce endogenous ceramide accumulation as well as inhibiting anti-apoptotic Bcl-2 protein. This cascade of proteins will eventually activate Bax in addition to other signaling molecules such as ceramide channel assembly that will permeabilize the mitochondria releasing cytochrome c and other apoptogenic molecules which in turn will activate caspase 9 followed by caspase 3 to induce apoptosis (caspase-dependent). Other apoptogenic proteins such as apoptosis-inducing factor (AIF) can initiate caspase-independent apoptosis. A modified figure of Morad *et al*. [14].

b. <u>Intrinsic apoptosis</u>

Intrinsic apoptosis or mitochondrial pathway is mainly triggered by non-receptor stimuli. As mentioned earlier, these include ionizing radiation, UV light, toxins,

depleting of growth factors, starvation and hypoxia/hyperoxia. These stimuli will trigger genotoxic stress leading to p53 upregulation [60] which in turn may regulate, apart from Bid, different Bcl-2 family members as well as ceramide biosynthesis. Ceramide and Bcl-2 family signaling molecules can cause mitochondrial pore formation resulting in mitochondrial outer membrane permeabilization (MOMP), loss of mitochondrial transmembrane potential and release of mitochondrial apoptogenic proteins from the transmembrane space into the cytosol [55]. These proteins consist of cytochrome c, SMAC/DIABLO and apoptosis-inducing factor (AIF). Cytochrome c in the cytosol will then bind and activate Apaf-1 protein which will sequentially oligomerize forming a platform for recruiting initiator pro-caspase 9 rendering it active (Figure 4). This formed complex is called apoptosome which in turn will cleave the executioner caspase 3 and subsequently apoptosis.

i. Tumor suppressor p53

Before discussing ceramide role in the mitochondrial pathway, we ought to emphasize on p53 role in apoptosis especially that p53 is thought as a main ceramide regulator. The tumor suppressor p53 is undoubtedly one of most studied proteins up to date, yet there is much to know about its regulation and activities within the cell. TP53 can be regulated at the level of transcription, translation and post-translation. However, phosphorylation is one of the crucial post-translational modifications in addition to acetylation and ubiquitination. For instance, p53 phosphorylation at Serine 46 (S46) residue was shown to correlate with the induction of specific proteins that promotes cytochrome c release during apoptosis [61]. Moreover, the phosphorylation of Serine 15 (S15) is considered a central event since its phosphorylation will trigger a series of succeeding phosphorylations and acetylations on p53 along with the inhibition

of the interaction with MDM2 (an E3 ligase targeting p53 degradation). These modifications are found fundamental for p53 activation upon DNA damage [62-63]. For example, γ-irradiation induces phosphorylation of p53 at (S15) residue in leukemic cells [64] as well as okadaic acid (a known phosphatase protein inhibitor) treatment was shown to provoke (S15) phosphorylation among other sites [65] in prior to apoptosis. The tumor suppressor p53 is a transcription factor that once activated can transcriptionally activate pro-apoptotic Bcl-2 genes such as p53 up regulated modulator of apoptosis (*PUMA*), phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1* or *Noxa*), Bcl-2 associated x (*Bax)* and Bcl-2 antagonist killer 1 (*Bak)* or repress anti-apoptotic Bcl-2 genes like *Bcl-2* and myeloid cell leukemia1 (*Mcl-1*) (for review see [61]). Despite the scope of its ample transcriptional activities, transcription-independent pro-apoptotic functions of p53 is evolving rapidly in the area of research, commenced by the identification of p53 protein in the cytoplasm and/or mitochondria as sites for its function [66]. In the mitochondrion, p53 can persuade Bax and Bak oligomerization by antagonizing the role of anti-apoptotic Bcl-2 protein through protein-protein interactions. Likewise, p53 can also prompt ceramide accumulation prior to apoptosis. Therefore, ceramide can initiate p53-dependent apoptosis, evidenced in both leukemia and fibrosarcoma cell lines [67]. Later on, the observed ceramide accumulation was denoted to *de novo* ceramide biosynthesis by the upregulation of ceramide synthase, specifically CerS5, known responsible for C_{16} ceramide accrual [68]. Nevertheless, radiation-induced apoptosis in lung and heart epithelium, the pleura and pericardium mesothelium, can also affect ASMase activity however in a p53-independent manner[69]. Alternatively, apoptosis that involve both ASMase and p53 is evidenced in extrinsic apoptosis *via* caspase 8 activation [70]. Therefore, presumably p53-dependent ceramide-induced

intrinsic apoptosis is mainly through *de novo* synthesis. Of note, ceramide can also be generated independently to p53 [71], concomitantly to p53upregulation [72] or even upstream of p53 such as in resuscitating wild-type p53 expression in p53 mutants through ceramide accumulation after treatment with glucosylceramide synthase inhibitor [73]. Taken together, p53, ceramide and Bcl-2 family proteins are essential regulators and apparently collaborators in mitochondrial-mediated apoptosis. Since both p53 and Bcl-2 family proteins are interrelated, it is fundamental to acknowledge the role of ceramide in context of Bcl-2 family proteins.

ii. Bcl-2 family proteins

All stressors-mediating apoptosis join in permeabilizing mitochondrial outer membrane conveyed by Bcl-2 family proteins. The Bcl-2 family proteins are divided into anti- and pro- apoptotic proteins, where any slight deviation in the dynamic balance between the two groups will result in inhibition or promotion of cell death. Bcl-2 family proteins are divided into three groups 1) Anti-apoptotic Bcl-2 proteins 2) BH3-only pro-apoptotic proteins and 3) Effectors pro-apoptotic Bcl-2 proteins. 1) The anti-apoptotic proteins have multiple Bcl-2 homology domains (BH1-4) and they consist of Bcl-2, Mcl-1 and Bcl-xL (long isoform) which are either located on cellular membranes or in cytosol prior to activation. For instance, inactive Bcl-2 is mainly found anchored on cellular membranes such as mitochondrial and ER membranes where upon activation it just undergo conformational changes, whereas, inactive Mcl-1 and Bcl-xL are located in the cytosol and once activated they translocate on to the mitochondrial membrane [74]. Bcl-2 anti-apoptotic proteins function in preventing MOMP by inhibiting the oligomerization of the effectors proteins Bax and Bak. Two competing models are suggested for their mode of action i) the depression model which is characterized by the constitutive activation of

anti-apoptotic Bcl-2 proteins thereby hindering Bax/Bak from initiating MOMP unless these anti-apoptotic Bcl-2 proteins are inhibited by BH3-only proteins ii) the activation model indicates that both Bcl-2 and effector proteins are inactive until apoptotic signaling is initiated where then the balance between anti-apoptotic and BH3-only pro-apoptotic proteins decides whether Bax/Bak oligomerization will get triggered or not [75]. 2) The BH3-only pro-apoptotic proteins, as their name indicates, is a group that possess only one of the BH domains and they involve Noxa, Puma, Bid, Bcl-2 antagonist of cell death (Bad), Bcl-2 interacting mediator of cell death (Bim) and others. They can be activated either at the transcriptional level expressed as protein level, post-translational modifications, caspase-dependent protein cleavage or by altering their subcellular localization. Specifically, Noxa and Puma were shown to be transcriptionally activated by p53 [76] whereas Bad is highly regulated by phosphorylation [65]. The BH3-only pro-apoptotic proteins are further divided, according to their function, into sensitizers and/or activators whereby the former inhibit anti-apoptotic Bcl-2 proteins while the latter directly stimulates executioner proteins [77] in either cases apoptosis will be initiated. For instance, Noxa was found to translocate to the mitochondria where it will recruit Mcl-1 found in cytosol resulting in its phosphorylation and subsequent ubiquitination prior to cell death [78]. Hence, Noxa exclusively functions as sensitizer rather than activator. However, Puma was found to operate as both activator and sensitizer [79-80]. 3) Finally are the executioner proteins which are also called effector proteins, they retain four BH domains and consist of Bax and Bak. The inactive form of Bax is located in the cytosol whereas Bak is present on the outer mitochondrial membrane (OMM) where upon activation Bax translocates to the OMM while Bak undergo conformational changes, these activated forms will insert and oligomerize on the mitochondrial

membraneand organizing channels that would lead to loss of mitochondrial potential releasing cytochrome c. Ceramide can play a pivotal role in the Bcl-2-mediated apoptosis pathway at any level of the hierarchy that is, anti-apoptotic, pro-apoptotic and/or effectors Bcl-2 proteins. One of the examples is in Aldh111-induced apoptosis (the enzyme involved in the regulation of folate metabolism), here ceramide accumulates in response to Puma upregulation in a p53-dependent manner and the silencing of Puma has abrogated ceramide generation [81]. Therefore, postulating Puma as a possible upstream activator of ceramide synthase, specifically CerS6. In other studies, ceramide was shown to functions upstream of BH3-only protein Bad in human corneal stromal fibroblasts [82]. On the other hand, p53 was shown to be upregulated in cases of exogenously added ceramide, which in turn regulates Bax and Bcl-2 proteins prior to apoptosis [83]. Hence, suggesting a role for ceramide upstream of p53, Bax and Bcl-2. These results contradict the former study which declares that ceramide is downstream of Puma and p53. Moreover, several studies have also shown that exogenously added ceramide dephosphorylates Bcl-2 protein thus rendering it inactive [65, 84]. On the other hand, the overexpression of Bcl-2 was found to inhibit ceramide biosynthesis such as in etoposide-induced C6 glioma cells [84]. These outcomes suggest that, exogenously added ceramide can inhibit Bcl-2 whereas the overexpression of Bcl-2 can in turn inhibit ceramide accumulation therefore, indicating that both molecules can influence each other and it is indeed the balance between the two molecules which decides on the cellular destiny. Furthermore, Bak was found to induce ceramide generation on the mitochondrial membrane *via de novo* synthesis [85], particularly $C_{18:0}$-, $C_{18:1}$-, $C_{20:0}$- and $C_{20:1}$-ceramides [86] which in turn induces Bax activation [87]. In fact, downregulation of ceramide synthase, specifically CerS5 or ASMase had significantly attenuated Bax

translocation. Altogether, depending on the encountered system ceramide can function upstream or downstream of p53 and Bcl-2 family proteins, however, in most cases it seems that endogenous ceramide accumulate downstream of p53, Bak and BH3-only proteins, at least in case of Puma, and upstream of anti-apoptotic Bcl-2 proteins. Although the cooperation between Bcl-2 family members and ceramides were only recently well-documented, still remains the ordering of ceramide in the Bcl-2-induced apoptotic machinery requires further studies and it seems more perplexing than one thought.

iii. Ceramide channels and Bcl-2 family members deep in mitochondria

Several ceramide enzymes are allocated on the mitochondria namely ceramidase and ceramide synthase [88]. These enzymes synthesize ceramide in both outer and inner mitochondrial membranes. Ceramide can be also synthesized from SMase, sphingomyeline synthase or ceramide synthase located on mitochondrial-associated membranes (MAMs). These enzymes in addition to MAM's generated ceramides, can be translocated to the mitochondria upon stress induction [14]. Actually, dihydroceramides are the ones present on the mitochondrial membranes of healthy cells but in apoptotic conditions these are converted to ceramide [89]. The produced ceramide will fuse with the mitochondrial membrane a mechanism known as 'the kiss of death' [14]. This ceramide will then affect mitochondria by generating reactive oxygen species (ROS) and decrease in inner mitochondrial potential resulting in MOMP. Similar machinery was attributed for Bak and Bax oligomerization. Their oligomerization on the mitochondrial membrane are mainly characterized by the assembly of dynamin-related proteins (Drp1), membrane curvature, formation of mitochondrial fission and fusion and finally mitochondrial fragmentation (for a detailed review see [77]). Intriguingly, a comparable

characteristics where also assigned for ceramide channels. As previously discussed, ceramide physico-chemical properties enhances membrane curvature and membrane fission and fusion. Furthermore, ceramide was also found to induce Drp1 expression in addition to its redistribution on the mitochondrial membrane which in turn mediates mitochondrial fission preceding mitochondrial fragmentation and apoptosis [90-91]. However, inhibiting Drp1 had prevented ceramide-induced mitochondrial fission. Accordingly, ceramide, Bak and Bax are profoundly found related to mitochondrial pore formation (Figure 5).

As discussed previously, ceramide forms barrels structured channels on the mitochondrial membrane. The anti-apoptotic Bcl-xL was found to directly bind and disrupts these channels whereas in presence of Bcl-xL inhibitor ceramide channels were recovered [92]. Alternatively, Bax interaction with ceramide channels favors the curvature of the channel thereby increasing its stability. Moreover, Bax affinity to ceramide channels increases with increase in ceramide concentration until a point of saturity, supporting the existence of an equilibrium state. It is also recently evidenced that Bax and ceramide works synergistically in initiating MOMP where the permeability exceeded that of either Bax or ceramide added alone. This interaction increases the size of formed channels allowing for larger proteins exchange. It was found that Bcl-xL proteins interact preferentially with the aliphatic chains of ceramide channels while Bax seems to interact with the amide nitrogen of ceramides. In another study, the specificity of the interaction between Bcl-xL and ceramide channels was tested using truncated version of Bcl-xL [93]. This truncated form was unable to disrupt ceramide channels or initite apoptosis, therefore it was suggested that the function of Bcl-xL is indeed specific. Hence, we can speculate that anti-apoptotic Bcl-2 proteins can have dual functions

consisting of inhibiting Bax and Bak in addition to disassembling ceramide channels or even inhibiting ceramide productivity, all will end up in inhibiting MOMP. The ability of Bcl-xL and Bax to interfere with MOMP was found optimal for C_{16}- and C_{18}-ceramides where these forms are also known as main regulators of apoptosis [87, 92]. Another line of evidence suggest that Bak but not Bax is the one responsible for ceramide generation [85], and Bak but not Bax plays an important role in mitochondrial fragmentation [94]. They have shown that in Bak-deficient primary neurons isolated from brain cortex of mice, mouse embryonic fibroblasts or baby mouse kidney cells have attenuated mitochondrial fragmentation following a mitochondrial injury. Alternatively, Bax-deficiency did not prevent mitochondrial fragmentation whereas the reconstitution of Bak in Bak/Bax double-deficient cells has restored mitochondrial fragmentation. In consistency, ceramide channels were shown responsible for Bax insertion, oligomerization and pore formation [95]. Taken together, mitochondrial stress induces p53, BH3-only proteins and Bak upregulation which seems to initiate mitochondrial fragmentation, at least in part, *via* ceramide accumulation and ceramide channel assembly which might sequentially triggers the activation and translocation of Bax to the mitochondria where then pore assembly will be enhanced and subsequent MOMP will be initiated (Figure 5) and this could be prevented by the Bcl-2 protein disassembling ceramide channels. From the above, we shall appreciate the importance, yet the complexity, of ceramide and Bcl-2 in cancer biology.

