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Sphingolipid metabolism and neutral sphingomyelinases

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Abstract

Sphingolipids are an important class of lipid molecules that play fundamental roles in our cells and body. Beyond a structural role, it is now clearly established that sphingolipids serve as bioactive signaling molecules to regulate diverse processes including inflammatory signaling, cell death, proliferation, and pain sensing. Sphingolipid metabolites have been implicated in the onset and progression of various diseases including cancer, lung disease, diabetes, and lysosomal storage disorders. Here we will review sphingolipid metabolism to introduce basic concepts as well as emerging complexities in sphingolipid function gained from modern technological advances and detailed cell and animal studies. Furthermore, we will discuss the family of neutral sphingomyelinases (N-SMases), which generate ceramide through the hydrolysis of sphingomyelin and are key enzymes in sphingolipid metabolism. Four mammalian N-SMase enzymes have now been identified. Most prominent is nSMase2 with established roles in bone mineralization, exosome formation, and cellular stress responses. Function for the other N-SMases have been more enigmatic and is an area of active investigation. The known properties and potential role(s) of each enzyme will be discussed to help guide to future studies.

Part one: Sphingolipid metabolism

Sphingolipids encompass a broad range of lipid molecules that elicit a wide range of signaling properties and cellular functions [1]. To understand the diverse functionality of sphingolipids in health and disease, it is important to be familiar with the chemical structure of these lipid molecules, their cellular metabolism, and the sphingolipid-metabolizing enzymes. Here we present a brief review of these areas in an effort to introduce the main points and emerging concepts in the field.

Introduction

Sphingolipids—At the most basic level, sphingolipids can be defined as any lipid molecule that contains the sphingoid backbone, derived from the condensation of an amino acid (predominantly serine) and a fatty acid (predominantly palmitate) (Figure 1B) [2]. The presence or absence of an acyl chain distinguishes ceramide (Cer) from sphingosine (Sph), while phosphorylation of the 1-hydroxy group generates ceramide-1-phosphate (C1P) or sphingosine-1-phosphate (S1P). Other common sphingolipids contain different head groups at this position. Sphingomyelin (SM) contains a phosphorylcholine headgroup, and the basic

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glycosphingolipids, glucosylceramide (GluCer) and galactosylceramide (GalCer), contain a single sugar molecule linked to ceramide.

De novo biosynthesis—Sphingolipids can be synthesized *de novo* by a single biosynthetic pathway, with the end product being Cer [3] (Figure 1A). The first step is catalyzed by the serine palmitoyl transferase (SPT) complex and condenses the amino acid serine and the fatty acid palmitate to form 3-keto-dihydrosphingosine. The carbonyl group of 3-keto-dihydrosphingosine is then reduced to form dihydrosphingosine, and the enzyme (dihydro)ceramide synthase (CerS) then adds a fatty acid chain (the acyl chain) by N-acylation to form dihydroceramide. Finally, desaturation of the 4,5 carbon-carbon bond on the sphingoid backbone generates Cer [4].

Sphingolipid metabolism—From Cer as a central point, sphingolipid metabolism branches out in four main directions (Figure 1A). Three of these reactions alter the head group of Cer. These include the phosphorylation of Cer by ceramide kinase (CerK) to produce C1P [5], the addition of phosphocholine by sphingomyelin synthase (SMS) to produce SM (via transfer of the phosphocholine headgroup from phosphatidylcholine) [6], and the addition of a sugar molecule by glucosyl- and galactosyl-ceramidesynthases to create GluCer or GalCer respectively [2].

Alternatively, Cer can be broken down by ceramidase (CDase), which removes the acyl chain, to produce the lyso-sphingolipid Sph [7]. Sph can be reconverted to Cer by CerS or phosphorylated by sphingosine kinase (SK) to produce S1P [8]. S1P can be either dephosphorylated back to Sph or broken down by S1P lyase to ethanolamine phosphate and hexadecenal [9]. Action by S1P lyase is notably the sole exit point for sphingolipid breakdown and is not reversible [1].

New bioactive sphingolipids—The advances in lipodomics and metabolomics provide a basis for the continuing discovery of new bioactive sphingolipids. For example, a recent study identified N,N-dimethylsphingosine (DMS) as a new bioactive molecule, inducing chronic pain in mice [10]. The pathway and enzymes responsible for DMS synthesis have not been established but may simply involve the di-methylation of Sph.

