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Visualizing sphingolipid biosynthesis in cells

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A B S T R A C T

Biosynthetic pathways play a fundamental role in the building and operation of the cell by synthesizing the constituents by which the cell is constructed, and by producing signalling intermediates that play a key role in cell regulation. While a lot is known about the metabolite profile of the cells and about the biochemical pathways through which these metabolites are produced, the cellular localization of the biosynthetic machineries and the importance of this localization to the regulation of the metabolism has often been given less attention. This derives from the fact that, for several of these pathways, the enzymes involved are found colocalized in one compartment where their specific localization is unlikely to influence their function. The sphingolipid (SL) metabolic pathway is a notable exception to this as SL synthetic enzymes are laid out on a specific pattern across the secretory compartments. Such compartmentalized organization of the SL synthesis has functional implications as it makes the fine-tuned regulation of the process possible by allowing cells to regulate specific segments of the pathway in response to stimuli and for adaptation. The organization, dynamics, and regulation of the SLs and their biosynthetic machinery have been investigated using imaging-based methods. Here we provide a brief introduction to the techniques that have been or that could be employed to visualize the SL biosynthetic machinery and SLs themselves and discuss the insights provided by these studies in understanding this metabolism.

1. Introduction

The plasma membrane of eukaryotic cells is enriched in sphingolipids (SLs), which serve both signalling and structural functions that are fundamental for cell organization and physiology (Hannun and Obeid, 2018). The production of SLs begins in the Endoplasmic Reticulum (ER) and continues at the Golgi complex with consecutive synthetic reactions being distributed along the different sub-compartments of the secretory pathway. Thanks to this organization, enzymes are restricted to specific compartments that provide them optimal physico-chemical conditions for their activities and segregate them from competing enzymes. The organelles hosting the SL synthetic enzymes, then, exchange metabolic intermediates through vesicular and non-vesicular means and SL trafficking influences metabolic fate of SL precursors. Given the importance of compartmentalization and trafficking for SL biosynthesis, we analyse the published data on the subcellular distribution of SLs, SL biosynthetic enzymes and of their regulators and the implications of the distinct localization to their function.

2. The SL biosynthetic pathway

The SL biosynthesis starts with the production of ceramide (Cer) that is then channelled across multiple mutually exclusive pathways (Fig. 1) ending up in hundreds of end products (Fig.1b) [see (Hannun and Obeid, 2018; Yamaji and Hanada, 2015) for detailed descriptions]. Cer biosynthesis starts with the action of serine palmitoyl transferases (SPT1-3) that condense the amino acid serine with palmitoyl CoA to produce 3-ketodihydrosphingosine. This is then reduced by 3-ketodihydrosphingosine reductase (KSR) to produce dihydrosphingosine (a.k.a. sphinganine). Dihydrosphingosine is then acylated by one of the six ceramide synthases (CerS1-6) using acyl CoA as the donor to produce dihydroceramide. The dihydroceramide thus produced is then desaturated by the dihydroceramide–D4-desaturases (DES1 and DES2) to produce Cer.

This Cer is processed along the secretory pathway and can meet with one of the following anabolic fates: **a.** it can be transported by CERT (ceramide transfer protein) in non-vesicular manner to sphingomyelin synthase (SMS) containing compartment, where a phosphocholine group is transferred from phosphtidylcholine to Cer to produce sphingomyelin (SM); **b.** galactosyl transferase can add galactose to Cer to produce galactosylceramide (GalCer); **c.** similarly, a glucosyl transferase can produce glucosylceramide (GlcCer); **d.** acylation of ceramide leads to the production of acylceramide which is then accumulated in lipid droplets and **e.** finally phosphorylation of Cer leads to the production of Cer-1-phosphate, an important mediator of inflammatory signalling.

While SM is not modified any further, GalCer and GlcCer are further modified to produce complex glycosphingolipids (GSLs). GalCer is sulphated by cerebroside sulfotransferase to produce sulfatide, galactosylated to produce Gb2 or sialylated to produce GM4. GlcCer on

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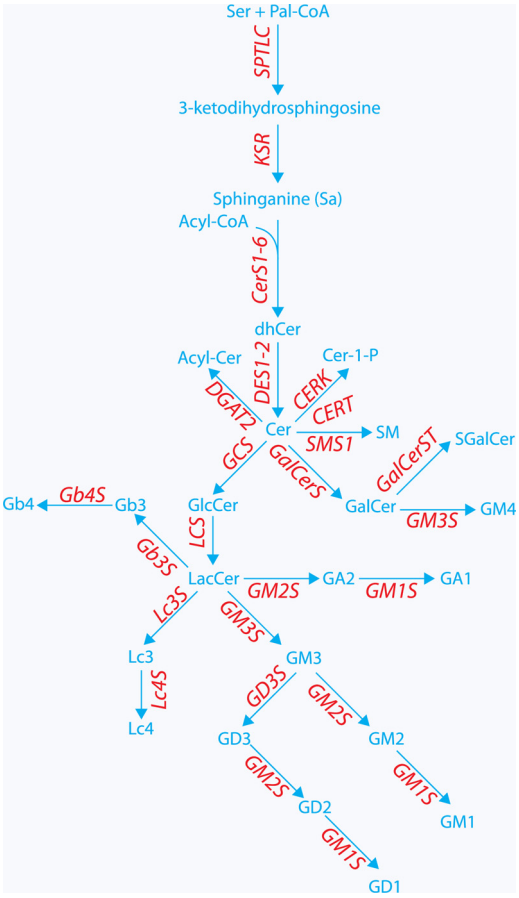