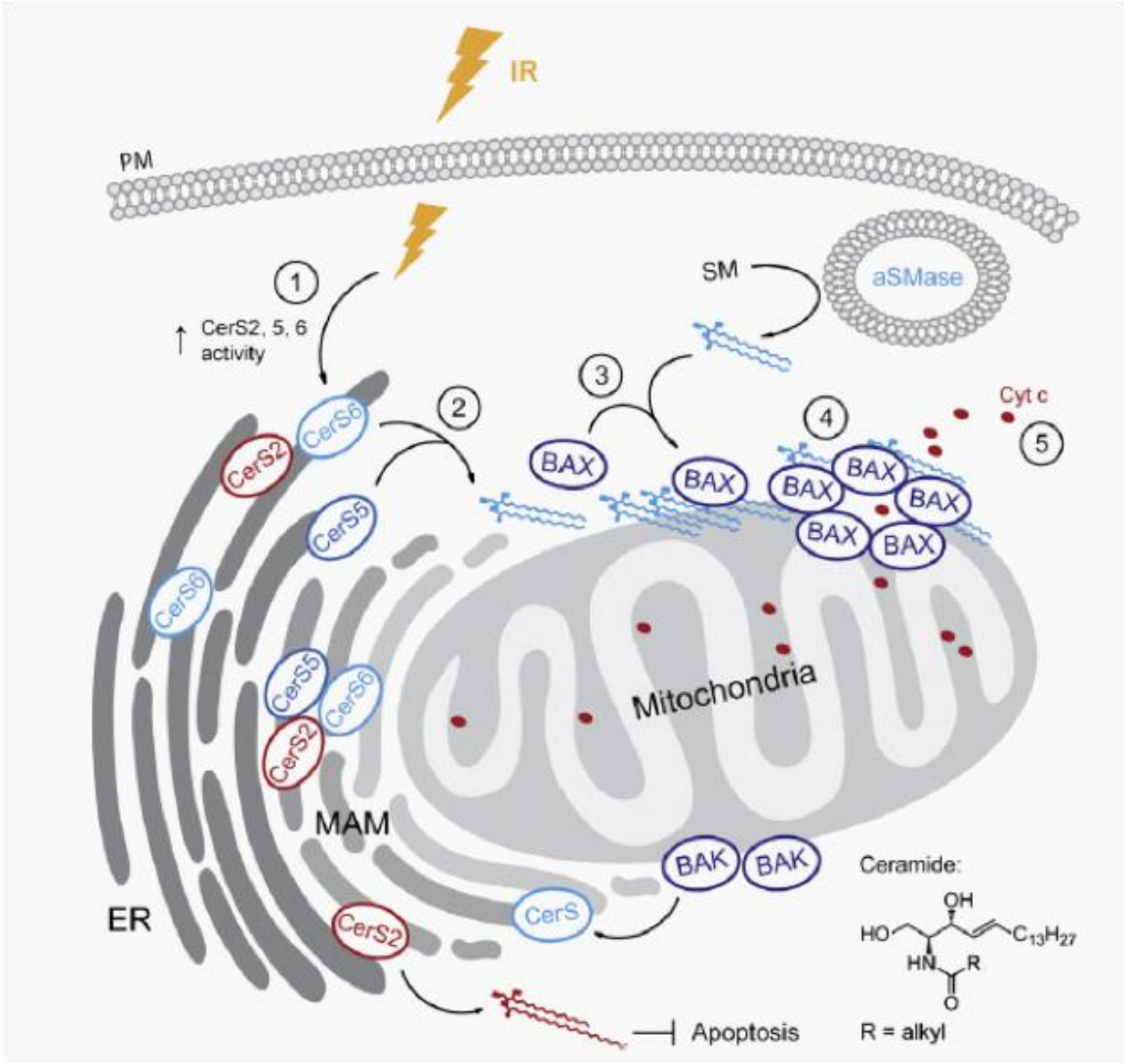


Figure 5. Ceramide and Bcl-2 family proteins in triggering mitochondrial apoptosis. DNA damage induced by irradiation initiates MOMP in the following sequence: **1)** CerS and ASMase activation **2)** ceramide generation **3)** Ceramide and Bax integration on mitochondrial membrane. **4)** Bax oligomerizes at ceramide rich-sites thus enhancing pore size **5)** permitting MOMP and subsequent cytochrome c release. Bak but not Bax is can induce ceramide generation. CerS2 synthesizes C_{24} (red) which functions as pro-survival molecule whereas CerS5 and CerS6 (blue) synthesize pro-apoptotic C_{16} ceramide. PM: plasma membrane [96].

c. Others: ER stress and mitotic catastrophe

ER is involved in many metabolic processes such as gluconeogenesis, lipid synthesis and as calcium reservoir. Upon ER stress series of proteins are activated that eventually will lead to apoptosis of damaged cells in a process called unfolded protein

response (UPR). Activating transcription factor 6 (ATF6) and protein kinase RNA-like ER kinase (PERK) are first proteins to be up regulated following ER stress. PERK and ATF6 will activate C/EBP-homologous protein (CHOP), ATF4 and p53 which in turn activate BH3-only proteins and repress anti-apoptotic Bcl-2 proteins herein joining the mitochondrial-mediated apoptosis pathway through the activation of caspase 4, a specific ER stress-activated caspase. For instance, ER stress was found to induce Bid cleavage in addition to activation of other BH3-only proteins such as Noxa, Puma and Bim. Activated BH3-only proteins will subsequently lead to Bak and Bax activation followed by MOMP and apoptosis (for detailed review see [97]). Ceramide levels perturbation was recently found to be associated with ER stress. For instance, knockdown of CerS6 or CerS2 has induced ER stress [21]. Another example is in glioma tetrahydrocannabinol treated cells, a known treatment to initiate ER stress, were found to induce *de novo* ceramide synthesis. Moreover, combination of vorinostat and sorafenib, a histone deacytelase and multi-kinase inhibitors, respectively, had triggered ceramide synthase and ASMase followed by PERK activation prior to apoptosis.

Mitotic catastrophe is another form of cell death characterized by cells with multiple micronuclei and hyper-condensed chromatin that reflects the features of both abnormal mitosis and apoptosis, respectively [98]. Mitotic catastrophe is a form of cell death resulted from abnormal mitosis. It occurs when DNA damage initiates G2-M arrest followed by generation of polyploidy cells, which are cells that harbors more than 4n of DNA content, corresponding to improper segregation and improper cytokinesis a process called mitotic failure, followed by apoptosis. This occurs when cancerous cells lack G2 phase checkpoints, specifically checkpoint kinase 2(Chk2), which found responsible for preventing damaged cells to enter M phase by inhibiting cell division cycle 25C

(CDC25C) phosphatase and from entering G1 phase by activating p53 [99]. Normally, in presence of p53, upon DNA damage cyclin-dependent kinase 1(CDK1), which permits cell progression from G2 to M phase, found to be inhibited by Chk2 and p21 (a G1 and S phase inhibitor) thus initiating either G0-G1 arrest or apoptosis depending on the activated signaling molecules. Cells that lack Chk2 inhibitor will proceed in the cell cycle but with aberrant mitosis. Therefore mitotic failure may occur and polyploidy cells would appear. However, in presence of p53, Chk2 deficient cells with abnormal mitosis will be halted at the G1 checkpoint and CDK1 will be negatively regulated by p53, thereby averting the propagation of errors, yet arrested at polyploidy in G1 state [98]. In contrast, p53 and Chk2 deficient cells will accumulate CDK1. This over accumulation can trigger apoptosis through phosphorylating Bad which in turn activate Bax/Bak oligomerization thus triggering MOMP and subsequently massive cellular apoptosis [98, 100]. As a consequence, presence of p53 can arrest polyploidy cells at G0-G1 phase whereas p53 deficient cells (or p53 wild type cells undergoing apoptosis instead of cell cycle arrest) have aberrant cellular segregation that is followed by cell death a form called mitotic catastrophe. Studies relating ceramide accumulation in response to mitotic catastrophe is still lacking far behind, however it can be partly explained by the mechanism of the cell undertaken in that either cell cycle arrest or apoptosis.

2. Cell Cycle Arrest

Ceramide can modulate other aspects of cellular destinies apart from apoptosis such as growth arrest and senescence. Cell cycle is regulated by cyclins and CDKs. CDK2, CDK 4 and CDK6 along with cyclin E and cyclin D control G1-S phase transition whereas CDK1, cyclin A and cyclin B regulates G2-M phase transition. Activation of

CDKs can phosphorylate retinoblastoma (Rb) protein rendering it inactive where then E2F transcription factor can be released and DNA synthesis is then initiated. A p53-dependent cell cycle arrest at G1 phase as a cellular response to stress has been well acknowledged, however p53-dependent G2-M arrest is still controversial. In p53-deficient cells G0-G1 arrest is aberrant whereas G2-M arrest remains, at least partially, operative [101-103]. Interestingly, arrested p53 mutant cells at G2-M phase have shown substantial mitotic cells in opposite to p53-contianing cells. Therefore, mutated cells are indeed undergoing mitosis rather than actual arrest. The mechanism of p53-mediated cell cycle arrest at G1 phase is through activation of CDK inhibitors such as p21 and p27 in addition to reduction in Rb protein. Exposure to exogenous ceramide can also induce cell cycle arrest at G1 or G2 phase. Ceramide initiating cell cycle arrest upregulates p21 and dephosphorylates and/or reduce Rb along with cyclin E and cyclin D1 [104]. Interestingly, ceramide initiating G1 arrest stimulates PPARγ [105] whereas in G2-M arrest ceramide was proposed to suppress survivin expression, a key regulator of mitosis, which is also regulated by p53 [14]. The promotion of cell cycle arrest by ceramide is one means of the diverse ceramide activities in halting tumor progression.

Several chemotherapeutic drugs have engendered *de novo* ceramide synthesis such as daunrobicin, doxorubicin, arsenic trioxide and gemcitabin [21]. Further on, overexpressing ceramide's precursors was shown to be related to cancer resistance to a variety of chemotherapeutics such as vinscristine, adriamycin and many others. The novel discovery of nanoliposomes would also remarkably enhance tumor therapy. Similarly, other drugs were shown to work through BH3-only proteins such as bortezomib which functions by upregulating Noxa in a p53-independent manner [106], gleevec which elicit Bad and Bim dependent apoptosis [107], paclitaxel, or taxol, which

depends on Bak for cytotoxicity [108] and etoposide which induce apoptosis through Noxa and Puma [109]. Accordingly, future studies deciphering these pathways would provide a novel platform for new combinational therapeutics discovery with enhanced efficacy and sensitivity.

In mice experiments, fatty acids called ceramides acted like a Trojan horse.

Tumor cells invited them in (they like an acid environment) and, in turn, the ceramides told lysosomes (tiny recycling centers inside cells) to burst, killing the cancer cell.

Ceramides also act as a protective shield on your skin. Someday ceramides might be the basis for a gentle, effective, integrative cancer treatment. Will they work against other cancers and not just in mice? We'll have to wait and see.

"Michael Roizen, MD,"

CHAPTER II

THESIS HYPOTHESIS, AIM, SIGNIFICANCE AND LIMITATIONS

A. Thesis Hypothesis, Aim and Objectives

1. *Thesis hypothesis*

Ceramide accumulation was evidenced in cells exposed to genotoxic stress prior to apoptosis. Likewise, Noxa stimulation by p53-mediated intrinsic apoptosis has been observed. Herein, a correlation between ceramide and Noxa is possible, yet not addressed. Hence, we examined if crosstalk between Noxa and ceramide existed or whether these were players in two parallel pathways initiated by p53 to mediate apoptosis. Therefore, we hypothesized that *IN THE CONTEXT OF p53-DEPENDENT APOPTOSIS, CROSSTALK EXISTS BETWEEN NOXA AND CERAMIDE ACCUMULATION.*

2. *Thesis aim, objectives and approach*

Our aim was to examine the ordering of ceramide accumulation with respect to Noxa in Molt-4 Human T-cell leukemia and HCT116 colon cancer cell lines.

We chose Molt-4 human T-lymphocytic leukemia cell line because it is highly radio-sensitive and hence it is a good model for radiation-induced p53-dependent apoptosis. On the other hand, HCT116 colon cancer cell line reflects a highly aggressive model for cancer and, thus, proving our hypothesis in this model will further validate it.

The following is the experimental approach we used to achieve our objectives:

a. The role of Noxa in p53-mediated apoptosis

To inspect the role of Noxa with respect to p53 in mediating apoptosis, Molt-4 cells and HCT116 cells were transfected with antisense Noxa oligonucleotide in order to "knockdown" the expression of Noxa. However, in order to study the role of Noxa in HCT116 cell line, we needed to identify a stressor that initiates p53-dependent apoptosis in this cell line, similar to γ-irradiation in Molt-4 cell line. Therefore, several experiments were done on HCT116 cells before proceeding with our main objectives that are as follows:

i. Does Noxa downregulation affect apoptosis level?

AnnexinV and/or Propidium iodide (PI) incorporation was assessed using flow cytometry analysis whereas PARP cleavage was inspected by Western Blot analysis, following γ-irradiation and okadaic acid treatment in Molt-4 and HCT116 cells, respectively. Apoptotic level was measured in silenced Noxa models and compared to their control counterpart. For HCT116 cell line we did not pursue further and the remaining objectives were addressed only in Molt-4 cells whereas similar work will be done on HCT116 cells in further experiments.

ii. What are the signaling molecules involved in p53 and Noxa-dependent apoptosis?

To investigate which of the intrinsic or extrinsic pathways is responsible for the observed apoptosis in γ-irradiated Molt-4 cells, we screened for activation of different caspases that are specific for different apoptotic pathway. Moreover, other Bcl-2 signaling molecules were examined to identify their ordering relative to Noxa expression using Western Blot analysis.

iii. How to relate Noxa expression to the mitochondrial pathway?

Isolated mitochondria were probed for Noxa and other Bcl-2 family members, in order to investigate the possible translocation of any these proteins to the mitochondria, as this is a well-known feature of Bcl-2 family proteins. In addition, we also examined cytochrome c release to evaluate mitochondrial permeability.

b. The role of ceramide in Noxa-dependent apoptosis

Previously, we had shown that ceramide accumulates in a p53-dependent manner prior to apoptosis in γ-irradiated Molt-4 cells. Therefore, it was important for us to study the ordering of ceramide in this pathway with respect to Noxa. To examine our hypothesis we aimed to knockdown Noxa, since it was well shown to have a role in p53-mediated apoptosis, and then measure ceramide levels in order to investigate its dependence on Noxa. The following experiments were conducted on γ-irradiated Molt-4 cells.

i. Where does ceramide align with respect to Noxa?

To answer such a question, we needed to examine if ceramide generation is affected in Noxa knockdown models. Ceramide was measured after γ-irradiation in both control and Noxa knocked down Molt-4 cells by the diacyl glycerol kinase (DGK) assay.

ii. Where does generated ceramide accumulate?

Ceramide was measured in purely isolated mitochondria with the intention of identifying whether ceramide accumulates there prior to apoptosis or not.

B. Thesis Significance

This is the first study, to our knowledge, that relates Noxa to ceramide generation hence providing a novel link between two apoptotic pathways previously thought to operate independently. Furthermore, our results suggest a role for ceramide in

the execution phase of apoptosis rather than downstream regulator. Taking into account the previous studies indicating that ceramide generation is dependent on Bak protein, our results confirm the possibility that Bcl-2 family and ceramide are interrelated in the intrinsic apoptosis. Such an evidence could contribute to the development of novel and emerging lines of research in addressing combined chemotherapeutic approaches, especially that several drugs where shown to act at the level of ceramide generation or Noxa upregulation individually such as in arsenic trioxide or ABT-737 drugs, respectively [12, 110].

C. Thesis Content and Scope

This thesis begins with an introductory chapter providing an overview of different concepts regarding the work done and it includes background information for the reader. The second chapter discusses the hypothesis, aims, objectives, significance and the limitations of the work. The third chapter explains the methodologies applied to attain the required experimentations. The fourth chapter provides with the preliminary results obtained that led to the development of the current hypothesis. Chapter five describes the experiments done to test our hypothesis and the achieved results. These results are discussed in chapter six and evaluated with respect to current research in the literature. Finally, chapter seven provides suggestions for future work.

D. Thesis Limitations

There are several limitations that could affect our results.

One of the limitations is that our study was conducted on immortal cell lines therefore they do possess some phenotypic mutations in order to maintain them immortal.

Hence, these mutations may affect our study and may not reflect *in vivo* cell signaling. A complementary approach could be conducted on primary cells.

Moreover, some of the performed experiments were done only once or twice, raising the possibility that they could not be reproduced. Thus, some of these experiments need to be repeated at least three times and to perform statistical analysis, to assure that our obtained results are significant.

Due to flawed antibodies, some of the Western blot images are not clear, although these results are repetition of previously done experiments in our lab, or that the same concept was proven using different experimental procedures to obtain the conclusion.

Although with okadaic acid treatment we found a model similar to that of Molt-4 cells, in which p53-dependent apoptosis is observed, we are bearing in mind that okadaic acid is a PP2A inhibitor (at 10 nM), which is known as one of the downstream molecules in ceramide signaling. However, we are suspecting ceramide channel assembly known to be independent of PP2A activity, to be the major mode of action in p53-dependent apoptosis. Moreover, there is no clear evidence found in literature relating endogenous ceramide and ceramide-activated protein phosphatases (CAPP) to regulate Rb or Bcl-2 family proteins, except in the case of exogenous ceramide stimulation or in protein kinase C (PKC) and SR proteins.

Stably transfected models are not 100% effective in inhibiting or expressing proteins. Therefore, faint expression of the protein in system being evaluated as "knockdown" or deficient can be seen and that should be taken into consideration. Further selection can be done although this can also exhaust our cells.

Furthermore, our isolated mitochondrial samples are not 100% pure and have MAMs contamination, however this contamination does not affect our results because MAMs containing ER membranes are a minority, and therefore, they do not significantly influence our measured ceramide levels in mitochondria.

Finally, some of the antibodies used do not bind the active form of the protein especially in the case of Bcl-2 family proteins known to undergo post-translational modifications without being transcriptionally altered. Therefore, some of our results reflect unchanged total protein levels that can be misleading. Hence, using specific antibodies that reflect only the active form of the protein are needed future examination.

CHAPTER III

MATERIALS AND METHODOLOGIES

A. Reagents

The chemicals used were growth media, fetal bovine serum, and percoll medium (Sigma); SDS-PAGE gels, SDS buffer, transfer buffer, skimmed milk, and PVDF membranes (Bio-Rad); protein ladder (Abcam); ECL (enhanced chemiluminescence) detection system (Invitrogen); anti-Bak, anti-Bcl-2, anti-Bax, anti-p53, anti-caspase 3, anti-caspase 8, anti-caspase 9 (Santa Cruz Biotechnology); anti-Noxa (CalBiochem); anti cytochrome c (Pharmingen) and anti-PARP and anti-phospho.p53 (Cell Signaling).

