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## Ceramide signaling in cancer and stem cells

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### Abstract

Most of the previous work on the sphingolipid ceramide has been devoted to its function as an apoptosis inducer. Recent studies, however, have shown that in stem cells, ceramide has additional nonapoptotic functions. In this article, ceramide signaling will be reviewed in light of ‘systems interface biology’: as an interconnection of sphingolipid metabolism, membrane biophysics and cell signaling. The focus will be on the metabolic interconversion of ceramide and sphingomyelin or sphingosine-1-phosphate. Lipid rafts and sphingolipid-induced protein scaffolds will be discussed as a membrane interface for lipid-controlled cell signaling. Ceramide/sphingomyelin and ceramide/sphingosine-1-phosphate-interdependent cell-signaling pathways are significant for the regulation of cell polarity, apoptosis and/or proliferation, and as novel pharmacologic targets in cancer and stem cells.

### Keywords

apoptosis; cancer; cell polarity; ceramide; ES cells; lipid rafts; sphingomyelin; sphingosine-1-phosphate

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### Ceramide & its derivatives: from metabolism to cell signaling

The structural core of ceramide is the sphingoid base sphingosine (Figure 1), a lipid that is synthesized from the amino acid serine and the activated fatty acid palmitoyl-CoA (Figure 2). Sphingosine (or sphingenine) was first isolated and characterized by the German pathologist and ‘father of neurochemistry’ Johann Ludwig Wilhelm Thudichum (1829–1901) [1,2]. He coined the term sphingolipids, which is derived from ‘sphingos’, the genitive of Sphinx. In Greek, ‘sphingo’ means ‘to strangle’. According to the legend, a fate of death was meant for everyone who could not solve the riddles posed by the Sphinx. Quite ironically, this is how many sphingolipid researchers may feel when faced with analyzing the function of ceramide. The riddle of ceramide, its function and how it acts as signaling lipid, is far from being solved. To ease our fears when confronted with the Sphinx in sphingolipid biochemistry, a related Greek word, ‘sphingein’, meaning ‘to bind tight’, alludes to the possibility of solving the enigma of ceramide by identifying its binding proteins.

In addition to ceramide, more than a hundred different sphingolipids are known. The most comprehensive collection of sphingolipids, with respect to their characteristics and structures, can be found at [401], a Webpage supported by the National Institutes of General Medical Sciences [3,4]. Sphingolipids are essential components of cellular membranes and have been

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implicated in a variety of biological functions (Figure 3) [5–27]. Among these, their roles as pro- or anti-apoptotic and pro- or anti-proliferative signaling lipids are the most important. Most recently, our group has found that establishment of cell polarity during embryonic development is another biological process regulated by sphingolipids [28]. To define a profile of functions for individual sphingolipids is difficult because of their rapid metabolic interconversion. To add to this predicament, several derivatives of ceramide have functions similar to ceramide itself. Hence, novel techniques are needed that will help to unravel the function of individual sphingolipids.

In the ‘protein world’, the function of a protein is defined by its interaction with another protein and the biological effect of this interaction. Because of this, knockout mice of two interacting proteins often show similar phenotypes, which is of tremendous help to achieve an ‘educated guess’ on candidate proteins to test for interaction. The characterization of protein interaction has become standard procedure: two-hybrid assays identify all associated proteins, ectopic expression and coimmunoprecipitation/pull-down assays confirm the interaction, and *in vitro* mutagenesis narrows down binding domains. Perhaps at the end, a pharmacologic approach to weaken or strengthen the interaction is tested to foster a translational goal. The scenario looks quite different in the ‘lipid world’. Certainly, it is still the interaction of a lipid with another lipid or a protein that defines its biological activity. However, there is not yet a protein–lipid two-hybrid assay, coimmunoprecipitation assays are difficult because of a lack of efficient antilipid antibodies, and mutagenesis of a lipid demands for rather complicated synthetic chemistry. Nevertheless, in order to define a cell-signaling function of a lipid, it is inevitable to identify the interface to the protein world – the initially interacting protein that induces a signaling cascade potentially regulated by the lipid.

A major drawback in identifying the interfacing protein between lipid and protein-dependent cell signaling is that the proteins with which they interact are known only for a few sphingolipids [29]. However, there have been successful studies to determine binding proteins and even specific binding domains for many other signaling lipids. For example, phosphatidylinositolphosphates (PIPs) bind to the pleckstrin homology (PH) domain and structurally related domains [30,31]. Diacylglycerol (DAG), an important pro-proliferative lipid, interacts with the C1 domain of classical PKC (PKC $\alpha$ ) and PKD [31–35]. Within the family of sphingolipids, binding partners have only been specified for ceramide [29,36–46], ceramide-1-phosphate [47–49], sphingosine-1-phosphate (S1P) [19,50–52] and some gangliosides (e.g., GM1).

## Ceramide-binding proteins: from interaction to action

Starting 20 years ago, landmark studies from Hannun’s and Kolesnick’s laboratories described the effect of ceramide on ceramide-activated protein phosphatase(s) [36,37,53–56] and kinase (s) [46,57–59], respectively. The activating effect of ceramide on protein phosphatase (PP)1 and PP2a is now well established. Recent studies have shown that ceramide activation of PP1 alters splicing of Bcl-x and caspase 9 from anti- to pro-apoptotic proteins [60,61]. Most recently, ceramide activation of PP1 $\gamma$  in a confluent cell culture has been shown to lead to dephosphorylation of  $\beta$ -catenin [62]. It has been known for a long time that ceramide-activated PP2a is involved in dephosphorylation of a variety of key factors in cell-signaling pathways regulating proliferation, apoptosis and differentiation [36,54,63,64]. Ceramide-induced repression of telomerase has been found to arrest cells in the G1 phase, an elegant way to explain the anti-proliferative effect of ceramide [65,66]. New studies suggest that this is caused by activation (possibly mediated by PP2a) of histone deacetylase by C18 ceramide, which deacetylates and inactivates SP3, a transcription factor that upregulates telomerase expression [67,68].

In addition to phosphatases, protein kinases have been suggested to be regulated by ceramide. Within the group of protein kinases, a ceramide-activated protein kinase, which was later characterized as kinase suppressor of Ras (KSR) was the first protein kinase found to be activated by ceramide [46,57]. It has been shown that dephosphorylation of serine 136 in the proapoptotic protein Bad is a mechanism by which ceramide activation of KSR triggers apoptosis via inactivation of the Ras–Raf1/MEK1 pathway [69]. Interestingly, ceramide-mediated suppression of a phosphorylation pathway (here from Ras to Bad) together with ceramide activation of phosphatases may complement each other in cell signaling, a principle on which we elaborate in one of the following sections.

Several laboratories have independently shown that the PKC family is regulated by ceramide and other sphingolipids [28,38–44,70–76]. Classical PKC $\alpha$  was the first PKC isoform found to be regulated by sphingolipids [70,73,75–77]. From early on, it was recognized that sphingosine could be an inhibitor of PKC because serine is phosphorylated by PKC and serine is a structural component in sphingosine (Figure 1). Indeed, *in vitro* data suggested that positively charged sphingosine inhibits PKC $\alpha$  by simple displacement and charge neutralization [77]. From there on, many studies used sphingosine as a specific inhibitor of PKC $\alpha$ , although the physiological function of this interaction is still unclear. Soon after these initial studies, PKC $\alpha$  was also found to be inhibited by ceramide in cells, although this inhibition appears to be mediated by ceramide activation of PP1-catalyzed dephosphorylation of PKC $\alpha$  [75,78].

In 1994, Moscat's group described for the first time that PKC $\zeta$  is activated by ceramide, which was found to inhibit nuclear factor (NF)- $\kappa$ B, a major cell-survival transcription factor in neural and other cell types [38]. Soon thereafter, these results were confirmed by Pfizenmaier's group, who proposed that ceramide may activate or inhibit PKC $\zeta$  depending on its concentration [39]. From there on, ceramide-dependent regulation of PKC $\zeta$  has been found by several other laboratories, including our group, which has specialized on the biological effect of this interaction in cancer and stem cells [28,40–43,72,79–82]. PKC $\zeta$  belongs to the group of atypical PKCs (aPKCs), a family of three highly similar isoenzymes termed PKC $\zeta$ ,  $\lambda$  and  $\tau$  [83–90]. Although identical in their activity *in vitro*, knockout mice for PKC $\zeta$  and  $\lambda$  have clearly demonstrated that these two isoforms have redundant as well as distinct functions. Loss of PKC $\lambda$  is embryonic lethal, while PKC $\zeta$  deficiency does not disrupt embryonic development, but affects NF- $\kappa$ B and insulin signaling and results in impaired glucose transport [91–94]. The function of aPKC for embryonic development and the potential involvement of ceramide in its regulation will be discussed in one of the following sections.

Considering the convincing evidence that ceramide activates aPKC, it is logical to speculate that there is a potential binding site for ceramide. aPKC contains three major protein domains that are common to all of the members of the PKC family, as shown in Figure 4 [84]. The N-terminal pseudosubstrate domain folds back onto the active site of the C-terminal catalytic domain. This 'self inactivates' aPKC, unless binding of an activating factor or phosphorylation 'opens up' the protein conformation and primes the enzyme for further activation. In PKC $\alpha$ , two factors are known to activate the enzyme: calcium and DAG [95]. Calcium binds to the C2 domain (absent in aPKC), which is part of a polypartite regulatory protein moiety located between the pseudosubstrate and catalytic domain. A second motif found in all of the members of the PKC family, KSR and DAG kinase, is the C1 domain, which in PKC $\alpha$  mediates activation of the enzyme by DAG and phorbol ester [33,95]. Since DAG is structurally similar to ceramide, it was suggested that a relative of the C1 domain, the modified phorbol ester/diacylglycerol binding (C1B) domain, may bind to ceramide in aPKC [32,96,97]. In addition to aPKC, the C1B domain is present in PKC $\delta$ , which has led to several studies determining the function of ceramide for the regulation of this enzyme [71,98]. It should be noted that in addition to ceramide-activated phosphatases or kinases, several other proteins have been found

to bind to ceramide [29]. Apart from enzymes using ceramide as a substrate, cathepsin D and ceramide transport protein (CERT) have been shown to associate with ceramide. Ceramide binding of endosomal cathepsin D is involved in the induction of apoptosis by acid sphingomyelinase (ASMase), an enzyme that will be discussed in one of the following sections [99,100]. In this section, we will also elaborate on the function of CERT. Among the ceramide-binding proteins, only ceramide-activated phosphatases and kinases have been found to participate in major regulatory cell-signaling pathways. Therefore, we have focused on the effect of ceramide on aPKC based on its involvement in embryonic development and stem cell differentiation [42,43].

It was not until recently that evidence for the direct binding of aPKC to ceramide was found [38,41–43,101]. Ceramide, and probably many ceramide analogues, form structurally organized lipid microdomains in the cell membrane [40,99,102–107]. These microdomains, also referred to as lipid rafts (note discussion on the nature of ceramide microdomains in the following section), may allow for the repeated or multiple binding of ceramide-associated proteins, such as aPKC, thereby enhancing the formation of protein complexes with cell-signaling functions. Therefore, it is tempting to speculate that ceramide-induced rafts and raft-associated proteins form an initial platform for growth factor or cytokine-dependent cell-signaling pathways.

