1. Ceramide: the center of sphingolipid biosynthesis

1.1. Introducing the sphingolipid family

The cell membrane contains three main classes of lipids: **glycerolipids**, **sphingolipids** and **sterols** (Futerman and Hannun 2004; Gulbins and Li 2006; Grassme *et al.* 2007). First discovered by J.L. W. Thudichum in 1876, sphingolipids were considered to play primarily structural roles in membrane formation (Zheng *et al.* 2006; Bartke and Hannun 2009). However, by the end of the twentieth century, sphingolipids were described as effector molecules which are involved in the regulation of apoptosis, cell proliferation, cell migration, senescence, and inflammation (Hannun 1996; Perry and Hannun 1998; Hannun and Obeid 2002; Futerman and Hannun 2004; Ogretmen and Hannun 2004; Reynolds *et al.* 2004; Fox *et al.* 2006; Modrak *et al.* 2006; Saddoughi *et al.* 2008).

Sphingolipid metabolism pathways have a unique metabolic entry point, serine palmitoyl transferase (SPT) which forms 3-ketosphinganine, the first sphingolipid in the *de novo* pathway and a unique exit point, sphingosine-1-phosphate (S1P) lyase, which breaks down S1P into non-sphingolipid molecules. In this network, ceramide can be considered to be a metabolic hub because it occupies a central position in sphingolipid biosynthesis and catabolism (Hannun and Obeid 2008) (Figure 1).

1.2. Ceramide structure and metabolism

Ceramide (Cer) from mammalian membranes is composed of sphingosine, which is an amide linked to a fatty acyl chain, varying in length from 14 to 26 carbon atoms (Mimeault 2002; Pettus *et al.* 2002; Ogretmen and Hannun 2004; Zheng *et al.* 2006) (Figure 1). Ceramide constitutes the metabolic and structural precursor for complex sphingolipids, which are composed of hydrophilic head groups, such as sphingomyelin, ceramide 1-phosphate, and glucosylceramide (Saddoughi *et al.* 2008).

Endogenous ceramide levels are regulated by integrated and metabolic pathways, involving a number of specialized enzymes (Futerman and Riezman 2005; Saddoughi *et al.* 2008) (Figure 1).

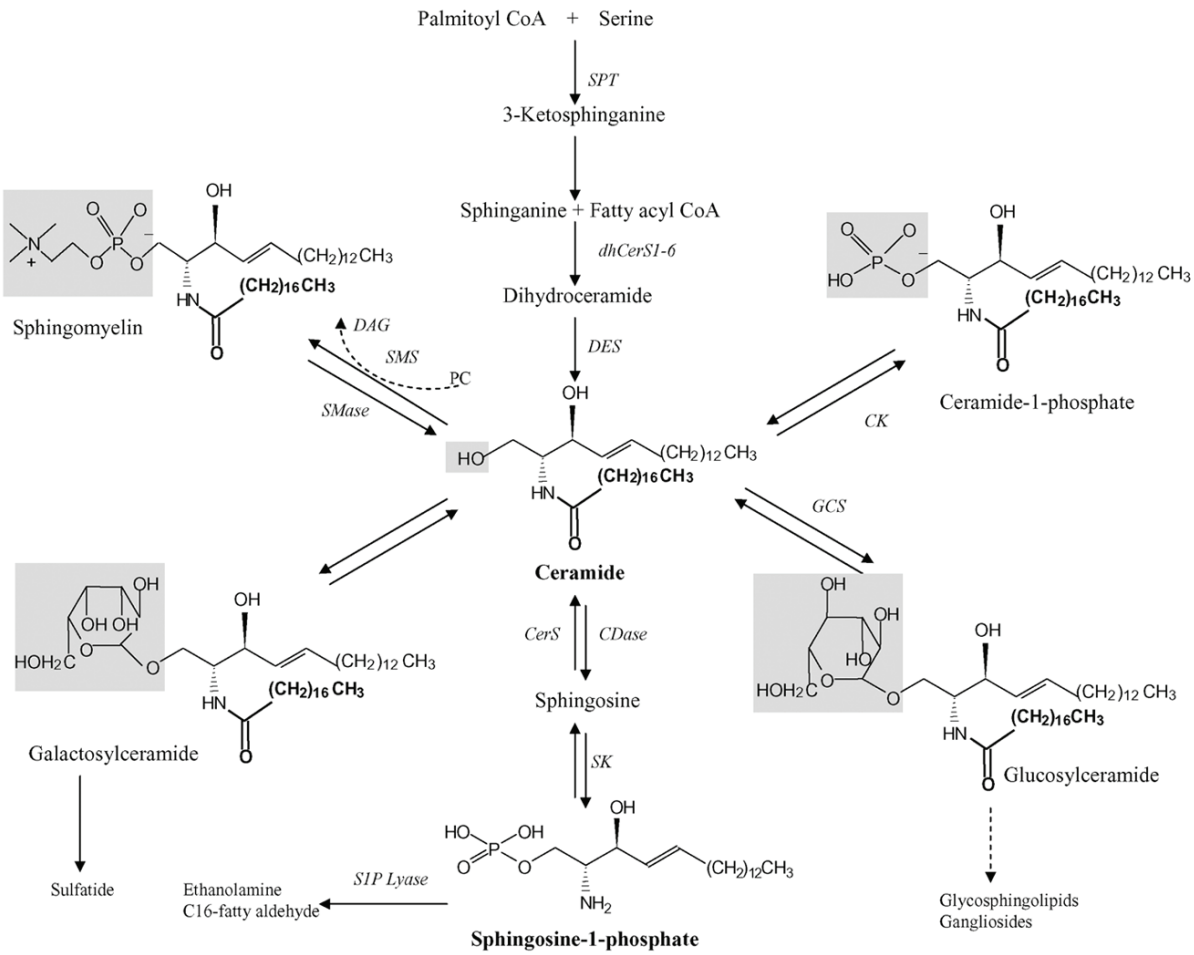

Figure 1: sphingolipid metabolism (Saddoughi *et al.* 2008).

Ceramide can be produced in at least two distinct ways. First, the generation of ceramide is triggered by the action of sphingomyelinases (SMases) which hydrolyze sphingomyelin (SM) to yield ceramide and phosphorylcholine. Several sphingomyelinases have been characterized and can be divided in three main groups depending on their pH optimum: acidic, neutral or alkaline. They show differential cellular localizations (Spence *et al.* 1983; Andrieu-Abadie and Levade 2002; Kolesnick 2002; Ogretmen and Hannun 2004; Bartke and Hannun 2009).

Second, endogenous ceramide can be generated *via* the *de novo* pathway. In this pathway, serine and palmitoyl CoA condense to form 3-ketosphinganine by serine palmitoyl transferase (SPT), followed by reduction of 3-ketosphinganine to sphinganine (dihydrosphingosine), which is then *N*-acylated by ceramide synthases to produce dihydroceramide (dhCer) (Mandon *et al.* 1992). Six mammalian genes that encode ceramide synthase have recently been cloned. Each isoform called longevity-assurance homolog (LASS1-6) renamed as ceramide synthases 1-6 (CerS1-6), shows substrate preference for specific chain length fatty acyl CoAs (Pewzner-Jung *et al.* 2006; Saddoughi *et al.* 2008) (Figure 2).

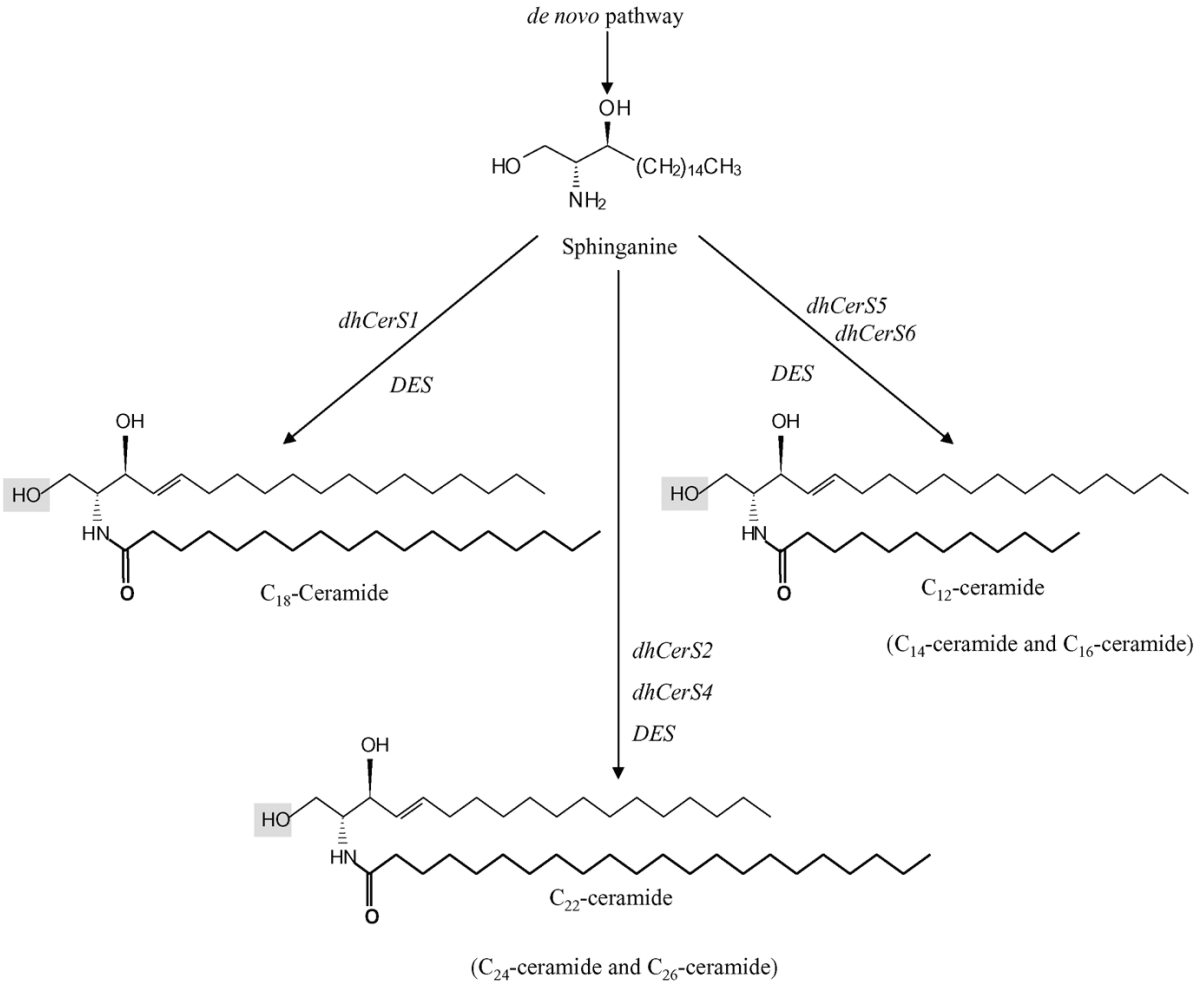

Figure 2: de novo generation of ceramide via the function of dihydroceramide synthases. Recently identified dhCerS1–6 are responsible for the generation and determining the fatty-acid chain length of ceramide in the *de novo* pathway. For example, dhCerS1, previously known as LASS1, is responsible for generating dihydro-C18-ceramide, whereas CersS2 and CerS4 synthesize dihydro-C22-, C24-, and C26-ceramides. In addition, dihydro-C12-, C14- and C16-ceramides are generated by CerS5 and CerS6. These dihydro-ceramides are then desaturated to form ceramides by dihydroceramide desaturase (DES) (Saddoughi *et al.* 2008).

Dihydroceramide is then desaturated by dihydroceramide desaturase (DES), generating a 4, 5-trans-double bond to form ceramide (Michel and van Echten-Deckert 1997).

Ceramide can then be utilized as a substrate by ceramidases (CDases) to liberate sphingosine, which is phosphorylated to generate S1P. Three types of CDases have been classified by their pH optimum and subcellular localization: acid CDase, neutral CDase, and alkaline CDase (Mao and Obeid 2008).

Ceramide can be phosphorylated by ceramide kinase (CERK), which in turn can be recycled by a ceramide-1-phosphate (C1P) phosphatase (Shinghal *et al.* 1993)

Ceramide can also be metabolized by SM synthase (Tafesse *et al.* 2006), which requires the transport of ceramide from the endoplasmic reticulum (ER) to the Golgi apparatus by ceramide transporter protein, CERT (Hanada *et al.* 2003; Kudo *et al.* 2008). Ceramide can

also be converted into glucosylceramide (GlcCer) in the Golgi, through a CERT-independent transport. GlcCer is metabolized into more complex glycosphingolipids. Importantly, this process requires the transport of GlcCer from its site of synthesis (early Golgi) to the distal Golgi compartment thanks to a non vesicular transport, the four-phosphate adaptor protein (FAPP2) (D'Angelo *et al.* 2007).

1.3. Ceramide compartmentalization and regulation

Enzymatic reactions in sphingolipid metabolism are distributed throughout different cellular compartments (Hannun and Obeid 2008). The initial step of sphingolipid *de novo* synthesis leading to ceramide formation takes place on the cytosolic surface of the endoplasmic reticulum (ER) and possibly on ER-associated membranes, such as the perinuclear membrane and mitochondria-associated membranes (MAMs) (Hannun and Obeid 2008; Bartke and Hannun 2009) (Figure 3).

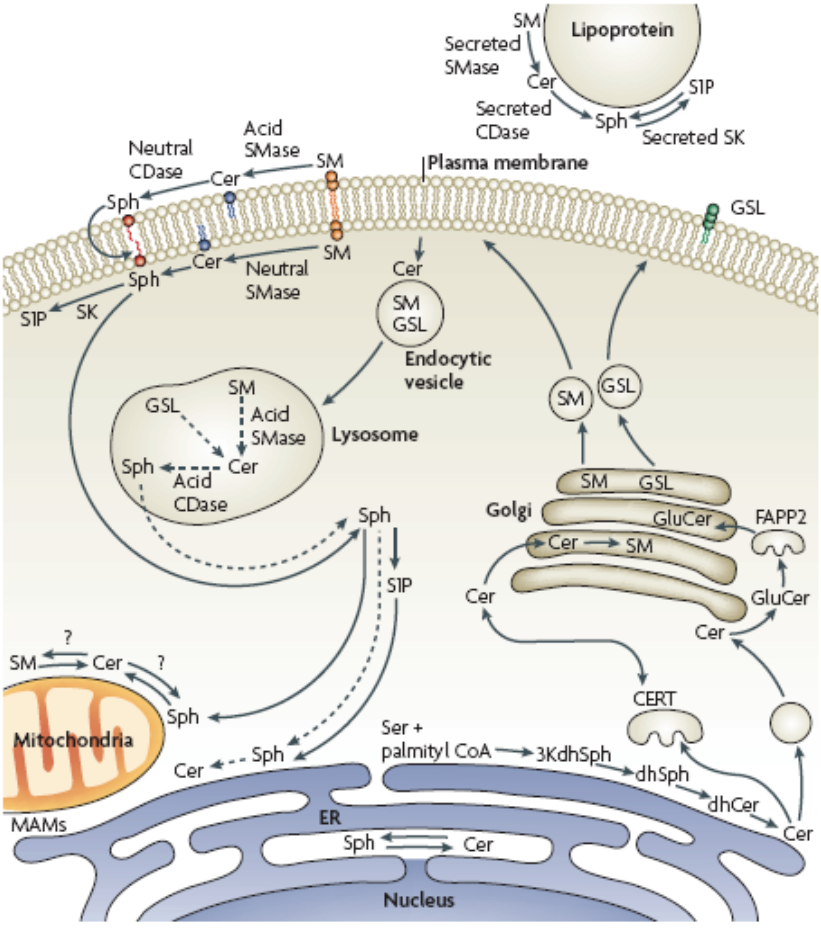

Figure 3: compartmentalization of pathways of sphingolipid metabolism (Hannun and Obeid 2008).

Ceramide formed in this compartment is transported to the Golgi, which is the site of synthesis of sphingomyelin and glucosylceramide (GlcCer). The Cer transport to the Golgi occurs either through the action of the Cer transfer protein CERT, which delivers Cer for SM synthesis (Hanada *et al.* 2003; Kudo *et al.* 2008), or through vesicular transport which delivers Cer for the synthesis of GlcCer. The transfer of GlcCer for glycosphingolipid (GSL) synthesis requires the action of the transport protein FAPP2. GlcCer appears to be synthesized on the cytosolic side of the Golgi, and needs to flip to the luminal side of the Golgi for the synthesis of complex GSLs (e.g., gangliosides) (D'Angelo *et al.* 2007). This mechanism is supported by the ABC transporter, P-glycoprotein (also known as MDR1) (Lannert *et al.* 1998)

Subsequently, SM and complex GSLs are delivered to the plasma membrane by vesicular transport. Acid sphingomyelinase (Acid SMase), present in the outer membrane leaflet or neutral sphingomyelinase (neutral SMase), present in the inner leaflet of the bilayer can metabolize SM to Cer and other bioactive lipids (sphingosine, S1P) (Hannun and Obeid 2008).

From the plasma membrane, SM and GSL may recirculated through the endosomal pathway and reach the lysosomal compartment, where they are degraded by acid SMase and glucosidases to form Cer. Cer is then hydrolysed by acid ceramidase (CDase) to form sphingosine (Sph). Sph may exit the lysosome and shows adequate solubility in the cytosol to move among membranes, including the ER, where it would be recycled into Cer (Riboni *et al.* 1998). This recycling of sphingosine from the catabolism of complex sphingolipids (SM and GSLs) is termed the “salvage pathway” (Gillard *et al.* 1998; Ogretmen *et al.* 2002; Tettamanti *et al.* 2003; Becker *et al.* 2005; Kitatani *et al.* 2008) (Figure 4). This pathway, taking place in the lysosome, involves a number of key enzymes that include acid SMase, exohydrolase, ceramidases, and dihydroceramide synthases. It should also be noted that the treatment of cells with exogenous ceramide may result in the generation of endogenous long chain ceramide *via* the sphingosine recycling pathway. In fact, exogenous C6-ceramide is subject to deacylation by ceramidase, releasing free sphingosine which in turn undergoes reacylation in a ceramide synthase-dependent manner (Wang *et al.* 1991; Ogretmen *et al.* 2002) (Figure 4).