B. Cell lines and cell culture

Cells were obtained from American Type Culture Collection: Human T cell leukemia cell line Molt4-LXSN SCR/shRNA Noxa cells (stably transfected with empty vector LXSN and scrambled vector SCR or shRNA Noxa) and Molt4-E6 SCR/shRNA Noxa (stably transfected with the human papilloma virus E6 protein, a kind gift from Dr. Denise Galloway, University of Washington, Seattle, WA and scrambled vector SCR or shRNA Noxa); Colon cancer cells HCT116 wild type (or p53+/+) and HCT116 p53-/- cells, were a kind gift from Dr. Nadine Darwiche, American University of Beirut, Beirut, Lebanon. Molt-4 cells were cultured in RPMI 1640 (supplemented with 10% FBS, 1% penicillin and streptomycin (P/S), 500 µg/ml geneticin (G418) and 500 µg/ml hygromycin). HCT116 p53+/+ cells were cultured in RPMI 1640 (supplemented with 10% FBS, 1% P/S, 1% sodium pyruvate and 500 µg/ml hygromycin). HCT116 p53-/- cells were cultured in DMEM (supplemented with 10% FBS, 1% P/S, 1% sodium

pyruvate, 1% non-essential amino acids (NEAA) and 500 µg/ml hygromycin). Cells were splitted and passaged twice per week.

C. shRNAs technology and stable transfection

Since the use of siRNA oligos induces a short-term silencing, short-hairpin RNA (shRNAs) are better silencing tools that can be smuggled into a cell *via* a vector or virus to induce transient or stable expression in both dividing and non-dividing cells. Noxa specific shRNA plasmids (PMAIP1; NM_021127) and one negative control plasmid were purchased from Qiagen (sample and assay technologies). Bacterial transformation was done and stocks of bacteria were stored at -80°C for future use. HCT116 cells were seeded in a 6-well plate at a density of 300,000 cells/well. Twenty-four hours post-seeding, cells were washed with PBS then adding 800 µl of Opti-MEM (Gibco) reduced serum media and then transfection mixture was added to the cells 200 µl/well drop wise. Transfection mixture should contain 1.5 µg of DNA and 4 µl of lipofectamine 2000 (Invitrogen, USA) added to 100 µl Opti-MEM each (as recommended by the manufacturer, Invirogen). Six hours post-transfection the media was replaced with either RPMI or DMEM complete media. Afterwards the cells were harvested 24 hours and 48 hours post-transfection and then transfection efficiency was examined by western blot. The transfected cells were selected with hygromycin to obtain stably transfected models with *Noxa* silenced. However, Noxa knockdown in Molt-4 cells was attained using nucleofector technique. As the manufacturer's instructions (Amaxa cell line nucleofector kit L, Lonza), 10^6cells were pelleted and resuspended in 100 µl of nucleofector solution supplemented with 2 µg of the required plasmid or pmax GFP vector, as positive control.

The obtained solution was carefully transferred to a certified cuvette where then nucleofection was initiated using the appropriate program. Cells were then incubated in 12-well plate containing 1.5 ml media. After 6 hours, cells were transferred into T-25 flask containing 15 ml media. Transfection efficiency can be then identified by Western Blot analysis. The obtained transfected cells were selected with hygromycin at a final concentration of 0.5 mg/ml in a weekly basis.

D. Irradiation

Molt-4 and HCT116 cells were irradiated with 5 and 10 Gy, respectively, of gamma rays using a Cesium source ^{137}Cs (244.1 cGy/min).

E. Okadaic acid treatment

HCT116 cells were treated with okadaic acid of a final concentration 9 nM. Cells were then harvested at the corresponding time points. For Tetrazolium (MTT) assay series of concentrations was prepared.

F. MTT assay

HCT116 cells were seeded at a density of 5,000 cells per well in 96-well plate where then they were treated with different concentrations of okadaic acid. Cells were then harvested at 24, 48 and 72 hours. Twenty micolitres of MTT solution was then added and the plate was then incubated for 2 hours at 37°C after which the media was aspirated and 100 μl of isopropanol with 10% HCl was added and left at room

temperature shaking for 15 min. The absorbance was then measured using a spectrophotometer at 595 nm wavelength.

G. Trypan Blue exclusion

Molt-4 cells were seeded at density of $5x10^6$ cells in T-25 flask. Irradiated cells were harvested at 6, 10 and 24 hours and then cells were pelleted at speed of 1500 rate per minute (rpm) for 5 minute at 4°C then cell pellets were resuspended in 1 ml media and 100 µl of cells were stained with trypan blue of ratio 1:1. Cells were counted with Hemacytometer. Cells which incorporated trypan blue are considered dead whereas shiny cells are not. Fold increase of dead cells was blotted.

H. Cell Cycle analysis

Molt-4 and HCT116 cells were seeded at a density of 10^6 per 100mm plate/ T-25 flask. Then, after 24 hours cells were irradiated or treated with okadaic acid and harvested at 24, 48 and 72 hours. Cells were pelleted at a speed of 2,000 rpm for 10 minutes at 4°C then resuspended with PBS (1x) and transferred to polystyrene tubes and repelleted at the same conditions. Cells were resuspended with 200 µl of 70% ethanol and stored at -20°C for later flow cytometry analysis. Before analysis, collected samples were centrifuged and washed with PBS (1x) to remove any traces of ethanol, then 800 µl of propidium iodide (PI) of concentration 50 µg/ml supplemented with RNAase was added per sample followed by incubation for 1 hour at dark before analyzing them.

I. Annexin V/PI assay

As the manufacturer's instructions the Annexin-V-FLOUS Staining Kit (Roche) with minor modifications was done as follows, collected cells at a density of 10^6 per condition were centrifuged at 2000 rpm for 10 minutes at 4°C and washed with PBS (1x), pelleted cells were resuspended and incubated with 100 µl of incubation buffer, 2 µl of Annexin and 2 µl of PI for 15 minutes, and then immediately analyzed by fluorescent-activated cell sorter (FACS).

J. Lipid extraction

Briefly, collected cells at a density of 10 x 10^6 or 3 x 10^6 in Molt-4 or HCT116 cells, respectively, per condition were transferred into borosilicate glass tubes, pelleted at 1,500 rpm for 5 minutes at 4°C, then resuspended in ice cold PBS (1X) and repelleted with same conditions. Afterwards cells were resuspended in 2 ml methanol, 1 ml chloroform and then left between 2-3 days for fixation at -80°C. Next, the aqueous and the lipid phases were separated following the addition of mixtures of methanol, chloroform and water. The resulting lower lipid phase was then transferred into a new glass tube to be dried by speed vacuum. Finally, the invisible pellet was resuspended in 1 ml chloroform [111].

K. Phosphate assay

Lipid phosphates were measured as in method of Rouser *et al*. [112]. Lipid aliquots (approximately 200 µl) that were previously extracted were dried and resuspended in 70 µl perchloric acid (70%). The reaction was allowed to proceed at

180ºC for 1 hour. After which the samples are left to cool down and then 830 µl of water was added per sample followed by 170 µl of ammonium molybdate (2.5%) and 170 µl of freshly prepared ascorbic acid (10%) were added. The samples were incubated in water bath at 50ºC for 15 minutes. The absorbance was then measured using a spectrophotometer at a wavelength of 820 nm and phosphate concentrations were calculated in comparison with Na_2HPO_4 of known phosphate standards.

L. DGK assay

It was performed as described by Preiss *et al*.[113] which is a modified version of diacyl glycerol kinase (DGK) assay. Briefly, aliquots of lipid samples were dried using speed vacuum, and then the invisible pellet was resuspended in 20 µl micelles (made of octyl-β-D-glucoside/dioleoyl phosphatidylglycerol). This was followed by sonication for 30 minute accompanied by vortexing each 10 minute interval. Meanwhile, a reaction mixture was prepared containing 2 x buffer, 0.2 µl of 1 M DTT, 5 µg of diacylglycerol kinase enzyme and dilution buffer. Seventy microliters of the mixture was added per sample, and then the reaction was initiated by adding 10 µl of the radioactive ATP mixture made of 2.5 mM cold ATP (non-radioactive), 1.3 µCi of [γ-^{32}P] ATP solution and water. The reaction was allowed to proceed for 30 minutes and then stopped by adding a mixture of methanol, chloroform and water. The lipid phase was then transferred into a clean glass tube and dried. Lipids were resuspended in 50 µl of chloroform: methanol mixture of ratio 9:1. Once the elution chamber is saturated, 25 µl of samples were spotted on a lane of silica gel thin layer chromatography plate. Samples will migrate and lipids will be separated. The plate was then exposed to X-ray film overnight at -70ºC. Ceramide phosphate bands were identified based on the ceramide

standards and then scrapped into scintillation vials filled with scintillation fluid and so to be read using liquid scintillation counter.

M. Crude and pure mitochondria isolation

As manufacturer's instructions (Abcam) with few modifications the following was done, concisely, collected cells at density of $2x10^8$ cells were pelleted. Frozen cells at -80°C were then thawed to weaken the cell membranes, and then resuspended in Reagent A, followed by homogenization and centrifugation at 1,000 g for 10 minutes at 4°C. The pellet contains unbroken cells and nuclei whereas the collected supernatant contains mitochondrial, microsomal and cytoplasmic fractions. This supernatant was centrifuged at 12,000 g for 15 minutes at 4°C to isolate crude mitochondrial fraction. Afterward, the pellet was washed twice with Reagent C to wash off any cytoplasmic contamination and then stored at -20ºC. To further purify the mitochondria from associated MAMs, protocol by Wieckowski *et al*. was followed [114] with fine modifications. The isolated crude mitochondria was layered above 8 ml of 30% percoll medium in an ultracentrifuge tube followed by a layer of mitochondrial resuspending buffer (MRB) was added gently on top of the mitochondrial sample to fill up the ultracentrifuge tube. A dense pure mitochondrial band was found at the bottom of the tube whereas MAMs was found as diffused band on top of the centrifuge tube. Mitochondrial and MAMs bands were collected and purified separately at 6,500 g and 100,000 g, respectively. Pure pellets were resuspended with MRB and stored at -20ºC for later investigations such as western blot or DGK assay.

N. Protein extraction and quantification

Treated and untreated cells were pelleted at speed of 1,500 rpm for 5 minutes then resuspended in lysis buffer (0.25 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol and 2 mg bromophenol blue) followed by boiling at 95ºC for 5 minutes. The extracted proteins were quantified using Bio-Rad Protein Assay as described by the manufacturer. The concentrations of proteins in the samples were identified using a standard of Bovine Serum Albumin (BSA, Amersco).

O. Western blotting

Equal amounts of proteins were loaded with 1:1 ratio (proteins: loading buffer) in an acrylamide gel of 100 μg for whole cell lysate and 25 μg for mitochondrial samples. Proteins were left to migrate, then transferred into a PVDF membrane and then blocked with 5% fat-free milk-TBS 1X (Tris 12 g, 87.8 g NaCl and 1 L DDW of pH8). Following blocking, the membrane was incubated with primary antibody overnight at 4ºC followed by incubation for 1 hour with suitable secondary antibody coupled to horseradish peroxidase. In between the incubations periods, the membranes were washed three times with TBS 1X for each for 10 minute interval. Finally, the protein bands were revealed using ECL western blotting reagent.

CHAPTER IV

PRELIMINARY RESULTS

A. γ-Irradiation triggers p53 expression

In our previous data we have shown that γ-irradiation stimulates p53 protein upregulation in a time and dose-dependent manner [115]. Molt-4 cells expressing either the LXSN retroviral vector or the papilloma virus E6 protein, which is responsible for p53 protein degradation and thus, its downregulation, were subjected to γ-irradiation at 5 Gy dose. It was found that p53 protein expression level increases with time as early as 2 hours post-irradiation in Molt-4 LXSN cells (Figure 6).

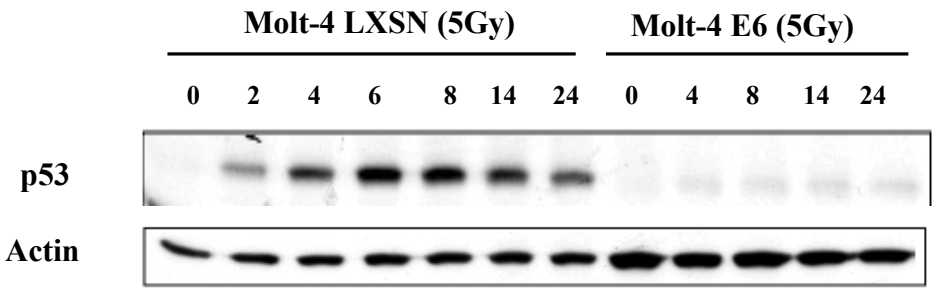


Figure 6. p53 expression increases upon γ-irradiation. γ-irradiation induces p53 transcription and upregulation in Molt-4 LXSN cells. Molt-4 LXSN and Molt-4 E6 cells were irradiated at a total dose of 5 Gy and collected at the specified time points, then protein expression levels was qualitatively measured using Western Blot.

B. γ-Irradiation triggers p53-dependent ceramide accumulation and cell death

Cell death was measured in γ-irradiated Molt-4 LXSN and Molt-4 E6 cells at 5 Gy dose using trypan blue. Twenty-four hours post-irradiation Molt-4 LXSN cells underwent cell death (approximately 45%) whereas, Molt-4 E6 cells were insensitive (Figure 7A) [67]. This death was accompanied by endogenous ceramide accumulation under similar conditions. Ceramide levels increased 10 fold at 24 hours compared to untreated Molt-4 LXSN cells, but no significant change was observed in Molt-4 E6 cells

(Figure 7B). These studies indicate that ceramide accumulation is reliant on p53 expression and is complemented by cell death in response to genotoxic stresses such as γ-irradiation.

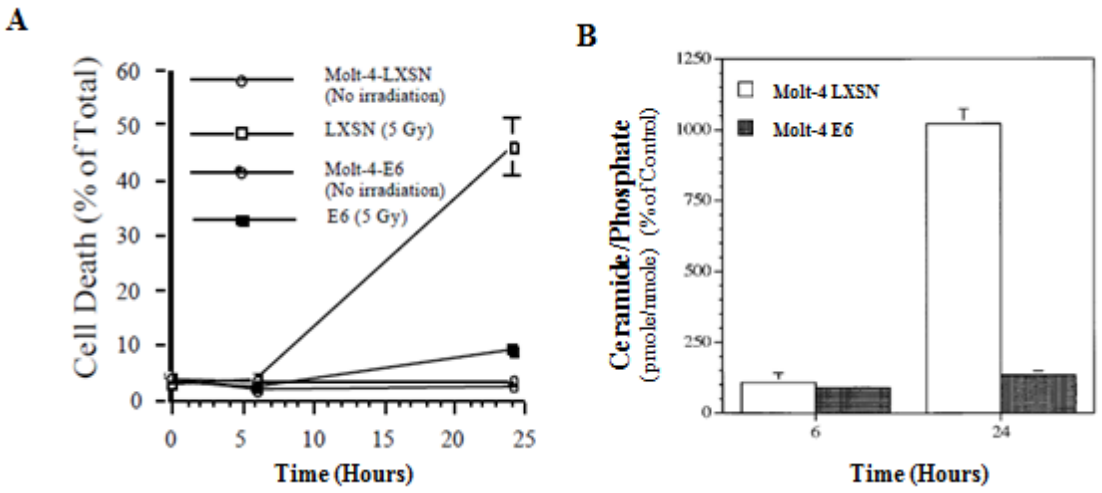


Figure 7. Genotoxic effects of γ-irradiation in Molt-4 LXSN and Molt-4 E6 cells on cell death and ceramide accumulation. **(A)** Requirement of p53 for cell death upon γ-irradiation. Molt-4 LXSN and Molt-4 E6 cells were γ-irradiated at a total of 5 Gy dose and then harvested and cell death was measured by trypan blue uptake at the indicated time points, results were compared with respect to their non-irradiated controls. **(B)** p53 is essential for ceramide accumulation in response to γ-irradiation. Molt-4 LXSN and Molt-4 E6 cells exposed to similar conditions were pelleted at a density of 10^7 cells at different time points as shown above and endogenous ceramide levels were measured. Ceramide levels are represented as percentage of control cells. The ceramide measurements are average of three independent experiments done in duplicates.