Complexity in sphingolipid metabolism

Variations in chemical structure—Each lipid species described above comprises a family of molecules that share the same basic framework (i.e. same head group) but can differ in their chemical structure in specific ways (Figure 1B). A common variation is the length of the acyl chain [2]. This occurs through utilization of different length fatty acyl-CoAs by CerS enzymes [11]. In addition, the length of the sphingoid base can vary if the SPT complex uses myristate or stearate as a substrate instead of palmitate [12]. Besides chain length, both the acyl chain and sphingoid base can be hydroxylated [13] and the sphingoid base can be saturated or desaturated [14]. Overall, these variations create a family of related molecules that have the potential for distinct molecular and cellular functions.

Compartmentalization and specificity—In mammalian sphingolipid metabolism, there is often more than one enzyme in the cell to catalyze each chemical reaction (Figure 1A) [3].

From a cellular perspective, the functional reasons for enzyme multiplicity are two-fold. First, isozymes can be localized in different cellular compartments. This allows organelle-specific activity and separate regulatory mechanisms. For example, there have been six identified SMase isoforms that localize to various compartments including the lysosome, inner or outer leaflet of the plasma membrane, ER, mitochondria, and nucleus [15,16]. Secondly, isozymes can have different substrate specificities to allow the formation or breakdown of particular molecular species. Representatively, the CerS isozymes appear to all localize to the ER but exhibit different preferences in fatty acid chain length [17].

Interconnectivity of metabolites—The metabolic network of sphingolipids is connected through a series of chemical reactions that transform one bioactive metabolite to another [3] (Figure 1A). Expanding beyond two-state equilibriums, we see a connectivity map that allows the production of distant metabolites from another; e.g. S1P from SM by the sequential action of SMases (SM to Cer), CDases (Cer to Sph) and SKs (Sph to S1P). Given the large excess of SM to S1P, it is a logical progression that a relatively small SM depletion has the ability to profoundly affect S1P production. Overall, this means the bioactive lipid generated by the activation of SMases or CDases, and the associated cellular response, does not have to directly correlate with the immediate reaction product [1].

Emerging concepts

Many ceramides—A new paradigm for sphingolipid metabolism and function has emerged that integrates the significant advances described above into a working model. In the *many ceramides* hypothesis, each Cer molecular species, and related bioactive sphingolipid, has the potential to illicit a unique cellular response [18]. Unique functions may arise from differences in the bioactive molecule's structure, protein targets, and/or subcellular localization.

Molecular specificity in cellular function—An elegant example of highly selective recognition of a lipid molecule was recently reported that embodies the essence of the *many ceramides* hypothesis [10]. The authors found that the COPI machinery protein p24 specifically recognized and bound SM with 18 carbons. This recognition was required for efficient COPI-dependent transport. SM-18 was previously reported to be enriched in the Golgi, which suggests that this molecular specificity drives a compartment specific cellular function [10].

Many of the other current examples include the chain-length dependent correlation of Cer molecules in various processes including autophagy, apoptosis, inflammation, and cancer [19]. In general, these processes have been associated with the action of CerS enzymes, which have different chain-length specificities. Based on their chain-length specificities, the involvement of individual CerS enzymes in these pathologies has been either inferred or tested experimentally [20–23].

As another example, the three-member family of alkaline ceramidases (AlkCDase1–3) has recently been characterized to have different specificities in the hydrolysis of Cer. AlkCDase1 hydrolyzes very long chain Cers [7], AlkCDase2 has broad substrate specificity

[24], and AlkCDase3 hydrolyzes long chain Cers [25] to regulate the levels of distinct Cer subclasses in the Golgi.

Mechanistic implications—It is interesting to consider how this molecular specificity is functionally translated. Consider that all the distinguishing structural characteristics of different Cer molecules are embedded in the membrane and should only be recognizable by protein transmembrane domains. Proteins working only at the membrane interface should not be able to distinguish these molecular differences. This should limit the recognition of sphingolipid specificities to integral membrane proteins or proteins with significant hydrophobic pockets or membrane insertions.

Conclusions

Although new sphingolipids and their functions are still being identified, the basic outline for sphingolipid metabolism is well established. Involvement of sphingolipids and sphingolipid metabolizing enzymes in new pathologies and signaling pathways continues to increase. Moving forward, the challenge is to understand the molecular mechanisms underlying sphingolipid functions using the advancing technology and our increased appreciation of sphingolipid complexities. Investigations of this nature will significantly advance our knowledge to benefit in the identification of novel targets and strategies for therapeutic intervention in health and disease.