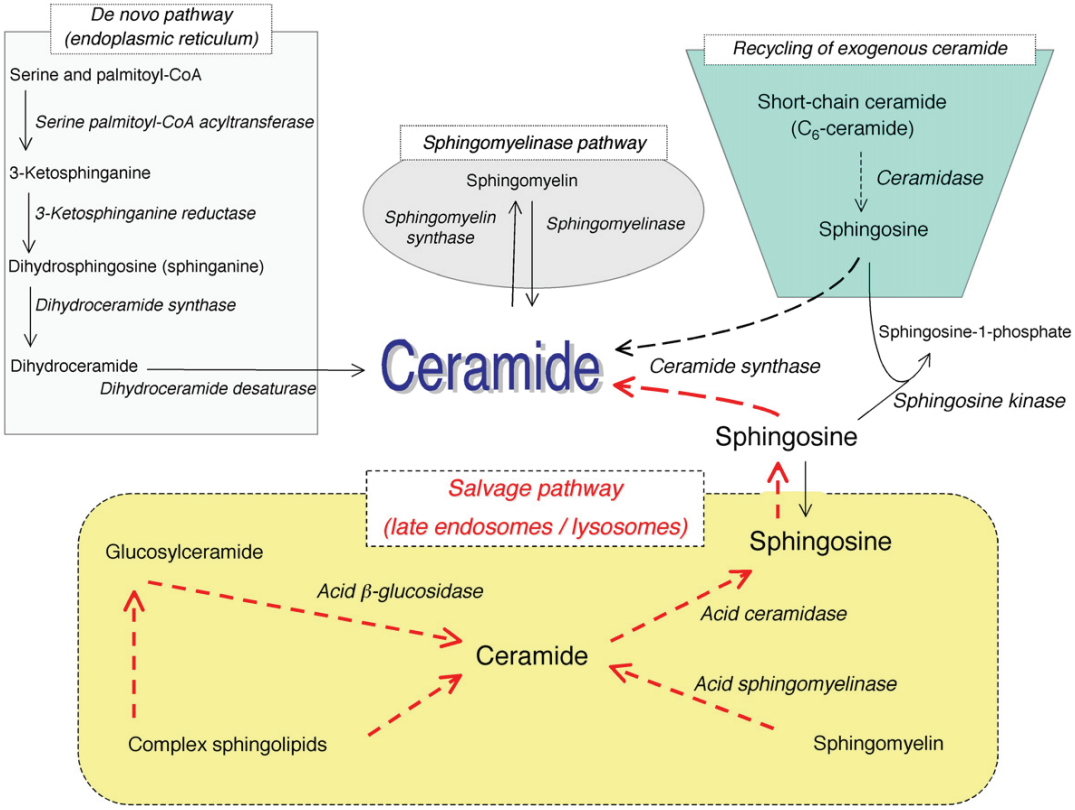

Figure 4: **ceramide synthesis**. The scheme shows metabolic pathways for ceramide synthesis composed of the sphingomyelinase pathway, the de novo pathway, the exogenous ceramide-recycling pathway, and the salvage pathway. Dotted lines indicate the pathway of ceramide synthesis resulting from recycling/salvaging sphingosine (Kitatani *et al.* 2008).

2. Ceramide and programmed cell death

2.1. Overview of programmed cell death

2.1.1. Introduction

The term apoptosis, which first appeared in 1972, was used to describe a morphologically distinct form of cell death (Kerr 2002). Apoptosis has since been recognized and accepted as a mode of "programmed" cell death, which involves the genetically determined elimination of cells. Apoptosis normally occurs during development and aging and is a mechanism to maintain cell populations in tissues. Moreover, apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged (Norbury and Hickson 2001). Finally, apoptosis is a coordinated and energy-dependent process that involves the activation of various cysteine proteases called caspases and a complex cascade of events that links the initiating stimuli to the final demise of the cell. However, inappropriate apoptosis may lead to neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer (Elmore 2007).

2.1.2. Morphological features of apoptosis

The various morphological changes that occur during apoptosis affect all aspects of the cell from the plasma membrane to the nucleus. During the early process of apoptosis, the cells are smaller in size, the cytoplasm is dense, the organelles are more tightly packed (shrinkage) and the chromatin is condensed (pyknosis). Another apoptotic change is the plasma membrane blebbing, followed by DNA fragmentation (karyorrhexis) and separation of cell fragments into apoptotic bodies (Hacker 2000). These bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment and are subsequently phagocytosed by macrophages, parenchymal cells or neoplastic cells and degraded within phagolysosomes. These engulfing cells do not produce anti-inflammatory cytokines. Moreover, apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue. Thus, there is no inflammatory reaction associated neither with the process of apoptosis nor with removal of apoptotic cells (Savill and Fadok 2000; Kurosaka *et al.* 2003).

2.1.3. Biochemical features of apoptosis

Apoptotic cells exhibit biochemical modifications such as protein cleavage through caspase activation. Caspases are expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. This proteolytic cascade amplifies the apoptotic signalling pathway and thus leads to rapid cell death. Caspases thus have a proteolytic activity and are able to cleave proteins at aspartic acid residues. To date, 14 different caspases have been identified in mammalian tissues and categorized into: initiators (caspases-2,-8,-9,-10), effectors (caspases-3,-6,-7) and inflammatory caspases (caspases-1,-4,-5) (Cohen 1997). The other caspases that have been identified include caspases 11,-12,-13,-14 (Elmore 2007).

Another characteristic of apoptotic cells is protein cross-linking which is achieved through the expression and activation of tissue transglutaminase (Nemes *et al.* 1996). DNA breakdown by endonucleases also occurs (Bortner *et al.* 1995).

Another biochemical feature is the externalization of phosphatidylserine (PS) that result in the phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue (Bratton *et al.* 1997). Other proteins are also exposed on the cell surface during apoptotic cell clearance. Calreticulin, a protein that binds to an LDL-receptor related protein on the engulfing cell, is suggested to cooperate with phosphatidylserine as a recognition signal (Gardai *et al.* 2005).

2.1.4. Distinguishing apoptosis from necrosis

Necrosis is an uncontrolled and passive process that affects large fields of cells whereas apoptosis is controlled and energy-dependent and can affect individual or clusters of cells. Necrotic cell injury is mediated by interference with the energy supply of the cell and direct damage to cell membranes (Elmore 2007). Some of the major morphological changes that occur with necrosis include cell swelling; cytoplasmic vacuoles; distended endoplasmic reticulum; cytoplasmic blebs; condensed, swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and eventually disruption of the cell membrane (Majno and Joris 1995). This loss of cell membrane integrity results in the release of the cytoplasmic content into surrounding tissue with eventual recruitment of inflammatory cells.

It also important to note that pyknosis and karyorrhexis are not exclusive to apoptosis and can be part of the cytomorphological changes that occur with necrosis (Elmore 2007) (Table 1).

Apoptosis	Necrosis
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage	Cell swelling
Pyknosis and karyorrhexis	Karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

Table 1: comparison of morphological features of apoptosis and necrosis (Elmore 2007).

Apoptosis and necrosis processes can occur independently, sequentially, as well as simultaneously. Whether a cell dies by necrosis or apoptosis depends in part on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and the physiological conditions (Zeiss 2003).

2.1.5. Mechanism of apoptosis

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. There are two main apoptotic pathways that are linked: the extrinsic and the intrinsic pathways (Figure 5). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell (Elmore 2007). We will not discuss this signalling pathway in this manuscript.

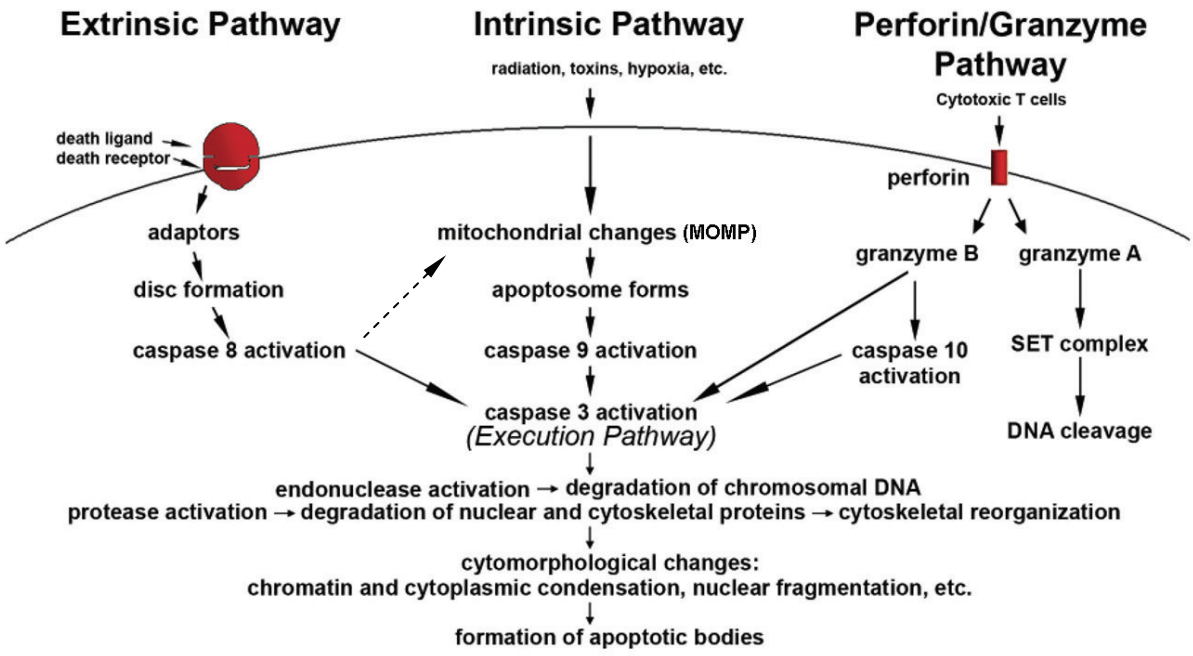

Figure 5: **schematic representation of apoptotic events**. The two main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/granzyme pathway. Each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the executioner caspase-3. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages (adapted from Elmore 2007).

- Extrinsic pathway

The extrinsic signalling pathway that initiate apoptosis involves transmembrane receptor-mediated interactions. Death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley *et al.* 2001). Members of this family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain". The best characterized ligands and corresponding death receptors include FasL/FasR, TNFα/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Ashkenazi and Dixit 1998). The sequence of events that defines the extrinsic phase of apoptosis were best characterized with the FasL/FasR model. In this model, there is clustering of receptors and binding with the homologous trimeric ligand. Upon binding of Fas to the Fas receptor, the cytoplasmic adapter protein FADD which contains a death domain that binds the Fas receptor, is recruited (Wajant 2002). FADD then associates with procaspase-8 via dimerization of the death effector domain, forming the death-inducing signalling complex (DISC). Once assembled, the DISC triggers the auto-catalytic activation of procaspase-8 and initiates a caspase apoptosis pathway (Kischkel *et al.* 1995).

- Intrinsic pathway

The intrinsic signalling pathways that initiate apoptosis involve non-receptor stimuli that produce intracellular signals. All of these stimuli cause changes in the inner mitochondrial membrane that result in the loss of the mitochondrial transmembrane potential, the opening of the mitochondrial permeability transition (MPT) pore, and the release of sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens *et al.* 2004) (Figure 6). Once released into the cytosol, these mitochondrial proteins activate both caspase-dependent and -independent cell death pathways.

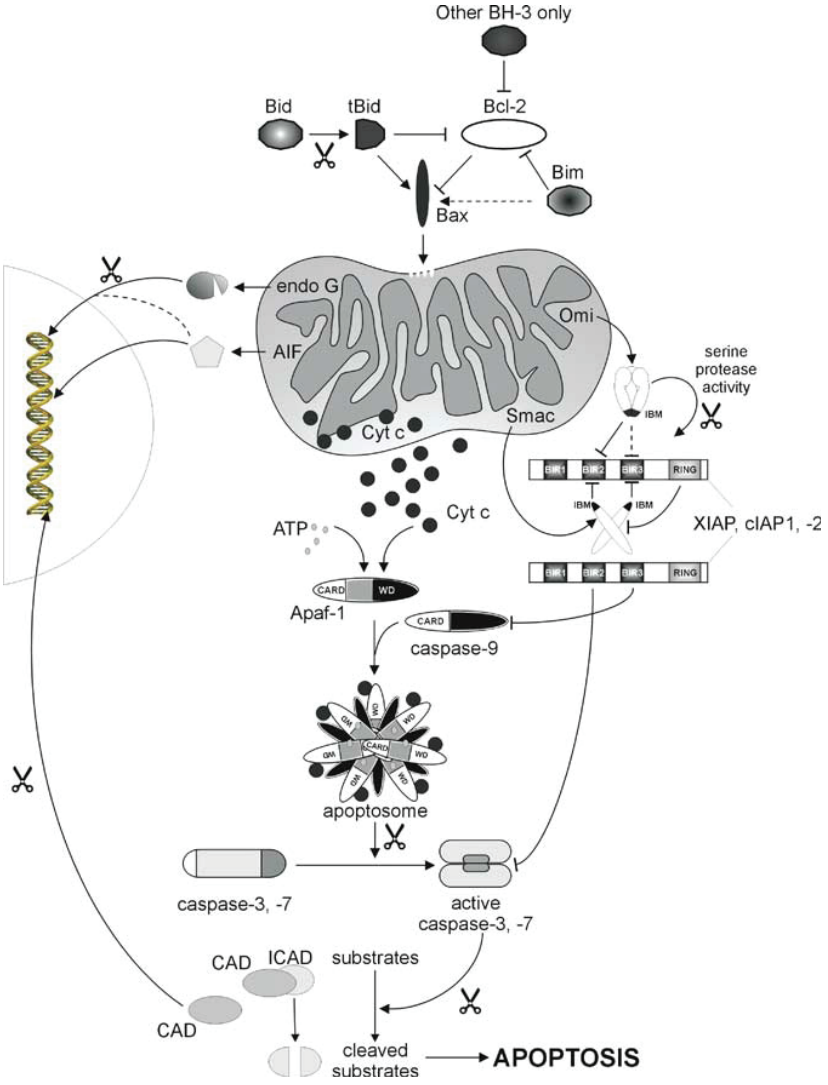

Figure 6: **multiple intermembrane space proteins are released when the intrinsic pathway is activated**. Outer mitochondrial membrane integrity is mainly controlled by the Bcl-2 family proteins (Saelens *et al.* 2004).

Cytochrome *c*, the most studied of these proteins (Sellers and Fisher 1999; Cain *et al.* 2000), binds and activates Apaf-1, which then activates procaspase-9, forming an "apoptosome" (Cain *et al.* 2000).

Smac/Diablo and the serine protease HtrA2/Omi, which are also released from the mitochondrion, neutralize inhibitors of apoptosis proteins (IAP) activity by binding to the BIR2 and BIR3 domains (Huang *et al.* 2003). In addition, Omi can cleave IAPs through its serine protease activity (Verhagen *et al.* 2002). Smac is subject to proteasome-mediated degradation, as it is targeted by the ubiquitin protein ligase activity residing in the C-terminal RING domain of XIAP, cIAP1 and cIAP2 (Hu and Yang 2003). Moreover, IAPs prevent caspase-9 dimerization and block caspases-3 and -7 (Saelens *et al.* 2004).

Multiple cellular substrates are targeted by caspases, including ICAD that becomes inactivated by cleavage, thereby liberating caspase-activated DNAse (CAD), which can translocate to the nucleus where it generates oligonucleosomal DNA fragmentation (Sakahira *et al.* 1998).

In addition, other released mitochondrial proteins include apoptosis-inducing factor (AIF) and endonuclease G (endo G), both of which contribute to apoptotic nuclear DNA damage in a caspase-independent way (Joza *et al.* 2001; Li *et al.* 2001).

Pro- and anti-apoptotic Bcl-2 family proteins control the release of these mitochondrial proteins by inducing or preventing permeabilization of the outer mitochondrial membrane (Siskind 2005). This proteins family will be discussed later in this manuscript.

- Execution pathway

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered as the final pathway of apoptosis. Execution caspases active cytoplasmic endonucleases, which degrades chromosomal DNA and proteases, that degrade nuclear and cytoskeletal proteins (Elmore 2007).

The effector or “executioner” caspases (caspases-3,-6,-7), cleaving various substrates including cytokeratins, PARP, and others, cause the morphological biochemical changes observed in apoptotic cells (Slee *et al.* 2001).

Caspase-3 is considered to be the most important of the executioner caspases and is activated by initiator caspases (caspases-8,-9,-10). As described above, in apoptotic cells, activated caspase-3 cleaves the inhibitor of the caspase-activated DNAse (ICAD) to release the endonuclease CAD (Sakahira *et al.* 1998). Caspase-3 can also cleave gelsolin, an actin binding protein. Fragments of gelsonin can then cleave actin filaments in a calcium independent manner. This results in cytoskeleton disruption (Kothakota *et al.* 1997).

Phagocytic uptake of apoptotic cells is the last apoptotic event. As explained above, the appearance of phosphatidylserine on the outer leaflet of apoptotic cells facilitates noninflammatory phagocytic recognition of these cells, allowing their early uptake and disposal with no release of cellular constituents (Fadok *et al.* 2001).

2.1.6. Physiological apoptosis

Apoptosis is critically important during various developmental processes such as nervous or immune system development.

This process is also necessary to rid the body of pathogen-invaded cells and is a component of wound healing (Greenhalgh 1998). Apoptosis is also needed to eliminate activated or auto-aggressive immune cells either during maturation in the central lymphoid organs or in peripheral tissues (Osborne 1996).

It is clear that apoptosis has to be regulated since too little or too much cell death may lead to diseases.

2.1.7. Pathological apoptosis

Abnormalities in cell death regulation can be a significant component of diseases such as cancers, autoimmune lymphoproliferative syndrome, AIDS, ischemia, and neuro-degenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and Amyotrophic Lateral Sclerosis (Elmore 2007). In this part, we will focus specifically on cancer. Cancer is an example where the normal mechanisms of cell cycle regulation are dysfunctional, with either an overproliferation of cells and/or decreased cells removal (King and Cidlowski 1998). Suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of various cancers (Kerr *et al.* 1994). There are a variety of molecular mechanisms that tumor cells use to avoid apoptosis. Tumor cells can acquire resistance to apoptosis by overexpressing anti-apoptotic proteins such as Bcl-2 or by the down-regulation or mutation of pro-apoptotic proteins such as p53 and BAX (Miyashita *et al.* 1994).

Another mechanism of apoptosis suppression in cancer involves evasion of immune surveillance. Certain immune cells (T cells and natural killer cells) normally destroy tumor cells *via* the granzyme B pathway or the death receptor pathway. In order to evade immune destruction, some tumor cells diminish the response of the death receptor pathway to Fas

ligand produced by T cells (Elmore 2007). This has been shown to occur in a variety of ways including down-regulation of the Fas receptor on tumor cells, expression of non-functioning Fas receptors or expression of Fas ligand on the surface of tumor cells (Elnemr *et al.* 2001).

Moreover, dysregulation of apoptosis, resulting from alterations of various signalling pathways, can also lead to cancers. For example, p53 can activate DNA repair proteins when DNA has substained damage, and can initiate apoptosis if the DNA damage proves to be irreparable (Pietenpol and Stewart 2002). Tumorigenesis can thus occur if this system goes awry. Indeed, if the TP53 gene is damaged (by radiation, various chemicals, or viruses for example), then tumor suppression is severely reduced (Ko and Prives 1996; Vogelstein *et al.* 2000).