C. Affymetrix studies reveal a possibility of Noxa protein involvement

A complete coverage of human genome of more than 47,000 transcripts was performed using Gene Chip Human Genome U133Plus 2.0 Array on Molt-4 LXSN and Molt-4 E6 cells exposed to 5 Gy of γ-irradiation followed by harvesting at 0, 3 and 8 hours (Figure 8). Totally, more than 2000 genes had displayed a significant differentially altered expression when Molt-4 LXSN and Molt-4 E6 compared among themselves at different time points or when compared to each other. Results were analyzed using Linear

Model for Microarray Data (limma) software that is based on empirical Bayes method. Transcripts showing logged fold change greater the 1 or lower than -1 were considered significant. Molt-4 LXSN cells 3 hours after irradiation, when compared to its control, showed gene expression elevation in apoptosis regulators such as *Noxa* and *Bcl2 L11* that decreases at later time points. In contrast, 8 hours after irradiation different sets of genes were expressed such as *Bik* and *Bax.* Moreover, several genes of different functions, other than apoptosis, displayed differential expression were identified. Many of these genes are involved in proliferation (such as signal-induced proliferation-associated 1 like 2, *SIPA1L2*), DNA repair (*Brca1* as a transcription regulator) and ceramide synthesis longevity assurance gene 5 (*LASS5*) (codes for ceramide synthase enzyme). Figure 8A-C presents a few of the apoptotic genes that are regulated in Molt-4 LXSN and Mol-4 E6 cells following 5 Gy dose of γ-irradiation at different intervals of a logged fold change greater than 1. Similarly to Molt-4 models, gene expression profiling was performed on EB and EB-1 (a stably transfected model with a p53 gene associated with MT-1 promoter, thereby expressing p53 upon zinc chloride induction) colon cancer cell line (results not shown). In parallel with gene profiling, Noxa protein levels were analyzed, displaying a transient increase in Noxa proteins 2 hours post-irradiation in Molt-4 LXSN and insignificant increase in Molt-4 E6 cells. This was followed by decrease after a 6 hours interval (Figure 9A). A similar trend of Noxa protein expression was observed in EB and EB-1 colon cancer cell models (Figure 9B). These results prompted us to further study Bcl-2 family proteins, specifically Noxa, to identify their role in the p53-dependent ceramide accumulation, in T-leukemic cell line and colon cancer cell line.

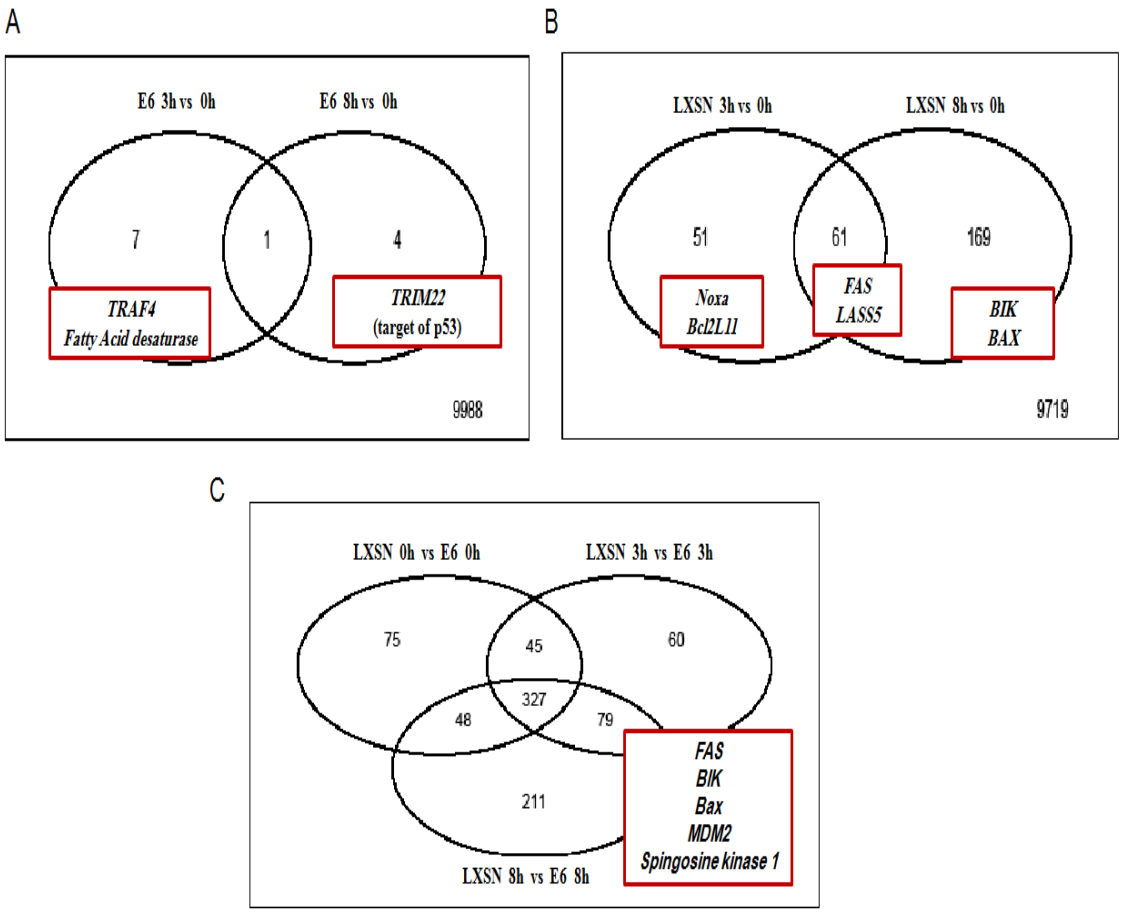


Figure 8. Affymetrix analyses of apoptotic transcripts in γ-irradiated Molt-4 LXSN and Molt-4 E6 cells. **(A)** A Venn diagram presenting the differential expression of specific transcripts in Molt-4 E6 cells. *TRAF4* and *Fatty Acid desaturase* genes were elevated in Molt-4 E6 cells after 3 hours of irradiation whereas *TRIM22* (possess an E3 ubiquitin ligase activity) was elevated after 8 hours post-irradiation. Gene expression was compared to its untreated counterpart. **(B)** A Venn diagram showing the differential expression of different genes in Molt-4 LXSN cells. *Noxa, BIK, BAX* and *Bcl211* transcripts were elevated transiently post-irradiation at the indicated time points, however *FAS* and *LASS5* (a ceramide synthase gene) was elevated in both time points. Gene expression was compared to their untreated counterparts. **(C)** A Venn diagram comparing Molt-4 LXSN cells to Molt-4 E6 cells at the given time points. *FAS, BIK, BAX, MDM2* and *SK1* genes were highly expressed in Molt-4 LXSN cells after 3 and 8 hours but not in Molt-4 E6 cells. The indicated values in the diagram represent the number of genes with altered expression either after 3 hours or after 8 hours or at both time points, when compared to their analogue counterparts. The above data is representative of three independent experiments.

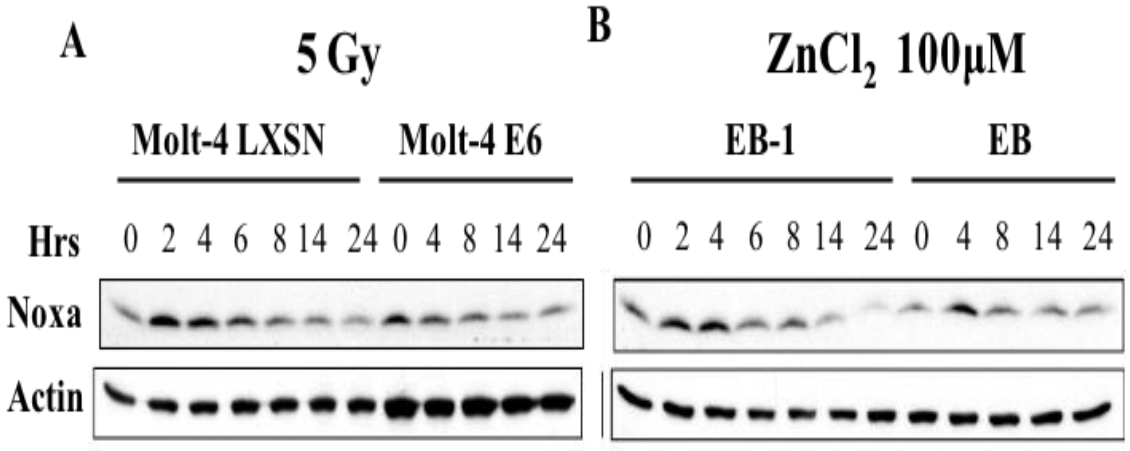


Figure 9. Genotoxic stress induces Noxa upregulation. **(A)** γ-irradiation of 5 Gy dose stimulates transient Noxa upregulation in Molt-4 LXSN and to lesser extent in Molt-4 E6 cells. Noxa expression increases rapidly following irradiation **(B)** $ZnCl_2$ treatment induces Noxa expression in EB-1 and to lesser extent in EB cells. Irradiated cells were harvested and proteins were extracted and probed for Noxa using Western Blot. Actin was used as an indicator of equal protein amount.

CHAPTER V

RESULTS

A. Experiments Conducted in Molt-4 cell line

In line with the preliminary results, we aimed to study the ordering of endogenous ceramide with respect to Noxa in cell death induction and whether Noxa plays a role in mediating the effect of p53 on ceramide accumulation. Also, we wanted to investigate how Noxa protein would affect apoptosis.

1. *Establishment of stable Noxa knockdown in Molt-4 cell line*

In order to study the role of Noxa protein in the apoptotic pathway, we transfected Molt-4 cells with either shRNA Noxa or scrambled vector, and assessed the intensity of knockdown by Western Blot (Figure 10). Thereafter, to achieve stably transfected models, Molt-4 LXSN SCR, Molt-4 E6 SCR, Molt-4 LXSN KD^{NOXA} and Molt-4 E6 KD^{NOXA} (cells transfected with scrambled vector and shRNA Noxa, respectively) were continuously selected with Hygromycin and Geneticin (at final concentration of 0.5 mg/ml).

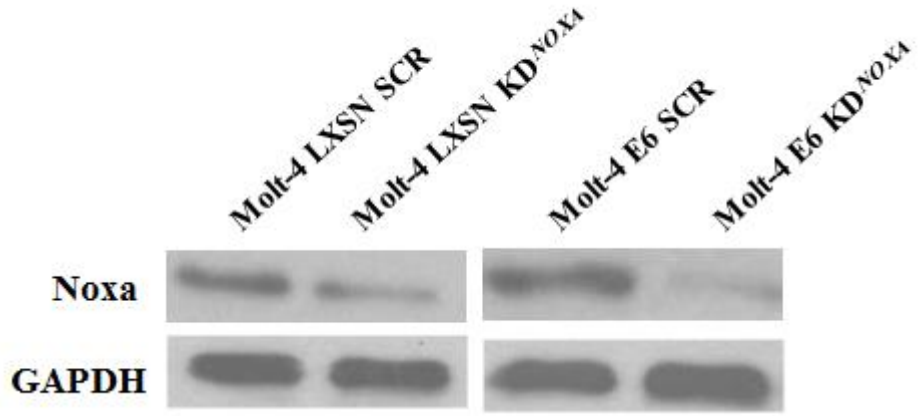


Figure 10. Stable transfectants of Molt-4 LXSN and Molt-4 E6 cells with shRNA Noxa. Molt-4 LXSN KD^{NOXA} and Molt-4 E6 KD^{NOXA} models were established showing significant Noxa inhibition using Noxa shRNA. These models were selected by hygromycin and geneticin on weekly basis of final concentration 0.5 mg/ml. Noxa inhibition was determined by Western Blot analysis. GAPDH was used as an indicator of equal protein loading.

2. ***Noxa protein partially influences p53-dependent apoptosis***

Molt-4 LXSN SCR and Molt-4 LXSN KD^{NOXA} cells showed comparable time-dependent elevation in p53 expression upon γ-irradiation at 5 Gy dose (Figure 11). Thus, demonstrating that p53 expression is unaffected by Noxa levels. Moreover, this elevation was accompanied by apoptosis in both models, yet Molt-4 LXSN KD^{NOXA} exhibited a slight decrease in apoptotic levels at 24 and 48 hours when compared to their control model counterpart (Figure 12A-D). Of particular interest, irradiated Molt-4 E6 SCR and Molt-4 E6 KD^{NOXA} were arrested at G2-M phase and this was shifted to polyploidy at 48 hours indicating a p53 and Noxa-independent mechanism. Similarly, a complete PARP cleavage, an indicator of apoptosis, was observed in Molt-4 LXSN SCR and Molt-4 LXSN KD^{NOXA} cells whereas partial cleavage and no cleavage were noticed in Molt-4 E6 SCR and Molt-4 E6 KD^{NOXA}, respectively (Figure 12D). Apoptosis was verified using trypan blue (Figure 12B), PARP cleavage (Figure 12D), Annexin V/PI assay (Figure 12C) and cell cycle analysis (Figure 12A).

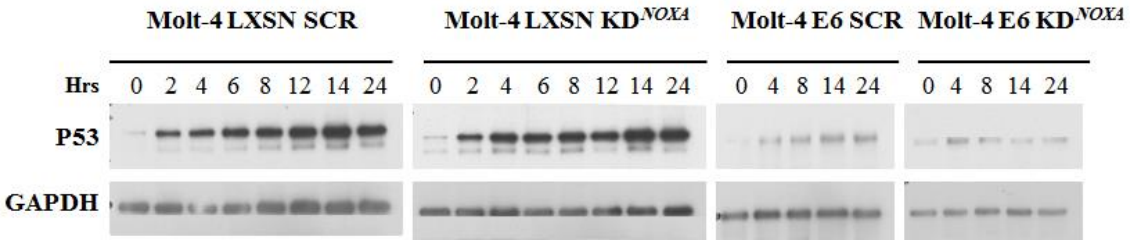
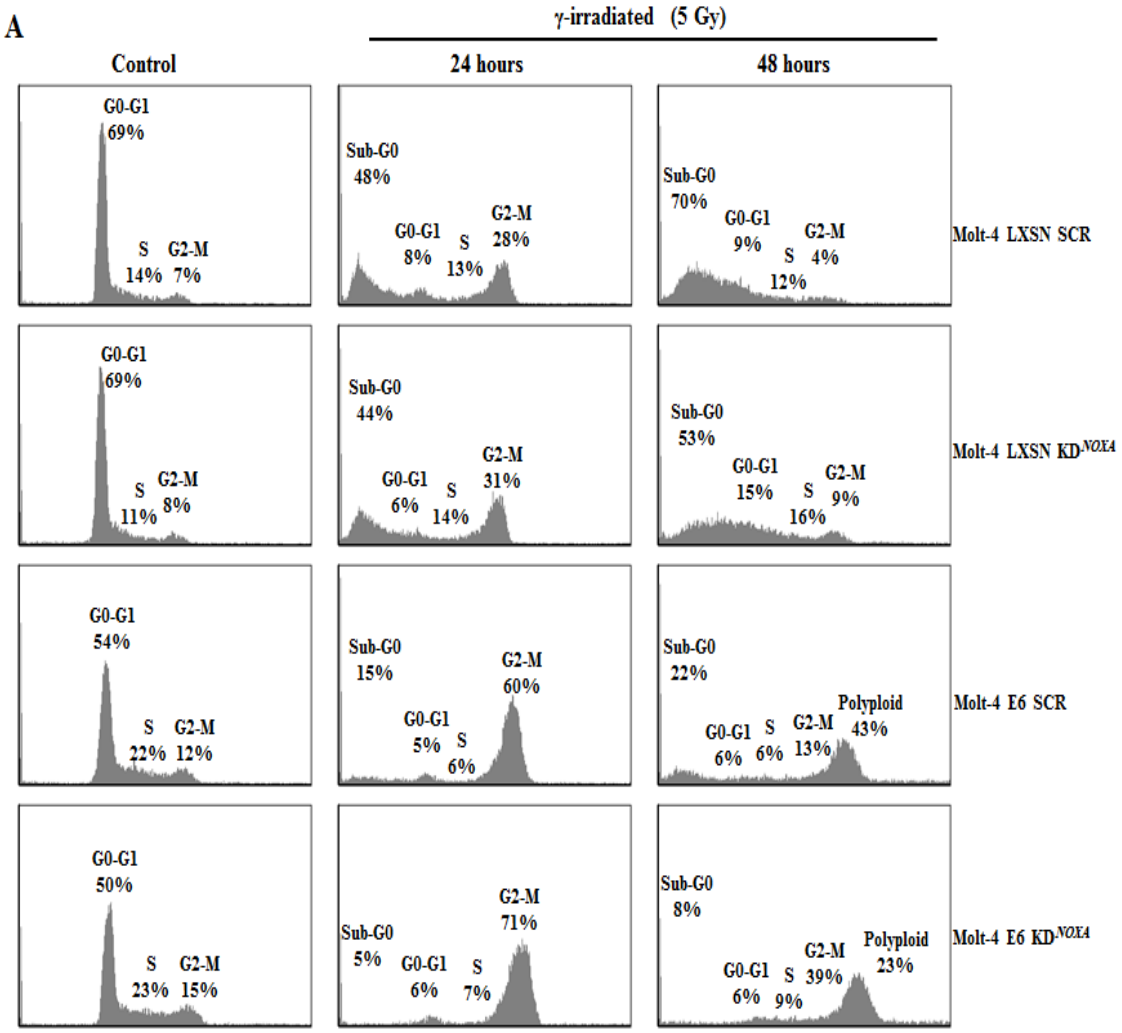


Figure 11. γ-irradiation stimulates p53 protein upregulation independent to Noxa status. p53 accumulates constantly after 2 hours of irradiation at 5 Gy dose in both Molt-4 LXSN SCR and Molt-4 LXSN KD^{NOXA}. Cells were pelleted at a density of 10^7 cells at and the whole cell extracts were probed for p53 by Western Blot. An antibody for GAPDH was used for measuring equal loading. Data shown is representative of four independent experiments of similar results.