Part two: Neutral Sphingomyelinases

Neutral Sphingomyelinases (N-SMases) are a family of related enzymes that catalyze the hydrolysis of SM to generate Cer and phosphorylcholine. Cer and sphingolipid metabolites are well-established regulators of many important cellular signaling pathways and are implicated in human health and disease [26]. In this section we will discuss the mammalian N-SMase enzymes highlighting biochemical properties, localization, and roles in lipid metabolism, cellular signaling, and physiology.

Introduction

The mammalian N-SMases—Since the original identification of N-SMase activity in 1967 [27] four mammalian N-SMase genes have been cloned or purified (Figure 2A). These include nSMase1 (gene name = *SMPD2*), nSMase2 (*SMPD3*), nSMase3 (*SMPD4*), and MA-nSMase (mitochondrial-associated nSMase) (*SMPD5*). NSMase2 is currently the best-studied isoform with established roles in bone mineralization, cell growth arrest, exosome formation, and the inflammatory response. The roles of the other N-SMases in mammalian physiology and biology are still ambiguous due to either an enigmatic function or an only recent identification.

Biochemical and structural features—Most N-SMases, from bacteria to mammals, share a DNase I type catalytic core suggesting a common catalytic mechanism for SM hydrolysis [28]. NSMase3 is an exception and will be discussed separately. N-SMases catalyze the hydrolysis of SM in a PLC-type manner to generate the reaction products: Cer and phosphorylcholine (Figure 2C). The active site is defined by 8 metal binding residues,

which together bind two Mg^{2+} ions [29]. Other highly conserved residues are found near the active site and may be important for catalytic activity. Two examples are Asp and Lys residues found in a P-loop like motif, which are both required for catalysis in the yeast N-SMase homolog Isc1 [30]. The crystal structures of two N-SMase bacterial homologs have defined the general protein fold and position of these conserved residues [29,31].

All mammalian N-SMases contain an extra hydrophobic domain that tethers the catalytic domain to the membrane (Figure 2A). The hydrophobic domain can also play other roles in phospholipid binding, subcellular localization, and enzyme activation to contribute to the activity and regulation of N-SMase enzymes *in vivo*.

Subcellular localization—In general, sphingolipids and sphingolipid metabolism are segregated into membrane compartments [1]. The localization of each N-SMase enzyme differs, suggesting each enzyme is responsible for SM hydrolysis and ceramide formation in specific organelles. In addition, enzyme localization constrains the biologically relevant activation mechanisms, as the concentration of different lipid molecules is also organelle dependent.

Neutral SMase2

NSMase2 is the best-studied mammalian N-SMase and has emerged as a key mediator of cellular stress-induced generation of Cer, as well as a somewhat surprising role in bone mineralization. Biochemical and physical characterization has identified a number of mechanisms for activation and regulation of nSMase2. These studies provide a template to investigate other N-SMase isoforms, as well as potential areas and modes for therapeutic intervention.

Biochemical properties—Two domains have been identified in nSMase2: an N-terminal domain that is hydrophobic and tethers nSMase2 to the membrane and a C-terminal domain encompassing the catalytic domain [32] (Figure 2B). Interestingly, the region separating these domains contains recently identified serine phosphorylation sites [33] and a Calcineurin binding site [34] suggesting this may be a major area for activation and regulation of nSMase2.

NSMase2 exhibits low basal SMase activity *in vitro* and requires activation by anionic phospholipids (APLs) [35]. The requirement of APLs is consistent with the location of nSMase2 at the plasma membrane (PM) [36,6], rich in the APL phosphatidylserine (PS) [37]. Another potential biologically relevant APL is the minor lipid phosphatidic acid (PA) transiently produced at the PM in low concentrations [38]. Given the different spatiotemporal dynamics of PS and PA at the PM, we speculate that constitutive function of nSMase2 may depend on PS, while PA generation may be one mechanism for acute activation.

The interaction of nSMase2 with the membrane has been studied in detail. The N-terminal domain contains two hydrophobic segments (Figure 2), predicted to be helical, that associate with but do not span the membrane [39] (Figure 2B). Additionally, nSMase2 harbors two palmitoylation sites that contribute to nSMase2 membrane association [40]. Recently, the

APL binding domain was found to localize exclusively to the N-terminal domain [41]. A binding motif consisting of three conserved Arg residues are necessary for APL binding, APL-mediated activation, and correct trafficking of nSMase2.