### The rising of the raft: interfacing membrane biophysics & cell signaling

In chemistry, an interface is defined as the surface boundary between two phases (e.g., solid and liquid). An interface is also the interconnection point between two signaling networks. In our concept of systems interface biology, the plasma membrane is viewed as a surface interface that converts extracellular cues into the activation of intracellular cell-signaling cascades. Lipids in the membrane support this process by generating a signaling platform that brings together receptors and other signaling molecules. Therefore, oligomerization and avidity (multiple binding) of receptors and other signaling molecules is likely to be catalyzed by lipid rafts, which, in principle, is based on a process of lipid aggregation and coalescence forming specific lipid microdomains in the cell membrane. Rafts are defined as lipid microdomains with ordered (Lo) structure in the liquid phase of the membrane [108–120]. The terms lipid microdomains, rafts and detergent-insoluble membranes are often used interchangeably. However, the characterization of detergent-insoluble membranes containing specific phospho- and sphingolipids occurred earlier than the concept of the lipid raft. It dates back to several studies from 1972 to 1974 showing that alkaline (carbonate) or cold Triton X-100<sup>TM</sup>-insoluble (Sigma Chemical Co., MO, USA) membrane fractions were enriched in cholesterol and sphingolipids, in particular sphingomyelin and glycosphingolipids [121,122]. 10 years later, Hakomori's group found that detergent-insoluble membranes that contain glycosphingolipids (i.e., the ganglioside GM3) sustain cell attachment [123]. In the following decade, detergent-insoluble membranes were linked to many processes of cell signaling and were suggested to represent the equivalent of lipid rafts or microdomains. In principle, all of the cell-signaling pathways known to be affected by sphingolipid and cholesterol metabolism or modification, such as Ras, TNF- $\alpha$ , IL-1, TGF $\beta$ , Fas/CD95, Sonic hedgehog, Wnt and insulin, are potentially regulated by lipid rafts [124–130].

Unfortunately, it has turned out to be difficult to directly visualize lipid rafts, which is important for demonstrating their biological significance. One of the main reasons for this shortcoming was the unavailability of raft-specific probes that can be used for fluorescence microscopy. While proteins known to segregate to detergent-insoluble membranes can be expressed as fluorescent protein (e.g., green fluorescent protein [GFP])-tagged probes, the lipid portion is more difficult to label. Nitrobenzoxadiazole (NBD) and borondipyrromethene (Bodipy)-labeled cholesterol or sphingolipids often behave distinctly from their nonconjugated

counterparts [131,132]. Antibodies against raft lipids or binding proteins such as cholera toxin subunit B (for GM1 binding) are not always specific and can only be used in fixed tissues because they change the distribution of their associated lipid by binding to it. In the last 5 years, 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan) has emerged as a raft-specific probe that shows a blue shift of fluorescence emission when packed in microdomains of living cells [133–137]. Another promising development to visualize lipid rafts in living cells and tissues is the generation of nonlytic, fluorescently labeled lysenin, an earthworm-derived protein that binds specifically to sphingomyelin microdomains [138]. However, a concern similar to that with lipid-specific antibodies arises with respect to the prolonged effect of binding proteins to the distribution of their associated membrane lipids. On the protein side, glycosylphosphatidylinositol-conjugated proteins (e.g., Thy-1, CD59 and neural cell adhesion molecule [NCAM]), caveolin and flotillin are generally used as raft markers [139–141]. However, regardless of the method of visualization, it is recommended to confirm the colocalization or codistribution of potential raft lipids and proteins using both fluorescence microscopy and density-gradient centrifugation of detergent- or alkaline-insoluble membranes.

Lipid microdomains have been found to be specific for individual sphingolipids. Gangliosides, such as GM3 and GM1, form glycosphingolipid rafts that are important for the activation of B cells via so-called ‘glycosynapses’ [142,143]. Other lipid microdomains are involved in caveolin-mediated internalization of endocytotic vesicles [125,143,144]. These caveolae are distinct from glycosphingolipid rafts [143].

Recently, evidence has amounted suggesting that ceramide forms specific microdomains termed ceramide rafts [145–147]. These rafts are segregated from sphingomyelin-containing microdomains, although they can be derived from them [103,104,127,148]. The raft nature and significance of ceramide microdomains is still a matter of debate. The ‘classical’ sphingomyelin/cholesterol-rich raft is commonly accepted as being in the Lo state with low membrane fluidity. By contrast, the biophysics of ceramide rafts is less clearly defined. In some studies, ceramide rafts are described as another, highly ordered state or, alternatively, as lipid microdomains that rather perturb sphingomyelin-rich rafts leading to increased membrane fluidity [102,103,149]. It is clear that these two models are distinct from each other. While highly ordered ceramide rafts may organize their own specific cell-signaling platforms, ceramide as a fluidizer inactivates pre-existing, raft-induced cell-signaling pathways. The final decision on which model is biologically significant has not yet been found because of the notorious intricacies involved in the visualization and detection of lipid rafts in living cells. However, it may very well be that both models are significant in different contexts. The variety of biological functions of ceramide, ranging from apoptosis to cell polarity, is unlikely to rely on a single biophysical process. Instead, the biophysical effect of ceramide, as a raft organizer or perturber, may be regulated by the fatty acid species within ceramide (e.g., variation in chain length and degree of saturation), the overall lipid composition of the membrane or localized areas of it (e.g., proportions of ceramide, sphingomyelin and cholesterol) and the proteins associated with rafts. Remarkably, the two models for ceramide microdomains have in common that ceramide and sphingomyelin microdomains are segregated, but can still be metabolically converted into each other.

## **Sphingolipid biosynthesis in the endoplasmic reticulum: enzymology & pharmacology**

Sphingolipid metabolism is maintained in different subcellular compartments [9,15,17,150–155]. It all starts in the endoplasmic reticulum (ER), where condensation of serine with palmitoyl-CoA generates 3-ketodihydrosphingosine (ketosphinganine) (Figure 2). This reaction is catalyzed by serine palmitoyltransferase (SPT), a membrane-resident enzyme consisting of two subunits, SPT1 (SPTLC-1) and SPT2 (SPTLC-2) [156–162]. Most recently,

a third subunit, SPTLC-3, has been described and suggested to form a high molecular mass complex with the other two subunits [163]. Conventional knockout mice with SPTLC-1 or -2 deficiency are embryonic lethal well before gestational day E14 [161]. Since embryos could not be recovered from these knockout mice at any developmental stage, the exact time point of embryonic lethality is unknown. It may be well before neural tube closure or gastrulation, suggesting an essential role of sphingolipid biosynthesis for embryo development. This significance appears to be evolutionarily well conserved, since SPT deficiency in *Saccharomyces*, *Aspergillus*, *Arabidopsis* and *Drosophila* leads to developmental abnormalities [164–169]. In humans, the only well-characterized congenital disease associated with mutant SPT is hereditary sensory and autonomic neuropathy type I, an autosomal-dominant disorder of the peripheral nervous system caused by mutations of the gene coding for SPTLC-1 on chromosome 9q22.1–22.3 [170–174]. Another disease recently found to be associated with abnormal SPT activity is psoriasis, an inflammatory skin overgrowth, which may result from perturbed ceramide levels in the skin [175,176]. However, many other diseases, ranging from atherosclerosis to cancer, appear to be linked to dysregulated sphingolipid biosynthesis, underlining once more the biological significance of ceramide and its derivatives [11,25,63,146,161,177–182].

Several pharmacologic inhibitors of sphingolipid biosynthesis are available. Myriocin, or ISP-1, was first isolated from thermophilic fungi such as *Mycelia sterilia*, and it is now commonly used in cell culture for loss-of-function studies by inhibiting *de novo* sphingolipid biosynthesis [183–187]. It is complemented by fumonisin B1 (FB1), an inhibitor of ceramide synthase, a later enzymatic step in the *de novo* and salvage pathways of sphingolipid biosynthesis (Figure 2) [188–190]. To clarify the definition of these two processes; *de novo* biosynthesis includes the initial generation of the sphingoid base (ketosphinganine) by SPT, while the salvage pathway reacylates the sphingoid base (sphingosine) from degraded sphingolipids, which is catalyzed by ceramide synthase (Figure 2). Its inhibitor, FB1, is a teratogen that causes neural tube defects in animals and humans who consume *Fusarium* mold-contaminated food [190–194]. Currently, several novel SPT and other enzyme inhibitors for sphingolipid biosynthesis are being tested for their pharmacologic relevance [185,195–198]. These inhibitors are likely to be effective in diseases accompanied by degenerative or inflammatory processes. For example, myriocin has been found to prevent ceramide-induced apoptosis in a lung emphysema mouse model [199,200], and it has also shown ameliorative effects in genetic mouse models for atherosclerosis [201]. Unfortunately, myriocin and FB1 are too toxic (mainly for liver and kidney) to find broad systemic application in humans. However, a structural analogue of myriocin, FTY720 (Figure 1), is of pharmacologic use in clinical trials, which will be discussed in the final section.

How about activators of sphingolipid biosynthesis that could induce apoptosis by ceramide elevation in cancer cells? A very effective activator of ceramide generation is UV and  $\gamma$ -irradiation, which is clinically relevant but does not necessarily rely on sphingolipid biosynthesis. The reactions underlying (ir)radiation-induced ceramide generation will be discussed in one of the following sections. Several pharmacologic anticancer compounds have been shown to elevate sphingolipid biosynthesis by upregulating the gene expression or activity of the respective enzymes. Prominent examples are daunorubicin, etoposide and tamoxifen, which appear, at least in part, to act via ceramide-induced apoptosis [65,181,202–206]. Further promising agents to treat cancer are inhibitors for enzymes that metabolize ceramide, such as ceramidase or glucosyl-transferase (Figure 2) [207–211]. Ceramide itself is rather difficult to use as a drug because of its poor water solubility and rapid physiological degradation. However, recent attempts to deliver ceramide in liposomes or nanovesicles may allow for new therapeutic strategies [212,213]. In addition, water-soluble analogues of ceramide may play an important role in eliminating cancer cells or replacing ceramide when it is needed [79,214]. Our laboratory has, for the first time, synthesized novel ceramide analogues on the serinol basis (*N*-acylated

2-amino-1,3-propanediol) (Figure 1), the application of which will be discussed in a later section [43,79].

Approximately 40 years ago, it was thought that sphingosine (Figure 1) was directly derived from sphinganine and that sphingosine was acylated by ceramide synthase to generate ceramide [215,216]. This view has changed more than 10 years after the original labeling experiments with radioactive serine and palmitoyl-CoA [217,218]. The commonly accepted pathway for *de novo* biosynthesis of ceramide is now based on the generation of dihydroceramide as the first step followed by reduction to ceramide by dihydroceramide desaturase (Figure 2). Sphingosine is derived from ceramide by hydrolysis of the amide bond, which is catalyzed by ceramidase.