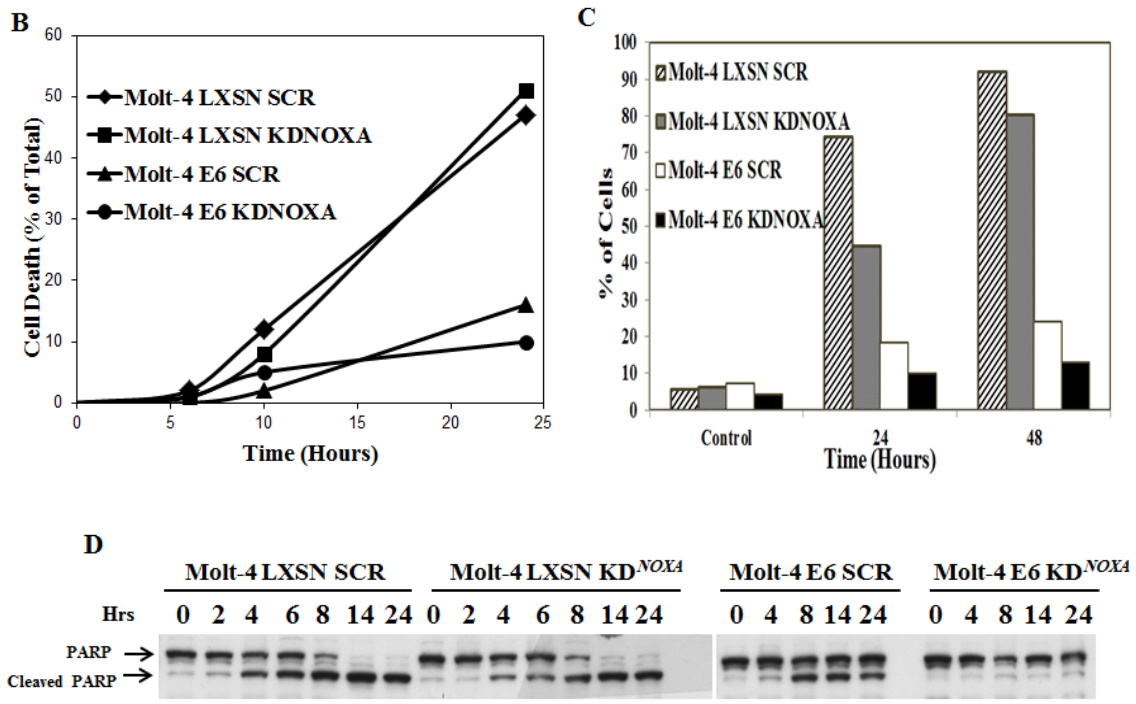


Figure 12. The requirement of p53 for γ-irradiation-induced apoptosis. **(A)** Molt-4 LXSN SCR and Molt-4 LXSN KD^{NOXA} underwent cell death whereas G2-M accumulation followed by polyploidy in Molt-4 E6 SCR and Molt-4 E6 KD^{NOXA}. Irradiated cells were collected at the indicated time points and fixed with 70% ethanol succeeding with PI staining, and cell cycle was analyzed by flow cytometry. A basal level of cell death was approximately 5% that were not compensated. Data shown represents two independent experiments. **(B)** A similar pattern of cell death was observed in Trypan blue analysis as shown above. Cells were collected and resuspended in 1 ml of media and Trypan blue exclusion was then performed, see materials and methods section for further details. Data is representative of one experiment. **(C)** The observed cell death is through the induction of apoptosis and not necrosis showed by Annexin V and PI positive cells. Molt-4 LXSN SCR, Molt-4 LXSN KD^{NOXA}, Molt-4 E6 SCR and Molt-4 E6 KD^{NOXA} cells were harvested after irradiation and then stained with a combination of Annexin V and PI to assay for viable, apoptotic and necrotic cells. The represented values show a total of both single stained Annexin V positive (A+P-) cells and double stained Annexin V and PI positive (A+P+) cells. Data shown is a representative of 2 independent experiments. **(D)** γ-irradiation induces PARP cleavage in Molt-4 LXSN SCR, Molt-4 LXSN KD^{NOXA} and Molt-4 E6 SCR but not in Molt-4 E6 KD^{NOXA}. Cells were collected at the indicated time points and whole cell lysate of 100 μg of proteins were loaded and SDS-PAGE gel was performed. The diagram shows uncleaved PARP (upper panel) of 116 kDa size and cleaved PARP (lower panel) of 89 kDa size. Data shown is a representative of four independent experiments.

3. Intracellular levels of γ-irradiated-signaling molecules in T-leukemic cells: Noxa initiating intrinsic apoptosis

Bcl-2 family proteins are known to be involved in both extrinsic and intrinsic apoptosis. In order to identify which of these two pathways is triggered upon γ-irradiation, different caspases were analyzed. In addition, we studied the expression profile of different Bcl-2 family proteins in response to p53 upregulation and/or Noxa silencing. Upon γ-irradiation (5 Gy) a cascade of caspases was activated. However, as expected, only Molt-4 cells possessing active p53 showed activation of the terminal caspase 3 (Figure 13A). Molt-4 LXSN SCR cells underwent caspase 3 cleavage 12 hours post-irradiation while Molt-4 LXSN KD^{NOXA} showed later caspase 3 cleavage at 14 hours. On the other hand, Molt-4 E6 SCR and Molt-4 E6 KD^{NOXA} displayed no caspase 3 cleavage (Figure 13A). To investigate whether extrinsic or intrinsic pathway are responsible for caspase 3 cleavage, Molt-4 LXSN KD^{NOXA} and Molt-4 LXSN SCR were probed for caspase 9 which is a key marker for intrinsic apoptosis. Caspase 9 was cleaved as early as 2 hours after irradiation in Molt-4 LXSN SCR whereas its cleavage was only initiated at 24 hours in Molt-4 LXSN KD^{NOXA} (Figure 13B). This is consistent with the observed delay in induction of apoptosis in Molt-4 cells with silenced Noxa. We can deduce that Noxa is essential for intrinsic apoptosis since silencing Noxa delayed caspase 9 cleavage, once stimulated by γ-irradiation in Molt-4 cells (Figure 13B).

Noxa is well known to activate Bak and Bax by inhibiting the anti-apoptotic Bcl-2 proteins such as Mcl-1 and Bcl-2, thus it was interesting for us to examine the expression of the different Bcl-2 family proteins. In Bcl-2, the expression was maintained at a constant level at all-time points in all our cell models except in Molt-4 LXSN SCR where, surprisingly, the expression increased gradually in a time-dependent manner. In

case of Bak, the expression increased in Molt-4 LXSN SCR and to a lesser extent in both Molt-4 LXSN KD^{NOXA} and Molt-4 E6 SCR but instead remained constant in Molt-4 E6 KD^{NOXA}, indicating an important role of p53 and Noxa together in Bak upregulation (Figure 14). Unfortunately, Bax and Mcl-1 were not detectable with the antibodies that were purchased (results are not shown).

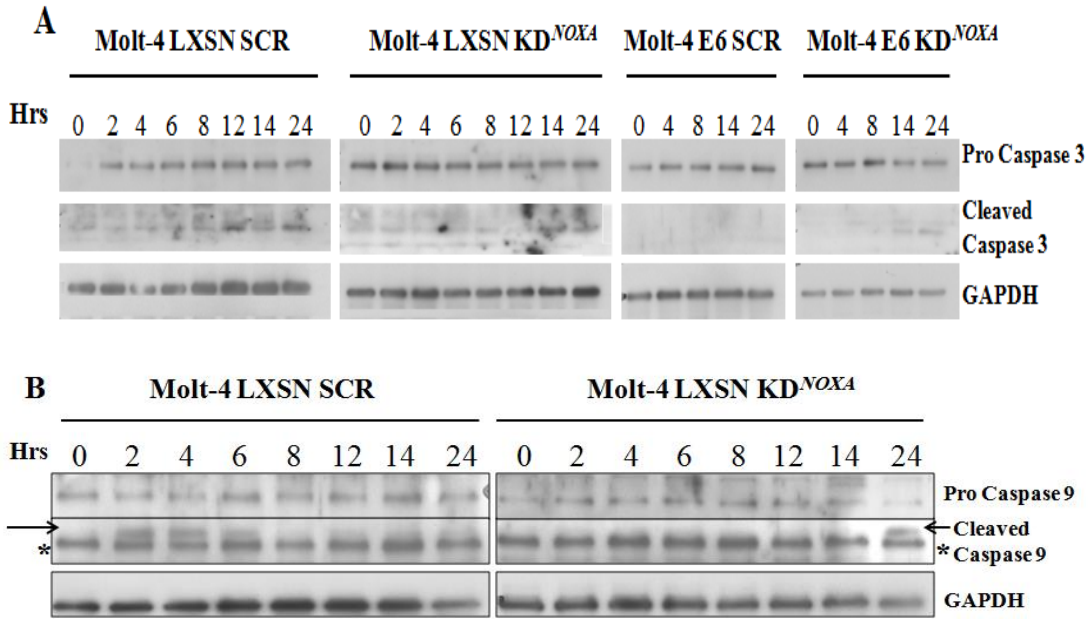


Figure 13. Induction of the initiator caspase 9 and terminal caspase 3 by γ-irradiation in the context of p53 and Noxa expression. **(A)** Noxa and p53 expression trigger terminal caspase 3 cleavage. Pro-caspase 3 and its cleaved form were clearly observed at the indicated time points. **(B)** Role of Noxa in triggering intrinsic apoptosis upon γ-irradiation. Pro-caspase 9 and its cleaved form were probed at the indicated time points. Cells were collected and whole cell lysate of 100 μg of proteins were loaded. GAPDH was used as loading control for protein samples. *Asterisk* represents a nonspecific band. Data are representative of one experiment.

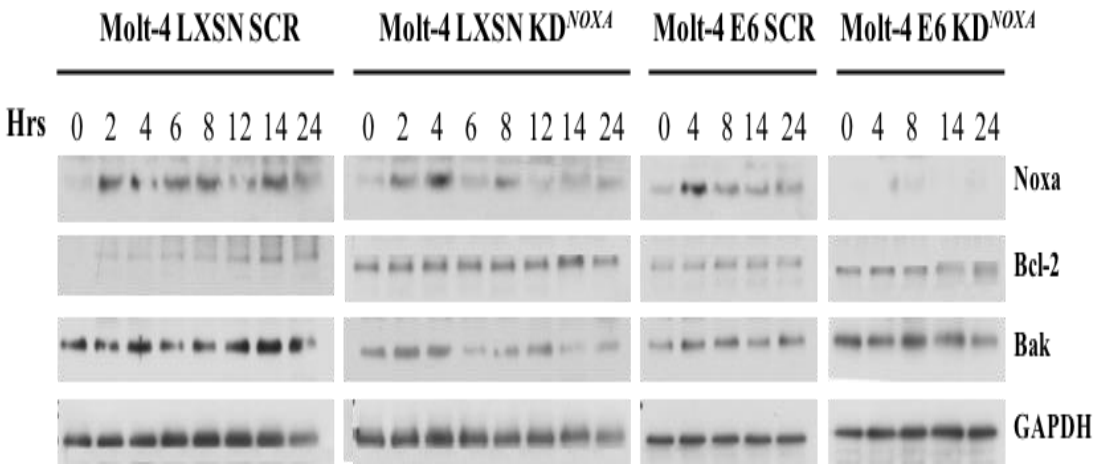


Figure 14. Noxa and p53 trigger Bcl-2 and Bak upregulation in γ-irradiated Molt-4 cells. Irradiated cells were harvested at the mentioned time points and 100 µg of whole cell lysate were loaded on 15% SDS-PAGE gel for Noxa, Bcl-2 and Bak. GAPDH was used as a loading control. Data are representative of two independent experiments.

4. ***Ceramide accumulation is p53 and Noxa-dependent***

Furthermore, we examined ceramide levels in our Noxa knocked down cell models under similar conditions using DGK assay. Strikingly, ceramide accumulated with 3 fold increase at 14 and 24 hours post-irradiation in Molt-4 LXSN SCR cells only (Figure 15). Hence, speculating the requirement of expressed p53 and Noxa proteins for endogenous ceramide synthesis.

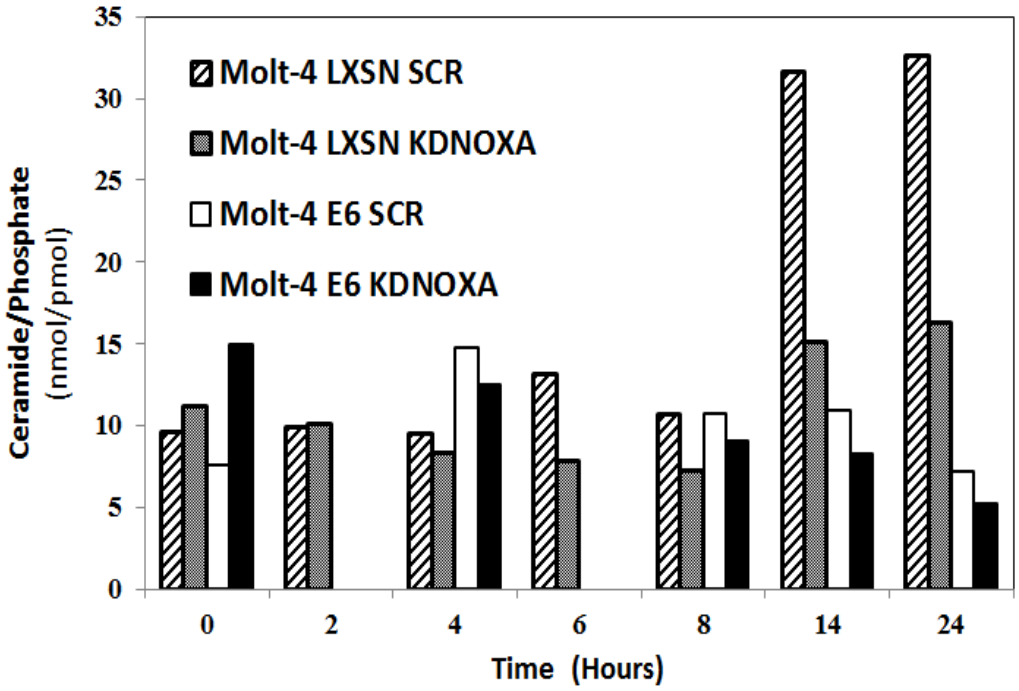


Figure 15. ***Ceramide synthesis requires p53 and Noxa expression.*** Cells at a density of 10^7 cells were harvested after γ-irradiation (5 Gy) and then ceramide was measured by the DGK assay. Ceramide values were normalized to lipid phosphates. Data shown is representative of three independent experiments.

5. ***Mitochondrial ceramide elevation is associated with Noxa translocation in irradiated Molt-4 LXSN cells***

The above studies demonstrated an important role of both p53 and Noxa in endogenous ceramide accumulation and subsequently intrinsic apoptosis; hence it was captivating for us to look at ceramide levels in the mitochondria. To quantify mitochondrial ceramide, we isolated pure mitochondria devoid from MAM contamination. Therefore, the measured ceramide reflects the level of ceramide present in the mitochondria and mostly lacks contamination from the ER origin. The purity of the mitochondria was verified by Western Blot analysis for the ER marker Calnexin. We extracted pure mitochondria of γ-irradiated Molt-4 LXSN cells followed by endogenous ceramide quantification in addition to Western Blot analysis. Irradiated cells were

collected at 0, 4, 8, 14 and 24 hours and then DGK assay was performed in both mitochondrial and cytosolic (containing light membranes such as ER and golgi apparatus) fractions. Two forms of ceramides species were detected on TLC plates, very-long and long ceramides collectively will be named long-chain ceramides and short-chain ceramides, identified based on co-migration with ceramide standards. Mitochondrial long-chain and short-chain ceramide accumulation began at 8 hours post-irradiation and drastically increased at 14 and 24 hours about 5 fold baseline levels by 24 hours. Notably, the absolute values of mitochondrial long-chain ceramide were much higher than those of short-chain ceramide (Figure 16A-C). On the other hand, cytosolic fractions displayed a 2 fold increase in long-chain ceramide levels at 14 hours that remained constant at 24 hours. However, a 2 fold increase in short-chain ceramide was only detected at 24 hours post-irradiation (Figure 16B-C). Although ceramide accumulation occurred in both fractions, mitochondrial ceramide levels were increased disproportionately compared to those in the cytosolic fraction. These results indicate that ceramide is either translocated to the mitochondria or synthesized within the mitochondria or both.