2.2. The role of ceramide in programmed cell death

2.2.1. Endogenous ceramide accumulation

Apoptosis can be induced by various factors including chemotherapeutic agents, CD95, tumor necrosis factor-1, growth factor withdrawal, UV radiation, hypoxia, or DNA damage. Many of these mediators of apoptosis are regulators of ceramide generation, suggesting a role for ceramide in apoptosis (Pettus *et al.* 2002). Indeed, several studies indicate that the changes in endogenous levels of ceramide in response to these agents occur before triggering an apoptotic cascade (Dbaibo *et al.* 1998).

In mammalian cells, *de novo* synthesis of ceramide, which resides in the ER, is enhanced in response to some chemotherapeutic agents, such as etoposide, daunorubicin, irinotecan, gemcitabine, N-4-hydroxyphenylretinamide (4-HPR), cannabinoids and Fas ligand, and also by treatment with palmitate (Bose *et al.* 1995; Brenner *et al.* 1998; Perry *et al.* 2000; Chalfant *et al.* 2001; Wang *et al.* 2001; Chalfant *et al.* 2002). For example, daunorubicin induces ceramide, by activation of dihydroceramide synthase whereas etoposide enhances serine palmitoyltransferase (SPT) in leukaemic Molt-4 cells (Bose *et al.* 1995; Perry *et al.* 2000).

Another mechanism for ceramide generation involves the activation of the sphingomyelin pathway. Several stress stimuli, such as ultraviolet (UV) and ionizing radiation, ligation of death receptors and chemotherapeutic agents (including platinum, paclitaxel and histone deacetylase inhibitors) have shown to activate acid SMase, usually within a few minutes of cell stimulation (Gulbins 2003; Kolesnick and Fuks 2003; Hannun and Obeid 2008). It was

shown that activation of acid SMase in response to phorbol esters and to UV radiation also leads to an increase in ceramide formation through the salvage pathway (Becker *et al.* 2005).

Multiple studies have coupled activation of neutral SMase to the action of several extracellular cytokines and stress responses (Clarke *et al.* 2006). Indeed, Liu *et al*. demonstrated that glutathione inhibits the tumor necrosis factor-alpha (TNFα) induced activation of neutral SMase in MCF7 cells, so linking oxidative damage to ceramide formation (Liu *et al.* 1998). Furthermore, another study reported that the regulation of neutral SMase in response to TNFα involves the TNF-receptor associated protein FAN (factor associated with N-SMase activation). The adapter protein FAN is recruited at the neutral SMase activation domain (NSD) of TNFα receptor (Segui *et al.* 2001).

Another neutral sphingomyelinase, neutral SMase2, identified as a bona fide sphingomyelinase (Marchesini *et al.* 2003), is activated by cytokines, TNFα and interleukin-1 (IL-1). Several studies have implicated neutral SMase2 in the cytotoxic action of amyloid peptide-β (Zeng *et al.* 2005). This sphingomyelinase appears to localize to the inner leaflet of the plasma membrane where a minor pool of SM may reside (Tani and Hannun 2007).

It is also evident that ceramidases (CDases) regulate the levels of ceramide. For example, nitric oxide (NO) induces degradation of neutral CDases, and thereby mediates ceramide accumulation and subsequent cell death (Franzen *et al.* 2002).

Additionally, the endogenous levels of ceramide can be increased by using inhibitors of ceramide metabolism enzymes or by overexpressing ceramide-generating enzymes. This point will be discussed later in paragraph 3.1.1.

2.2.2. Indirect apoptosis: formation of ceramide-enriched membrane microdomains

Ceramide can exert its physiological effects either through changes in membrane properties or through binding to specific target proteins (Kolesnick *et al.* 2000). Many studies indicate a critical role of ceramide in the reorganization of the cell membrane and membrane rafts. The generation of ceramide within the cell membrane alters its properties. Indeed, ceramide molecules spontaneously self-aggregate and are tightly packed in homodimers/multimers in association with other sphingolipids (van Blitterswijk *et al.* 2003), leading to the formation of ceramide-enriched membrane domains that fuse to large ceramide-enriched membrane platforms (Holopainen *et al.* 1998; Kolesnick *et al.* 2000; Grassme *et al.* 2007).

These ceramide enriched-membrane platforms serve to the reorganization and clustering of receptor molecules such as CD95, CD40, DR5/TRAIL, CD20, CD28, TNFα, interleukin-1 receptor, FCγRII and the PAF-receptor (Gulbins and Li 2006; Grassme *et al.* 2007) (Figure 7).

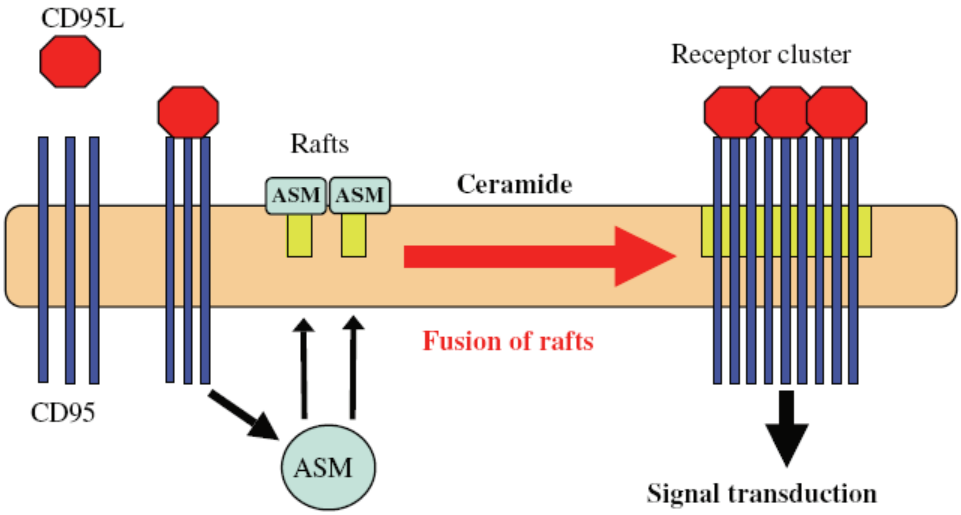

Figure 7: ceramide-enriched membrane platforms serve clustering of receptors and amplify signals. Stimulation of cells via a receptor, for instance CD95, activates the acid sphingomyelinase and induces a translocation of the enzyme onto the extracellular leaflet of the cell membrane. The acid sphingomyelinase releases ceramide from sphingomyelin finally resulting in the formation of ceramide-enriched membrane platforms. These membrane domains serve the clustering of receptor molecules, i.e., CD95, the re-organization of the intracellular signalosome and, thus, the amplification of the primary signal (Grassme *et al.* 2007).

It was shown that receptor activation triggers a translocation of acid SMase onto the extracellular leaflet of the plasma membrane resulting in the release of ceramide into the outer leaflet of the cell membrane (Grassme *et al.* 2001; Grassme *et al.* 2001). At this location, ceramide facilitates raft clustering (Grassme *et al.* 2001).

Gulbins and Kolesnick and co-workers showed that ceramide generated by acid SMase in the lipid rafts on the surface of T and B cells following activation of the TNF receptor family members CD95/Fas or CD40 respectively can facilitate the ligand-induced clustering of these receptors residing in rafts (Vidalain *et al.* 2000; Grassme *et al.* 2001; Grassme *et al.* 2002; Hueber *et al.* 2002). Receptor clustering was associated with recruitment to these rafts of downstream signalling proteins: Fas-associated death domain (FADD) and caspase-8 in the case of CD95/Fas (Hueber *et al.* 2002) and TNF-receptor-associated factor (TRAF) in the case of CD40 (Vidalain *et al.* 2000).

Moreover, ceramide-enriched membrane platforms are also involved in the mediation of stress signals into cells. Thus, it was demonstrated that γ-irradiation, UV light, some chemotherapeutics (cisplatin, doxorubicin, gemcitabine, paclitaxel) as well as bacterial and

viral infections activate the acid SMase triggering the release of ceramide and the formation of ceramide-enriched membrane platforms (Gulbins and Li 2006; Grassme *et al.* 2007).

2.2.3. Direct apoptosis: second messenger

- Kinases, phosphatases and proteases

In addition to its function in membrane platforms, ceramide interacts and activates a variety of intracellular targets, including kinases, phosphatases, and proteases (Pettus *et al.* 2002; Ogretmen and Hannun 2004; Modrak *et al.* 2006; Saddoughi *et al.* 2008) (Figure 8).

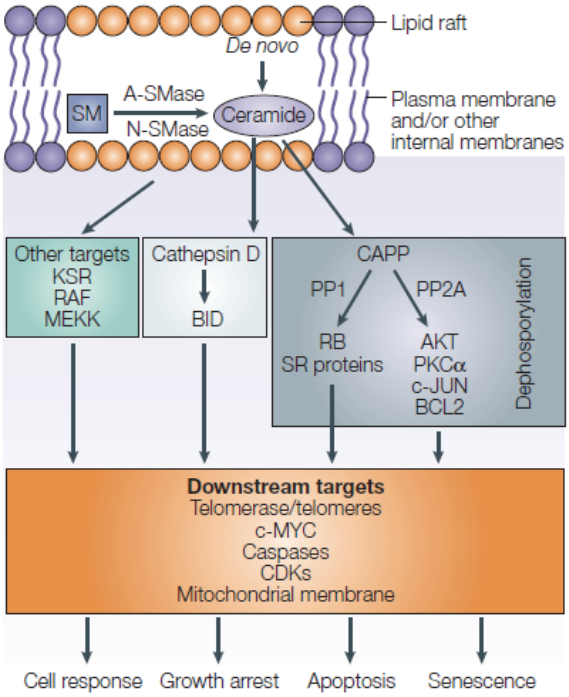

Figure 8: ceramide-regulated targets and pathways (Ogretmen and Hannun 2004).

The biological roles of ceramide might be controlled by its subcellular localization (Saddoughi *et al.* 2008). Indeed, in the nucleus, ceramide activates protein phosphatase-1 (PP1), which then dephosphorylates serine/arginine-rich proteins (SR-proteins) that mediate the alternative splicing of Bcl-XS or caspase-9 (Chalfant *et al.* 2001; Chalfant *et al.* 2002) (Figure 9).

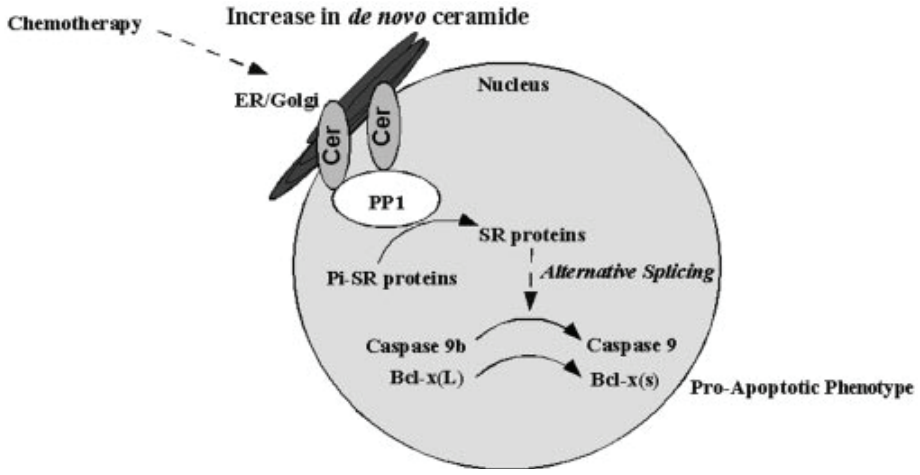

Figure 9: hypothetical schematic of the signal transduction pathway mediating the alternative splicing of Bcl-x and caspases-9 (Chalfant *et al.* 2002).

The PP1 can also dephosphorylate the retinoblastoma protein (Rb) (Kishikawa *et al.* 1999).

Whereas in mitochondria, ceramide generated by neutral SMase, which can be activated by reactive oxygen species (ROS), can activate protein phosphatase 2A (PP2A). This phosphatase can then dephosphorylate anti-apoptotic proteins such as Bcl-2, Akt, leading to loss of mitochondrial membrane potential, cytochrome *c* release, and intrinsic cell death (Ruvolo *et al.* 1999). Activation of PP2A can also induce apoptosis by activating the pro-apoptotic proteins BAD and BAX (Xin and Deng 2006).

Another ceramide target, the kinase suppressor of Ras (KSR), may be downstream of PP2A (Zhang *et al.* 1997). Ceramide may also interact with KSR directly to activate Raf-1 (Zhou *et al.* 2002). Subsequently, the stress pathways mediated by MAP kinases cascades become activated leading to apoptosis (Basu and Kolesnick 1998; Yan and Polk 2001).

Ceramide was shown to associate with protein kinase zeta (PKC-ζ) (Saddoughi, 2008) and to activate it, which in turn activates the stress-activated protein kinase (SAPK/JNK) (Bourbon *et al.* 2000). Moreover, the ceramide-PKCζ binding is important for its activation leading to the formation of a pro-apoptotic complex between PKC-zeta and prostate apoptotic response-4 (PAR-4) in differentiating stem cells (Wang *et al.* 2005).

Also, ceramide directly binds to stress-activated protein kinase (SAPK/JNK) and induces glomerular epithelial cells to undergo apoptosis (Huwiler *et al.* 2004).

Another ceramide binding protein is the non-caspase protease, cathepsin D (Heinrich *et al.* 1999), which is activated by ceramide generated in lysosome membranes through of acid

SMase. This interaction leads to activation and cleavage of BID, followed by caspase-9 and caspase-3, resulting in apoptosis (Heinrich *et al.* 2004).

- Caspases

Ceramide has been shown to activate others proteases, the caspases, especially caspase-3, a PARP-cleaving protease (Smyth *et al.* 1996). Extensive works showed that ceramide accumulates in apoptotic cells prior to the activation of execution caspases, but downstream of initiator caspases (Hannun and Luberto 2000; Sawada *et al.* 2004). Indeed, Sawada *et al.* demonstrated that ceramide generation *via* p53-mediated ROS-dependent and independent pathways are initiated by the activation of caspase-8 in response to TNFα. Then, ceramide generated through neutral SMase or acid SMase, induces mitochondrial depolarization and cytochrome *c* release, leading to caspase-3 activation and subsequent apoptotic cell death in human glioma cells (Sawada *et al.* 2004) (Figure 10).

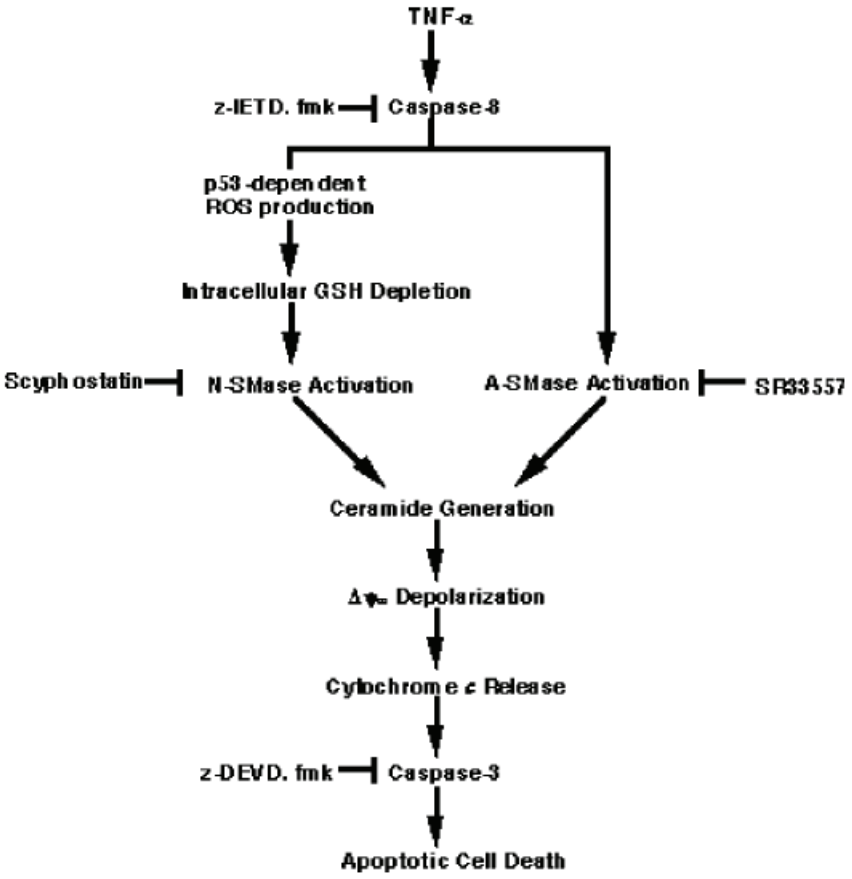

Figure 10: a hypothetical scheme for TNFα-induced signalling cascades in human glioma cells (Sawada *et al.* 2004).

Alternatively, UV-C induced acid SMase translocation into rafts and its activation. In this case, ceramide generation is caspase-8 independent. Nonetheless, ceramide-rich platforms induce caspase-9 and caspase-3 activation, leading to apoptosis of Jurkat T cells (Rotolo *et al.* 2005).

2.2.4. Ceramide and the mitochondrion

As stated above, increase in cellular ceramide levels during apoptosis have been shown to occur prior to the mitochondrial phase of apoptosis (Siskind 2005).

Mitochondria contain the enzymes responsible for ceramide synthesis and hydrolysis, namely, ceramide synthase and ceramidase. Bionda *et al.* showed that both mitochondrial outer and inner membranes are capable of generating ceramide (Bionda *et al.* 2004). Moreover, several studies demonstrated that CD95-, TNFα-radiation, and UV-induced apoptosis occur *via* an increase in mitochondrial ceramide levels (Garcia-Ruiz *et al.* 1997; Birbes *et al.* 2005).

Ceramide causes the change of mitochondrial transmembrane potential (MTP) by targeting MTP-controlling proteins, such as Bcl-2 family members, resulting in the release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria to cytoplasm, followed by caspase-3 activation (Birbes *et al.* 2002) (Figure 11).

Moreover, ceramide potentiates the induction of mitochondrial permeability transition by BAX (Pastorino *et al.* 1999; von Haefen *et al.* 2002). In addition, ceramide induces a transitory increase of intracellular pH (pHi) in relation to the permeability transition pore. This increase in pH led to conformational changes in BAX which is responsible for further apoptosis (Belaud-Rotureau *et al.* 2000) (Figure 11).