In addition to sphinganine, ceramide synthase accepts sphingosine as a substrate for the acylation reaction, which is an essential step in the previously mentioned salvage pathway of ceramide biosynthesis (Figure 2). Recently, this pathway has gained attention since many processes involving ceramide as an apoptosis inducer appear to be related to ceramide in a perinuclear, possibly endosomal, compartment [219]. The salvage pathway is also interesting with respect to the fatty acid specificity of ceramide synthase. To date, six ceramide synthase isoenzymes have been described, which are encoded by the longevity assurance (*lass*) genes 1–6 [220–223]. Each isoenzyme shows group specificity for particular fatty acids, leading to the *lass* gene-controlled biosynthesis of ceramide and ceramide derivatives with a specific fatty acid composition. This composition may very well determine the biophysical properties of ceramide, as discussed in the previous section.

C16 ceramide (Figure 1) is a prevalent physiological ceramide species, the biosynthesis of which is catalyzed by the gene products of *lass 5* and *6*. We have found that in hippocampal tissue derived from a mouse model of early-onset Alzheimer's disease (PS1M146V presenilin 1-knockin mouse), the gene expression of *lass 2* and *4* is upregulated [224]. The tissue consequently contains four- or 8.5-fold more long chain ceramide of the C20 and C24 type, respectively. This ceramide species induces apoptosis in PS1 (PSEN1) mouse-derived astrocytes, which may contribute to the etiology of Alzheimer's disease. The salvage pathway could rapidly convert various ceramide species and derivatives to proapoptotic ceramide, thereby enhancing degenerative processes.

## Spingolipid metabolism in the Golgi: ceramide transport & exit to the cell membrane

Before the salvage pathway can utilize ceramide, it has to be converted into its derivatives. To achieve this, ceramide is transported from the ER to the Golgi apparatus following two distinct transport routes (Figure 2). Until recently, ceramide was thought to be transported exclusively via lipid vesicles. However, 5 years ago, Hanada showed that a cytosolic protein, namely Goodpasture antigen-binding protein, picks up ceramide from the ER and delivers it to the Golgi [225,226]. From there on, Goodpasture antigen-binding protein has been termed CERT. The two important binding domains of CERT are the pleckstrin homology (PH) domain, which anchors the protein to specific sites within the Golgi, and the steroidogenic acute regulatory protein (STAR)-related lipid transfer (START) domain, a conserved lipid-binding domain that associates with sterols and ceramide [227]. It is now thought that ceramide transported by CERT is used for sphingomyelin biosynthesis while that converted to glycosphingolipids follows the classical vesicular route [225,226]. At this point, it should be noted that ceramide may also distribute throughout specialized ER compartments, such as the perinuclear membrane and the mitochondrial-associated ER membrane (MAM). In the ER, ceramide is known to affect calcium hemostasis, which has been suggested to induce ER (stress)-dependent apoptosis [228–230].

Using ceramide-specific antibodies, we have shown that ceramide is present in ER-associated membranes, such as the perinuclear membrane and MAMs [42,231]. The nucleus and mitochondria themselves contain ceramide; although it is not entirely clear whether ceramide is synthesized in these organelles or transported from the ER as indicated in Figure 2 [232–236]. Recent studies suggest that ceramide is involved in the initiation of apoptosis by forming pores in the outer mitochondrial membrane [237].

After ceramide has been transported to the Golgi, the polar head group flips from the outer (cytosolic) to the inner (luminal) leaflet before sphingomyelin is synthesized. This transbilayer movement appears to occur at a rapid rate, although it may be regulated and will then become a rate-limiting step [27]. In the Golgi lumen, sphingomyelin (Figure 1) is synthesized by sphingomyelin synthase (SMS)1 and then transported to the cell membrane (Figure 2) [238–240]. SMS1 is distinct from SMS2, which regenerates sphingomyelin from ceramide in the cell membrane [241,242].

## Ceramide & sphingomyelin: interfacing sphingolipid, phospholipids & C1 metabolism

Sphingomyelin synthase 1 and 2 utilize phosphatidylcholine (PC) and ceramide as substrates to generate sphingomyelin and DAG in a phospho(ryl)choline transfer reaction. The SMS reaction links major metabolic pathways with cell-signaling function (Figure 5). We have discussed that DAG is an activator of PKC $\alpha$ . It can be used to regenerate PC from CDP-choline (CDP-choline or Kennedy pathway), which can be regenerated from phospho(ryl)choline (via CTP:phosphocholine cytidyltransferase), a product of sphingomyelin hydrolysis by sphingomyelinase (SMase) (Figure 5A) [243–248].

Interestingly, *de novo* synthesis of PC as a substrate for sphingomyelin biosynthesis is immediately linked to methylation reactions of C1 (one carbon unit) metabolism (Figure 5B). One would not expect a shortage of PC since it is abundant in our nutrition and is also synthesized by the liver. However, studies on primary-cultured oligodendrocytes have shown that a major portion of PC is *de novo* synthesized from phosphatidylserine by decarboxylation and methylation reactions (Figure 5B) [249,250]. Therefore, a supply of methyl group donors appears to be essential for sphingomyelin biosynthesis and could explain why deficiency of folate, an essential cofactor for the generation of the methyl group donor *S*-adenosyl methionine (Figure 5B) contributes to neural tube defects. In fact, studies in our laboratory [Bieberich, Unpublished Data] show that embryonic stem (ES) cell-derived neural progenitor cells that express folate-binding protein 1 are also enriched for sphingomyelin (Figure 5C). These results are consistent with studies from our [Bieberich, Unpublished Data] and other groups demonstrating that perturbation of C1 and choline metabolism reduces the level of PC and sphingomyelin in stem cells and mouse embryos, and leads to neural tube defects [251,252]. In summary, concurrent PC and sphingomyelin biosynthesis sustained by C1 metabolism and the postulated CDP-choline/sphingomyelin cycle appear to be important pathways to regenerate sphingomyelin and to ‘detoxify’ and recycle pro-apoptotic ceramide. Perturbation of these pathways may lead to aberrant stem cell differentiation and embryo malformation. CDP-choline or citicoline is currently in clinical trials to treat stroke and cognitive impairment, which may be based on its function as a precursor substrate for sphingomyelin biosynthesis [253].

Another interesting area pursued in our laboratory is the regulation of sphingomyelin biosynthesis by cholesterol derivatives such as oxysterols and bile acids. We found that sphingomyelin biosynthesis in human breast cancer cells is elevated by incubation with 25-hydroxy-cholesterol, concurrent with increased migration of these cells [254]. These results were consistent with previous studies reporting coregulation of cholesterol and sphingolipid



transport and metabolism [255]. In a follow-up study, cell survival and migration of human breast cancer (MDA-MB-231) cells was also found to be enhanced by the secondary bile acid deoxycholate at physiological plasma concentrations (5–10  $\mu\text{M}$ ) [256]. This was very surprising since a wealth of literature describes induction of apoptosis in cancer cells by deoxycholate, probably by activation of SMase and elevation of ceramide [257,258]. However, these studies used deoxycholate at concentrations that were more than tenfold higher than the concentration used in our study. It is quite possible that depending on its concentration, deoxycholate activates or inhibits cell survival in breast cancer cells, which may be mediated by the regulation of ceramide and sphingomyelin biosynthesis.

Previously, ceramide and sphingomyelin have been described as part of the sphingomyelin cycle, however, without invoking CDP-choline and DAG in the Kennedy pathway for PC biosynthesis as intermediate substrates (Figure 5A) [56]. The sphingomyelin cycle links growth factor and cytokine-induced activation of SMase to the interconversion of sphingomyelin and ceramide microdomains/rafts and vice versa [259,260]. There are two main groups of SMases, depending on their enzyme activity at neutral or acidic pH [261]. ASMases are localized in lysosomes or secreted via the Golgi secretory pathway [147]. Deficiency of lysosomal ASMase causes Niemann–Pick disease, a lysosomal storage disease. The secreted form of ASMase associates with the outer leaflet of the cell membrane and is activated by proapoptotic cytokines, such as TNF- $\alpha$  or Fas ligand [105,147]. In addition, ASMase is activated by UV or  $\gamma$ -irradiation, probably via the radiation-induced generation of reactive oxygen species [146, 262,263]. Neutral SMases (NSMases) can also be activated by TNF- $\alpha$ ; however, these enzymes are located at the inner leaflet. Any activation of NSMase will require flipping the polar head group of sphingomyelin toward the inner leaflet before hydrolysis to ceramide can occur. Currently, it is not completely understood how this transfer of the polar head group is achieved. It is thought that a phospholipid-binding protein, a ‘scramblase’ or ‘flippase’, transfers sphingomyelin to the inner membrane leaflet, while at the same time externalizing phosphatidylserine [264–266]. This model would be consistent with phosphatidylserine externalization being an early event in the onset of apoptosis induced by ceramide.

The activation of NSMase has found recent attention owing to its potential link to the binding of NGF to the low affinity neurotrophin receptor p75NTR [259,267–269]. In contrast to the cytokine-activated receptors (e.g., Fas/CD95 and TNF receptor), the p75NTR cell-signaling pathway is not *a priori* proapoptotic. p75NTR is expressed by many neural cell types and induces axonal outgrowth in the peripheral nervous system when stimulated with NGF [267, 270–273]. However, evidence has amounted that p75NTR-induced apoptosis is a major factor in neuro-degenerative diseases such as Alzheimer’s disease [269,274–281]. This apparently paradoxical, dual function of p75NTR, proapoptotic or pro-outgrowth, has been explained by a model suggesting that the effect of p75NTR activation depends on heterodimerization with other neurotrophin receptors [282–292]. If p75NTR forms heterodimers with tropomyosin-related kinase A, a tyrosine kinase receptor, activation of the chimeric receptor induces axonal outgrowth. However, if p75NTR forms homodimers, binding of NGF to p75NTR activates NSMase, which then generates ceramide and may induce apoptosis.

Most recently, our group has found that p75NTR is highly expressed in the hippocampus of the PS1M146V (early-onset Alzheimer’s disease model) mouse. p75NTR has been discussed as a substrate for the  $\gamma$ -secretase subunit of the PS1 or 2 protein complex [293,294]. A deficiency of this secretase could lead to accumulation of p75NTR and, in turn, increased activation of NSMase. In conjunction with elevation of the *lass 2* or *4* gene products, this would be consistent with elevation of long-chain fatty acid ceramide from sphingomyelin and ceramide-induced apoptosis of astrocytes as discussed in the preceding section [224]. Dysregulated receptor turnover may lead to conversion of sphingomyelin to ceramide rafts and, in turn, induce apoptosis in a neurodegenerative disease process. In the following section,

further examples for the cell-signaling function of ceramide will be discussed as well as how the generation of a ceramide microdomain is translated into a protein-driven cell-signaling cascade.