Localization—NSMase2 localizes to the plasma membrane (PM), Golgi, and recycling compartments. TNF-α, PMA, H_2O_2, and cell confluence all induce nSMase2 translocation from the Golgi to the PM, and correlate with increased N-SMase activity. This suggests the major site of nSMase2 action is the inner leaflet of the PM. Translocation may regulate activity by controlling access to substrate SM, activating APLs, or involve other mechanisms.

GW4869: an nSMase2 specific inhibitor—NSMase2 is the only N-SMase with a specific inhibitor, GW4869 [42,43]. GW4869 has been widely used as a tool to identify and confirm nSMase2-specific functions [26]. Mechanistically, GW4869 is thought to inhibit nSMase2 by interfering with APL activation [42]. We note that any inhibitory effect of GW4869 on the recently identified MA-nSMase has not been assessed and should be tested.

Cellular signaling—NSMase2 has been implicated in the response to various cellular stresses and cytokines to affect a diverse set of signaling pathways including cancer pathogenesis [44,45], growth and development [46,47], and inflammatory responses [48,49]. It was originally cloned as a confluence arrest gene [50] and has a demonstrated role linking confluence to cell cycle arrest [51]. Most recently, it has been linked as an upstream regulator of the mTOR/S6 Kinase pathway [52] and shown that this pathway regulates the biosynthesis of the extracellular matrix component hyaluronan, which is a glycosaminoglycan produced at the PM [53].

Activation by TNF-α is currently the best-studied activation pathway and occurs through PKC-δ [54] and p38 MAPK dependent mechanisms [55]. Direct protein interaction with embryonic ectodermal development (EED) couples nSMase2 to TNF receptor 1 through FAN (Factor Associated with N-SMase activation) [56] and the scaffolding protein RACK1 [57].

Phosphorylation has emerged as one mechanism regulating nSMase2 activity. Phosphorylation has been shown to modulate the activity of nSMase2, to be regulated by the phosphatase Calcineurin [34], and to occur through p38 MAPK and PKC dependent pathways [54,55]. Recently, two clusters of specific serine residues were identified as phosphorylation sites and found to effect protein stability [33]. The actual kinase responsible for directly phosphorylating nSMase2 is unknown.

A role for nSMase2 in Cer-mediated lung injury has emerged [58]. Oxidants in cigarette smoke and hydrogen peroxide increase nSMase2 activity and Cer levels leading to increased apoptosis in bronchial epithelial cells and the lung tissues of rodents [59–61]. The effects of cigarette smoke can be blocked by the antioxidants glutathione and N-acetyl cysteine. In addition, the expression of nSMase2 was increased in lung tissues of smokers with emphysema [61].

NSMase2 may play a role in tumorigenesis with mutations identified in the SMPD3 gene in several human leukemias [44]. Two of these mutations affect function by either reducing protein stability or altering localization. Other studies suggest nSMase2 may be important for cell death in transformed cells [26]. For example, NSMase2 mRNA and protein levels were induced by the anti-cancer drug daunorubicin, leading to increase N-SMase activity, Cer levels, and cell death [45].

Several studies have now demonstrated that ceramide and nSMase2 are key regulators of exosome formation and microRNA (miRNA) secretion [62–64]. miRNAs are gene regulatory elements found both intra- and extra-cellularly [62]. It is thought they are encapsulated in exosomes, leading to their secretion outside the cell. Interestingly, overexpression of nSMase2 increased miRNA secretion, while inhibition by GW4869 or knockdown by siRNA of nSMase2 decreased miRNA secretion. This process was not dependent on the endosomal sorting complex required for transport (ESCRT) implicating a novel ceramide-dependent, ESCRT-independent secretion mechanism. In addition, these studies suggest the Golgi may be a second site of action for nSMase2 SM hydrolysis.

Physiological role in skeletal development—Animal studies of nSMase2-deficient mice have identified a role for nSMase2 in bone homeostasis. The nSMase2 knockout mice suffer from short stature [63], while the nSMase2-inactivating *fro/fro* (for *fragilitas ossium*) mutation results in bone fragility [47]. The observed skeletal abnormalities include short and bent limbs, as well as deformations in rib cages, long bones, and growth plate cartilage [64].

Recently, it was shown that nSMase2 plays cell-specific roles in skeletal development [64]. Fro/fro mice are defective in both bone and cartilage mineralization and these two events are regulated by the different cell types: osteoblasts and chondrocytes, respectively. By selectively expressing nSMase2 in the osteoblasts of fro/fro mice, the authors were able to correct the osteoblast-specific bone defects but not affect the cartilage defects [64].