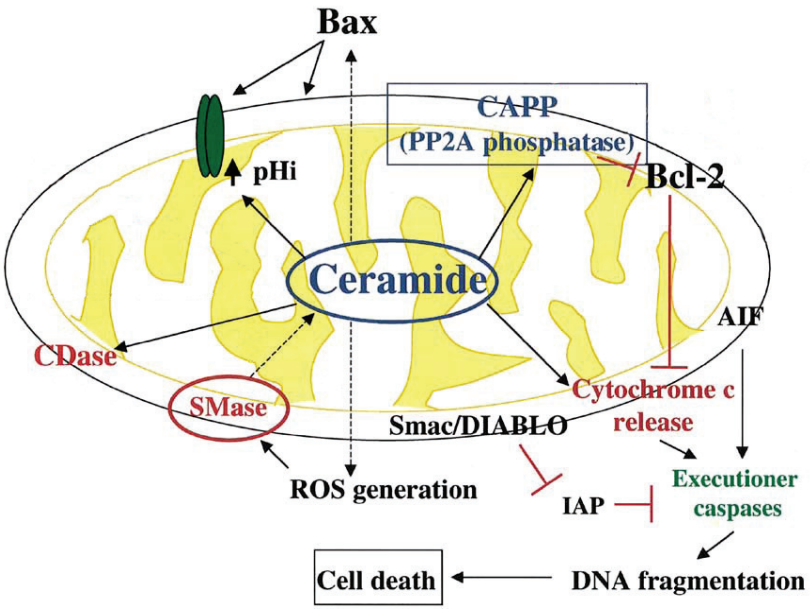

Figure 11: scheme summarizing proposed interactions of ceramide with the apoptotic machinery in the context of mitochondria (adapted from Birbes *et al.* 2002).

In addition, exogenous C2-ceramide can induce mitochondrial damage through caspase-2 and caspase-8 activation. This leads to BID cleavage and translocation, mitochondrial transmembrane potential ($\Delta\psi_m$) reduction, cytochrome *c* release, caspase-9 and -3 activation and PARP cleavage, resulting in apoptosis (Lin *et al.* 2004).

Ceramide has also been reported to have numerous effects on mitochondria, including enhanced generation of reactive oxygen species, alteration of calcium homeostasis at the mitochondrial and endoplasmic reticulum, ATP depletion, collapse of the inner mitochondrial membrane potential, and inhibition and/ or activation of various components involved in the mitochondrial electron transport chain (Arora *et al.* 1997; Garcia-Ruiz *et al.* 1997; Di Paola *et al.* 2000; Siskind 2005).

Furthermore, Siskind and Colombini showed that C2- and C16-ceramides increased the permeability of the mitochondrial outer membrane through the formation of large protein permeable channels that would allow the release of intermembrane space small proteins, including cytochrome *c* (Siskind and Colombini 2000) (Figure 12). It is noteworthy that ceramide channel formation requires the presence of the 4-5 *trans* double bond as dihydroceramide does not form channels and does not induce cytochrome *c* release (Siskind and Colombini 2000). Moreover, C2- and C16-dihydroceramides inhibit permeabilization induced by ceramide (Stiban *et al.* 2006).

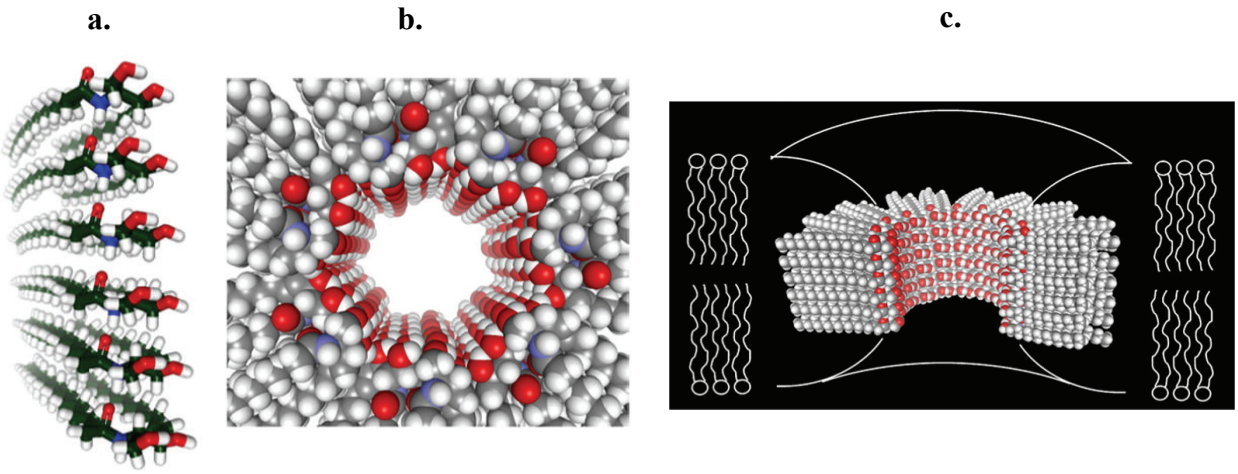

Figure 12: structural model for ceramide pore formation. **a.** A column of ceramide residues held together by intermolecular hydrogen bonds between amide and carbonyl groups. This column would span the hydrophobic portion of the membrane and, in association with other columns, would form pores of various sizes. **b.** Top view of a ceramide channel consisting of 12 columns of ceramide molecules. Adjacent columns are oriented in an antiparallel fashion so that amide dipoles attract. The columns are held together *via* intermolecular hydrogen bonds between hydroxyl groups proposed to line the channel lumen. **c**. A longitudinal cut-away of a ceramide channel, consisting of 14 columns of ceramide molecules, where 4 columns have been removed to show the interior of the channel. The curvature of the phospholipids of the membrane at the channel interface would minimize the exposure of the hydrophobic regions of the outer surface of the channel to the aqueous solution. The ceramide molecules form an annulus that encloses a polar inner space lined with hydroxyl groups (Siskind 2005).

2.2.5. Ceramide and its metabolites: a balance between survival and cell death

Dynamic sphingolipid equilibrium has been described where pro-apoptotic sphingolipids exist in a balance with pro-survival sphingolipids (Cuvillier *et al.* 1996) (Figure 13). The most common example of this equilibrium is the balance between ceramide and sphingosine-1-P (S1P). Ceramide and sphingosine mediate apoptosis, cell cycle arrest, and differentiation, whereas S1P promotes proliferation, survival, and inhibition of apoptosis. The ratio between the intracellular level of ceramide and sphingosine-1-P determines cell fate (Spiegel and Milstien 2003).

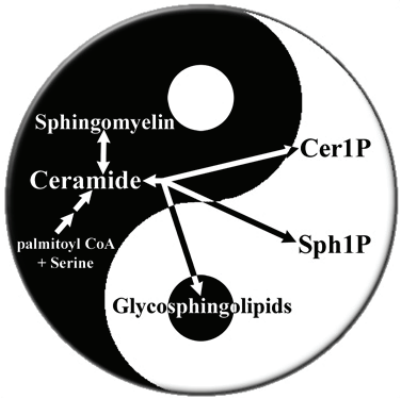

Figure 13: the "Tao" of sphingolipid metabolism (Fox *et al.* 2006).

The generation of S1P from ceramide requires the action of two enzymes, ceramidase which deacylates ceramide to form sphingosine, and sphingosine kinase (SK), which phosphorylates sphingosine to sphingosine-1-phosphate (S1P) (Huwiler and Zangemeister-Wittke 2007) (Figure 14).

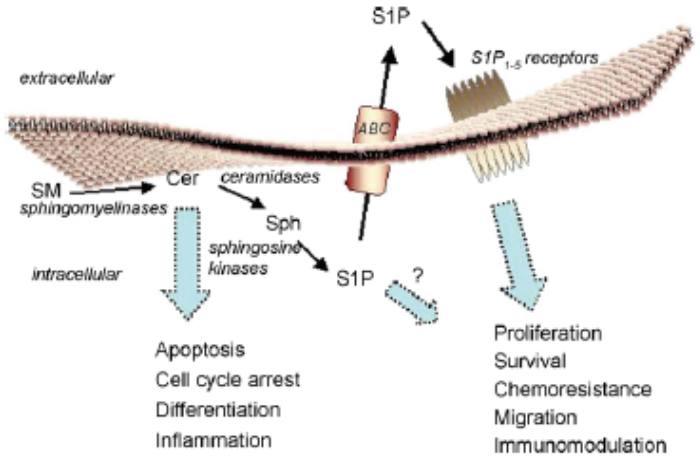

Figure 14: schematic signalling pathway from sphingomyelin to S1P. Cer, ceramide; ABC, ATP binding cassette transporter; SM, sphingomyelin; S1P, sphingosine-1-phosphate (Huwiler and Zangemeister-Wittke 2007).

S1P has mitogenic effects, both as an extracellular signalling molecule and as an intracellular second messenger (Modrak *et al.* 2006). In the cells, sphingosine kinase is activated by growth stimuli and cytokines, increasing intracellular levels of S1P. S1P can act in autocrine/paracrine manner to stimulate S1P receptors present at the cell surface (signalling "inside-out") and initiate downstream G protein-mediated signalling pathways, leading to a variety of cellular responses such as increased Ca^{2+} mobilization and cell proliferation, inhibition of apoptosis and cell migration, induction of stress fiber formation and up-regulation of adhesion molecules (Maceyka *et al.* 2002; Hla 2003; Hait *et al.* 2006; Young and Van Brocklyn 2006; Huwiler and Zangemeister-Wittke 2007; Kihara *et al.* 2007).

One of the putative anti-apoptotic mechanisms of S1P is to prevent ceramide-induced mitochondrial events such as cytochrome *c* release and caspase activation (Cuvillier and Levade 2001; Cuvillier *et al.* 2001). Indeed, Cuvillier and co-workers reported that S1P prevents caspase-3 activation by inhibiting the cytochrome *c* and Smac/Diablo release from mitochondria induced by apoptotic stimuli (anti-Fas, TNFα or C6-ceramide) (Cuvillier and Levade 2001).

S1P can also exert its proliferative and survival effects by activating a known anti-apoptotic transcription factor, NF-κB and pro-survival mediators like Akt, nitric oxide, and the extracellular signal-regulated kinase (ERK) or by counteracting the ceramide-induced activation of stress-activated protein kinase (SAPK) (Maceyka *et al.* 2002; Taha *et al.* 2006) (Figure 15).

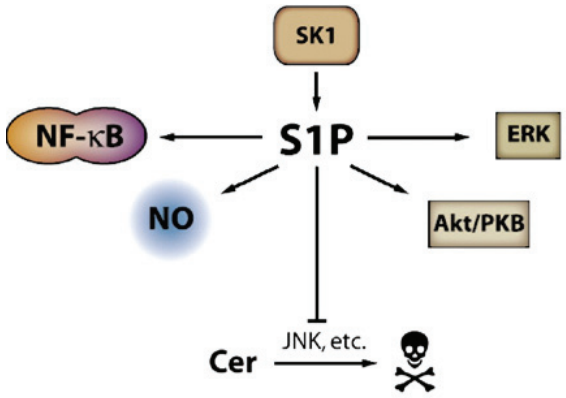

Figure 15: S1P inhibits apoptosis by activating pro-survival factors (Taha *et al.* 2006).

In addition to these direct effects on tumor cells, S1P alters several mechanisms that trigger cell survival and growth. Thus, S1P promotes tumor angiogenesis, a vital process for the development of aggressive tumors (English *et al.* 2002). By stimulating inflammatory responses, S1P could also participate to tumor development (Pettus *et al.* 2003).

The levels of ceramide and S1P can be directly regulated not only by sphingosine kinases, but also by S1P phosphohydrolases and S1P lyase (Segui *et al.* 2006). These enzymes regulate in opposite directions the intracellular content of sphingosine that can be converted to ceramide by ceramide synthase. The balance between ceramide and S1P is also likely to be influenced by the rate of conversion of ceramide to sphingosine, i.e., by the activity of ceramidases, which can affect the induction of cancer cell death (Strelow *et al.* 2000).

Furthermore, increased S1P levels promote proliferation and survival in human glioma and breast cancer cells (Sarkar *et al.* 2005). For example, Xia and collaborators found that overexpression of SK1, the immediate regulator of S1P, results in malignant transformation in NIH3T3 fibroblasts and tumour formation in SCID mice (Xia *et al.* 2000). Reciprocally, knockdown of SK1 in MCF-7 human breast cancer cells causes a significant increase in ceramide levels in the mitochondrion. This ceramide accumulation initiates the mitochondrial events leading to apoptosis: BAX oligomerization, cytochrome *c* release and caspase activation (Taha *et al.* 2006).

Interestingly, Johnson *et al.* showed that siRNA inhibition of sphingosine-1-phosphate phosphatase-1 (S1PP1), which converts S1P to sphingosine, results in increased intracellular and extracellular levels of S1P and causes resistance of MCF-7 cells to TNFα and daunorubicin treatment (Johnson *et al.* 2003).

During the synthesis of sphingomyelin, a phosphocholine group is transferred from phosphatidylcholine to ceramide, leading to the liberation of diacylglycerol (DAG). DAG is a known activator of PKC, which promotes cellular proliferation (Blatt and Glick 2001; Hannun and Obeid 2008).

Also, ceramide can be metabolized into glucosylceramide, by a glucosylceramide synthase (GCS) on the cytosolic surface of the Golgi. Specially, increased activity of GCS attenuates ceramide levels and contributes to protect cancer cells from chemotherapy-induced apoptosis; therefore, changes in sphingolipid metabolism play functional roles in conferring drug resistance (Liu *et al.* 2001; Ogretmen and Hannun 2001; Bleicher and Cabot 2002; Gouaze-Andersson and Cabot 2006). After translocation into the Golgi lumen, GlcCers are further converted to lactosylceramides by a galactosyltransferase. The action of different sialyltransferases then converts lactosylceramide into the gangliosides GM3, GD3, and GT3, through addition of sialic acid residues to the galactose unit (Malisan and Testi 2002; Bektas and Spiegel 2004) (Figure 16).

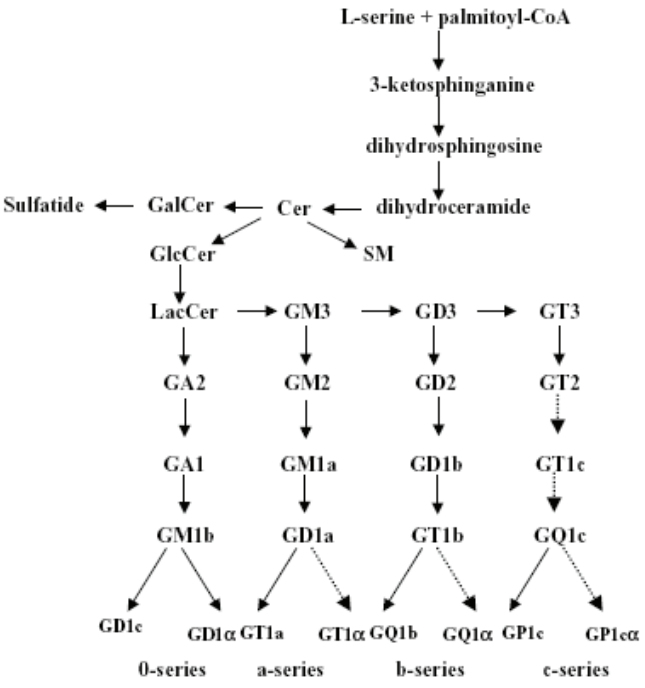

Figure 16: simplified scheme of glycosphingolipid biosynthesis (Bektas and Spiegel 2004).

Most gangliosides are known to protect cells from apoptosis (Bektas and Spiegel 2004). For example, GM1 has been shown to prevent apoptotic cell death in growth factor- deprived neuronal PC12 cells (Ferrari *et al.* 1995). Similar results were obtained in a study conducted in rat heart fibroblasts where GM1 was shown to enhance S1P production through the activation of sphingosine kinase and to protect cells from C2-ceramide or staurosporine-induced cell death (Cavallini *et al.* 1999).

Moreover, it has been shown that ceramide-1-phosphate (C1P) blocks apoptosis in bone-marrow derived macrophages through inhibition of acid SMase, thereby reducing ceramide generation (Gomez-Munoz *et al.* 2004).

On the other side, some metabolites are pro-apoptotic. For example, sphingosine (Sph) has been shown to affect various signalling pathways, including activation of c-Jun N-terminal kinase (JNK) and retinoblastoma gene product (pRb), stimulation of poly (ADP-ribose) polymerase (PARP) cleavage, induction of BID and BAX truncation, inhibition of protein kinase C, Akt kinase, ERK1/2 kinases, c-Src/v-Src protein kinases and the Ca^{2+}/calmodulin-dependent protein kinase (Cuvillier 2002).

Sphingosine can also induce cell death *via* a truncated version of PKCδ, the sphingosine-dependent protein kinase (SDK1), which leads to BAX activation through inhibition of 14-3-3 proteins (Taha *et al.* 2006) (Figure 17).

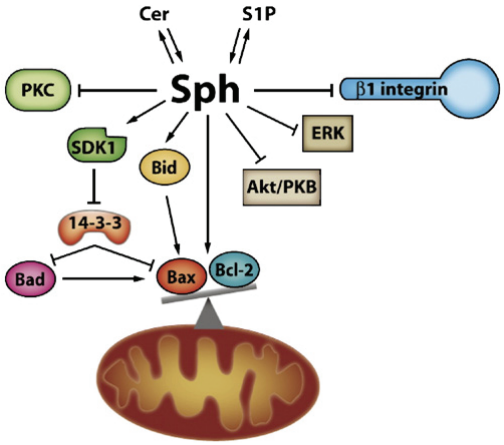

Figure 17: sphingosine in apoptosis (Taha *et al.* 2006).

In addition, ceramide promotes apoptosis through conversion into ganglioside GD3, which contributes to the mitochondrial changes. GD3 perturbs the mitochondrial membrane, leading to release of ROS, AIF, cytochrome *c* and caspase-9 activation, which activate the execution phase of apoptosis (Garcia-Ruiz *et al.* 2000; Rippo *et al.* 2000) (Figure 18).

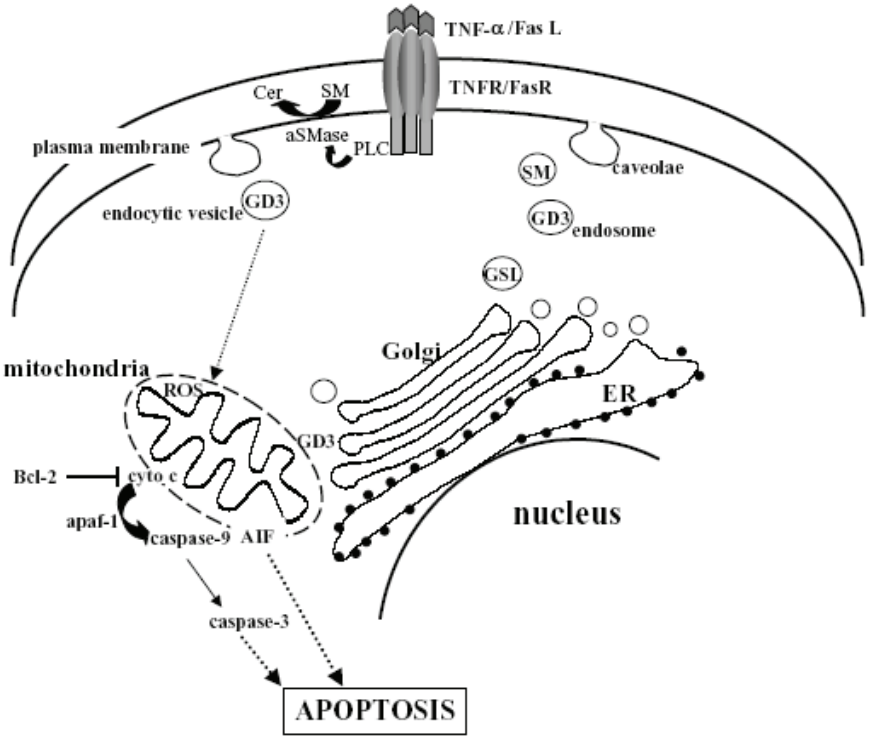

Figure 18: signalling pathway of GD3-induced apoptosis. After oligomerization of the Fas or TNFα receptors, acid SMase (aSMase) is activated in a phosphatidylcholine- phospholipase C (PC-PLC-)-dependent manner. Ceramide accumulates and activates GD3 synthesis. GD3 is targeted from the plasma membrane by vesicle transport or alternatively by physical redistribution from the Golgi to the mitochondria. There, GD3 perturbs the mitochondrial membrane leading to release of cytochrome c and AIF and caspase-9 activation, which activate the execution phase of the apoptotic program leading to demise of the cell (Bektas and Spiegel 2004).