Because we are interested in studying the role of Noxa in stimulating ceramide accumulation it was important to examine the translocation of Bcl-2 family proteins. Extracted crude mitochondria and cytosolic fractions at the time points 0, 4, 8, 12, 14 and 24 hours post-irradiation were immunoblotted for Bax, Noxa, Bak and cytochrome c. Significantly, Noxa was translocated from the cytosolic fraction to the mitochondria by 4 hours and this became more prominent at 8 hours to decrease at 12 hours (Figure 17). When compared to the time course of ceramide levels above, it was noted that Noxa translocation precedes mitochondrial ceramide accumulation. When combined with the absence of ceramide accumulation after is Noxa knocked, this may suggest that Noxa

translocation to the mitochondria regulates the observed mitochondrial ceramide increase. Moreover, Bax expression increases in a time dependent manner in the cytosolic fraction and appears in the crude mitochondrial fraction by 8 hours post-irradiation (Figure 17). As for Bak protein, a faint increase was detected in the cytosolic fraction that was not reflected in the mitochondrial fraction. Cytochrome c release was also examined, and its time-dependent release into the cytosolic fraction is coincided with ceramide accumulation (Figure 17). Consequently, these results indicate that, for at least, the three molecules ceramide, Noxa and Bax function together in mediating MOMP and subsequently cytochrome c release.

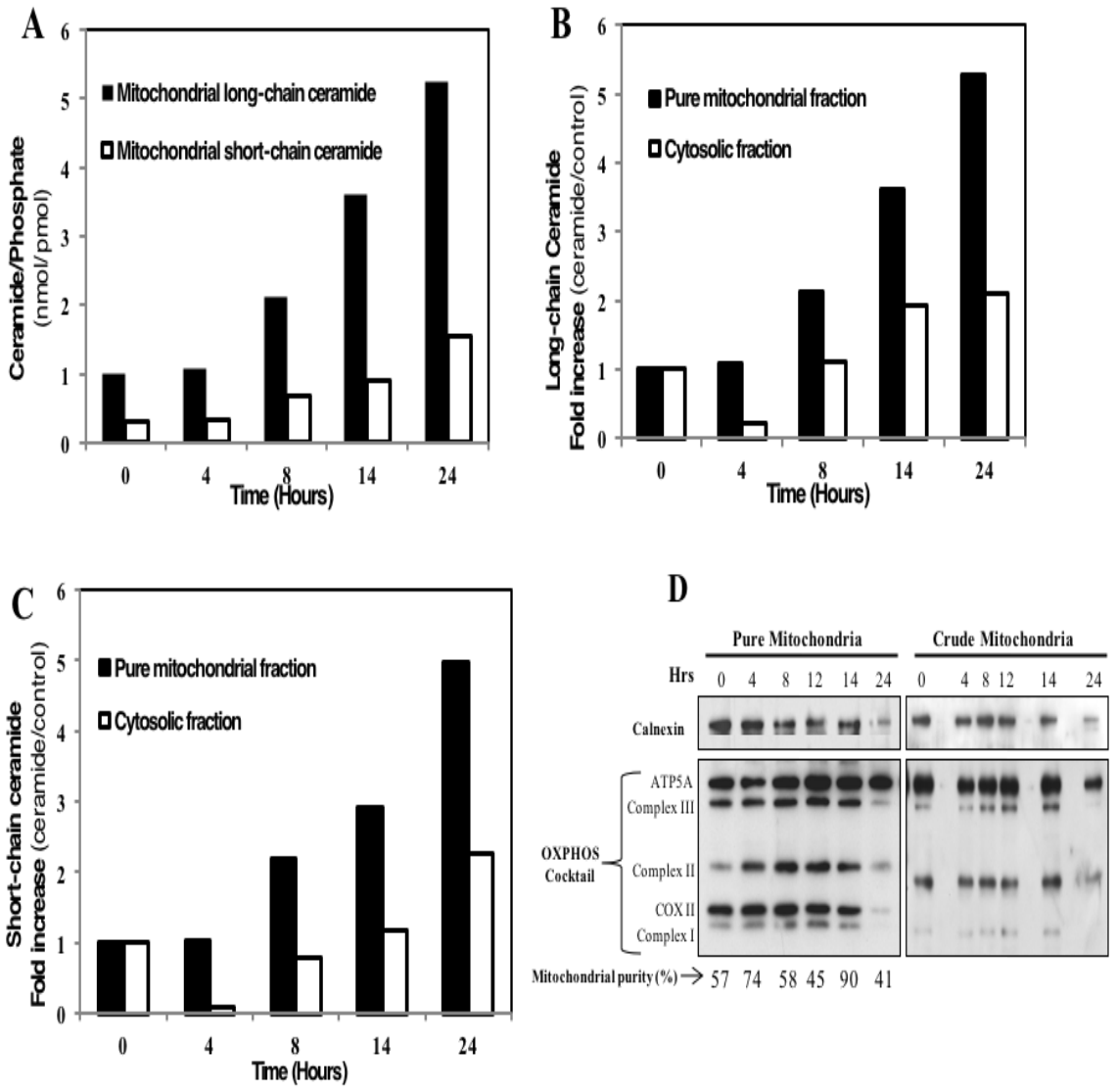


Figure 16. Time-course of ceramide accumulation in the mitochondrial and light membranes of γ-irradiated Molt-4 LXSN cells. **(A)** Absolute values of ceramides in purely isolated mitochondria are showing two forms of ceramides, long and short-chain ceramides. **(B)** Fold increase of long-chain ceramides in mitochondrial and cytosolic fractions compared to their untreated controls. **(C)** A similar fold increase was observed in short-chain ceramides in the mitochondrial and cytosolic fractions. Cells were seeded at a density of 10^8 cells per time point. Collected samples were homogenized and crude mitochondria were isolated by differential centrifugation followed by separation of MAMs from the mitochondria by ultracentrifugation using discontinuous percoll gradient (see materials and methods for further details). 1 ml of cytosolic fractions and 400 μl of mitochondrial fractions were used for lipid extraction and subsequently for the DGK analysis Ceramide values were normalized to their corresponding lipid phosphates. **(D)** Immunoblotting of the ER marker calnexin was used for testing mitochondrial purity, devoid from MAMs contamination, by comparing it to crude mitochondria. OXPHOS (a cocktail of mitochondrial markers) was used as equal loading indicator. Calnexin was normalized to its corresponding OXPHOS (the band of complex II was chosen for

normalization) and then using Image J software percentage of mitochondrial purity was measured. Indicated values represent the percentage of mitochondrial purity where pure mitochondrial samples (devoid from MAMs) divided by their corresponding crude mitochondria (containing MAMs) for each time point. Data are representative of one experiment.

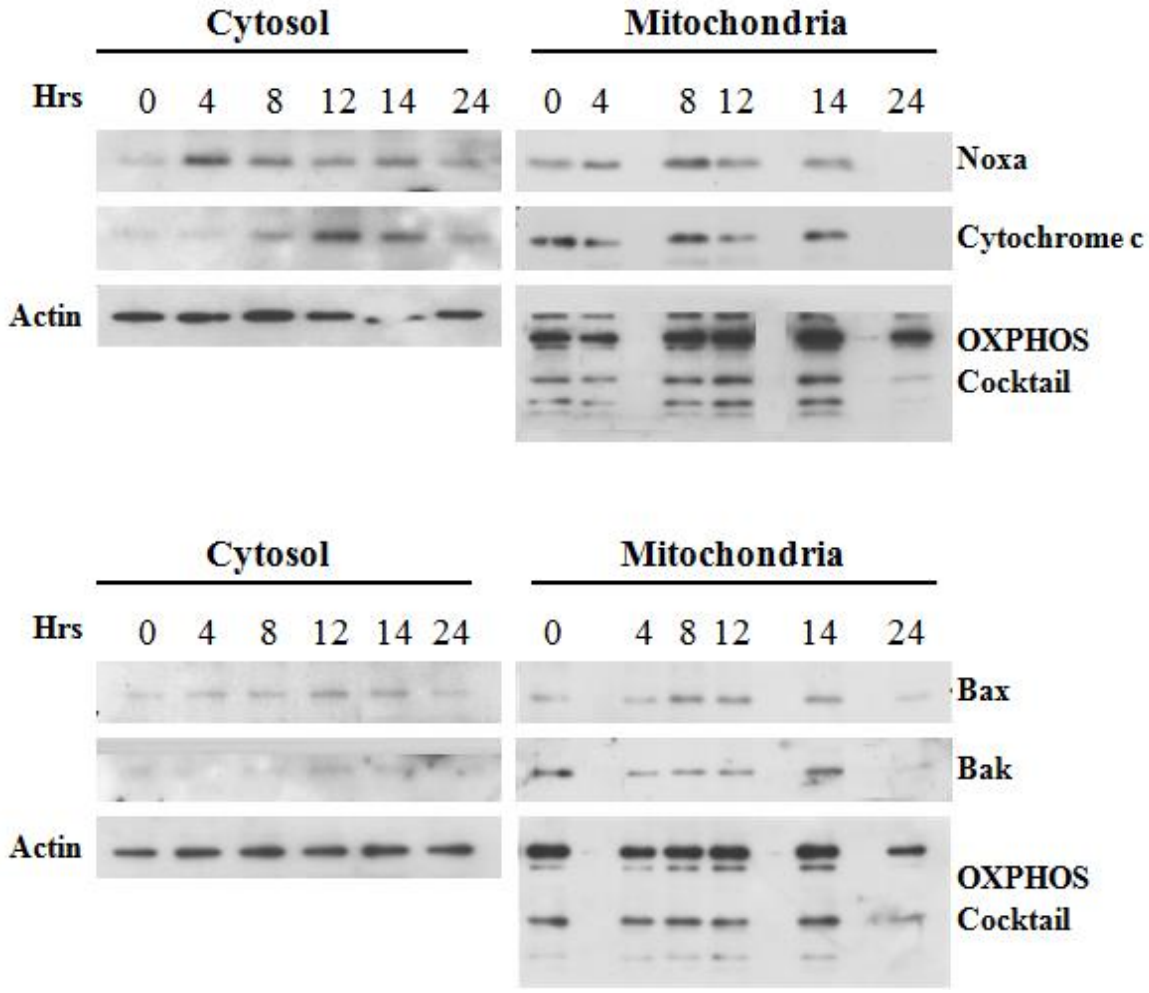


Figure 17. The effect of γ-irradiation on Bcl-2 family proteins translocation and initiation of MOMP in Molt-4 LXSN cells. Proteins inducing MOMP were identified by immunoblot analysis using antibodies against the indicated proteins. Noxa, Bax and Bak translocation into the mitochondria upon γ-irradiation was examined at the denoted time points. Cytochrome c release an indicator of MOMP induction was also examined. Crude mitochondria and cytosolic samples were prepared by homogenizing whole cell samples followed by differential centrifugation. Cytosolic and mitochondrial fractions were stored at -80˚C for Western analysis. 25 μg of proteins were loaded on SDS-PAGE gel and proteins were probed accordingly. Actin and OXPHOS cocktail antibodies were used as loading controls for cytosolic and mitochondrial fractions, respectively. Data are representative of one experiment.

B. Experiments Conducted on HCT116 cell line.

To further support our hypothesis, we were interested in studying endogenous ceramide regulation in HCT116 cell line. This cell line was chosen because, based on our preliminary results, we have observed a simultaneous increase of Noxa and ceramide levels in EB colon cancer cell line. However, these cells were difficult to handle in culture due to their slow growth rate so we shifted to HCT116 cell line. Before pursuing ceramide ordering in the apoptotic pathway, we sought to establish a p53-dependent system in this cell line.

1. ***γ-irradiation initiates mitotic catastrophe: a failed model***

Both HCT116 wild type p53-containing (p53 +/+) cells and HCT116 genetically disrupted p53 alleles (p53-/-) cells were exposed to γ-irradiation at 10 or 15 Gy (results not shown) dose and this was followed by measurement of cell cycle distribution by flow cytometry. A substantial G2-M arrest was observed in both genotypes at 24 hours post γ-irradiation. However, sooner this was shifted to polyploidy at a later time points (Figure 18A). Unexpectedly, HCT116 p53-/- cells quickly escaped this arrest starting at 48 hours into Sub-G0 phase, indicative of apoptosis, similarly did HCT116 p53+/+ cells but at a slower pace. Our findings are compatible with the characteristics of mitotic catastrophe in HCT116 p53-/- cells and polyploidy arrest in G1 state in HCT116 p53+/+ cells [99]. Furthermore, these results were associated with early ceramide increase by 24 hours in HCT116 p53+/+ cells, in contrast to HCT116 p53-/- cells where ceramide accumulation was delayed (Figure 18B). Despite these intriguing results of γ-irradiation inducing p53-independent mitotic catastrophe associated with ceramide accumulation, we felt that

γ-irradiation did not induce a clear p53-dependent apoptotic system. Therefore, other treatments were examined.

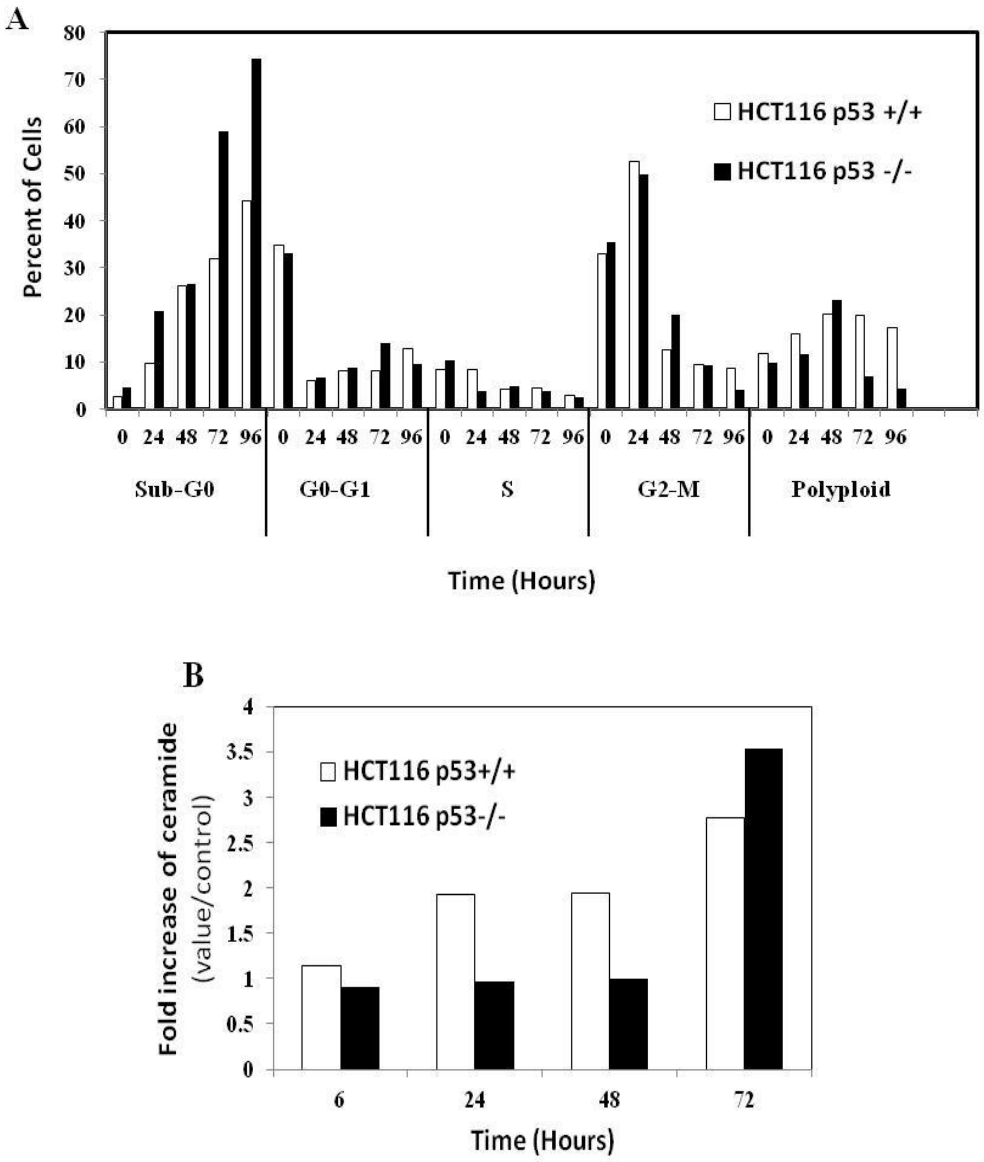


Figure 18. γ-irradiation triggers p53-dependent polyploidy phase accumulation and p53-independent mitotic catastrophe where both processes are ceramide-dependent. **(A)** HCT116 p53-/- cells underwent mitotic catastrophe upon γ-irradiation whereas HCT116 p53+/+ cells were arrested at polyploidy in G1 state. Cells were γ-irradiated at a total of 10 Gy dose then harvested at the indicated time points. 10^6 cells per time point were fixed in 70% ethanol and cell cycle was measured using flow cytometry after PI staining. Data is representative of two independent experiments done in duplicates. **(B)** Ceramide accumulates in both models but in dissimilar patterns. Cells were seeded at a density 10^6 per plate and then harvested for DGK analysis at the mentioned time points. Ceramide results were normalized to lipid phosphates. Data is representative of one experiment done in duplicates.