The role of nSMase2 appears to involve a novel mechanism that does not involve the typical factors such as calcium, phosphate, and alkaline phosphates. This suggests a novel, nSMase2-dependent mechanism gating proper bone mineralization and may help in understanding cases of osteogenesis imperfecta in human patients that also share similar phenotypes and undetectable differences in typical mineralization parameters [64,65]. Overall, these discoveries are exciting and future work deciphering the molecular mechanisms of nSMase2 in bone mineralization is of great interest.

Neutral SMase1

Biochemical properties—NSMase1 was the first identified and cloned mammalian N-SMase based on sequence homology to bacterial SMases [66]. The domain architecture of nSMase1 is identical to Isc1, the yeast homologue to N-SMases [67], with a catalytic domain followed by two C-terminal transmembrane helices (Figure 2A). Unlike other N-SMases, nSMase1 is not activated by phospholipids [66].

Localization—The localization of nSMase1 appears to vary when comparing endogenous to overexpressed protein. Overexpressed nSMase1 mainly colocalizes with endoplasmic

reticulum (ER) markers [68]. However, endogenous nSMase1, in contrast to overexpressed nSMase1, was reported to localize to the nuclear matrix [69].

A role for nSMase1 in lipid metabolism?—Despite the activity of nSMase1 on SM *in vitro*, overexpression in cells does not affect SM metabolism [66,70]. This ambiguity casts doubt on the role of nSMase1 in sphingolipid metabolism. Lyso-platelet activating factor (lyso-PAF) may be a biologically relevant substrate, with nSMase1 displaying both PLC-lyso-PAF activity *in vitro* and in cells [70]. However, nSMase1 KO mice had no detectable changes in sphingolipid metabolism by high performance thin layer chromatography (HPTLC), a relatively insensitive method compared to mass spectrometry, including no alterations in SM or lyso-PAF metabolism.

Although a function for nSMase1 is not apparent, this does not preclude a role for nSMase1 in lipid metabolism. Given the significant advancements in lipid quantification by mass spectrometry and the emerging *many ceramides* model [18], it would be interesting to measure lipid levels in nSMase1 KO mice to determine if discrete SM, Cer, or lyso-PAF species, undetectable by HPTLC, are altered. Quantification of ceramide levels in nSMase1 transiently transfected MCF7 cells did result in slight increases in some ceramide levels [71]. Correct substrate identification may require *in vivo* measurements from KO animal tissues, as recently demonstrated for a different lipid metabolizing enzyme [72]. With regards to nSMase1, this may be particularly relevant given the different subcellular localizations of endogenous and overexpressed proteins.

MA-nSMase (Mitochondrial-Associated Neutral SMase)

Biochemical properties and localization—MA-nSMase is the most recently identified mammalian N-SMase being discovered in 2010 [16] by sequence homology with nSMase2 and a zebrafish mitochondrial N-SMase [73]. The subcellular localization of overexpressed MA-nSMase protein varied with cell type, showing strong or partial co-localization with mitochondrial markers, in addition to co-localization with ER markers [16]. The mouse MA-nSMase protein conserves a mitochondrial signal peptide with the zebrafish N-SMase, which may be responsible for its mitochondrial localization.

MA-nSMase has comparable domain architecture and biochemical properties with nSMase2. Catalytic activity requires Mg^{2+} or Mn^{2+} ions and is strongly increased by the presence of the phospholipids cardiolipin (CL), PS, or phosphatidylglycerol (PG) (Figure 2). Both CL and PG are present in mitochondria providing a putative mechanism for activation and/or regulation of MA-nSMase activity *in vivo* [16]. The N-terminal hydrophobic domain of MA-nSMase is similar to nSMase2 by sequence homology, but the membrane topology and APL binding motif has yet to be characterized.

Human vs. murine MA-nSMase—The human homolog to murine MA-nSMase has yet to be cloned and characterized. However, an open reading frame for the human SMPD5 gene (XP_001714084) is present in the NCBI database. Surprisingly, the human and mouse proteins contain key differences in the N-terminal APL activation domain. Importantly, the putative mitochondrial signal peptide is conserved suggesting the human protein will also

localize to mitochondria. In the future it is important to clone the human gene and compare the amino acid sequence, biochemical properties, localization, and cellular functions.