### **Rafts-to-SLIPS hypothesis: nucleating the lipid–protein interactome**

The translation of lipid formation into the activation of a particular cell-signaling pathway demands for a mechanism that engages raft-associated lipids into a complex with proteins, the lipid–protein interactome. We introduce the term nucleation to describe a two-step mechanism that is initiated by the association of a lipid with a protein (step 1), which induces a protein–protein interaction to form a larger lipid–protein complex, or interactome (step 2). The working models discussed in this section are based on ideas and studies that originated in our laboratory; however, they should be considered hypothetical until substantiated by further experiments. To date, there are two processes known by which membrane lipids nucleate this interactome resulting from the initial association of a lipid with a protein. Proteins themselves can be covalently conjugated to lipids such as the glycosylphosphatidylinositol-anchor and cholesteryl, geranyl, farnesyl, myristyl or palmitoyl residues [125,139]. Prominent examples relevant to stem cell differentiation and cancer cell proliferation are the morphogen Sonic hedgehog, which is covalently linked to cholesterol and palmitic acid, and the small GTPase Ras, which is modified by farnesylation [125,295]. We and other groups have shown that Ras codistributes with glycosphingolipid rafts that contain GM1 and cholesterol [254]. Blocking farnesylation with farnesyltransferase inhibitors or statins that inhibit hydroxymethyl glutaryl CoA reductase, the enzyme essential for the biosynthesis of farnesyl and cholesterol precursors, will prevent raft localization of Ras [254]. We have shown that these pharmacologic inhibitors reduce migration of breast cancer cells, which indicates that farnesyltransferase inhibitors as well as statins are potential anticancer drugs. Indeed, farnesyltransferase inhibitors as well as statins are currently being tested in clinical Phase I, II and III trials to treat various types of cancer, including lung and mammary carcinoma, lymphoma and melanoma [296–300].

Nucleation of a lipid–protein interactome by lipid modification and raft association of Ras is one way of activating client cell-signaling pathways that induce cell proliferation and migration. The second mechanism relies on nucleation by noncovalent interaction of a protein with a lipid. Approximately 10 years ago, the PH domain was described as the first protein domain specifically binding to PIPs, in particular PIP2 and 3 [301,302]. Currently, a variety of additional PIP-binding domains have been characterized and found to be a part of essential signaling factors, such as small GTPases, Akt/PKB and many others [303–305]. Consistent with the function of these factors, PIPs have been known for a long time to activate cell-signaling pathways for cell polarity, migration and proliferation. However, in contrast to *bona fide* raft lipids such as sphingomyelin, which contain only saturated or monounsaturated fatty acid residues, PIPs are mostly endowed with polyunsaturated fatty acids [306]. These fatty acid chain ‘kinks’ prevent the proper alignment and clustering of PIPs in rafts. To date, it is still not clear whether raft or nonraft association of PIPs is important for their function.

One of the proteins regulated by PIPs is aPKC, the PKC isoform that is also associated with ceramide as discussed in one of the preceding sections. The N terminus of aPKC contains a protein-binding (PB1) domain that interacts with the polarity protein Par6, which associates with the Rho type GTPase Cdc42 (Figure 4). Recently, conditional knockout of Cdc42 in murine neural progenitor cells has shown that it is indispensable for cell polarity in the neuroepithelium and normal brain development [307]. Cdc42 is endowed with a PH domain, which allows for binding to PIP and recruitment of the Par6–aPKC complex (Figure 4) [308–313]. Conditional knockout of aPKC in neural progenitor cells leads to a phenotype that is similar to that of the Cdc42 knockout, suggesting that cell polarity is dependent on a Cdc42–Par6–aPKC polarity complex, the formation of which is regulated by PIPs [91].

Based on the fundamental significance of cell polarity for development, the Cdc42–Par6–aPKC complex should already be initiated when cell polarity is established in the first primitive germ layer, the primitive ectoderm. Recently, we found that this polarity complex is critical for primitive ectoderm formation and depends on ceramide [28]. Inhibition of *de novo* sphingolipid biosynthesis with myriocin prevented primitive ectoderm formation, while incubation with C16 ceramide or the novel ceramide analogue S18 restored this process. In the absence of ceramide, aPKC and Cdc42 were no longer translocated to the membrane and they did not form a protein complex. These results suggested that ceramide is critical for the formation of the aPKC and Cdc42-containing polarity complex. We then performed an *in vitro* binding assay using ceramide vesicles and purified proteins (Figure 6A, left panel). Ceramide vesicles bound aPKC, Par6, and Cdc42 when Cdc42 was activated with GTP $\gamma$ S, but not when it was inactivated with GDP [28]. A similar assay is currently used in our group to identify additional ceramide raft-binding proteins (Figure 6A, right panel). These results confirmed the importance of ceramide for the formation of a polarity complex, which is depicted in Figures 4 & 6B. These figures show our proposed model involving PIPs and ceramide in activation of Cdc42 and recruitment of the polarity complex.

A polarity complex, the nucleation of which is initiated by ceramide, is the role model for a sphingolipid-induced protein scaffold (SLIPS) (Figure 6B), a term we introduced several years ago to explain how ceramide may regulate the cytoskeleton [25]. A SLIPS is an interface linking the ‘lipid world’ (ceramide microdomain) to the ‘protein world’ (cytoskeleton or cell-signaling cascades). Although this is still a matter of our ongoing research, the ceramide-associated Cdc42–Par6–aPKC complex converting ceramide raft formation into the stabilization/regulation of F-actin or microtubules is one of the proposed functions of SLIPS. The immunocytochemistry for ceramide and  $\alpha$ -tubulin (detected with anti-ceramide and antitubulin antibodies) in PKC $\zeta$ –GFP-expressing neural progenitor cells is shown in Figure 6C. There is significant codistribution of ceramide and tubulin (arrows), which confirms previously published data that suggest ceramide/aPKC-dependent regulation of microtubule formation [25]. Other functions of SLIPS depend on the ceramide/aPKC interactome, a group of aPKC-associated proteins, the interaction of which is potentially regulated by binding of ceramide to aPKC. A selection of proteins that are mainly involved in the regulation of cell polarity, migration and adhesion is depicted in Figure 7A. Additional factors associated with or phosphorylated by aPKC are involved in vesicular transport (e.g., p62), alternative splicing of mRNA (e.g., caspase 2) and receptor activation (e.g., ZIP3) [72,83,314–316]. Some of these functions may very well be regulated by the association of aPKC with ceramide. Our group has focused on the effect of ceramide on aPKC-dependent regulation of cell polarity and apoptosis in stem and cancer cells [25,28,42,43,79–82]. The proapoptotic effect is mainly based on the ceramide-induced association of aPKC with prostate apoptosis response (PAR)-4 and Akt, two signaling factors that will be discussed in the following section.

## Ceramide & S1P: close relatives, but conditional antagonists

From the previous sections, it is clear that the function of ceramide is modified by the cell signaling context. The main theme and hypothesis originally developed in our group is that the outcome of ceramide elevation depends on the protein, the association of which with aPKC is induced or strengthened by ceramide. The lipid vesicle-binding assays with purified or cell lysate-derived proteins show that ceramide-associated aPKC will bind to Par6–Cdc42 or PAR-4, most likely depending on their expression level, activation state or accessibility [28, 42]. We termed the mechanistic model describing the outcome of ceramide-induced association of aPKC with either Par6–Cdc42 or PAR-4 the ‘flipside model of ceramide/aPKC interaction’ (Figure 7B). Binding to Par6–Cdc42 will initiate the polarized stabilization of actin filaments or microtubules and sustain cell polarity or cell migration, while association with PAR-4 will lead to cell death. This opposite effect is not surprising since Cdc42 is known to

activate aPKC, while PAR-4 inhibits the enzyme. Hence, even ceramide-induced pre-activation of aPKC may eventually lead to its inhibition if PAR-4 is expressed (Figure 7B). As with SLIPS, this model is considered to be hypothetical until further experimental evidence has been achieved.

Our model is consistent with studies from our laboratory showing that the nonapoptotic function(s) of ceramide depend on a low expression level of PAR-4 [28,42,80,82]. PAR-4 is a leucine zipper protein with several functions. It was discovered by differential hybridization assays to identify proapoptotic genes expressed in androgen-dependent prostate cells [317]. Using two-hybrid assays it was soon found to be an inhibitor of aPKC and a transcriptional corepressor of Wilms' tumor suppressor 1 [318,319]. PAR-4 has recently gained attention owing to its multifaceted function in neural cells. It has been suggested to contribute to neurodegeneration in Alzheimer's and Parkinson's diseases, and to the etiology of amyotrophic lateral sclerosis and stroke [291,320–324]. We have found that there are three stages during ES cell differentiation at which expression of PAR-4 is absent or low: undifferentiated ES cells, ES cell-derived suspension embryoid bodies (EBs) and EB-derived neural progenitor cells [28,42,80–82]. In these differentiation stages of stem cells, ceramide or ceramide analogues are nonapoptotic (undifferentiated ES cells), sustain cell polarity and primitive ectoderm formation (suspension EBs), or induce sphingopodia and process formation (neural progenitor cells). In other differentiation stages, such as early pluripotent EB-derived cells, PAR-4 is highly expressed and conveys sensitivity to ceramide or its analogues. In PAR-4(-) suspension EBs, ceramide is indispensable for normal development. Ceramide depletion prevents membrane association of aPKC, disrupts the interaction between aPKC and Cdc42 and results in decreased phosphorylation of glycogen synthase kinase (GSK)-3 $\beta$  [28]. The novel ceramide analogue S18 restores primitive ectoderm formation, suggesting that it is ceramide and not one of its derivatives that regulates cell polarity. Although this implies a regulatory effect of ceramide on the noncanonical Wnt or cell-polarity pathway, further studies are needed to clarify the significance of ceramide for cell polarity in a wider spectrum of cells.

We propose the working model to explain the function of ceramide for cell polarity in neural progenitor cells and other cell types as shown in Figure 6B. Based on our observation that ceramide microdomains codistribute or even associate with microtubules (Figure 6C) [25, 231], we focus on the effect of ceramide-associated aPKC on GSK-3 $\beta$ . GSK-3 $\beta$  phosphorylates many proteins that regulate cell adhesion or formation of microtubules. Key factors are  $\beta$ -catenin, adenomatous polyposis coli (APC) and tau protein (Figure 8A) [309,325–332]. Phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$  renders it susceptible to proteolytic degradation [309], while hyperphosphorylation of tau causes its aggregation in tauopathy, a neurodegenerative disorder that is also involved in the etiology of Alzheimer's disease [325, 329,330]. Phosphorylation of APC by GSK-3 $\beta$  disrupts its function in stabilizing the plus end of microtubules [309]. According to our model, ceramide-induced activation of aPKC will result in phosphorylation and/or inactivation of GSK-3 $\beta$  (Figure 8A). Hence, ceramide and S18 should stabilize microtubules, while ceramide depletion will destabilize them.

Among the factors that regulate adherens junctions and microtubules, ceramide-activated aPKC and PP2a may complement each other (Figure 8A). It is known that PP2a dephosphorylates  $\beta$ -catenin, APC and tau [326,330]. Hence, loss of phosphorylation by ceramide-mediated inactivation of GSK-3 $\beta$  and enhanced dephosphorylation by ceramide-activated PP2a should act synergistically on promoting the stability of microtubules. Interestingly, GSK-3 $\beta$  can also phosphorylate and inactivate PP2a [333]. Therefore, ceramide can activate PP2a in two ways: by direct binding to PP2a and by inactivating GSK-3 $\beta$  (via aPKC). By contrast, ceramide-activated PP2a may also dephosphorylate GSK-3 $\beta$ , thereby antagonizing phosphorylation of GSK-3 $\beta$  [334]. Clearly, more studies on the ceramide-

dependent interplay of aPKC, GSK-3 $\beta$  and PP2a are needed to unravel these intricate regulatory cell-signaling pathways.