2.3. p53: "the guardian of the genome"

2.3.1. Introduction

The p53 protein was first identified in 1979 as a protein interacting with the simian virus 40 (SV40) T antigen and was originally believed to be an oncogene because overexpression of p53 appeared to cause oncogenic transformation of cells (Lane and Crawford 1979; Yu and Zhang 2005). However, subsequent studies demonstrated that initial observations were made with a mutated p53 instead of the wild-type protein and ten years after its discovery, p53 was described as a tumour suppressor (Ko and Prives 1996).

Moreover, it was found that the p53 protein does not function correctly in most human cancers. Indeed, in about half of these tumours, p53 is inactivated directly as a result of mutation in the TP53 gene. In the other cases, it is inactivated through binding to viral proteins or Mdm2 that increases p53 degradation or its nuclear exclusion (Ko and Prives 1996; Vogelstein *et al.* 2000). Most mutations are clustered in the DNA binding domain as shown in Figure 19.

In contrast to other tumor suppressor genes like APC and RB whose expressions are lost in tumorigenesis, about 80% of TP53 mutations are missense mutations that lead to amino acid substitutions in proteins and can alter the protein conformation (Vousden and Lane 2007). The presence of mutant p53 proteins in tumors might result from selection for dominant-negative mutants that cause a loss of wild-type function. Additionally, some p53 mutants are capable of conferring increased tumorigenicity, metastasic potential, and/or tissue invasiveness (Hsiao *et al.* 1994; Strano *et al.* 2007).

Moreover, mice deficient in TP53 are viable but highly prone to spontaneous tumour formation (Jacks *et al.* 1994; Ko and Prives 1996). Indeed, a germline mutation in TP53 leads to Li-Fraumeni syndrome, predisposing patients to develop multiple primary cancers (Varley 2003).

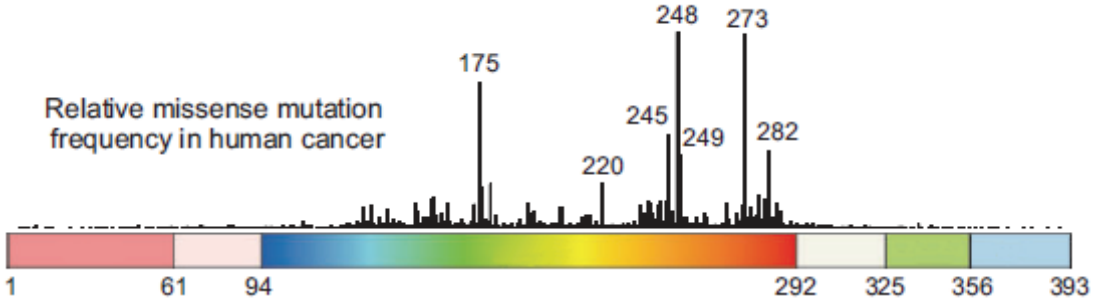

Figure 19: relative mutation frequency of p53 in human cancer. The bars above the diagram indicate the relative frequency of oncogenic mutations at each residue of p53. Most cancer-associated p53 mutations are located in central DNA-binding domain, represented with a rainbow color gradient from blue at its N terminus to red at its C terminus (adapted from Joerger and Fersht 2008).

Therefore, wild-type p53 is essential for the prevention of cancer development. In normal cells, p53 activity is kept low. This is achieved by its interaction with Mdm2, a negative regulatory partner. When the cellular DNA is damaged by ionizing radiation or chemical agents, p53-dependent cell-cycle arrest allows enough time for DNA damaged to be repaired. If the damage cannot be repaired, p53-dependent apoptosis occurs to prevent transfer of new mutations to daughter cells (Meek 2004; Bose and Ghosh 2007).

Following DNA damage, hypoxia or oncogene overexpression, wild-type p53 protein undergoes extensive post-translational changes which result in the increase of p53 binding to DNA and transcriptional activation of genes that are involved in mediating key cellular processes, such as DNA repair, cell cycle arrest, senescence and apoptosis (Vogelstein *et al.* 2000; Vousden and Lu 2002; Yu and Zhang 2005; Riley *et al.* 2008).

2.3.2. p53 stabilization and activation

p53 is active as a tetramer, with four identical chains of 393 residues. The N-terminal region consists of an intrinsically disordered transactivation domain and a proline-rich region. It is followed by the central, folded DNA-binding core domain that is responsible for sequence-specific DNA binding. This domain is connected to a short tetramerization domain that regulates the oligomerization state of p53. At its C terminus, p53 contains the so-called regulatory domain. This natively unfolded region is rich in basic amino acids (mainly lysines) and binds DNA non specifically (Joerger and Fersht 2008) (Figure 20).

A variety of different stresses are responsible for a series of post-translational modifications on p53 such as phosphorylation, sumoylation, acetylation, methylation and ubiquitination that govern p53 stability and activity (Vogelstein *et al.* 2000; Lavin and Gueven 2006; Kruse and Gu 2008). These modifications are located at the amino (N)-terminus and carboxy (C)–terminus of the protein as shown in Figure 20.

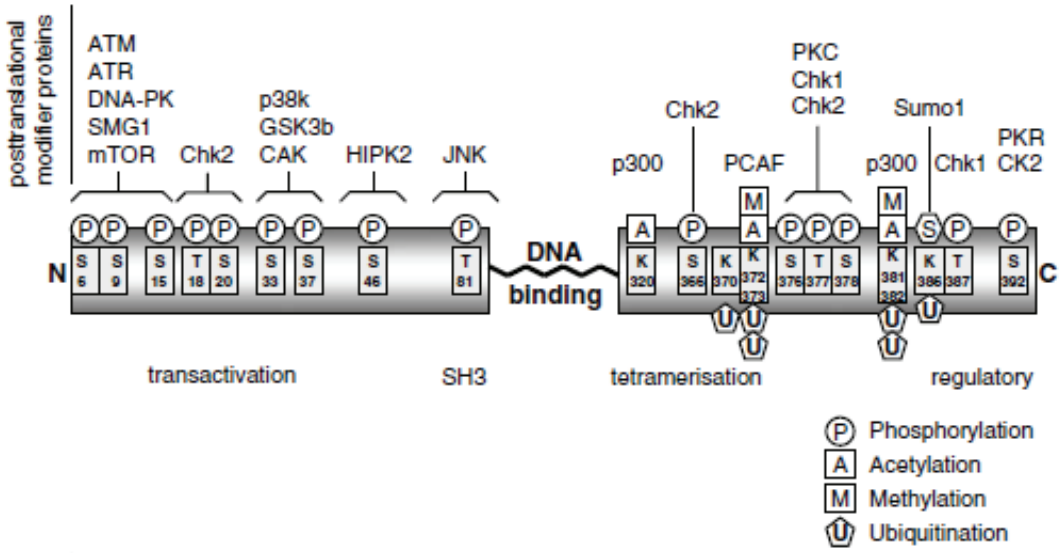

Figure 20: post-translational modifications of p53. In the upper part of the schematic representation of p53, phosphorylation, acetylation, methylation and sumoylation are indicated with their corresponding modifier protein(s). Ubiquitination is represented under the p53 scheme. The transactivation, SH3, DNA binding, tetramerization and regulatory domains of p53 are outlined (adapted from Lavin and Gueven 2006).

It is not possible in this introduction to present all post-translational modifications and protein interactions that contribute to the stabilization of p53 in response to DNA damage. We will provide an overview of possible ways of p53 stability control.

The major mechanism for control of p53 stabilization is dependent on its interaction with and ubiquitination by Mdm2. Indeed, the transcriptional activity of p53 is controlled by the

protein Mdm2, which acts as an E3 ligase and targets p53 for ubiquitin-dependent degradation, acting as a critical negative regulator (Bond *et al.* 2005). However, p53 can be degraded in an Mdm2- and ubiquitin independent manner (Asher *et al.* 2002). This process is mediated by the 20S proteasome and is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1). Interestingly, exposure to γ-irradiation increased the binding of p53 to NQO1, protecting p53 from degradation by the 20S proteasome (Asher and Shaul 2005).

Moreover, p53 can also be stabilized by removing the ubiquitin modification. In fact, the herpesvirus-associated ubiquitin-specific protease (HAUSP) stabilizes p53 by deubiquitination or by regulation of the Mdm2 ubiquitination level (Li *et al.* 2004).

Besides Mdm2 (Hdm2), the proteins Mdm4 (Hdm4, MdmX, HdmX) and ARF ($p14^{ARF}$ in humans and $p19^{ARF}$ in mouse) also play an important role in controlling p53 stability. Mdm4 is a structural homologue of Mdm2 that does not target p53 for degradation but inactivates p53 transcriptional activity (Finch *et al.* 2002), while ARF is a tumour suppressor that interacts with Mdm2 and inhibits p53 degradation, thereby stabilizing it (Dey *et al.* 2008).

Multiple protein kinases including ATM, ATR, Chk1 and Chk2, JNK, p38 and others that respond to different stress stimuli have been described. These proteins phosphorylate p53 on a single or several serine or threonine residues and influence the stabilization of p53 (Lavin and Gueven 2006).

Some sites of p53, Ser 376 and Ser 378, are constitutively phosphorylated and are dephosphorylated in response to ionizing radiation. For example, dephosphorylation from serine 376 creates a consensus binding site for 14-3-3 proteins and increases affinity of p53 for specific DNA sequences (Waterman *et al.* 1998).

Furthermore, phosphorylation of p53 on Ser33, Thr81 and Ser315 in response to stress leads to interaction with the propyl isomerase Pin1. Then, Pin 1 generates conformational changes in p53, enhancing its transactivation activity (Ryan and Vousden 2002; Zacchi *et al.* 2002).

It was demonstrated that acetylation of several lysine residues in the C-terminal region enhances DNA-binding activity *in vitro*. The histone acetylase p300/CBP and P/CAF acetylate p53 (Gu and Roeder 1997; Sakaguchi *et al.* 1998). It has been suggested that lysine acetylation of p53 protects it from degradation since some residues are also targets for ubiquitination by Mdm2 (Li *et al.* 2002). Moreover, p53 acetylation is influenced by phosphorylation. For instance, p53 acetylation at lysine 320 and 382 by P/CAF and p300/CBP

has been shown to require p53 phosphorylation at serine 15 by the kinase ATM in response to ionizing radiation (Sakaguchi *et al.* 1998; Saito *et al.* 2002).

p53 activity can also be regulated through methylation. In fact, the methyltransferase Set 7/9 was found to be able to methylate p53 at lysine 372 resulting in increased nuclear p53 stability and activity (Chuikov *et al.* 2004). This p53 lysine methylation has been shown to precede acetylation and stabilization of p53 (Chuikov *et al.* 2004; Ivanov *et al.* 2007).

2.3.3. p53-dependent apoptosis

Apoptosis induction is an essential function of p53 as a tumor suppressor (Schmitt *et al.* 2002). p53 can promote apoptosis through activation of the death receptors located at the plasma membrane, including Fas, death receptor 4 and 5 (DR4 and DR5). DR4 and DR5 are two of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors and trigger apoptosis on ligation with TRAIL (Wajant *et al.* 2002). Moreover, both DR4 and DR5 have been demonstrated to be induced by chemotherapeutic agents such as doxorubicin and their expression was shown to be regulated by p53 through an intronic p53 binding site (p53BS) (Sheikh *et al.* 1998; Liu *et al.* 2004).

Moreover, several Bcl-2 family proteins are implicated in p53-dependent apoptosis. Zhang *et al.* demonstrated that HCT116 lacking functional BAX are partially resistant to the apoptotic effect of 5-fluorouracil (5-FU) but apoptosis is not abolished (Zhang *et al.* 2000). Nevertheless, in mice, BAX is dispensable for the p53-dependent apoptosis induced by 5-FU or γ-irradiation in thymocytes and intestinal epithelial cells (Knudson *et al.* 1995; Pritchard *et al.* 1999). p53 regulates also several BH3 domain-only proteins that function upstream of BAX/BAK to induce apoptosis. For instance, PUMA and NOXA are activated in a p53 dependent manner following DNA damage (Oda *et al.* 2000; Yu *et al.* 2001).

In addition, following severe damage, p53 can down-regulate apoptosis repressors such as BCL2, BCLXL and SURVIVIN (Vousden and Lu 2002; Rodier *et al.* 2007).

p53 also has some transcription-independent activities (Moll *et al.* 2005). Indeed, a study of Chipuk and collaborators showed that cytosolic p53 can activate BAX in the absence of any others proteins or transcriptional mechanism and thereby involve BAX translocation to mitochondria, cytochrome *c* release and apoptosis (Chipuk *et al.* 2004).

Moreover, following DNA damage (for example, by γ-irradiation), p53 itself can translocate to the mitochondria and interact with Bcl-XL and Bcl-2 and antagonize their anti-apoptotic functions at the outer mitochondrial membrane (Mihara *et al.* 2003). Furthermore, it has been reported that, *in vitro*, purified wild-type p53 induces mitochondrial outer membrane permeabilization (MOMP) and promotes cytochrome *c* release by forming complex with Bcl-2 or Bcl-XL (Mihara *et al.* 2003). Mitochondrial p53 also directly promotes the pro-apoptotic activities of BAK and induces BAK oligomerization (Mihara *et al.* 2003; Leu *et al.* 2004; Murphy *et al.* 2004). Indeed, p53 interacts with the outer membrane protein BAK, thereby liberating BAK from its complex with the anti-apoptotic Bcl-2 family members Mcl-1 (Leu *et al.* 2004; Murphy *et al.* 2004).

In mice, upon γ-irradiation, p53 rapidly translocates to mitochondria (in thymus and spleen) and triggers a first wave of caspases-3 activation that precedes the transcriptional p53 response (Erster *et al.* 2004).

Although apoptosis induced by mitochondrial p53 differs mechanistically from apoptosis induced by cytosolic p53, both pathways converge to pro-apoptotic Bcl-2 family members that initiate the intrinsic apoptotic pathway (Moll *et al.* 2005).

2.3.4. Ceramide and p53

Both p53 and ceramide have been implicated in the regulation of growth suppression (Dbaibo *et al.* 1998). Several studies have generated diverging data on the relationship between ceramide and p53. Indeed, some reports have shown that p53 is upstream of ceramide in tumor stress responses. For instance, Dbaibo *et al.* demonstrated that actinomycin D and γ-irradiation cause p53 activation followed by an increase in endogenous ceramide levels and Molt-4 cell death (Dbaibo *et al.* 1998). Sawada *et al.* showed that p53-dependent production of reactive oxygen species (ROS) induced by etoposide may trigger neutral SMase activation and then causes ceramide accumulation in human glioma cells (Sawada *et al.* 2001). Moreover, another study of Sawada and collaborators showed that p53-induced ROS-dependent pathway may contribute to the TNFα-induced ceramide generation *via* neutral SMase leading to caspases-3 activation and apoptotic cell death in human glioma cells. It is noteworthy that another signalling cascade, p53-induced ROS-independent pathway can lead to TNFα-induced ceramide formation *via* acid SMase (Sawada *et al.* 2004).

By contrast, other reports implicated p53 as a downstream target of ceramide. Indeed, it has been demonstrated that cell proliferation was abolished by treatment with neutral SMase or by C2-ceramide only in wild-type p53 mouse fibrosarcoma cells. These results imply that p53 is involved in the cellular response to ceramide (Pruschy *et al.* 1999). Furthermore, Kim *et al.* reported that C2-ceramide-induced apoptosis in human neuroblastoma SKN-SH cells is accompanied by accumulation of p53 followed by a reduction in the ratio Bcl-2/BAX and activation of caspases (Kim *et al.* 2002).

Other observations place ceramide and p53 in two separate and independent pathways in the apoptotic process. For example, treatment with exogenous C2-ceramide leads to cell death in both LM (expressing p53) and LME6 (lacking p53) (Yang and Duerksen-Hughes 2001). Similarly, another study showed that C2-ceramide treatment induces similar levels of TK6 ($p53^{+/+}$) and WTK1 ($p53^{-/-}$) lymphoblastoid apoptotic cells indicating that p53 does not play a significant role in the induction of apoptosis by ceramide (Shi *et al.* 2001).

Moreover, one study has demonstrated that ceramide generated through the activation of acid SMase in response to γ-radiation, triggers caspases-3 activation and apoptosis only in human glioma cells lacking functional p53, suggesting that p53 can inhibit acid SMase activation and ceramide generation (Hara *et al.* 2004; Hara *et al.* 2004).

Therefore, it remains unclear how ceramide and p53 are linked in programmed cell death. Moreover, it seems to be cell-type dependent (Taha *et al.* 2006).

2.4. The Mdm2 oncoprotein

2.4.1. Introduction

The MDM2 (murine double minute 2) gene was originally identified as the product of an oncogene found overexpressed by amplification in a spontaneously transformed mouse BALB/c cell line (3T3-DM) (Cahilly-Snyder *et al.* 1987).

Both the MDM2 gene and its human counterpart, consist of 12 exons that can generate many different proteins. There are two different promoters, the second of which is responsive to p53. Alternative splicing of Mdm2 and the generation of short proteins occurs in many human and mouse tumors. In humans, Mdm2-A and Mdm2-B are the two major splice variants that delete exons 4-9 and 4-11, respectively. Mdm2-B, also named Mdm2-alt1,

interacts with full-length Mdm2 and sequesters it in the cytoplasm (Iwakuma and Lozano 2003) (Figure 21).