Future directions—At present, little is known about MA-nSMase beyond basic properties. However, the identification of a mammalian mitochondrial N-SMase presents another potential endogenous mechanism, in addition to action of ceramide synthases, for mitochondrial ceramide generation. This is exciting considering the numerous studies linking ceramide to mitochondrial activation of apoptosis [74].

Another potential role for MA-nSMase is in fertilization. Activation of an unidentified SMase during fertilization was inferred by a corresponding decrease in SM and increase in Cer levels [75]. In support of this hypothesis, MA-nSMase gene expression in organ tissues is the highest in testis [16] and the MA-nSMase gene appears to be bicistronic with a spermatogenesis associated factor (NCBI database, unpublished observation). It is our hope that future studies taking advantage of gene silencing, antibodies to endogenous proteins, and knockout animals will better illuminate the role of MA-nSMase in mammalian physiology and biology.

Neutral SMase3

Biochemical properties—Although human nSMase3 was identified in 2006 [76], relatively little work has been reported since regarding further biochemical and functional characterization. Identification was accomplished by sequence comparison using a peptide (KGLPYLEQLFR) from a previously purified N-SMase from bovine brain [77]. The peptide sequence only matches 7 of the 11 residues in the identified human protein and bovine homolog. Given the short peptide sequence and low identity, this raises the question if the original purified bovine and identified human proteins correspond to the same protein.

NSMase3 shares no sequence homology with any N-SMases or any other characterized type of enzyme catalytic domain. The region comprising the catalytic domain is yet to be identified. A C-terminal transmembrane helix is predicted to embed nSMase3 in the membrane [76].

Two conflicting reports have characterized nSMase3 activity. In the original identification, nSMase3 activity is reported to occur at neutral pH and require Mg^{2+} or Mn^{2+} [76]. The observed activity was slightly enhanced, approximately two-fold, by the phospholipid PS. In a later study, MCF-7 cells transiently and stably overexpressing nSMase3 did not have significant N-SMase activity over vector controls [71]. We hope that future studies will determine the underlying reasons behind this major discrepancy.

Localization—Subcellular localization studies found nSMase3 to display an ER distribution pattern [76]. Later work confirmed this and found localization was not dependent on a putative ER localization motif or the C-terminal transmembrane helix [78].

Cellular signaling—It has been suggested nSMase3 may play a role in TNF-α mediated signaling [76,78]. However, another report found that nSMase2 was the primary N-SMase activated by TNF-α in MCF-7 cells [71]. In support of this, a comparison of mouse

fibroblasts from normal and nSMase2 deficient *fro-/fro-* mice found that nSMase2 deficiency abrogated TNF-α induced increases in N-SMase activity [49] suggesting nSMase3 is not required in these cells.

The other identified putative function of nSMase3 involves a role in tumorigenesis [78]. In this study, nSMase3 expression was found to be both upregulated and downregulated to varying degrees (−70 to +70%) in different tumor samples. In addition, nSMase3 mRNA levels were down regulated by the tumor suppressor p53.

Overall, a clear functional role for nSMase3 has yet to emerge. Further studies investigating the biological function and molecular mechanisms of nSMase3 are required to validate the current findings.

Conclusions

Four N-SMase isoforms have now been cloned and purified. However, beyond nSMase2 there is relatively little known about N-SMase physiology and cellular function. In addition, at a biochemical and mechanistic level, there is still much to learn about N-SMase activation and regulation. The roles of nSMase2 in bone homeostasis, exosome secretion of miRNAs, and cigarette-induced lung injury are essential and provide the potential for nSMase2-targeted therapies.