When the proapoptotic form of PAR-4 is expressed, the nonapoptotic effect of ceramide changes fundamentally. Using lipid vesicles consisting of ceramide and phospholipids as described before (the lipid vesicle-mediated affinity chromatography [LIMAC] technique, Figure 6A) we have found that association of aPKC with ceramide will enhance the affinity of aPKC to its inhibitor PAR-4 [42]. In the presence of PAR-4, ceramide will not activate aPKC, but on the contrary, enhance its inhibition by PAR-4 (Figures 4 & 8B). Because of this, an initial non-apoptotic or even prosurvival function of ceramide can rapidly turn into the induction of apoptosis. Most recently, our group has found that in astrocytes from the PS1M146V mouse, the expression of PAR-4 was almost tenfold higher than in wild-type astrocytes [224]. These cells showed tremendously enhanced apoptosis (>tenfold) when incubated with C20 ceramide, which was elevated in the hippocampus of the early-onset Alzheimer's disease mouse model, as discussed previously. Wild-type astrocytes (and neurons) were not affected, clearly showing that the elevated expression of PAR-4 sensitizes neural cells toward ceramide-induced apoptosis. These data are consistent with previous reports demonstrating the involvement of PAR-4 in the etiology of Alzheimer's disease [335]. However, our data extended the earlier studies, in that we demonstrated that aberrant expression of PAR-4 contributes to ceramide-induced glial cell death in a mouse model for Alzheimer's disease.

Our data are also consistent with a recent study reporting that transgenic expression of an active fragment of PAR-4 (SAC domain) protects mice from induced and spontaneous tumors as a result of apoptosis-mediated elimination of cancer cells [336]. We have found that the expression of PAR-4 not only sensitizes stem cells and astrocytes toward ceramide-induced apoptosis, but also particular cancer cells. Interestingly, the aPKC isoform PKC $\lambda/\tau$  has been identified as an oncogene in multiple forms of human cancer [86]. The striking difference between PKC $\lambda/\tau$  and PKC $\zeta$  is that the first one does not contain the caspase 3 cleavage site that converts PKC $\zeta$  into the catalytically active 50 kDa form. Therefore, it is possible that PKC $\lambda/\tau$  can be inhibited by ceramide-induced apoptosis when the expression of PAR-4 is elevated. Hence, concurrent elevation of ceramide and expression of PAR-4 may be a potential strategy to treat cancer by inducing apoptosis.

In addition to its immediate cell-signaling function, ceramide serves as a metabolic precursor for another important cell-signaling lipid, S1P. Ceramide is hydrolyzed by ceramidase to sphingosine, which is then phosphorylated by sphingosine kinase (SK)1 or 2 to S1P (Figures 1 & 2) [13,337–343]. S1P is a soluble ligand that can bind and activate five isoforms of the S1P receptor [29,50–52,109,344–346]. Knockout mice for SK1 and 2 or S1P receptors have clearly demonstrated the essential function of S1P for vascular and neural development [339]. Recent studies indicate that one of the functions of S1P is to counterbalance ceramide-induced apoptosis [341,347,348]. S1P is known to increase phosphorylation of p42/44-MAPK and Akt/PKB, two important protein kinases that inactivate the proapoptotic proteins Bad and Bax (Figure 8B) [25,69,81,344,349–354]. Unlike S1P, ceramide has been shown to reduce the activity of p42/44-MAPK and Akt [40,81,354,355]. Hence, it is tempting to speculate that ceramide and S1P counter-regulate the phosphorylation of Bax and Bad, thereby controlling apoptosis and cell survival (Figure 8B). This may be of importance for the pharmacologic interference with neurodegenerative diseases linked to ceramide-induced apoptosis and for treatment of cancer. In the first case, FTY720 or S1P-receptor agonists may counteract ceramide-induced neurodegeneration, while in the second case, cancer cells may be sensitized to ceramide-induced apoptosis by concurrent inhibition of S1P biosynthesis or S1P-receptor antagonists. Indeed, FTY720 has been shown to promote oligodendrocyte differentiation, and it is currently being tested in Phase III clinical trials to treat multiple sclerosis [356,357].

Although this effect has been attributed to the immunosuppressive activity of the drug, neuroprotection may be another beneficial outcome of FTY720 treatment.

This hypothesis is consistent with data from our laboratory showing that S1P and FTY720 protect a small population ( $\leq 5\%$ ) of neural precursor cells (NPCs), termed NPC2, from ceramide-induced apoptosis [358]. We routinely incubate EB-derived cells with S18 to eliminate residual pluripotent stem cells that will form stem cell-derived tumors if they contaminate the stem cell graft [82]. This method has been recognized as a significant step toward safer stem cell therapy [359–362]. In contrast to the major population of nestin(+)/PAR-4(-) NPC1 cells, NPC2 cells maintain the expression of PAR-4 and are eliminated by incubation with S18 [358]. These cells are protected by adding S1P or FTY720, which underlines the importance of S1P as a metabolically derived antagonist of ceramide (Figure 8B). We are currently investigating the potential of NPC2 cells for neural differentiation after grafting.

While FTY720 may be useful to boost the survival and differentiation of transplanted or endogenous stem and progenitor cells to treat neurodegenerative diseases, inhibition of SK will be a critical step toward cancer treatment by inhibiting cell S1P-dependent survival pathways (Figure 8B) [363–365]. The classical SK inhibitor dimethylsphingosine has already been shown to reduce tumor growth in mouse models, and novel SK inhibitors are currently being developed and tested [366–368]. However, while SK1 generates antiapoptotic S1P, SK2 has been found to generate S1P that is converted to proapoptotic ceramide. Therefore, cancer therapy by inhibition of S1P biosynthesis should specifically target SK1. In conjunction with drugs that elevate ceramide, SK1 inhibitors are promising agents for future cancer therapy. Taken together, the examples discussed in this article clearly demonstrate that ceramide and its derivatives have taken center stage in cell signaling and will certainly surprise us with novel therapeutic interventions in stem cell and cancer therapies.

## Conclusion

The discussion of various aspects of ceramide and sphingolipid biology has demonstrated that research in this field is indeed an example of systems interface biology, a genuine multi-disciplinary approach that attempts to explain cell signaling by sphingolipid metabolism, membrane biophysics, lipid–protein binding and downstream effects on cell polarity, proliferation and apoptosis. Ceramide as a structural interface has been recognized to generate cell-signaling platforms in the plasma membrane (and other subcellular compartments) that translate cues from diffusible factors (e.g., growth factors and cytokines) into the localized and organized activation of intracellular cell-signaling cascades. Ceramide as a metabolic interface has been suggested to interconnect C1, cholesterol, phospholipid and sphingolipid metabolism. Finally, ceramide has been shown to be a signaling interface in that its immediate derivatives are co- or counter-players in regulating cell survival, polarity and differentiation versus apoptosis.

### Executive summary

Ceramide & its derivatives: from metabolism to cell signaling

- Many metabolic derivatives of ceramide have similar cell-signaling functions. To identify a cell-signaling pathway that is specifically regulated by ceramide, it is indispensable to define the signaling proteins with which ceramide interacts.

Ceramide-binding proteins: from interaction to action

- Among ceramide-binding proteins, ceramide-activated protein phosphatases and kinases are the most important. Specific protein phosphatases and kinases have been shown to be activated by ceramide, which includes atypical PKC (aPKC)-dependent cell-signaling pathways for cancer and stem cell polarity and apoptosis.

#### The rising of the raft: interfacing membrane biophysics & cell signaling

- Lipid microdomains or rafts have been proposed to constitute cell-signaling platforms, although their function in living cells is still debated. Ceramide microdomains have been suggested to be segregated from sphingomyelin (SM) rafts, although they can be derived from them. Ceramide microdomain-induced cell-signaling pathways are implicated in the induction of apoptosis.

#### Sphingolipid biosynthesis in the endoplasmic reticulum: enzymology & pharmacology

- Following biosynthesis by the *de novo* or salvage pathway in the endoplasmic reticulum, ceramide is converted to SM in the Golgi. Sphingolipid biosynthesis can be blocked by two pharmacologic inhibitors, myriocin and fumonisins B1. Ceramide analogues can restore the function of ceramide.

#### Sphingolipid metabolism in the Golgi: ceramide transport & exit to the cell membrane

- SM is transported to the cell membrane and serves as a substrate for the generation of ceramide microdomains, which is regulated by the SM cycle. This cycle can be interconnected with major pathways for lipid metabolism.

#### Ceramide & sphingomyelin: interfacing sphingolipid, phospholipid & C1 metabolism

- The interconnection of the SM cycle with lipid metabolism involves the biosynthesis of CDP-choline and phosphatidylcholine (Kennedy pathway). The proposed 'CDP-choline/SM cycle' uses methyl donor groups provided by the C1 (one carbon unit) metabolism and regulates phospholipid and sphingolipid biosynthesis, which is important for the generation of lipid rafts.

#### Rafts-to-SLIPS hypothesis: nucleating the lipid-protein interactome

- When generated by hydrolysis of SM, a ceramide raft-signaling platform will still need to interface with intracellular signaling pathways. Our group has proposed, for the first time, the term sphingolipid-induced protein scaffolds (SLIPS) that link ceramide microdomains via aPKC (or other ceramide-associated proteins) to polarity protein complexes and regulation of the cytoskeleton.

#### Ceramide & sphingosine-1-phosphate: close relatives, but conditional antagonists

- Sphingosine-1-phosphate (S1P) is another derivative of ceramide with pro-proliferative and antiapoptotic function. It may antagonize ceramide by preventing inactivation of cell-survival pathways such as Akt/PKB. This antagonism can be used to prevent apoptotic, degenerative processes (using S1P analogues and neutralizing ceramide) or to eliminate cancer cells (enhancing ceramide and inhibiting S1P) in future combination therapies.

## Future perspective

Translational research has always been important but has gained tremendous attention in the past few years. The purpose of translational research is to translate the results of basic research into clinical application. Ceramide and other sphingolipids are ideally suited for translational research in that they are small, lipophilic molecules regulating basic cell biology. Once the function of a sphingolipid has been determined, it is an immediate structural lead for drug development. In comparison, finding a pharmacological effector for a specific protein-protein

interaction can be a daunting task. Ceramide is already known to regulate protein–protein interactions. Therefore, lipidology of ceramide and other sphingolipids is rewarding and will be full of surprises. To be of translational relevance, future research will also focus on the *in vivo* function of ceramide. Most of our knowledge on ceramide was obtained from cell culture experiments. However, genetic models, such as knockout mice for enzymes in sphingolipid metabolism, will be significant to define novel functions and pharmacologic targets in ceramide biology. From the examples discussed in this article it is clear that understanding sphingolipids will be an important avenue for future therapeutics in cancer and stem cell biology.