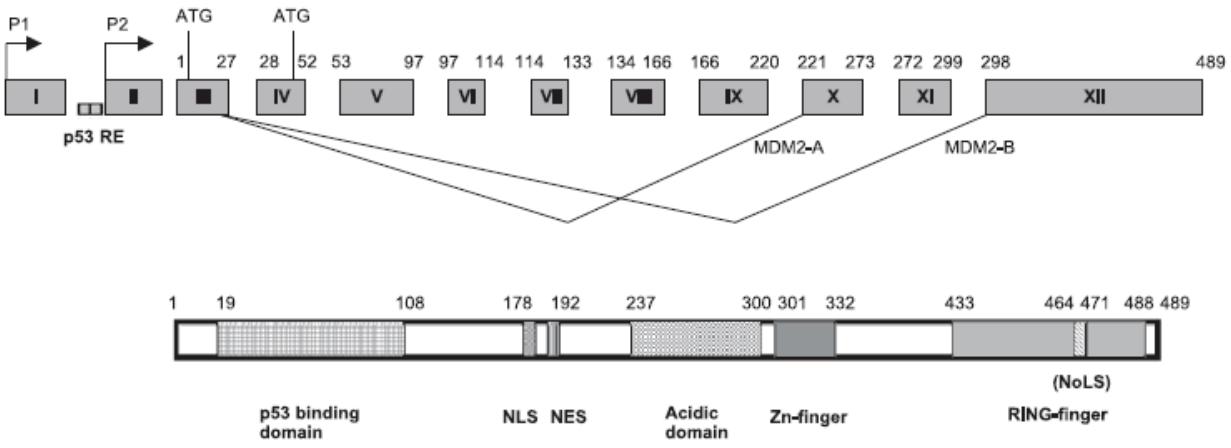

Figure 21: structure of MDM2 gene and protein. MDM2 gene consists of 12 exons and two p53 responsive elements (p53 RE) in intron 1. Two promoters are shown by arrows. Full-length MDM2 p90 is translated from the first start codon ATG in exon 3 and the short form, p76, is translated from the second ATG in exon 4. Two major alternative splice variants in the human genes MDM2-A and MDM2-B are shown. Full-length Mdm2 and known motifs are also represented. NLS, nuclear localization signal; NES, nuclear export signal; Zn-finger, zinc-finger domain; NoLS, nucleolar localization signal; RING-finger, ring-finger domain. The numbers above the drawings denote amino acid numbers and roman numerals are exon numbers (Iwakuma and Lozano 2003).

MDM2 is an essential regulator of p53 in normal cells, but its deregulated expression provides growth advantage to cells. In fact, overexpression of Mdm2 (also known as Hdm2) due to the amplification of the MDM2 gene has been found in approximately one-third of human sarcomas retaining wild-type p53, which indicates that the overexpression of Mdm2 is a molecular mechanism by which cells can inactivate p53 during tumor formation (Oliner *et al.* 1992). In combination with the TP53 mutation, the prognosis is worse than alone. This amplification is observed in approximately 10% of all human cancers (Rayburn *et al.* 2005).

Mdm2 possesses an E3 ubiquitin ligase activity, capable of self ubiquitination, as well as targeted ubiquitination of its substrates, among which p53. The E3 ligase activity of Mdm2 is centered within the RING finger domain (Honda and Yasuda 2000). Moreover, the Mdm2 protein contains nuclear localization and nuclear export signals within its structure, explaining the shuttle of Mdm2 between the nucleus and the cytoplasm (Roth *et al.* 1998).

2.4.2. Regulators of Mdm2

The MDM2 gene is transcriptionally activated by p53 (Barak *et al.* 1993). In fact, p53 binds the MDM2 P2 promoter and transcriptionally up-regulates MDM2 expression. Because

Mdm2 inhibits p53 activity, this forms a negative feedback loop that regulates p53 function. In turn, decreased p53 activity results in decreased Mdm2 to constitutive levels. Moreover, Mdm2 can also ubiquitinate itself and induces its own degradation (Honda and Yasuda 2000).

A variety of factors other than p53 regulate Mdm2 activity. One of the first proteins discovered to interact with Mdm2 is the tumor suppressor, ARF (or $p14^{ARF}$ in humans). This protein sequesters Mdm2 in the nucleolus and blocks nuclear export of Mdm2 and p53, thus resulting in p53 activation (Kamijo *et al.* 1998; Honda and Yasuda 1999; Tao and Levine 1999; Weber *et al.* 1999).

Like ARF, the ribosomal proteins L5, L11, and L23 bind to the central acidic and zing finger region in Mdm2 and can sequester it in the nucleolus, resulting in p53 stabilization (Coutts *et al.* 2009).

Moreover, Yin Yang 1 (YY1), a multifunctional transcription factor, can enhance Mdm2-mediated p53 poly-ubiquitination both *in vivo* and *in vitro*. In fact, YY1 increases the physical interactions between Mdm2 and p53, leading to decreased p53 levels and activity. Furthermore, ARF has been shown to interact with YY1, disrupting the interaction between YY1 and Mdm2 (Sui *et al.* 2004).

Also, the nuclear co-repressor KAP1 has been described as an Mdm2-interacting protein. KAP1 interacts with Mdm2 via the central acidic domain and stimulates p53 ubiquitination and degradation. KAP1 was shown to reduce YY1-Mdm2 binding, and ARF competes with KAP1 for Mdm2 binding (Wang *et al.* 2005).

Furthermore, the Mdm2-related protein, Mdmx (also called Mdm4) interacts with Mdm2 *via* their RING finger domains, and this interaction protects Mdm2 from degradation (Sharp *et al.* 1999; Tanimura *et al.* 1999).

Several kinases phosphorylate Mdm2 and modulate interactions with p53 (Coutts *et al.* 2009). The ATM kinase phosphorylates Mdm2 on serine 395 and impairs the nuclear export and degradation of p53 by Mdm2 (Maya *et al.* 2001). Moreover, the tyrosine-protein kinase ABL1 or c-abl neutralizes the inhibitory effect of Mdm2 on p53 *via* phosphorylation of Mdm2 on tyrosine 394 (Goldberg *et al.* 2002).

In contrast, the phosphatidylinositol 3-OH-kinase (PI3-kinase) and its downstream target, Akt/PKB serine-threonine kinase, bind and phosphorylate Mdm2 on serines 166 and 186 (Ogawara *et al.* 2002). It results in translocation of Mdm2 to the nucleus, where it can regulate TP53 (Mayo and Donner 2001). Moreover, phosphorylation of Mdm2 by Akt

enhances its interaction with the coactivator p300/CREB binding protein (CBP) that cooperates with Mdm2 in polyubiquitination and degradation of p53 (Grossman *et al.* 1998). It is noteworthy that the central acidic domain of Mdm2 is necessary for interaction with p300/CBP. However, the RING finger domain of Mdm2 can be acetylated by p300/CBP, which is thought to compromise Mdm2 E3 ligase activity, leading to decreased p53 poly-ubiquitination and increased p53 levels (Wang *et al.* 2004). Thus, p300 may play a dual role in p53 regulation.

Phosphatases are also involved in regulating Mdm2. Indeed, Cyclin G, a p53 target, recruits the active protein phosphatase 2A (PP2A) holoenzyme, which binds and activates Mdm2 through dephosphorylation (Okamoto *et al.* 2002).

2.4.3. The p53/Mdm2 network: the affair that never ends

As mentioned above, Mdm2 protein promotes proteasome-mediated degradation of p53 by functioning as an E3 ubiquitin ligase. In fact, p53 is mono- or poly-ubiquitinated on lysine residues in its C-terminal domain and it depends on the Mdm2 expression level. In unstressed cells, low levels of Mdm2 promote mono-ubiquitination of p53 and its nuclear export, whereas high levels of Mdm2 result in poly-ubiquitination and nuclear degradation of p53 (Li *et al.* 2003; Coutts *et al.* 2009) (Figure 22).

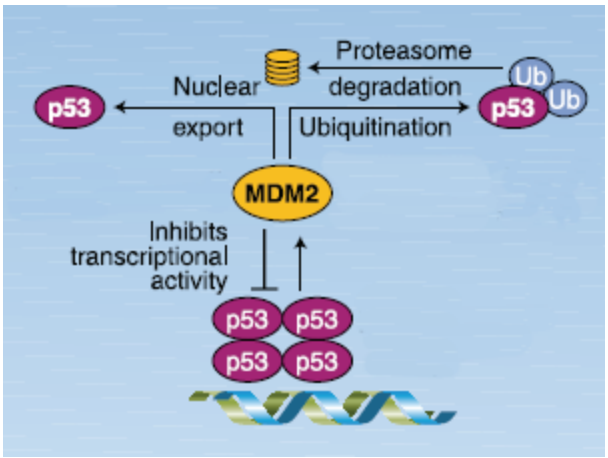

Figure 22: mdm2 inhibits p53 through an autoregulatory loop (adapted from Shangary and Wang 2008).

Alternatively, several other p53 ubiquitin ligases such as Pirh2, COP1, Yin Yang 1 (YY1) or ARF/BP1, have been discovered and shown to function in an Mdm2-independent manner (Leng *et al.* 2003; Dornan *et al.* 2004; Chen *et al.* 2005). However, none of these proteins can sufficiently compensate for a loss of Mdm2 function *in vivo*.

In addition to promote p53 degradation, Mdm2 binds to the transactivation domain of p53. It has been proposed that this interaction might interfere with the recruitment of the basal transcription machinery and/or essential co-activators. The Mdm2-p53 interaction was mapped to the first 120 NH2-terminal amino acids in Mdm2 and the amino terminal transactivation domain of p53 (Figure 23).

Figure 23: schematic model depict p53 structure. p53 is comprised of an N-terminal transcriptional activation domain (TAD), a proline rich domain (PRD), a central DNA-binding domain (DBD), and C-terminal tetramerization (TD) and regulatory (CRD) domains. Some regions of some p53-interacting proteins are demonstrated (adapted from Coutts *et al.* 2009).

Whereas Mdm2 is a major regulator of p53 stability, other proteins are implicated in the regulation of p53 stability including JNK, human papilloma virus E6, and the COP9 signalosome complex, as described previously in the p53 chapter (Alarcon-Vargas and Ronai 2002).

2.4.4. Other proteins regulated by Mdm2

Many other proteins than p53 appear to be affected by Mdm2. Recent studies indicate that Mdm2 regulates proteins involved in DNA repair, cell cycle control, DNA replication, ribosome biosynthesis, and apoptosis pathways independent of p53 (Ganguli and Wasylyk 2003; Bouska and Eischen 2009) (Figure 24). We will discuss only the interactions of Mdm2 with proteins that have effects on cell cycle control and apoptosis.

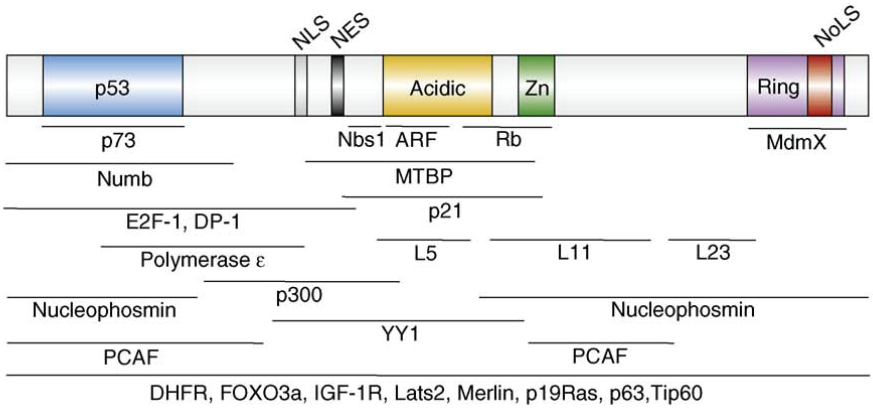

Figure 24: schematic of Mdm2 and the location of proteins that bind. The blue, orange, green and pink boxes represent the p53 binding (p53), acidic, zinc finger (Zn) and E3 ubiquitin ligase ring finger (ring) domains from N to C terminus, respectively. The locations of the nuclear localization signal (NLS), the nuclear export signal (NES) and the nucleolar export signal (NoLS) are indicated. The region(s) of Mdm2 where specific proteins bind are indicated with black lines. Proteins whose Mdm2-binding domains have not been determined are listed at the bottom (Bouska and Eischen 2009).

As discussed above, elevated levels of Mdm2 can inhibit apoptosis through its negative regulation of p53. However, Mdm2 also seems to influence apoptosis through its interaction with other proteins than p53 (Bouska and Eischen 2009).

Indeed, Mdm2 overexpression inhibits apoptosis induced by overexpression of E2F-1 and its binding partner, DP-1, in osteosarcoma cells lacking p53 and Rb (Loughran and La Thangue 2000).

Another mechanism by which Mdm2 could inhibit apoptosis is through the regulation of FOXO3. It is a transcription factor that controls the expression of genes that inhibit proliferation and induce apoptosis. In MEFs and breast cancer cells, increasing levels of Mdm2 lead to a decrease in FOXO3 levels through the ubiquitin-proteasome degradation pathway (Yang *et al.* 2008).

Several studies focused on the impact of Mdm2 expression on NF-κB activity (Gu *et al.* 2002; Cheney *et al.* 2008). NF-κB is a heterodimeric, anti-apoptotic transcription factor. The most common form comprises a p50 subunit and a p65 subunit. Interestingly, in a rhabdomyosarcoma cell line that expresses mutant p53 and in which the NF-κB pathway is constitutively active, Mdm2 overexpression decreased p65 protein levels and activity and increased apoptosis (Gu *et al.* 2002). In contrast, in another p53 mutant rhabdomyosarcoma cell line lacking constitutive NF-κB activation, Mdm2 overexpression activated p65 transcription, increased p65 protein levels, and reduced apoptosis (Cheney *et al.* 2008).

Mdm2 also interacts with several proteins that influence the G1-S-phase transition independently of p53.

Specifically, using immunoprecipitation experiments with U937 cell lysates, Mdm2 was identified as a pRb-binding protein. pRb is a tumor suppressor that is mutated in different cancers. Mdm2 forms a complex with pRb *in vivo*, and inhibits the pRb-mediated G1 arrest (Xiao *et al.* 1995). Moreover, pRb impairs certain functions of Mdm2, through the formation of a trimeric complex with p53. Indeed, this trimeric complex protects p53 from Mdm2-targeted degradation. pRb thereby overcomes the ability of Mdm2 to inhibit p53-mediated apoptosis (Hsieh *et al.* 1999).

Martin *et al.* showed that, in immunoprecipitation experiments in NIH3T3, Mdm2 also interacts with E2F-1/DP-1, a pRb-regulated heterodimeric transcription factor, involved in S-phase progression. Mdm2 induces E2F-1 transcriptional activation and stimulates the growth-promoting activity of E2F-1 (Martin *et al.* 1995; Loughran and La Thangue 2000).

Mdm2 is known to influence p21 expression through its regulation of p53, because p21 is a p53 transcriptional target gene. p21 belongs to the cip/kip family ($p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$) of the cyclin-dependent kinase inhibitors (CDKI) and inhibits the G1 to S-phase transition (Bond *et al.* 2005). However, Mdm2 can also regulate p21 expression independently of p53. In cells lacking p53, Mdm2 overexpression leads to increased p21-C8 proteasome subunit association and a subsequent decrease in p21 protein levels. This effect on p21 was independent of the Mdm2 E3 ubiquitin ligase domain (Jin *et al.* 2003; Zhang *et al.* 2004).

As discussed above, p300/CBP regulates Mdm2 stability and cooperates with Mdm2 to degrade p53 (Grossman *et al.* 1998; Grossman *et al.* 2003; Zeng *et al.* 2003). But, the activity of p300 is also regulated by Mdm2. It inhibits the interaction of p53 with p300, resulting in reduced activity of p53 (Wadgaonkar and Collins 1999). Additionally, Kobet *et al.* showed that Mdm2 inhibits p300/CBP-dependent p53 acetylation and thus p53 activation (Kobet *et al.* 2000).

Studies have also shown that Mdm2 is involved in the regulation of Mdmx levels (Kawai *et al.* 2001; Pan and Chen 2003; Marine *et al.* 2007).

2.4.5. Ceramide and Mdm2

A study of Phillips and co-workers described a novel role for ceramide accumulation in mediating G_2 arrest in Rhabdomyosarcoma (RMS) cells before the induction of apoptosis. In this study, the authors show that treatment of the RMS cell line RD with C6-ceramide or acid sphingomyelinase induces $p21^{Cip1/Waf1}$ accumulation independently of DNA damage, G_2 arrest and late induction of apoptosis (Phillips *et al.* 2007). In addition, they also demonstrate that the down-regulation of $p21^{Cip1/Waf1}$ expression by using a specific siRNA results in the inhibition of C6-ceramide-mediated G_2 arrest, confirming the requirement for $p21^{Cip1/Waf1}$ in ceramide-mediated G_2 arrest.

Interestingly, the authors showed that Mdm2 overexpression in RD cells prevents the C6-ceramide-induced up-regulation of $p21^{Cip1/Waf1}$, reduces C6-ceramide-mediated cell cycle arrest and the ensuing late apoptosis (Phillips *et al.* 2007). Indeed, as described above, turnover of $p21^{Cip1/Waf1}$ can be negatively regulated by Mdm2 by promoting its proteosomal degradation independently to ubiquitination and also p53/Rb status (Jin *et al.* 2003; Zhang *et al.* 2004).

This inhibition by Mdm2 overexpression is reversed by pretreatment with the proteasome inhibitor MG132 or by siRNA targeted against Mdm2 (Phillips *et al.* 2007).

2.5. The Bcl-2 protein family

2.5.1. Introduction

The extended Bcl-2 family of proteins controls the function of the mitochondrion in the healthy vs. the apoptotic state. In mammalian cells, the five pro-survival proteins (Bcl-2, Bcl-XL, Bcl-W, Mcl-1 and A1) and the pro-apoptotic proteins, BAK and BAX share four domains of sequence homology known as Bcl-2 homology 1 (BH1), BH2, BH3 and BH4. They also have a C-terminal membrane-anchoring sequence (Lessene *et al.* 2008).

Other key players that orchestrate apoptosis are the pro-apoptotic BH3-only proteins (BIM, BID, BAD, PUMA, NOXA, BMF, HRK and BIK) (Figure 25).