2. Okadaic acid induces a p53-dependent cytotoxicity

Among the different treatments that were examined such as calyculin A and doxorubicin, okadaic acid stood out as a p53-dependent stressor. Using the MTT assay, an indicator of mitochondrial function, we demonstrated that continuous okadaic acid treatment (whereby the treated media kept on cells continuously), caused loss of cell viability in HCT116 p53+/+ and not in HCT116 p53-/- cells. The IC50 was 9 nM at 48 hours, a time point where the most inhibition difference between the two models was detected at a differential concentration range between 6 and 20 nM (Figure 19).

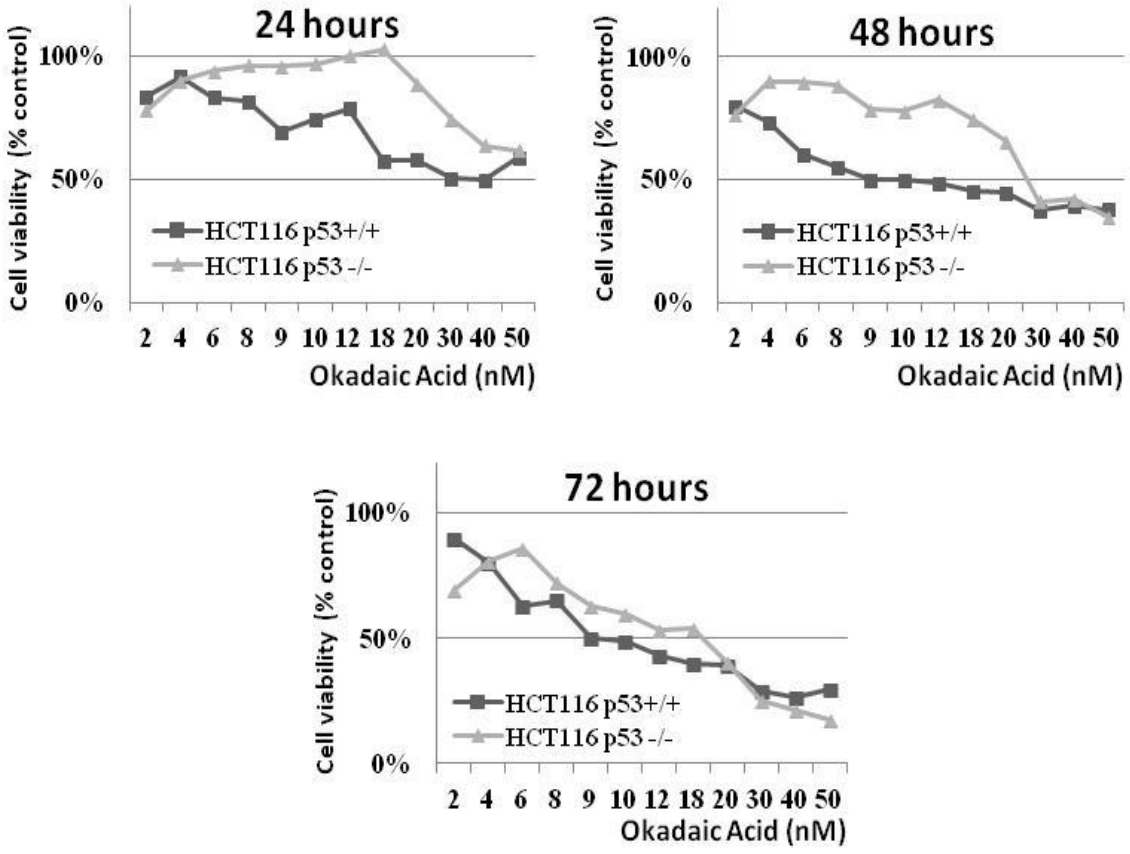


Figure 19. Cytotoxic effects of okadaic acid treatment are p53-dependent. Cells were treated with okadaic acid of series dilutions as indicated above. MTT assay was done and cell viability was measured using spectrophotometer at a wavelength 595 nm. The

obtained values were normalized to their untreated control counterparts and percentage of viability was calculated. Data shown is representative of one experiment.

3. Okadaic acid triggers p53-dependent apoptosis

To distinguish whether the observed cytotoxicity is due to apoptosis, cell cycle arrest or even necrosis, apoptotic cells were measured using Annexin V/ PI analysis. Depending on our MTT assay results, we chose to proceed with our experimental procedures with 9 nM concentration of okadaic acid. Around 10% of HCT116 p53+/+ cells underwent apoptosis after 24 hours of treatment and this increased gradually up to 72 hours reaching 27% apoptotic cells. In contrast, HCT116 p53-/- cells, negligible apoptosis was noticed upon okadaic acid treatment in the total time course experiment, thus confirming our MTT results, and indicating p53-dependent apoptosis (Figure 20). These results were similar to what we observed in Molt-4 cells and thus encouraged us to proceed with our experiments to study the role of Noxa in this pathway.

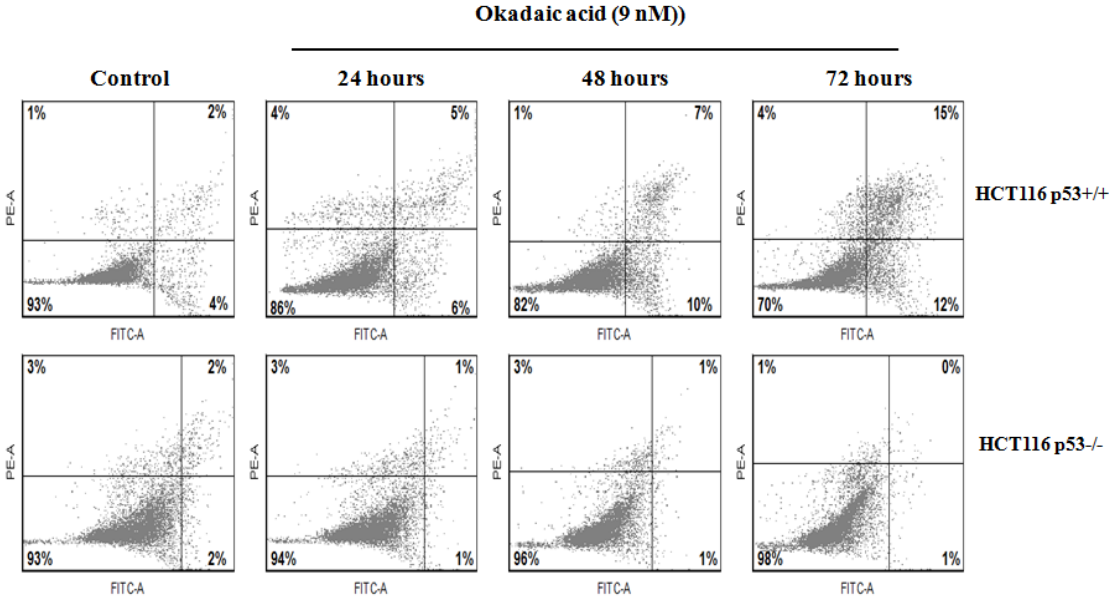


Figure 20. Okadaic acid initiates p53-dependent apoptosis. Annexin V-FITC / PI flow cytometry analysis was done following treatment with okadaic acid (9 nM) at the indicated time points. Collected cells were analyzed and the numbers of apoptotic cells

were identified. Lower left quadrant represents healthy cells with both negative Annexin V and PI staining (A-P-), lower right quadrant represents cells in early apoptosis with positive Annexin V and negative PI staining (A+P-), upper right represents cells in the late apoptosis with both positive Annexin V and PI staining (A+P+) and upper left quadrant contains necrotic cells with negative Annexin V and positive PI (A-P+). Cells were seeded at a density of 10^6 and harvested at 24, 48 and 72 hours post-treatment. Data shown is a representative of two independent experiments.

4. Okadaic acid initiates p53-independent Noxa expression

After determining okadaic acid as a p53-dependent stressor in HCT116 cell line, it was of particular importance to examine Noxa levels upon treatment. Noxa expression steadily increased in HCT116 p53-/- cells after 2 hours of okadaic acid treatment (9 nM) and decreased after 12 hours of treatment. Surprisingly, this was not observed in HCT116 p53+/+ cells, where the latter showed high Noxa levels in control cells that remained elevated at all the examined time points following treatment (Figure 21). Therefore, Noxa expression in HCT116 cells upon okadaic acid treatment is p53-independent.

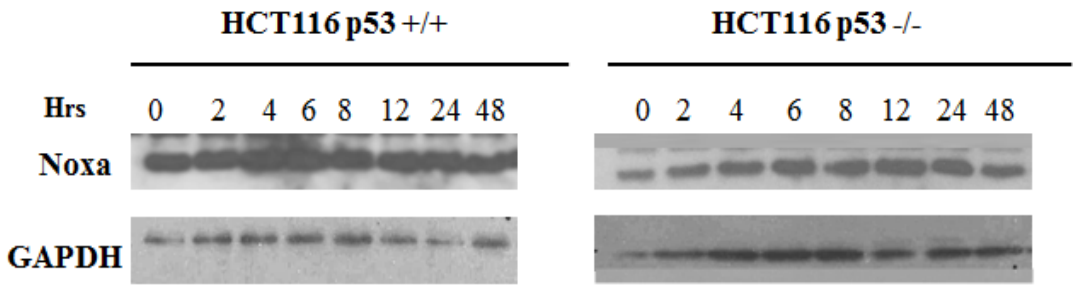


Figure 21. Noxa expression post okadaic acid treatment. Cells at a density of 6 x 10^5 per time point were treated with okadaic acid or with DMSO as vehicle control of final concentration of 9 nM and then harvested at the indicated time points. Whole cell lysates were quantified and accordingly 100 ug of proteins was loaded on SDS-PAGE followed by Noxa antibody incubation. GAPDH was used as a loading control. Data presented is of two independent experiments.

5. Establishment of stable Noxa knockdown HCT116 cell line

We next asked whether knockdown of Noxa would affect cell death and ceramide generation. Using shRNA Noxa or scrambled vector we stably knocked down

Noxa expression and assessed the degree of knockdown by Western Blot (Figure 22). Stably transfected HCT116 p53+/+ SCR, HCT116 p53-/- SCR, HCT116 p53+/+ KD^{NOXA} and HCT116 p53-/- KD^{NOXA} were selected with hygromycin (at a final concentration 0.5 mg/ml).

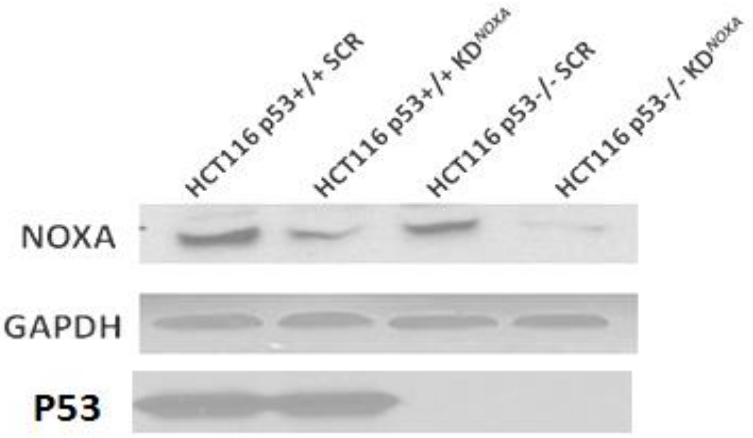


Figure 22. Stably transfected HCT116 cell with shRNA Noxa. Significant down regulation of Noxa expression in both HCT116 p53+/+ KD^{NOXA} and HCT116 p53-/- KD^{NOXA} models. Inhibition was examined after each thawing following selections with hygromycin at a final concentration of 0.5 mg/ml. p53 expression was also examined. GAPDH was used as a loading control.

6. Okadaic acid induces p53 upregulation and hyperphosphorylation

Upon treatment of HCT116 p53+/+ SCR and HCT116 p53+/+ KD^{NOXA} with okadaic acid, p53 was up regulated and hyperphosphorylated as early as 1 hour post-treatment and this increased steadily up to 48 hours (Figure 23). Interestingly, both p53 and its phosphorylated form (p.p53) transiently decreased at 8 hours and recovered by 18 hours following treatment.

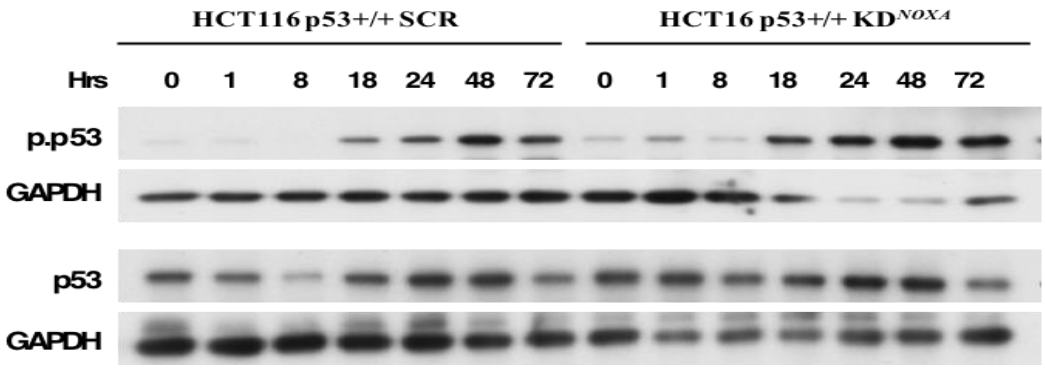


Figure 23. Effect of okadaic acid treatment on p53 expression and phosphoryation. HCT116 p53+/+ SCR and HCT116 p53+/+ KD^{NOXA} were seeded at a density of 6 x 10^5 cells/condition and treated with okadaic acid or with DMSO as a vehicle control at a final concentration of 9 nM. Cells were harvested at the specified time points and then protein expression was determined by Western Blot. GAPDH was used as a loading control. Data shown is representative of two independent experiments.

7. Okadaic acid initiates p53, and to certain extent, Noxa-dependent apoptosis

To study the role of Noxa in the apoptotic pathway, HCT116 cells were treated with okadaic acid and harvested at various time points. Apoptotic cells were determined by PARP cleavage and by cell cycle analysis. PARP cleavage was observed in both HCT116 p53+/+ SCR and HCT116 p53+/+ KD^{NOXA} cells. However, HCT116 p53+/+ KD^{NOXA} had delayed PARP cleavage compared to their controls (Figure 24A). On the other hand, HCT116 p53-/- SCR and HCT116 p53-/- KD^{NOXA} had negligible, if any, PARP cleavage. Furthermore, cell cycle analysis had demonstrated apoptotic cells in both HCT116 p53+/+ SCR and HCT116 p53+/+ KD^{NOXA} models whereby cells at G0-G1 phase had exited cell cycle into Sub-G0. Again, and consistent with PARP cleavage profile, HCT116 p53+/+ KD^{NOXA} showed lesser apoptotic cells when compared to its control counterpart cell model (Figure 24B). Treated HCT116 p53-/- SCR and HCT116 p53-/- KD^{NOXA} cells experienced, to some extent,

G2-M arrest, and a slight increase in Sub-G0. These results are more or less commensurate with what was observed in Molt-4 cells.