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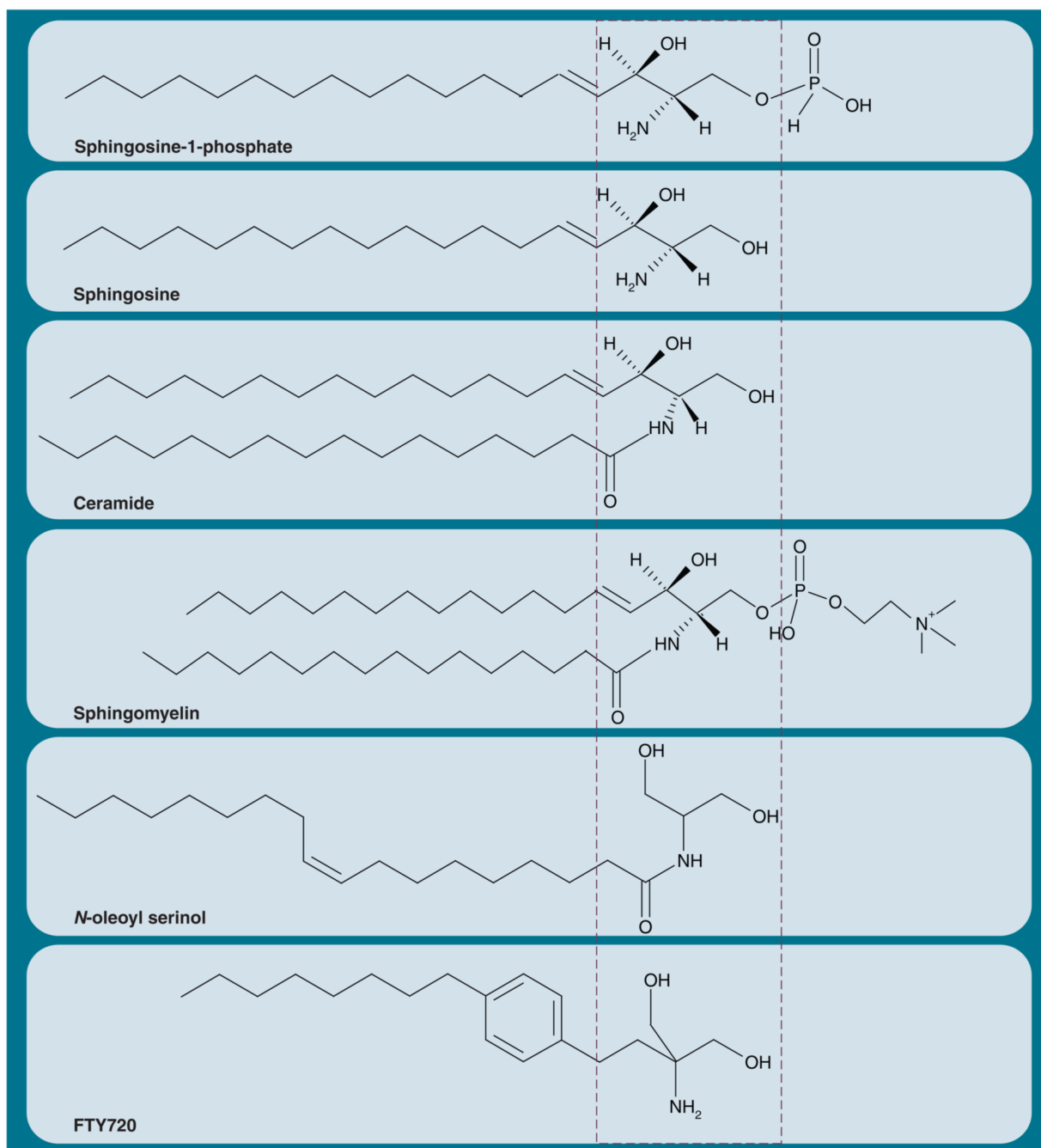
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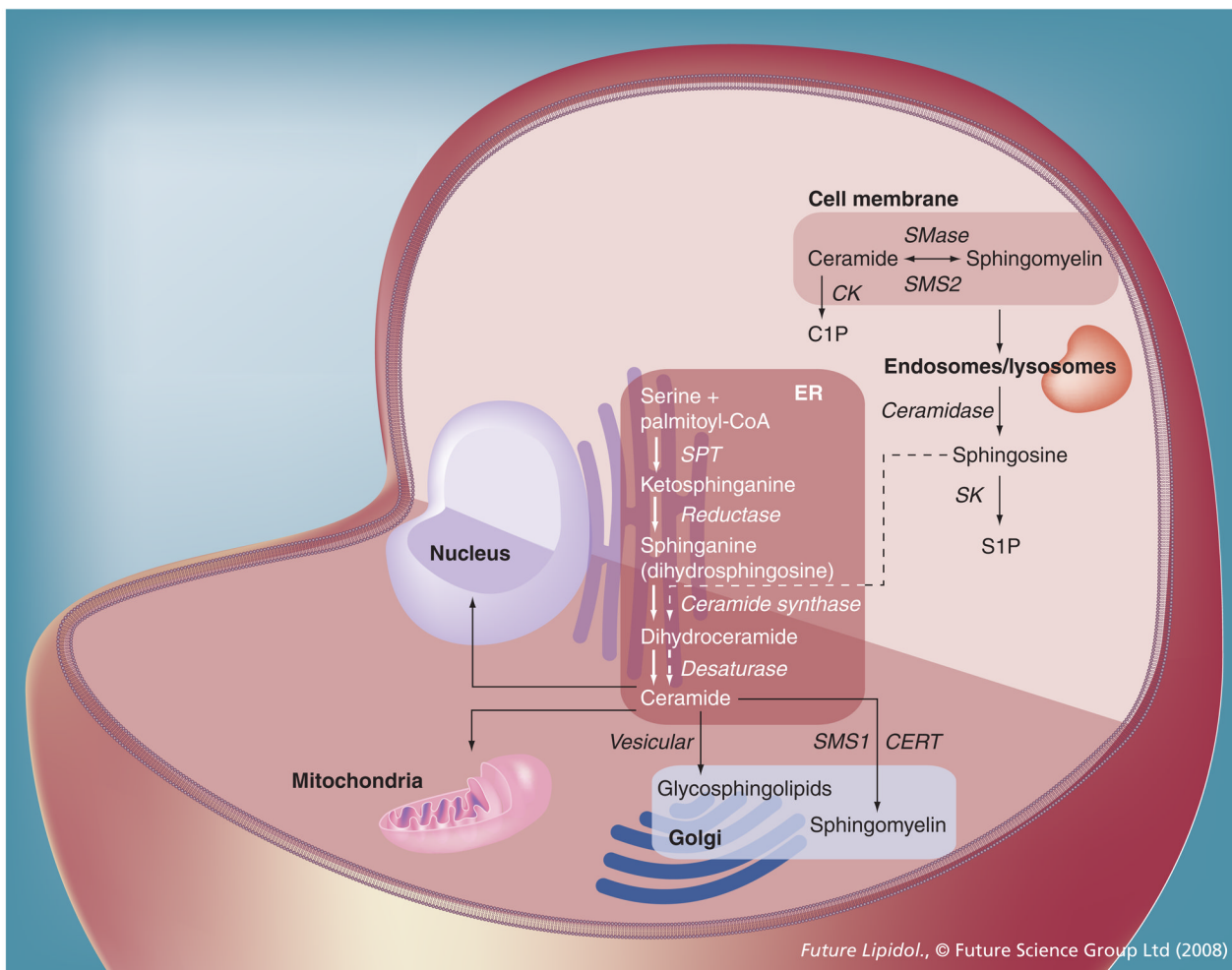


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**Figure 1. Ceramide and its derivatives**

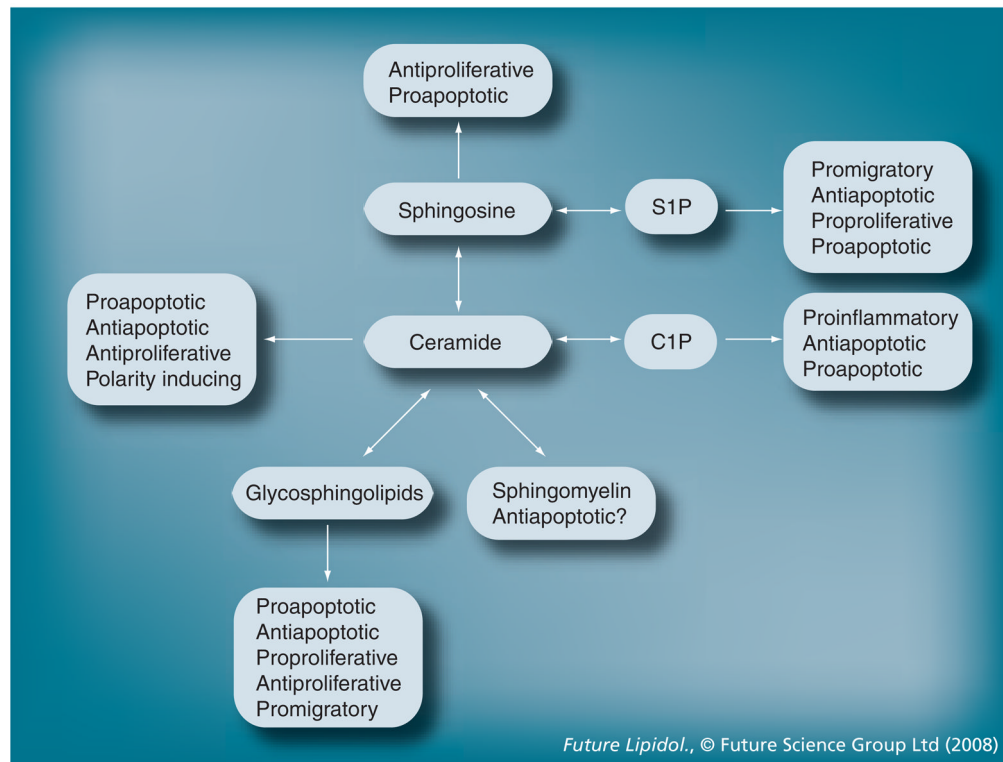
The sphingoid base (sphingosine portion) of all of the sphingolipids is derived from the condensation reaction of serine with palmitoyl-CoA. The minimal structural motif of many active sphingolipid analogues is composed of two hydroxyl groups  $\beta$ -positioned to an amino group or an imino group linked to a hydrocarbon chain (dashed box, S18 and FTY720 are closely related and derivatives of 2-amino 1,3-propanediol [serinol]).



### Figure 2. Metabolism and function of sphingolipids

*De novo* biosynthesis of sphingolipids is initiated in the ER, followed by transport of ceramide to the Golgi (vesicular or via CERT). In the Golgi, ceramide is used as precursor substrate for glycosphingolipid and sphingomyelin biosynthesis. Sphingomyelin is then transported to the cell membrane, where it can be hydrolyzed to ceramide (sphingomyelin cycle). In endosomes, ceramide can be hydrolyzed to sphingosine and is then recycled in the ER, which is mediated by the ‘salvage pathway’ (dashed arrows). Ceramide generated in the ER can be transported to the nucleus (via perinuclear ER cisternae) and the mitochondria (via mitochondrial-associated ER membrane). Ceramide can also be converted to C1P or S1P.