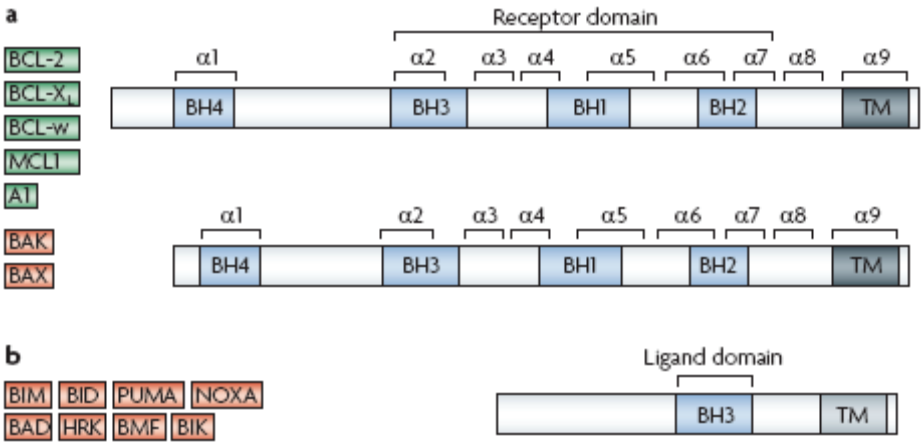

Figure 25: the extended Bcl-2 proteins family. a. Family members sharing four Bcl-2 homology (BH) domains are the multidomain proteins. **b.** Family members displaying only the BH3 domain are the BH3-only proteins. A1, also known as BFL-1/Bcl2-A1; Bcl-W, also known as Bcl2-L-2; Bcl-XL, also known as Bcl2-L-1; BAD, Bcl-2 antagonist of cell death; BIK, Bcl-2-interacting killer; BIM, also known as Bcl2-L-11; BMF, Bcl-2-modifying factor; Mcl-1, myeloid cell leukaemia sequence 1; PUMA, also known as Bcl-2-binding component 3; NOXA, also known as Phorbol-12-myristate-13-acetate-induced protein; TM, transmembrane (Lessene *et al.* 2008).

Different Bcl-2 like proteins have different cytoplasmic distributions. Both Bcl-2 and Bcl-XL are found to be associated with membranes of the mitochondria, endoplasmic reticulum, and nucleus. The pro-apoptotic members, BAD, BAX, and BID, in their inactive state, have a cytoplasmic location. However, upon activation, they translocate to the mitochondria (Wolter *et al.* 1997; Khaled *et al.* 1999). *In vitro* studies indicated that a rise in intracellular pH triggered the change in BAX conformation and then the translocation of BAX from the cytosol to mitochondria (Khaled *et al.* 1999).

2.5.2. Functions of individual family members

The mitochondrion is a converting point upon which apoptotic signals occur and by which apoptosis is initiated. In fact, the mitochondrion is the target of numerous pro-apoptotic signal transducing molecules such as ceramide, ganglioside GD3, fatty acids (e.g. palmitate), reactive oxygen species, nitric oxide, and calcium. These death signals converge on the mitochondria through the activation of pro-apoptotic members of the Bcl-2 family, including BAK, BAD, BAX and BID (Birbes *et al.* 2002).

The integrity of the mitochondrion depends on a critical balance between the pro-apoptotic and the antiapoptotic members of the Bcl-2 family (Chipuk and Green 2008) (Figure 26).

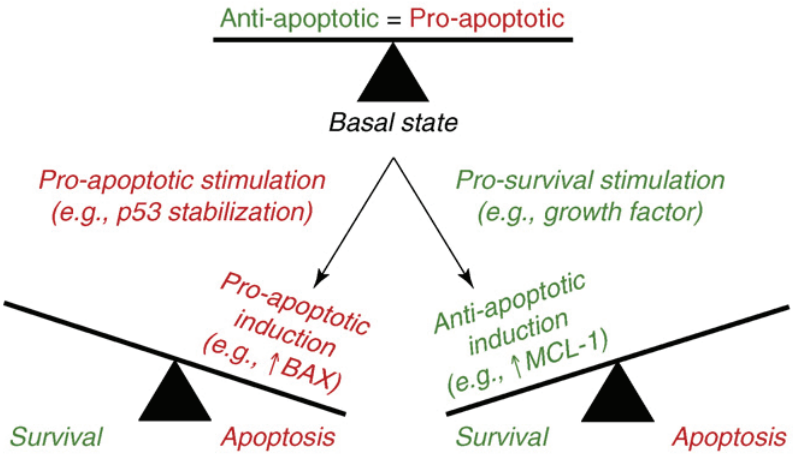

Figure 26: in a hypothetical basal state, the number of anti-apoptotic and pro-apoptotic molecules is equal; tipping this balance dictates cellular fate. If a stress (e.g. DNA damage) is applied, the induction of pro-apoptotic molecules provides the signal to engage mitochondrial outer membrane permeabilization (MOMP). On the contrary, growth factor addition would promote cellular survival by increasing the amount of anti-apoptotic proteins (Chipuk and Green 2008).

Bcl-2 proteins modulate apoptosis by permeabilization of the inner and/or outer membrane, leading to release of cytochrome *c*. In cell-free systems, Bcl-2 and Bcl-XL favour closure of the pore (PT) that controls the mitochondrial permeability transition (MPT), while pro-apoptotic BAX has the opposite effect, interacting with the adenine nucleotide translocase (ANT) and voltage-dependent anion channel (VDAC) to favour pore opening and cytochrome *c* release (Shimizu *et al.* 1999; Bouchier-Hayes *et al.* 2005) (Figure 27b).

In contrast, BH3-only proteins, BID and BIK, induce also cytochrome *c* release but they do not interact with either ANT or VDAC. Thus, they induce apoptosis without mitochondrial membrane potential ($\Delta\Psi$) loss (Shimizu and Tsujimoto 2000). In fact, BH-3-only proteins become active by different stress conditions. Indeed, BID is cleaved by a number of proteases including caspases-8, granzyme B and cathepsin B into tBID, which can directly activate BAX, leading to create pores in the outer mitochondrial membrane that permit release of intermembrane space proteins to the cytosol (Li *et al.* 1998; Bouchier-Hayes *et al.* 2005; Chipuk and Green 2008) (Figure 27a).

Also, the BH3-only proteins BID, BIM and PUMA engage mitochondrial outer membrane permeabilization (MOMP) because they bind and neutralize all anti-apoptotic Bcl-2 members. A combination of other BH3-only proteins is required to promote apoptosis because each neutralizes only a subset of antiapoptotic Bcl-2 proteins (e.g. BAD and NOXA neutralize Bcl-2/Bcl-XL/Bcl-W and Mcl-1/A1, respectively) (Chipuk and Green 2008).

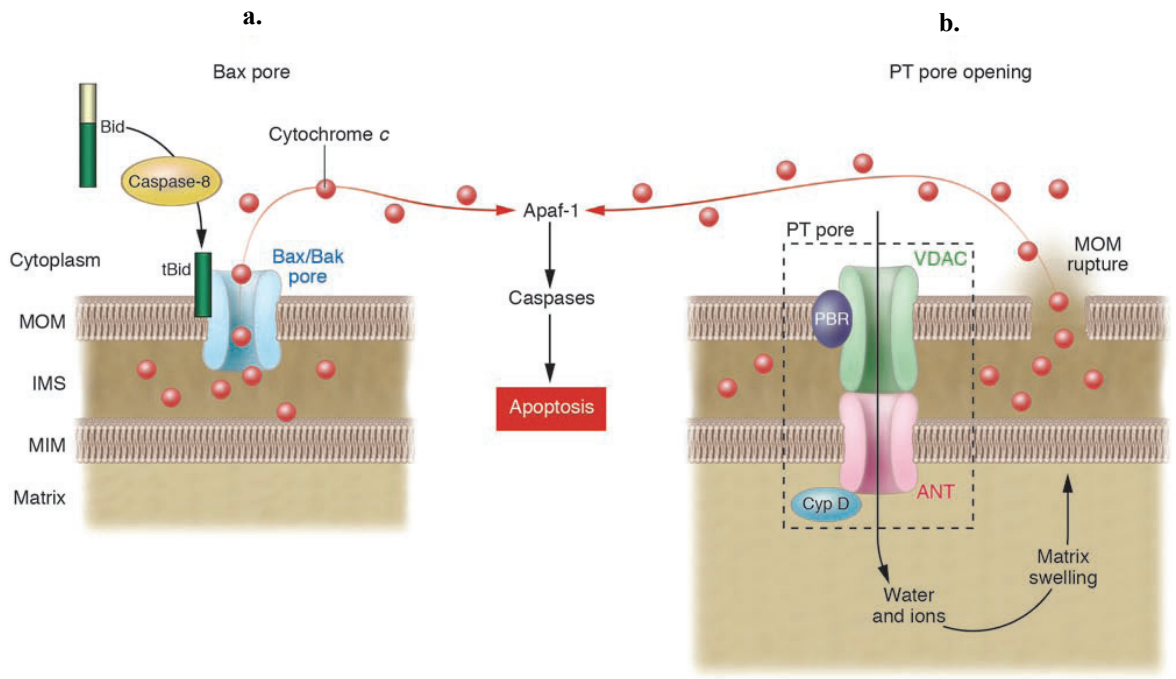

Figure 27: **molecular mechanisms of mitochondrial outer membrane permeabilization (MOMP)**. The proposed models of MOMP leading to cytochrome *c* release are represented. **a. BAX pore**. BAX or BAK forms a pore in the MOM after activation by a BH3-only protein such as BID. **b. Permeability transition (PT) pore opening**. Opening of the PT pore allows an influx of water and ions into the matrix, causing matrix swelling; this leads to rupture of the MOM, releasing intermembrane space (IMS) proteins such as cytochrome *c*. MIM, mitochondrial inner membrane; PBR, peripheral benzodiazepine receptor; Cyp D, the soluble matrix protein cyclophilin D (Bouchier-Hayes *et al.* 2005).

Most Bcl-2 proteins are capable of physical interactions, forming homodimers or heterodimers, and functioning as agonists or antagonists of each other (Blatt and Glick 2001). In fact, the BH3 domain of pro-apoptotic proteins is the mediator of interaction with pro-survival family members (Minn *et al.* 1998). Thus, Bcl-2 dimerizes with pro-apoptotic BAX to neutralize its activity. As a heterodimer, BAX is inactive, but once free to dimerize with itself, BAX is able to induce apoptosis (Gross *et al.* 1998; Antonsson and Martinou 2000).

2.5.3. Ceramide and the Bcl-2 family members

The position of ceramide with respect to Bcl-2 is variably reported. A number of groups have shown that ceramide is upstream of Bcl-2 in the apoptotic pathway. Indeed, in ALL-697 leukemia cells, the ceramide response to vincristine was not modified by Bcl-2 overexpression, indicating that Bcl-2 does not interfere with ceramide formation. However, Bcl-2 overexpression prevents apoptosis in response to vincristine, demonstrating that Bcl-2 acts upstream of ceramide (Zhang *et al.* 1996). Identically, Bcl-2 overexpression prevents apoptosis induced by ceramidase inhibitors in malignant melanoma and HaCaT keratinocytes (Raisova *et al.* 2002).

Moreover, El-Assaad *et al.* have shown that, in MCF7 breast carcinoma cells, only Bcl-XL abrogates ceramide generation in response to TNFα, while Bcl-2 does not, implying a pathway where ceramide is downstream of Bcl-XL but upstream of Bcl-2 (El-Assaad *et al.* 1998).

On the other hand, another study has shown that Bcl-2 overexpression in C6 rat glioma cells attenuates ceramide accumulation following etoposide treatment by repressing neutral sphingomyelinase. Thus, Bcl-2 can also act upstream of the lipid. The same study has shown that BAX overexpression does not affect ceramide production in response to etoposide (Sawada *et al.* 2000).

Additionally, another report showed that C2-ceramide treatment in a prostate (DU 145) and colorectal (HCT116) cancer cell line induces apoptosis only when BAX is overexpressed (von Haefen *et al.* 2002).

Furthermore, Birbes *et al.* showed that the generation of ceramide in mitochondria in response to TNFα is sufficient to induce BAX translocation to mitochondria, BAX oligomerization, cytochrome *c* release and cell death (Birbes *et al.* 2005).

Another study has also shown that ceramide production *via* activation of acidic sphingomyelinase by UV treatment or C16-ceramide treatment of HeLa cells, mediates BAX conformational change leading to the release of cytochrome *c* from mitochondria (Kashkar *et al.* 2005).

Finally, ceramide may regulate Bcl-2 and BAX by modulating their phosphorylation states. It was established that Bcl-2, whose phosphorylation by PKCα at serine 70 in the ER is required for its anti-apoptotic function, becomes dephosphorylated and inhibited in response to the ceramide-activated protein phosphatase PP2A (Ruvolo *et al.* 1999; Lin *et al.* 2006).

Xin *et al.* showed that BAX is dephosphorylated following C2-ceramide treatment in a PP2A-dependent manner. Dephosphorylation of BAX is associated with its conformational change that promotes insertion of BAX into mitochondrial membranes, formation of BAX oligomers, leading to cytochrome *c* release from mitochondria and apoptosis (Xin and Deng 2006).

As discussed above, C2-ceramide caused caspase-2 activation, mitochondrial damage followed by downstream caspase-9 and -3 activation, and cell apoptosis in human lung

epithelial cell line A549. Moreover, Lin *et al.* reported that overexpression of Bcl-2 inactivates caspase-2, which rescues ceramide-induced apoptosis (Lin *et al.* 2005).

3. Ceramide and its metabolites in therapeutics

3.1. Ceramide metabolism in cancer therapeutics

3.1.1. Targeting the ceramide/S1P balance in cancer therapy

As discussed above, the balance between ceramide and S1P is a critical determinant of a cell's life death decision (Figure 28). It has been well established that increases in intracellular ceramide will promote apoptosis (Huwiler and Zangemeister-Wittke 2007). To this end, certain chemotherapeutics agents such as daunorubicin, vincristine, camptothecin, fludarabine, etoposide, and gemcitabine increase ceramide generation through the *de novo* pathway, or *via* activation of SMases (Futerman and Hannun 2004; Ogretmen and Hannun 2004; Ogretmen 2006). Moreover, combining sphingomyelin with doxorubicin, allows increase of the cellular uptake of doxorubicin *via* alteration of cell membrane permeability, leading to increased accumulation, bioavailability and therapeutic effects of the drug in MCF-7, KB and A431 carcinoma cells (Veldman *et al.* 2004).

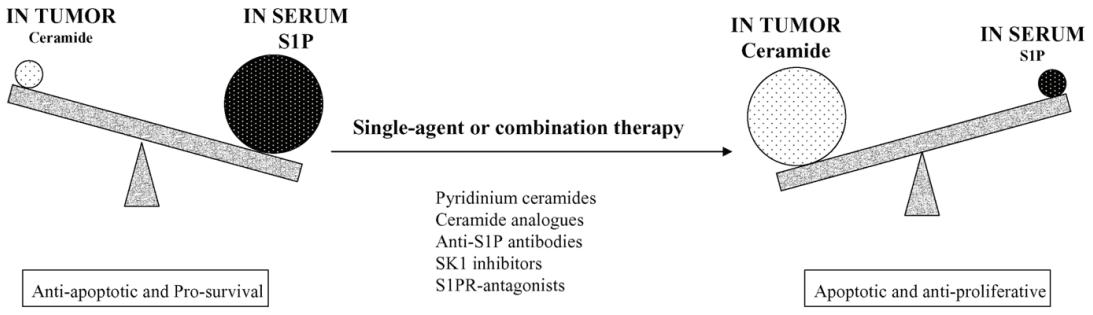

Figure 28: roles of ceramide and sphingosine-1-P in anti-cancer therapeutics. The cellular balance between ceramide in tumor samples and S1P is believed to determine the fate of cancer cells. Often, within a tumor, there are altered levels of ceramide and high S1P levels, which are often secreted into the serum at relatively high concentrations. This causes the metabolic balance to favor S1P resulting in a pro-survival outcome. However, new therapies are being explored to shift this balance in favor of ceramide. Ultimately, these new treatments are aimed at increasing ceramide levels while inhibiting S1P generation, secretion, or signalling through S1PRs (Huwiler and Zangemeister-Wittke 2007).

Additionally, small molecule inhibitors of several enzymes of ceramide clearance (SM synthases, CDases, CK) may serve as novel therapeutic targets to induce the accumulation of ceramide (Kok and Sietsma 2004; Ogretmen and Hannun 2004; Reynolds *et al.* 2004; Fox *et*

al. 2006; Modrak *et al.* 2006; Ogretmen 2006). For example, two neutral ceramidase inhibitors D-erythro-MAPP and B13 up-regulate ceramide levels in colon carcinoma cells (SW403) but also potently induce apoptosis (Selzner *et al.* 2001) (Figure 29).

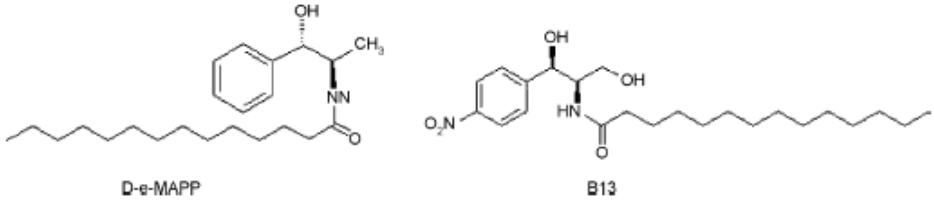

Figure 29: chemical structures of neutral ceramidase inhibitors. D-e-MAPP: 1*S*, 2*R*-d-erythro-2-*N*-myristoylamino-1-phenyl-1-propanol; B13: (1*R*, 2*R*-D-erythro 2-*N*-myristoylamino-1-nitrophenyl-propan-1, 2-diol) (Huwiler and Zangemeister-Wittke 2007).

In a complementary approach, the use of exogenous ceramide analogues as therapeutic agents could also promote apoptotic pathways in cancer cells (Saddoughi *et al.* 2008) (Table 2). For example, novel pyridinium ceramide (Pyr-Cer) has been synthesized with greater water solubility and cell-membrane permeability (Novgorodov *et al.* 2005; Rossi *et al.* 2005; Dindo *et al.* 2006; Szulc *et al.* 2006). The positive charge of the pyridinium ring in these structures permits targeting and accumulation of these ceramide analogues into negatively charged intracellular compartments, especially the mitochondrion and nucleus. These properties might be exploited for preferential targeting into tumor sites. Indeed, there are studies that suggest that cancer cells acquire, in general, a more negative charge in their sub-cellular structures (in mitochondria and/or nuclei) (Modica-Napolitano and Aprille 2001). The accumulation of Pyr-Cer in the mitochondria causes dramatic alterations in the structures and functions of mitochondria, resulting in a decrease of the mitochondrial potential, release of cytochrome *c*, activation of caspase-3 and caspase-9 and apoptotic cell death (Novgorodov *et al.* 2005; Dindo *et al.* 2006).

Other structural analogs of ceramide, such as C16-serinol and (2S, 3R)-(4E, 6E)-2-octanoylamidooctadecadiene-1, 3-(4, 6-diene-ceramide) induces apoptosis in neuroblastoma, and breast cancer cells (Struckhoff *et al.* 2004). Other ceramide analogs, 5R-OH-3E-C8-ceramide, adamantyl-ceramide and benzene-C4-ceramide selectively inhibit the growth of drug resistant human breast cancer cell lines (SKBr3 and MCF-7/Adr) (Crawford *et al.* 2003).

In an alternative approach, delivery of exogenous natural ceramide in pegylated liposomes, which are generally more effective at crossing the cell membrane, increases growth inhibitory effects in human breast cancer cells, *via* enhanced accumulation of ceramide and inhibition of phosphorylated Akt and stimulation of the activity of caspases-3/7 more effectively than non-

liposomal ceramide (Stover and Kester 2003). *In vivo* therapeutic efficacy of the pegylated ceramide for the delivery of exogenous ceramide that results in slower tumor growth in murine models of breast cancer, was also demonstrated (Stover *et al.* 2005).