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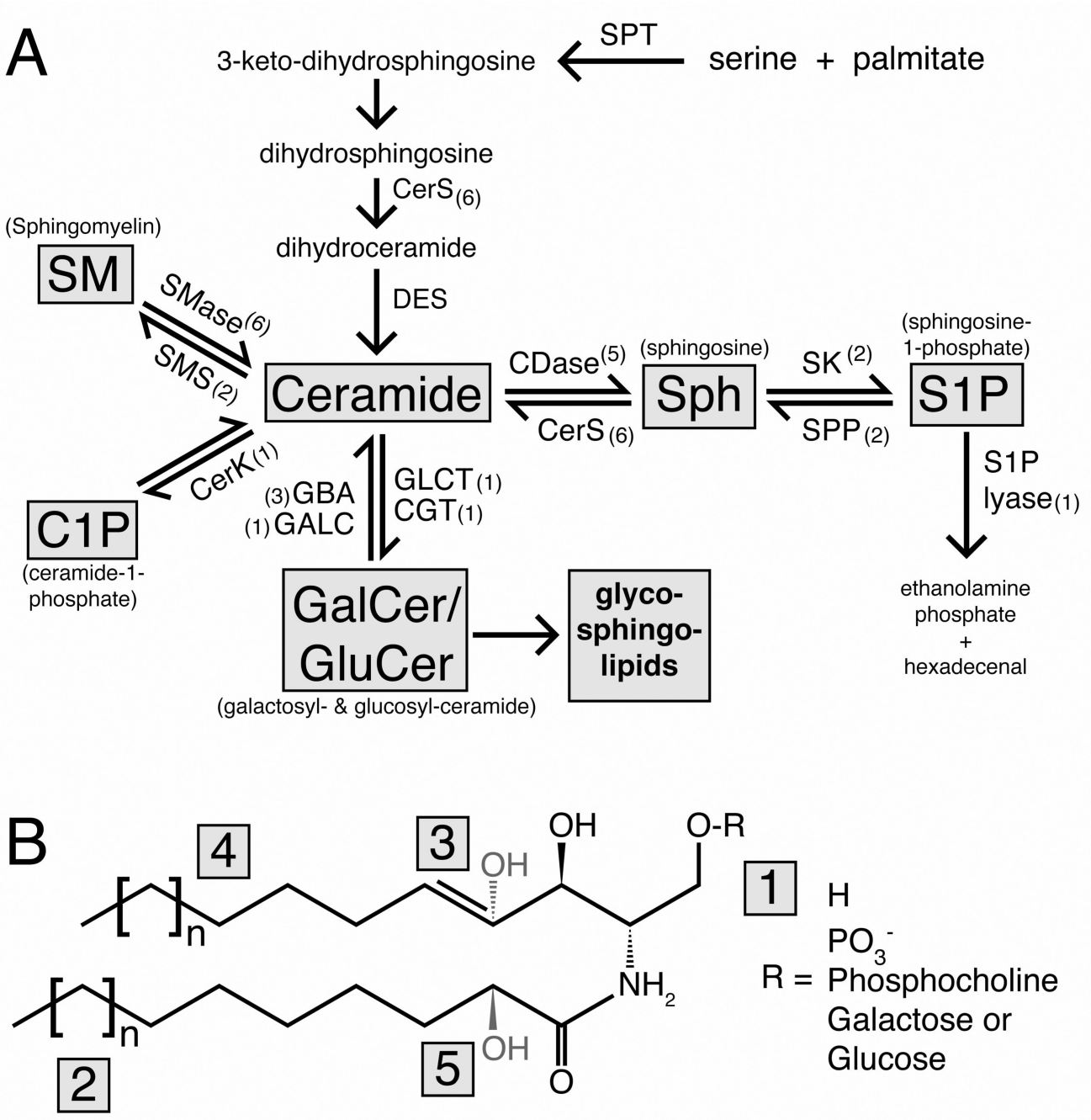

Figure 1. Sphingolipid metabolism and chemical structure

(A) Diagram of sphingolipid metabolism showing the major lipid species in grey boxes and sphingolipid metabolizing enzymes. The number of mammalian genes that catalyze each conversion are denoted by brackets. (B) Generic structure for sphingolipid molecules with modification points. The presence or absence of the acyl chain (lower chain) distinguishes ceramide (shown) from sphingosine. Variation in the headgroup (1), attached to the terminal 1-oxygen, distinguishes each family of sphingolipids. The length of the sphingoid backbone (4) or acyl-chain (2) generates subspecies within each family. Further modification at the 4,5

position on the sphingoid backbone (3) can occur with different saturation levels (e.g. dihydroCer vs. Cer) and hydroxylation. In addition, the acyl chain can be hydroxylated at the 2-position (5). Abbreviations: SPT = serine phosphoryltransferase, CerS = (dihydro)ceramide synthase, DES = dihydroceramide desaturase, SMase = sphingomyelinase, SMS = sphingomyelin synthase, CerK = ceramide kinase, GBA = glucosylceramidase, GALC = galactosylceramidase, GCLT = ceramide glucosyltransferase, CGT = ceramide galactosyltransferase, CDase = ceramidase, SK= sphingosine kinase, SPP = S1P-phosphatase.