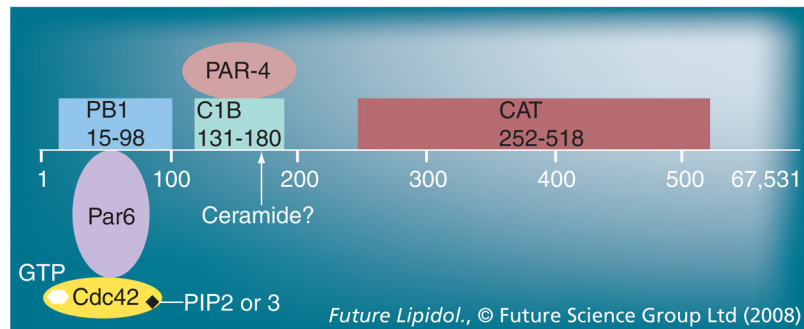
C1P: Ceramide-1-phosphate; CERT: Ceramide transport protein; CK: Ceramide kinase; ER: Endoplasmic reticulum; S1P: Sphingosine-1-phosphate; SK: Sphingosine kinase; SMase: Sphingomyelinase; SMS: Sphingomyelin synthase; SPT: Serine palmitoyltransferase.



**Figure 3. Metabolism and function of sphingolipids**

Ceramide and its derivatives have similar as well as opposite cell-signaling functions.

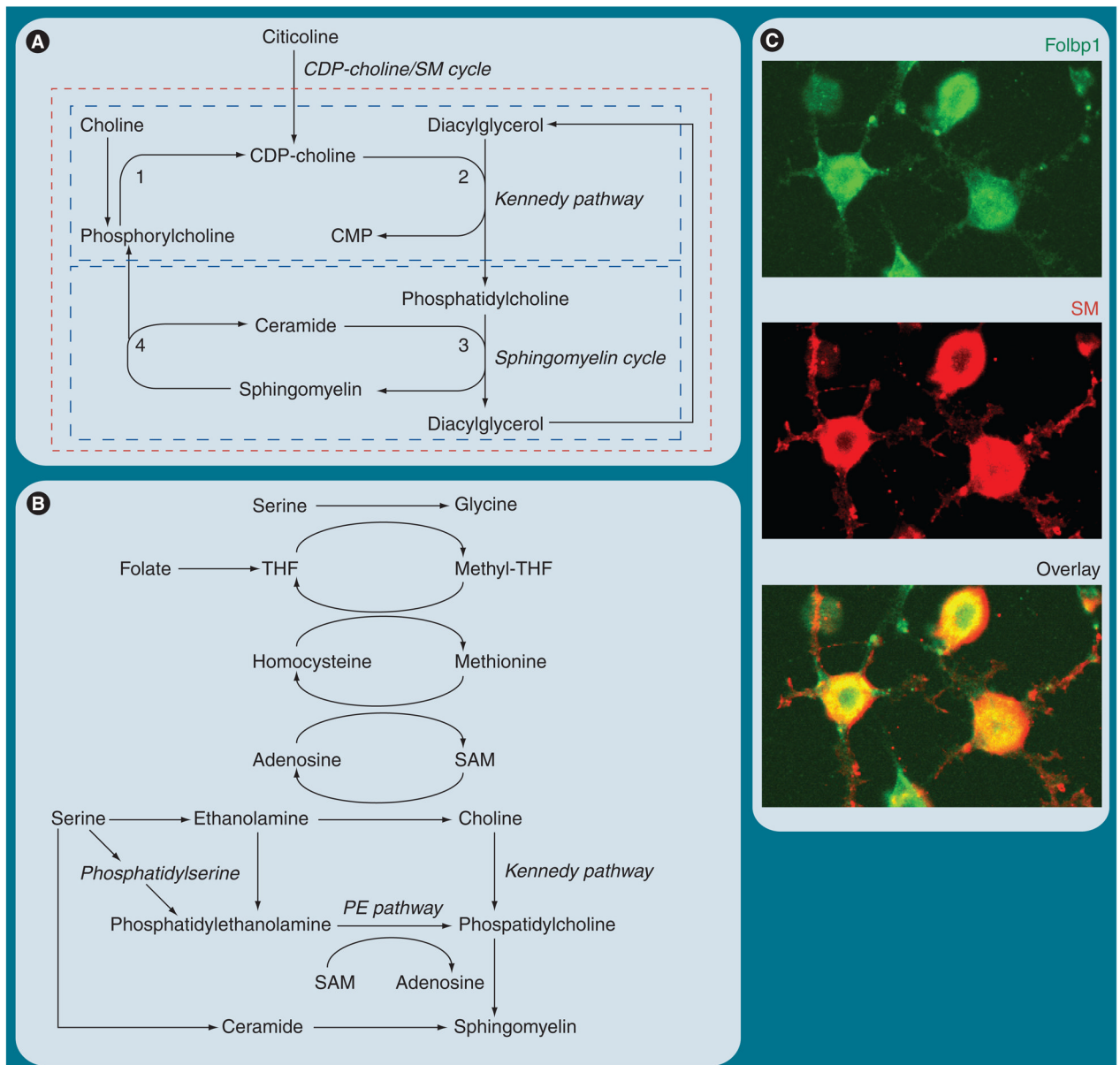
C1P: Ceramide-1-phosphate; S1P: Sphingosine-1-phosphate.



**Figure 4. Structure and binding domains of atypical PKC**

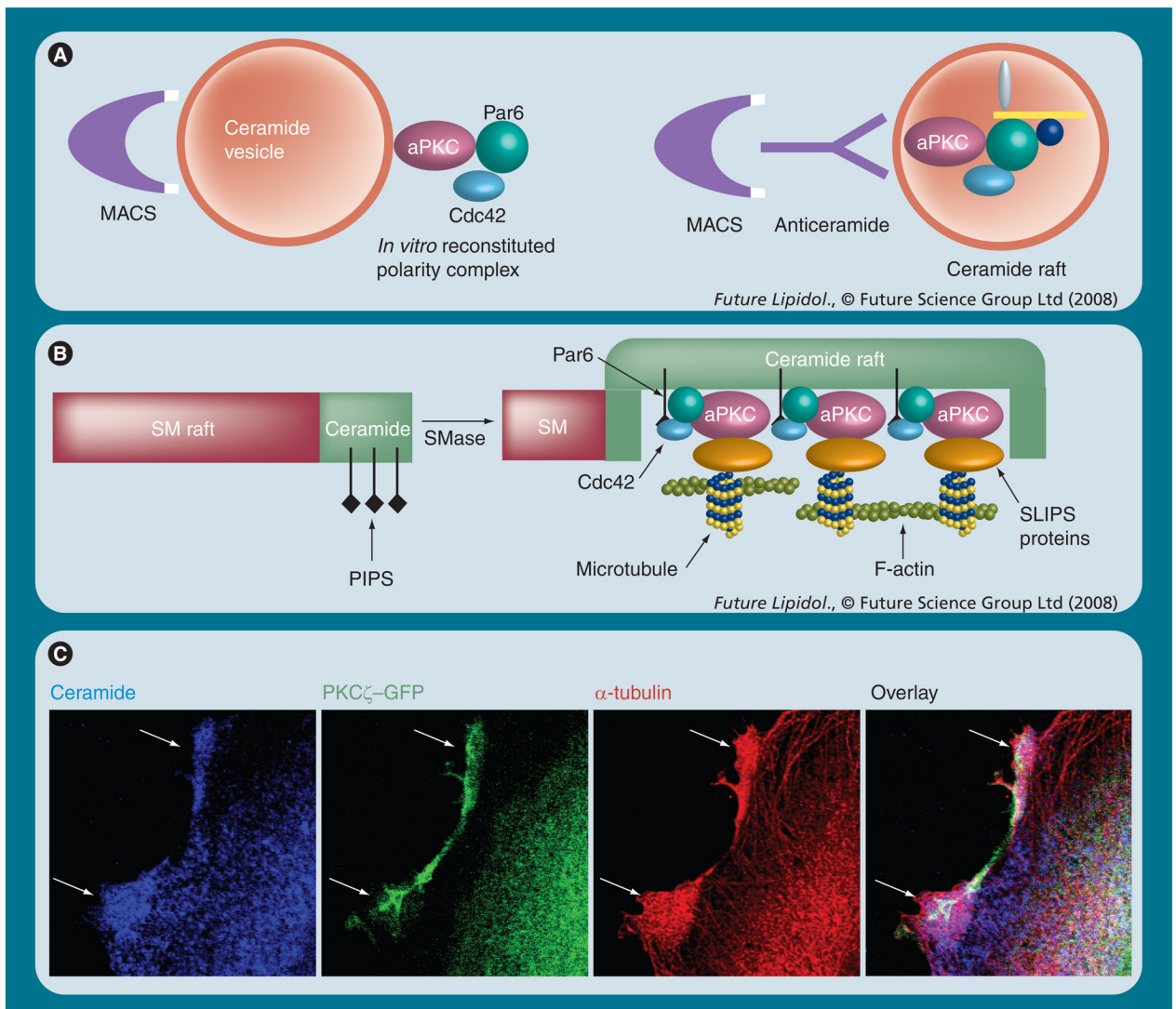
Atypical PKC (aPKC; figure shows PKC $\zeta$ ) consists of an N-terminal (regulatory) and C-terminal (catalytic) moiety connected by a hinge region. The N-terminal moiety contains a pseudosubstrate (PS) motif and a PB1 domain. The PB1 domain associates with the polarity protein Par6 that itself binds to Cdc42. The hinge region contains a C1B domain that has been suggested to associate with ceramide, and a putative binding site for PAR-4. The C-terminal moiety contains the catalytic domain and several sites, the phosphorylation of which stably activates the enzyme. In the inactive state of aPKC, the N-terminal PS motif 'folds back' onto the C-terminal catalytic domain and blocks its access to protein substrates. Binding of effectors to the N-terminal moiety 'opens up' aPKC and primes its phosphorylation and activation. In our model, ceramide binding, followed by the association with Par6 and Cdc42, and phosphorylation of the catalytic moiety are consecutive steps of robust aPKC activation. PIP2 or 3 may participate in aPKC activation and subcellular translocation by binding to active (GTP-associated) Cdc42 in this complex.

C1B: Modified phorbol ester/diacylglycerol binding; PAR-4: Prostate apoptosis response-4; PB: Protein binding; PIP: Phosphatidylinositol phosphate.



**Figure 5. Interconnection of phospholipid, C1 (one carbon unit) and sphingolipid metabolism**  
**(A)** The ‘CDP-choline/sphingomyelin (SM) cycle’ is a potential interconnection of the Kennedy (CDP-choline) pathway for phosphatidylcholine biosynthesis and the SM cycle. This cycle is sustained by supply with choline or CDP-choline (citicoline), which converts ceramide into SM. 1: CTP:phosphocholine cytidyltransferase. 2: CDP-choline:DAG cholinephosphotransferase. 3: SM synthase. 4: Sphingomyelinase. **(B)** The biosynthesis of choline is mediated by C1 metabolism, which depends on the supply of folate as cofactor for methylation reactions. Folate deficiency or perturbation of C1 metabolism may impair SM biosynthesis via the proposed CDP-choline/SM cycle. **(C)** Embryonic stem cell-derived neural progenitors express Folbp1 concurrent with SM. The immunocytochemistry was performed with anti-Folbp1 antibody and lysenin/anti-lysenin antibody [Bieberich, Unpublished Data]. Lysenin is an earthworm protein that binds specifically to sphingomyelin microdomains.