Conversely, suppression of S1P generation/accumulation could suppress tumor growth. Thus, one anti-cancer therapeutic strategy is to use inhibitors of SK1 (Saddoughi *et al.* 2008). Importantly, 4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol (SKI-II) showed a significant inhibition of tumor growth in mice (French *et al.* 2006).

There are also S1P analogs that are antagonists at two of the five S1P receptors, S1PR1 and S1PR3. Inhibition of these receptors with these compounds may inhibit cancer cell growth (Davis *et al.* 2005).

Another approach is the use of a monoclonal antibody that binds S1P with high affinity and specificity. The anti-S1P monoclonal antibody prevents S1P-induced cell proliferation, release of pro-angiogenic cytokines, and protection of tumor cells from apoptosis by S1P (Visentin *et al.* 2006).

Compound	Mode of Action	Cancer Type
B13	Acid ceramidase inhibitor	Prostate, Colon and HNSCC
D-MAPP	Neutral/Alkaline ceramidase inhibitor	Squamous cell carcinoma
Pyridinium ceramide	Mitochondrial targeting	HNSCC, lung, colon and breast
4,6-diene-ceramide	Ceramide analogue	Breast
C16-serinol	Ceramide analogue	Neuroblastoma
PPMP, PPPP	GCS inhibitors	Solid tumors
Dimethylsphingosine	Sphingosine Kinase inhibitor	Leukemia, colon and breast
Anti-S1P monoclonal antibody	Binds S1P	Solid tumors
Pegylated lyposomes with ceramide	Improved delivery	Breast
Vincristine in sphingomyelin- liposomes	Improved delivery	Acute lymphoid leukemia
Safingol (L-t-dihydro- sphingosine)	Sphingosine kinase inhibitor	Solid tumors
FTY-720	Myriocine analogue	Bladder, prostate, breast, lymphoma

Table 2: sphingolipid analogues and inhibitors of ceramide metabolism. D-MAPP: D-*erythro*-2-(N-myristoylamino)-1-phenyl-1-propanol; HNSCC: Head and neck squamous cell carcinoma; PPMP: 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; GCS: glucosylceramide synthase (Saddoughi *et al.* 2008).

3.1.2. Role of ceramide metabolites in cancer drug resistance

Mutidrug resistance (MDR) of cancer represents a major obstacle to successful chemotherapy. Recent studies indicate that one mechanism of resistance that cancer cells develop against chemotherapy is the alteration of ceramide accumulation (Saddoughi *et al.* 2008). Indeed, in some cancers, ceramide is highly metabolized into GlcCer due to an

increase in GCS activity (Gouaze-Andersson and Cabot 2006). Specifically, increased activity of GCS attenuates ceramide levels and contributes to the resistance of several human cancer types to many chemotherapeutic agents (Ogretmen and Hannun 2001).

Several studies revealed that there is a link between GCS and the ABC transporter (P-gp). In fact, Gouaze *et al.* reported that increased accumulation of GlcCer is found in cells overexpressing P-gp (Gouaze *et al.* 2004). Interestingly, P-glycoprotein has been proposed as a specific transporter for glucosylceramide that translocates this molecule across the Golgi to deliver it for the synthesis of glycosphingolipids (De Rosa *et al.* 2004).

Additionally, a study showed that SDZ PSC 833, an inhibitor of P-gp, inhibits also GCS (Goulding *et al.* 2000). Moreover, these data are consistent with another study demonstrating that knockdown of GCS expression with small interfering RNA (siRNA) inhibits the expression of MDR1, a gene that encodes for P-gp (Gouaze *et al.* 2005; Gouaze-Andersson *et al.* 2007). These results suggest that inhibitors of GCS such as PPMP may be useful in preventing chemotherapy resistance.

Therefore, changes in sphingolipid metabolism play functional roles in conferring drug resistance. Drug resistance of cancer cells also correlates with increased SK-1 expression. Conversely, the sensitivity of A-375 melanoma cells to Fas- and ceramide-mediated apoptosis can be restored by inhibition of SK-1 (Bektas *et al.* 2005).

Moreover, the role of S1P lyase in increased sensitivity to cisplatin was confirmed in human A549 lung cancer and HEK 293 cells (Min *et al.* 2005).

3.2. Emerging roles of ceramide and ceramide metabolites in diseases

3.2.1. Sphingolipids storage diseases

Sphingolipids storage diseases were associated with the defective activity of a particular lysosomal hydrolase involved in sphingolipids degradation. Currently, more than 40 lysosomal storage disorders (LSDs) are known (Futerman and van Meer 2004), of which at least 9 or 10 are due to defective sphingolipid degradation. For example, Gaucher disease is caused by the defective activity of glucosylceramide-β-glucosidase, and Fabry disease by defective α-galactosidase activity (Kacher and Futerman 2006).

A number of therapeutic options are available, which are discussed briefly below, including enzyme replacement therapy (ERT), substrate reduction therapy (SRT), enzyme enhancement therapy (EET) (Fox *et al.* 2006; Kacher and Futerman 2006; Kolter and Sandhoff 2006).

For over a decade now, enzyme replacement therapy (ERT) is the most successful available treatment for any sphingolipid storage diseases. ERT acts by supplementing defective enzyme with active enzyme, and has proved to be safe and effective over a period of 14 years (Kacher and Futerman 2006). Cerezyme®, a recombinant acid β-glucosidase, is used in treating patients with type 1 Gaucher disease. This enzyme targets macrophages by remodelling its oligosaccharide chains as to expose mannose residues, permitting uptake via macrophage mannose receptors. ERT is also used for a number of other sphingolipid storage diseases like Fabry disease, Niemann-Pick B disease (deficiency of acid SMase) (Desnick and Schuchman 2002). Unfortunately, these drugs do not cross the blood-brain barrier and are less effective against the neuropathic forms of these diseases (Kacher and Futerman 2006).

The substrate reduction therapy (SRT) is an alternative strategy. This therapeutic approach is based on preventing the accumulation of the undegraded substrates by partial inhibition of their synthesis. This approach has been used clinically for type 1 Gaucher disease patients, by using N-butyldeoxynojirimycin (Zavesca®), an inhibitor of glucosylceramide synthase (Cox *et al.* 2003). These molecules, in contrast to the enzymes used in ERT, could pass the blood-brain barrier. However, it is unclear whether deleterious side-effects might be observed in patients after extended use (Futerman *et al.* 2004).

Another therapeutic option is the enzyme enhancement therapy (EET) or chaperone therapy. The treatment is based on the concept that some mutations in SLs diseases cause misfolding of lysosomal enzymes. When this occurs, most of the newly synthetized enzyme is degraded in the ER rather than being transported to the lysosome. Thus, this strategy is based on the enzyme stabilization during its synthesis by use of small chemical chaperones (Pastores and Barnett 2005). This therapy has been shown to be successful in various disease models (e.g., Gaucher's disease) (Sawkar *et al.* 2002).

Additional strategies for the treatment of sphingolipidoses consist in the gene therapy and the cell-mediated therapy (CMT). In this case, cells are used as therapeutic agent to replace or compensate the defective cell population with normal equivalents, so that tissue or organ function might be restored, or to release enzymes for uptake by deficient cells. This can be

achieved by bone marrow transplantation (BMT), or by the use of neural progenitor cells as examples of this method (Kolter and Sandhoff 2006).

Four therapeutic options for sphingolipidoses are illustrated in Figure 30.

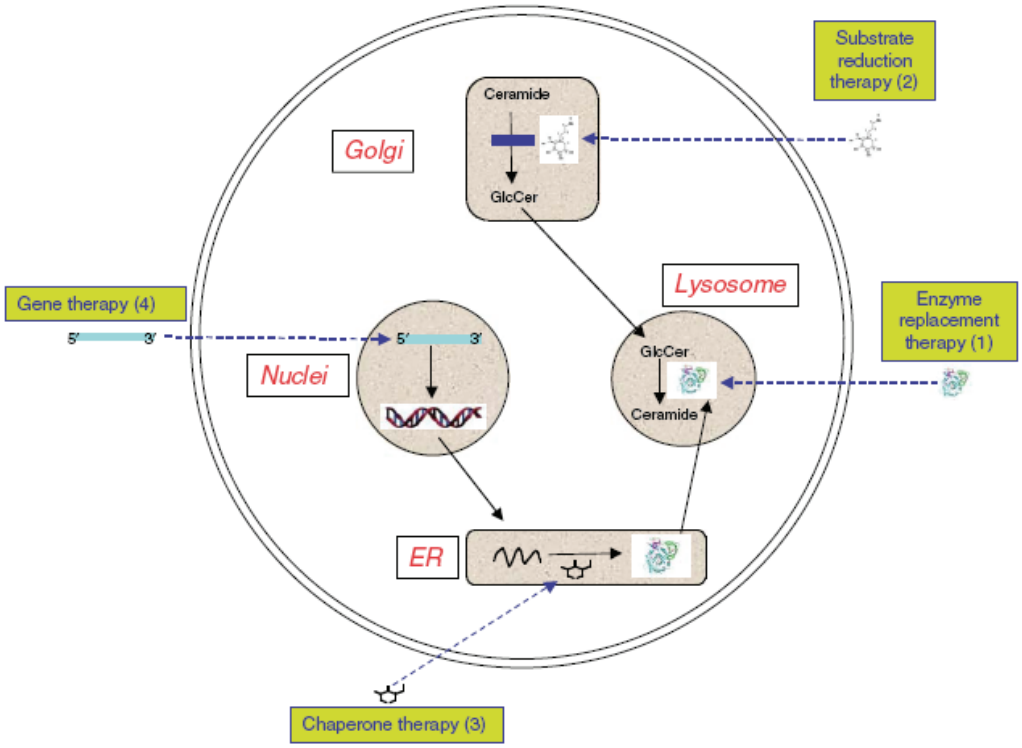

Figure 30: therapeutic options in sphingolipids storage diseases. Examples are shown for Gaucher disease, in which GlcCer accumulates (Kacher and Futerman 2006).

3.2.2. Sphingolipids in neurodegeneration

Sphingolipids are enriched within neural tissue and have been studied for their involvement in neurodegenerative disorders, especially Alzheimer's disease (AD) (Zeidan and Hannun 2007). This illness is characterized by the presence of amyloid plaques and extensive neuronal apoptosis. Complex glycosphingolipids were shown to mediate transport of amyloid precursor protein (APP) to the cell surface, and this transport then promotes proteolytic processing and β-amyloid (Aβ) formation (Tamboli *et al.* 2005). Moreover, several studies indicate that ceramide level is increased at the very earliest clinical stage of the disease, promoting amyloid beta-peptide biogenesis (Satoi *et al.* 2005; Jana *et al.* 2009). Therefore, in the current absence of effective therapies for AD, targeting sphingolipid metabolism affords a promising strategy for future drug design.

In addition to adult neurodegenerative diseases, sphingolipids have been implicated in various forms of childhood neurodegeneration. Neuronal ceroid lipofuscinoses (NCL, also

known as Batten's disease), a major cause of neurodegeneration in childhood, is characterized by mental and motor deficits, seizures and early lethality (Zeidan and Hannun 2007). Of the nine variants of the disease (CLN1–9), six genes have been identified thus far (Mole *et al.* 2005). A recent study identified a defect in dihydroceramide synthase activity in cells derived from CLN9 patients, suggesting that the unidentified CLN9 protein could be a regulator of dihydroceramide synthase. Importantly, transfection of either mammalian dihydroceramide synthase (LASS) or its yeast homolog (Lag1) rescued the abnormal growth phenotype observed in CLN9-deficient fibroblasts (Schulz *et al.* 2006). Therefore, manipulation of dihydroceramide synthase activity might be a novel therapeutic approach for CLN9 patients.

Additional evidence supporting the role of sphingolipid metabolism in neural development comes from studies on genetic lipid-storage disorders in humans. Indeed, deficiency of specific enzymes of sphingolipid metabolism has drastic effects on the nervous system and often leads to neurodegeneration, shortened lifespan and early lethality (Kolter and Sandhoff 2006).

3.2.3. Sphingolipids in microbial pathogenesis

Ceramide has been shown to facilitate the entry of a variety of pathogens. Thus, it was demonstrated that different bacterial, parasitic and viral infections activate the acid sphingomyelinase, induce the translocation of the enzyme onto the cell surface and trigger the release of ceramide and the formation of ceramide-enriched membrane platforms that facilitate invasion (Fox *et al.* 2006; Schenck *et al.* 2007; Zeidan and Hannun 2007). Indeed, receptors for certain pathogens (such as GalNAc glycoproteins for *Cyptosporidium parvum* and CEACAM receptor for *Neisseria gonorrhoeae*) were found to be enriched within ceramide domains and thus to facilitate microbial entry (Grassme *et al.* 1997; Nelson *et al.* 2006). Ceramide-enriched membrane domains were also shown to mediate *Pseudomonas aeruginosa* internalization, a critical step for clearance of this Gram-negative pathogen by bronchial epithelial cells. Indeed, internalization of *P. aeruginosa* leads to apoptosis of the infected cells, limiting systemic inflammatory responses and interleukin-1-induced septic death of infected mice (Grassme *et al.* 2003).

Furthermore, viruses such as HIV and rhinoviruses appear to utilize ceramide-enriched microdomains to invade epithelial cells and consequently manipulate the host machinery to replicate, survive, and finally infect neighboring cells (Zeidan and Hannun 2007). But, this

ceramide-induced increased infectivity has been linked to inactivation of the pathogen. Indeed, complementary studies have reported that treatment, with fenretinide which increases *de novo* ceramide biosynthesis or with exogenous sphingomyelinase, increased ceramide and inhibits HIV infection (Finnegan *et al.* 2004; Finnegan and Blumenthal 2006).

Interestingly, current evidence indicates that certain pathogens continue to modulate enzymes of the sphingolipid metabolism after the internalization step. Several studies have demonstrated that the intracellular pathogen *Mycobacterium tuberculosis* can inactivate SK within phagocytic cells of the host, favorising survival of *M. tuberculosis* in its host. Whereas other pathogens like *Cryptococcus Neoformans* utilize their own sphingolipid metabolism as virulence factors (Heung *et al.* 2006).

Therefore, one could envisage rational modulation of the sphingolipid metabolism within the host or pathogen as a novel antimicrobial therapy (Heung *et al.* 2006).

3.2.4. Sphingolipids in inflammation and immune response

Inflammation is a local and systemic response to infectious, allergic or injurious agents that can lead to pathological consequences when left uncontrolled. A growing body of literature supports a pleiotropic role for S1P and C1P in the inflammatory cascade (Chalfant and Spiegel 2005).

Coordination of S1P and C1P production by their respective kinases (SK and CK) is essential for the production of eicosanoid inflammatory mediators such as prostaglandins (Figure 30). Via IL-1β and TNFα treatments in cell-culture models, it was shown that the CK/C1P and SK1/S1P pathways independently activate two key rate-limiting enzymes, cytosolic phospholipase A2 (cPLA2) and cyclooxygenase 2 (COX-2), respectively, in prostanoid production (Chalfant and Spiegel 2005). Whereas C1P appears to regulate cPLA2 activity via direct binding, S1P exerts indirect regulation of COX-2 mRNA levels. The role of sphingolipids in the TNF/PGE2 pathway (Figure 31) was extended in a recent study, which demonstrated that breakdown of ceramide by acid ceramidase is essential for providing SK1 with sphingosine as a substrate (Zeidan *et al.* 2006).

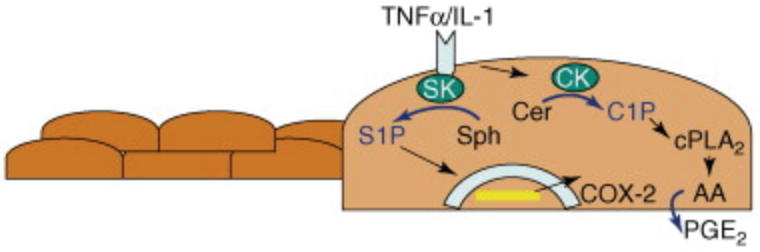

Figure 31: **role of S1P and Cer1P in inflammation.** In response to an inflammatory insult, sphingosine kinase (SK) and ceramide kinase (CK) coordinate eicosanoid biosynthesis (PGE2) by regulating COX-2 and cPLA2, respectively (Zeidan and Hannun 2007).

Besides prostaglandin production, S1P and C1P also regulate the inflammatory cascade. Neutrophil migration, extravasation and priming are early inflammatory events involving S1P generation (Ibrahim *et al.* 2004). Mast-cell degranulation, a critical step in allergic responses, requires S1P and C1P production downstream of Fc epsilon receptor I (FcεRI) crosslinking with IgE (Prieschl *et al.* 1999) (Figure 32).

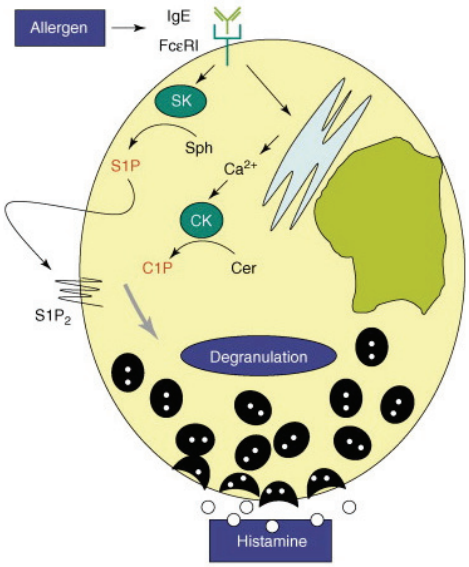

Figure 32: role of S1P and Cer1P in allergic response. In response to allergen exposure, activation of SK and CK downstream of FcεRI are essential steps during mast-cell degranulation and subsequent histamine release (Zeidan and Hannun 2007).

Therefore, based on the literature, one could envision SK, CK and S1PRs as potential targets for anti-inflammatory therapy.

A leading example in sphingolipid-based anti-inflammatory therapeutics is FTY720 (also called fingolimod), a sphingoid base and synthetic analog of myriocin. *In vivo*, FTY720 is phosphorylated by SK2 (and SK1 at higher concentrations). FTY720P induces lymphopenia by sequestering lymphocytes in lymphoid tissues by acting as a high-affinity agonist for S1P

G protein-coupled receptors (GPCRs) (Graler and Goetzl 2004; Matloubian *et al.* 2004). Remarkable effects of FTY720 have recently been demonstrated in a randomized clinical trial involving patients with relapsing multiple sclerosis (Kappos *et al.* 2006). Therefore, modulation of S1P signalling by FTY720 is becoming a promising intervention for treatment of immune-related disorders. Currently, FTY720 is undergoing clinical trials for its utilization as an immunosuppressant for treatment of autoimmune diseases (Zeidan and Hannun 2007).