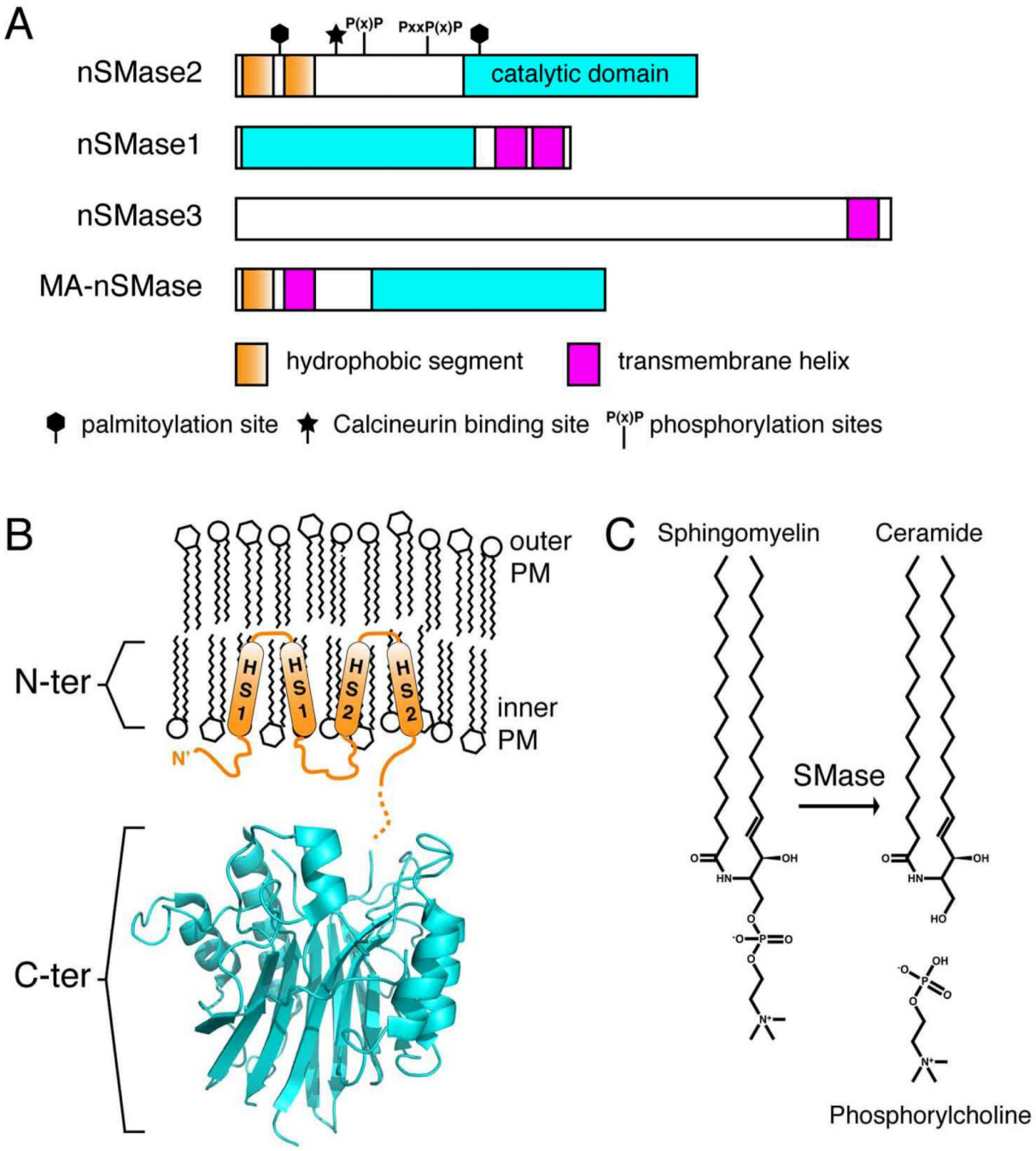

Figure 2. Domain architecture and topology of N-SMase isoforms

(A) Domain architecture of N-SMase isoforms highlighting the catalytic domain, membrane-associated or transmembrane regions, and sites of protein binding or post-translational modifications. (B) nSMase2 contains two domains: an N-terminal domain with two hydrophobic segments (HS1 & HS2) that associate with but do not span the membrane and a C-terminal catalytic domain (blue). For the catalytic domain, the structure of a bacterial homologue (bSMase, PDB: 2DDR) is shown with the active site towards the

membrane. (C) Sphingomyelinases catalyze the hydrolysis of SM to generate Cer and phosphocholine.