CMP: Cytidine monophosphate; Folbp1: Folate-binding protein 1; PE: Phosphatidyl ethanolamine; SAM: S-adenosyl methionine; THF: Tetrahydrofolate.



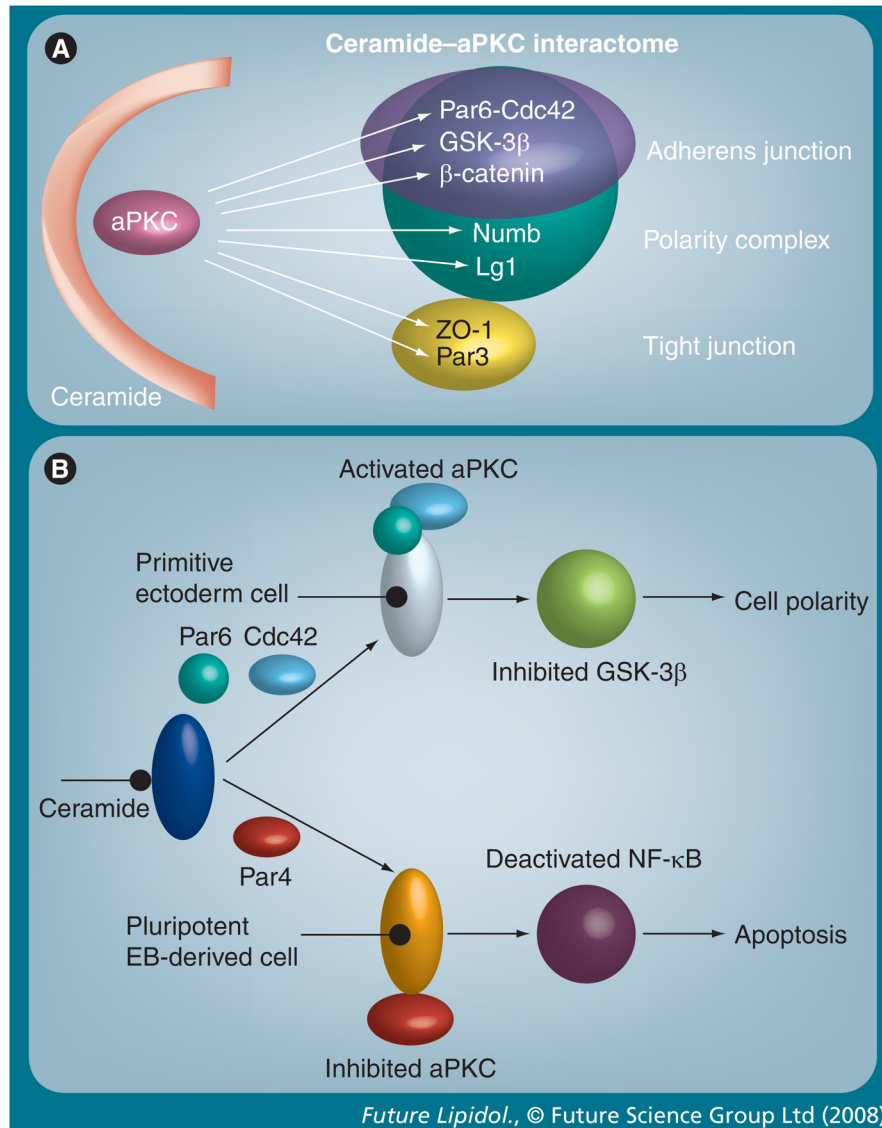
**Figure 6. Isolation, metabolic integration and visualization of sphingolipid rafts and SLIPS**

(A) The lipid vesicle-mediated affinity chromatography (LIMAC) technique was developed in our laboratory to isolate ceramide-associated protein complexes. Originally, the lipid vesicles were made of ceramide and phosphatidylserine (PS), which allowed for MACS using magnetic particle-conjugated annexin V (highly affine to PS) to isolate the vesicles and their associated proteins. We have used the LIMAC technique for the *in vitro* reconstitution of a ceramide-associated polarity complex (left panel) and the isolation of ceramide-binding proteins from cell lysates. LIMAC can be modified using other interaction partners for the isolation of the vesicles (e.g., [glycol]lipid-specific lectins or antibodies; right panel). (B) Hypothesis: activation of SMase converts SM into ceramide, which segregates into lipid microdomains with higher membrane fluidity. PIP2 or 3 embedded in the ceramide microdomain may bind to Cdc42 (via PH domain), while ceramide-associated aPKC binds to Par6. The formation of a SLIPS then regulates the dynamics of the cytoskeleton via additional proteins such as glycogen synthase kinase-3 $\beta$  or adenomatous polyposis coli (Figure 8). This may sustain ceramide-rich platforms (e.g., in the apical membrane of primitive ectoderm cells) or initiate processes (sphingopodia) in neural stem and progenitor cells. (C) Imaging of ceramide, aPKC and associated microtubules using the respective antibodies for confocal

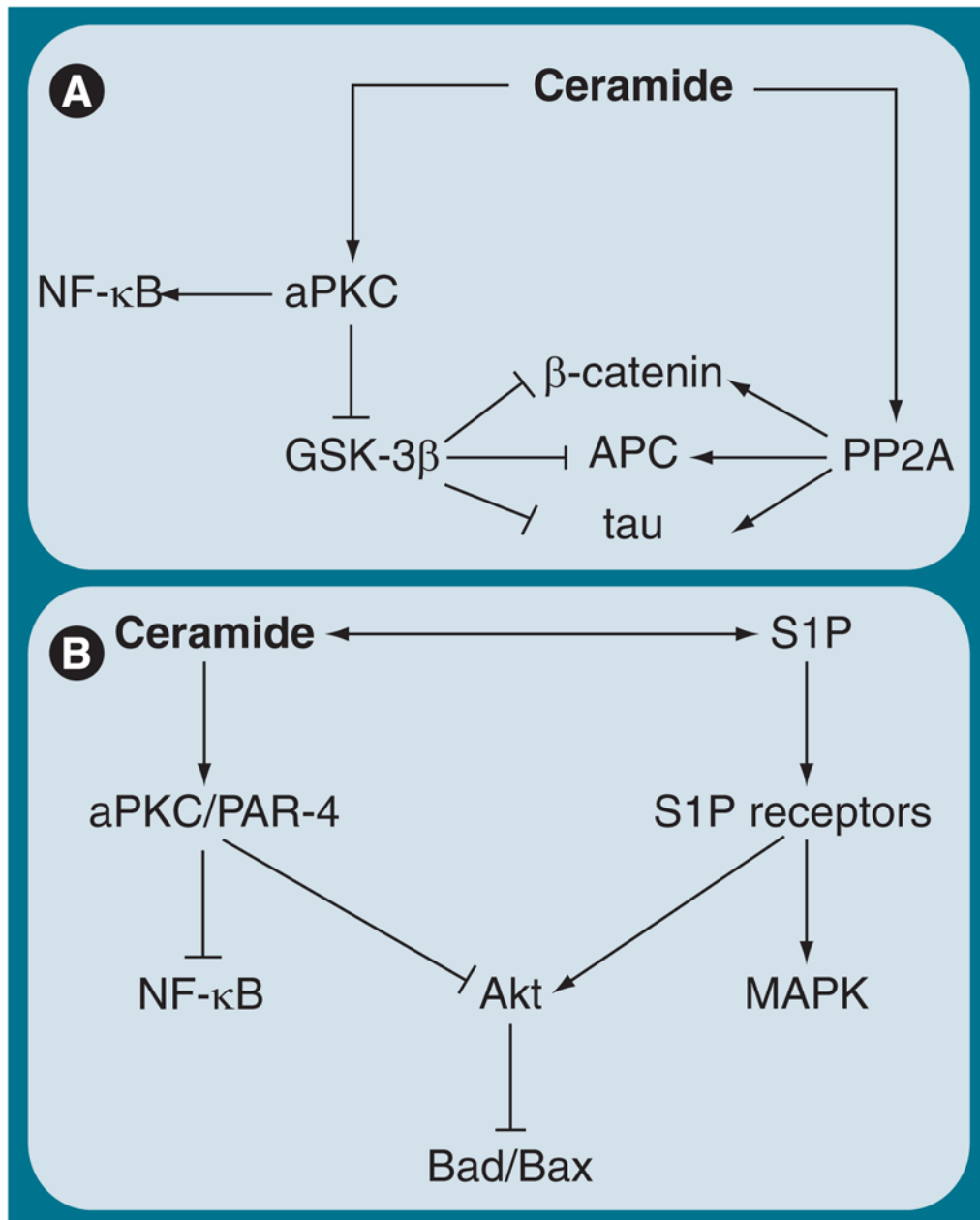


immunofluorescence microscopy with fixed neural progenitor cells [Wang, Krishnamurthy, Bieberich, Unpublished Data]. Ceramide-rich processes have been termed 'sphingopodia' and are proposed to be initiated by SLIPS.

aPKC: Atypical PKC; GFP: Green fluorescent protein; MACS: Magnetic-activated cell sorting; PIP: Phosphatidylinositolphosphate; SLIPS: Sphingolipid (here ceramide)-induced protein scaffold; SM: Sphingomyelin; SMase: Sphingomyelinase.



**Figure 7. Ceramide-atypical PKC interactome and the flipside model of ceramide (inter)action**  
**(A)** Ceramide-associated aPKC binds and/or phosphorylates a variety of junctional or polarity proteins, which is suggested to regulate cytoskeletal dynamics and cell adhesion. **(B)** ‘Flipside’ model of ceramide-induced aPKC activation or inhibition. If prostate apoptosis response (PAR)-4 is not expressed, ceramide-associated aPKC forms a polarity complex with Par6 and Cdc42 (upper panel). This may inhibit GSK-3β and control cell polarity or process formation as described in the legend of Figure 8 (top panel). In the presence of PAR-4, ceramide-associated aPKC binds to PAR-4, which inhibits its activity and induces apoptosis (bottom panel).  
 aPKC: Atypical PKC; EB: Embryoid body; GSK-3β: Glycogen synthase kinase 3β; NF-κB: Nuclear factor-κB.



**Figure 8. Interdependence of ceramide and S1P metabolism and cell-signaling pathways (hypothesis)**

**(A)** In the absence of PAR-4, ceramide activates aPKC, which may inactivate GSK-3 $\beta$  and stabilize  $\beta$ -catenin, increase binding of APC to the plus ends of microtubules and prevent aggregation of tau. Ceramide-activated PP2a may complement this effect of ceramide on aPKC. **(B)** In PAR-4-expressing cells, ceramide induces inhibition of aPKC by PAR-4, which may inactivate PI3K/Akt and NF- $\kappa$ B, two major cell-signaling pathways for cell survival in cancer and stem cells. Inhibition of Akt prevents inactivation of Bad/Bax and induces apoptosis. This is antagonized by S1P-mediated activation of Akt.

aPKC: Atypical PKC; APC: Adenomatous polyposis coli; GSK-3 $\beta$ : Glycogen synthase kinase 3 $\beta$ ; NF- $\kappa$ B: Nuclear factor- $\kappa$ B; PAR: Prostate apoptosis response; PP2a: Protein phosphatase 2a; S1P: Sphingosine-1-phosphate.