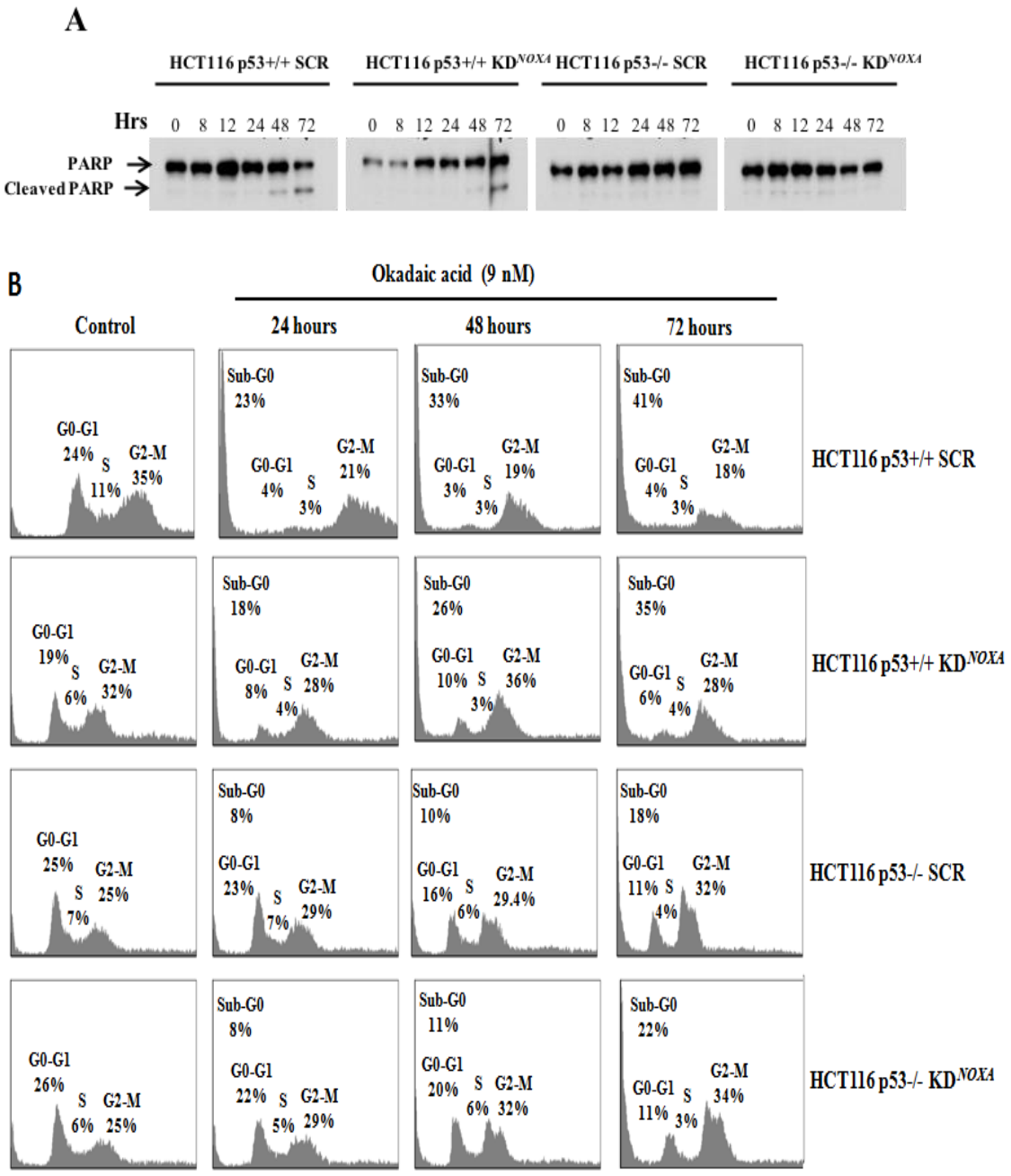


Figure 24. Induction of apoptosis by okadaic acid treatment depends primarily on p53 and secondary on Noxa. **(A)** p53 expression is required for PARP cleavage. Western Blot analysis for PARP and its cleavage in HCT116 cells was performed using 100 µg of cell lysates. Data is representative of three independent experiments. **(B)** p53 and Noxa-dependent cell death. Fixed cells were analyzed using flow cytometer following incubation with PI staining solution. Cells were seeded at a density of 10^6 cells/sample and harvested at the corresponding time points. A basal level of cell death was of

approximately 8% that was not compensated. The data presented is typical of three independent experiments.

CHAPTER VI

DISCUSSION

A number of recent studies have shown that γ-irradiation induces p53-dependent apoptosis regulated by Bcl-2 family proteins [116]. In fact, BH3-only pro-apoptotic proteins are found as main regulators in the p53-mediated apoptosis such as Puma [81] and Noxa [116]. TP53 has been shown to directly stimulate Noxa [117], and any defect in p53 has displayed two fold decreases in Noxa expression [118]. Similarly, we have shown that silencing of p53 has drastically down regulated Noxa levels whereas transfecting with antisense Noxa oligonucleotide showed no change in p53 expression thereby agreeing with previous studies that p53 is upstream of Noxa. Stress signaling can increase the activity of BH3-only pro-apoptotic proteins either at the level of transcription such as Puma, decreased turnover such as Noxa, post-translational modifications such as phosphorylation of Bad, caspase-dependent cleavage such as Bid or change in subcellular localization such as Bim [75]. We have noticed that Noxa is transiently expressed in response to γ-irradiation. Therefore, they can be regulated at the level of transcription evidenced by increase in protein expression, increase in turnover indicated by their transient expression and their subcellular localization where we have observed Noxa translocation from the cytosol to the mitochondria.

Once BH3-only pro-apoptotic proteins are stimulated, they can facilitate apoptosis either directly through the activation of Bax and Bak or indirectly through inhibiting Bcl-2 anti-apoptotic proteins. In all cases, the liberated Bax and Bak will translocate to the mitochondria where then they would oligomerize and initiate MOMP leading to cytochrome c release followed by caspase 9 cleavage and subsequently caspase

3 and PARP cleavage where then apoptosis will be terminated. To examine the role of Noxa in this pathway we silenced Noxa expression and measured apoptosis (Figure 12). Noxa knocked down cells showed higher resistance than did the similarly treated cells with wild type Noxa. On the other hand, cells with only knocked down Noxa were still more sensitive to apoptosis than were p53 knocked down cells, suggesting the involvement of other p53-dependent mechanisms. Although apoptosis was inhibited, p53-silenced cells were instead arrested at polyploidy, an indication of mitotic failure due to improper segregation and cytokinesis in response to γ-irradiation [104]. To study which mechanism of apoptosis Noxa is involved in, we investigated the levels of caspases 9, 8 and 3. Interestingly, and in consistency with the observed partial apoptosis, Noxa was found responsible only for caspase 9 cleavage and not caspase 8 (results are not shown) where the latter found cleaved irrespective to Noxa expression status, thus indicating that another mechanism, excluding Noxa-dependent apoptosis, is initiated upon γ-irradiation and that this mechanism is p53-dependent. In support with our observations, several studies have denoted p53-dependent caspase 8 and caspase 9 cleavages in Molt-4 cells upon γ-irradiation or X-ray irradiation [119-120], however none has emphasized on the role of Noxa in these distinct pathways. Hence, we can speculate that γ-irradiation induces p53-dependent intrinsic and extrinsic pathways but Noxa is accountable only for intrinsic apoptosis whereas other mechanism, presumably dependent on Fas expression and DISC formation, is responsible for the observed extrinsic apoptosis [120]. At the end both pathways will meet in caspase 3 cleavage although delayed in Noxa depleted cells.

To study the role of Noxa on possible downstream candidates, we investigated the expression levels of both Bak and Bcl-2 proteins. Bak and Bcl-2 expression levels

were found to be p53 and Noxa-dependent. These results confirm previous studies that indicate Noxa as upstream regulator of Bak protein [74]. Surprisingly, the anti-apoptotic Bcl-2 protein was also found to be upregulated in p53 and Noxa expressing cells, though, it is widely known that Noxa inhibits Bcl-2 anti-apoptotic proteins and not *vice versa*. A reasonable explanation for such observation is the "embedded together model" suggested by Leber *et al*. [75]. This model proposes that anti-apoptotic Bcl-2 proteins do not only possess the two functions previously described, the depression and the activation model, but also have two additional features, namely, that BH3-only proteins can inhibit and activate anti-apoptotic Bcl-2 proteins and that many of the functional interactions between Bcl-2 family members rely on membranous binding. Bcl-2 protein expression increases 12 hours post-irradiation which is the time point that Noxa expression was downregulated thus suggesting a possible negative feedback loop. However, such postulation would require further evidence. Before discussing the second feature of Bcl-2 family proteins we are first ought to emphasize on another important issue that is Noxa are commonly known to regulate Mcl-1 but not Bcl-2 protein, however, only recently research has appreciated the role of Noxa in Bcl-2 regulation [121].

The second feature of Bcl-2 family proteins is the requirement of membrane interaction for their activation this brings us to the mitochondrial translocation machinery of Bcl-2 family members. Interestingly, Noxa was found to translocate from the cytosol to the mitochondria preceding Bax translocation and cytochrome c release, thus confirming a role of Noxa upstream of Bax and MOMP initiation. However, Bak was preferentially expressed only in the mitochondria and not in the cytosol in agreement with the suggestion that Bak is mitochondrial bound protein in its inactive form and it possess only conformational changes for activation [74]. Therefore, to test for the

activation of Bak we need to measure specifically its active form or its oligomerization. Taken together, these results coincides with the embedded model, which demonstrates that Noxa transcriptional levels does not reflect its functional role instead it is the translocation of Noxa to the mitochondria was found responsible for sequential Bax translocation and cytochrome c release. Noxa translocation significance was also perceived in others' studies [78].

Our novel identification is that Noxa downstream candidate, ceramide, was only noticed to accumulate in cells possessing both p53 and Noxa prior to apoptosis. Ceramide accumulation was shown to follow Noxa stimulation however, no accumulation was observed in cells lacking p53 and/or Noxa therefore indicating that the observed ceramide participates specifically in intrinsic apoptosis and not otherwise. There are several reports showing that Fas-mediated apoptosis occurs independently to ceramide generation [122-123] thus strengthening our observations that the observed ceramide is probably involved only in intrinsic apoptosis rather than extrinsic apoptosis. On the other hand, others have reported that inhibiting ceramide by fumonisin B_1 did not affect apoptosis in X-irradiated Molt-4 cells. Therefore, they deduced that ceramide has no role in apoptosis [124]. Although we have also observed apoptosis in cells lacking Noxa and ceramide, however this is presumably because irradiated Molt-4 cells are encountering two distinct forms of apoptosis and only one of them is ceramide-dependent. Therefore assuming ceramide has no role in apoptosis in irradiated Molt-4 cells is an underestimation presumption.

Because ceramide plays an important role in mitochondrial polarization and in initiating MOMP, we investigated ceramide levels in isolated mitochondria to account for ceramide function in triggering mitochondrial apoptosis upon irradiation. Notably, early and a gradual ceramide accumulation was realized in mitochondrial fractions that was not

reflected in whole cell lysates. This could be explained by the presence of other membranous structures that are also capable of ceramide synthesis, thus, decreasing the difference of mitochondrial ceramide accumulation between treated and untreated cells. Moreover, Noxa translocation to the mitochondria was found to precede mitochondrial ceramide accumulation. As a result, we can speculate that Noxa translocation is crucial for ceramide accumulation. However, whether this accumulation is through mitochondrial ceramide synthesis or translocation by CERT or vesicles from other organelles such as ER still is unknown. Mitochondrial ceramide accumulation, Bax translocation and cytochome c release has occurred simultaneously. If mitochondrial ceramide acts as a regulator for induction of other downstream proteins, then ceramide should accumulates prior to cytochrome c release, especially if ceramide functions in protein transcriptional activation. However, for ceramide channels to initiate MOMP and subsequently cytochrome c release is just a matter of minutes [125]. Hence, we propose a role for ceramide in channel assembly as one of the final executioners rather than downstream activator of other proteins prior to apoptosis. Ceramide-mediated intrinsic apoptosis was reported to accumulate upon Bak stimulation and prior to Bax activation [85]. In addition, it was shown that Noxa translocation will result in Mcl-1 phosphorylation and thus its ubiquitination relieving Bak and thereby rendering it active [78]. Thus, when bearing in mind that Noxa activates Bak and that Bak in turn is responsible for ceramide accumulation (especially long chain ceramides), it is not surprising that ceramide accumulation is also Noxa-dependent. To our knowledge, this is the first study to implicate ceramide accumulation to Noxa levels.

Okadaic acid is a potent inhibitor of protein phosphatases, PP1 and PP2A, which functions by dephosphorylating serine/threonine residues. Okadaic acid is a non-selective

inhibitor; however, at concentrations lower than 10 nM it will selectively inhibit PP2A. We have shown that the inhibition of PP2A induces apoptosis only in cells possessing p53 whereas it induces cell cycle arrest at G2-M phase in cells lacking p53 owing to the fact that PP2A may function in G2-M transition [126]. In cell cycle arrest, several studies have implicated p53-dependent G1 arrest rather than G2-M arrest [103]. Hence, the reason behind the observed G2-M arrest is due to the lack of both PP2A and p53 proteins. On the other hand, inhibiting PP1 protein by calyculin A (results not shown) or okadaic acid treatment, at concentrations greater than 10 nM, induced p53-independent cell death, indicating that PP1 has a distinct function from that of PP2A. Indeed, PP1 was found to induce a p53-indepedent apoptosis by activating the transcription factor, EGR-1, which in turn elicits PTEN activation that will consequently lead to apoptosis [127]. We have shown that the inhibition of PP2A by okadaic acid has induced p53 hyperphosphorylation prior to apoptosis. Several studies have reported the role of okadaic acid in caspase-dependent apoptosis [128]. Therefore, the remarked p53 hyperphosphorylation and the observed PARP cleavage suggest that okadaic acid functions by triggering caspase-dependent apoptosis. Surprisingly, Noxa expression was not altered in p53 wild type cells but unpredictably it was transiently expressed in p53-deficient cells. A relation between okadaic acid and BH3-only proteins was only recently postulated whereby okadaic acid was found to induce p53 and Puma-dependent apoptosis [129]. We have also observed a similar phenomenon regarding Noxa, using flow cytometry analysis and PARP cleavage, but not at the level of transcription. We have observed that Noxa knocked down cells showed higher resistance to apoptosis than did the similarly treated cells with wild type Noxa. Hence, we can speculate that it is not the transcriptional activity of Noxa that plays an important role in apoptosis instead it is probably the other post-translational

modifications that have role in p53-mediated apoptosis. As long that we have achieved a system with p53 and Noxa-dependent apoptosis it would be interesting in future studies to look at the relation between Noxa and ceramide accumulation in apoptosis and to correlate it with our results in leukemic Molt-4 cells.

The implications of the trio p53, Noxa and ceramide in mediating apoptosis have been studied in tissue physiology and pathophysiology. Several studies have indicated the contribution of Noxa to the p53 response *in vivo*. For instance, Noxa-/- mice showed resistance to X-ray irradiation in gastrointestinal and epithelial cells of small intestinal crypts [130]. It was shown that Noxa is involved in the DNA-damage induced, p53-dependent apoptosis. Therefore, indicating that Noxa-mediated apoptosis is dependent on p53 expression. On the other hand, ceramide accumulation in mediating apoptosis was also identified in several organs. For example, whole body mice X-ray irradiation has induced ceramide accumulation in the alveolar septi of the lung, the intestinal mucosa and the central nervous system [131]. It was found that ceramide biosynthesis depends on the activity of ASMase and its inhibition has abrogated ceramide generation. Moreover, surprisingly, the observed ceramide was independent of p53 expression. Therefore, suggesting an important role of ceramide in inducing apoptosis independent to p53 levels. In fact, ceramide-mediated apoptosis was encountered in several human diseases. For instance, serum ceramides identification was found to increase the risk of Alzheimer's disease [132]. Moreover, ceramide mediating liver diseases was appreciated. For instance, a link between ceramide accumulation and diabetes was identified. Specifically, ceramide accumulation was found to interfere with liver cells ability to uptake insulin. In addition, ceramide significance was noticed in nonalcoholic fatty liver diseases (NFLD) [133]. Several chemotherapeutics were found to stimulate Noxa [106] or ceramide

synthesis [21] directly or indirectly in cancerous cells. These two molecules are known to locate downstream of p53. Therefore, the discovery of novel treatments that could trigger Noxa and ceramide accumulation would cause apoptosis in p53-defecient cancer cells. Accordingly, combined chemotherapeutics discovery would enhance drug efficacy and sensitivity.

Relating Noxa to ceramide biosynthesis provides a novel link between two apoptotic pathways that previously thought to operate independently. In most cases ceramide channel formation is restricted to artificial models or exogenous ceramide. Thus, the data discussed here point out to a possible function of endogenous ceramide in channel formation in cooperation with Bcl-2 family proteins, in mediating MOMP, cytochrome c release, caspase activation and the execution of apoptosis. Such observation will add one step further in future combination chemotherapeutics discoveries.

CHAPTER VII

FUTURE PERSPECTIVES

Our results suggest a correlation between Bcl-2 family proteins and ceramide do exists in mediating intrinsic apoptosis. Therefore, based on these results and as explained previously, ceramides are probably pledged in intrinsic apoptosis through forming channels on the outer mitochondrial membrane with the aid of Bax and probably Bak. In near future we aim to study channel assembly and/or protein oligomerization on the mitochondrial membranes in addition to measuring mitochondrial polarization. Moreover, it would be interesting to find out which of the downstream Bcl-2 family proteins are fundamental for ceramide accumulation as this would aid us in understanding the ordering of intrinsic apoptosis, such experiments can be done by silencing Bax and/or Bak using antisense oligonucleotides. As for the far future, hopefully our results will aid in new therapeutics discovery with enhanced efficacy and sensitivity, especially that several tumors have shown to exhibit defects in specific ceramide enzymes.

CHAPTER VIII

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