Fig. 1. Schematic representation of SL biosynthetic reactions:
In cyan are SL metabolites: Ser, Serine; Pal-CoA, Palmitoyl-CoA; dhCer, dihydroceramide; Cer, ceramide; Cer-1-P, cearmide-1-phosphate; SM; sphingomyelin; GalCer, galactosylceramide; SGalCer, sulfo- galactosylceramide; GM4, NeuAc-GalCer; GlcCer, glucosylceramide; LacCer, lactosylceramide; GM3, NeuAc-LacCer; GM2, GalNAc-GM3; GM1, Gal-GM2; GD3, NeuAc-GM3; GD2, GalNAc-GD3; GD1, Gal-GD2; GA2, GalNAc-LacCer; GA1, Gal-GA2; Lc3, GlcNAc-LacCer; Lc4, Gal-Lc3; Gb3, Gal-LacCer; Gb4; GalNAc-Gb3. In red are SL synthetic enzymes: SPTLC, Serine palmitoyltransferase; KSR; 3-Ketosphingosine Reductase; CerS, Ceramide synthase; DES; sphingolipid-delta-4-desaturase; CERK; Ceramide kinase; CERT, Ceramide transfer protein: SMS1, sphingomyelin synthase 1; GalCerS, GalCer synthase; GAlCerST, GalCer sulfo-transferase; GCS, GlcCer synthase; LCS, LacCer synthase; GM3S; GM3 syntahse; GM2S; GM2 synthase; GM1S, GM1 synthase; GD3S, GD3 synthase; LC3S, Lc3 synthase; Lc4S, Lc4 synthase; Gb3S, Gb3 synthase; Gb4S, Gb4 synthase.

the other hand is galactosylated to produce lactosylceramide (LacCer) by lactosylceramide synthase (LCS). LacCer has multiple fates and is further glycosylated to produce one of the following three series of GSLs: a. Lacto/neolacto series initiated by the N-acetylglucosaminyl transferase B3GNT5 (a.k.a. Lc3 synthase; Lc3S) that produces Lc3 b. Globosides initiated by the galactosyl transferase A4GALT (a.k.a. Gb3 synthase; Gb3S) that produces Gb3 from LacCer and c. Ganglioseries initiated by the sialyltransferase ST3GAL5 (a.k.a. GM3 synthase; GM3S) that produces GM3. These GSLs are then processed by additional glycosyltransferases to produce nearly 500 varieties of GSLs belonging to the different series (Fig. 1).

Individual tissues and cell types produce their own specific set of SLs depending on the relative expression of SL synthetic enzymes (Handa and Hakomori, 2017). Nonetheless, not all of the diversity in SL production is explained by variations in gene expression (Nairn et al., 2012). Thus, other factors acting at the post-transcriptional level affect SL production, which include specific conformations in the spatial arrangement of the SL synthetic machinery and in SL trafficking (Yamaji et al., 2008). Moreover, the production of the same bioactive SLs at different subcellular locations triggers specific cellular responses due to the distinctive subcellular distribution of SL signalling targets emphasizing the importance of a space resolved analysis while studying SL biology (Hannun and Obeid, 2011).

2.1. Localization of the SL synthetic machinery

The localization of enzymatic activities to the sub-cellular compartments was traditionally evaluated by sub-cellular fractionation followed by assays for their activity (De Duve, 1965). In subcellular fractionation approaches cells are homogenized and organelles are separated due to their different physical properties by the use of differential centrifugation and density gradients. Individual fractions are then tested for enzymatic activities or for immunoreactivity of antibodies recognizing specific antigens (De Duve, 1965). While this method is indeed powerful, the spatial resolution obtained is limited and it was soon superseded by immunolocalization coupled to microscopy. Immunohistochemistry and immunofluorescence microscopy have revolutionised our understanding of subcellular organization of the cellular compartments. These coupled to electron microscopy, with its unparalleled resolution and contextual information, have provided most of the information about the organization of the sub-cellular compartments and the localization of specific protein and metabolites including those involved in SL biosynthesis.

2.1.1. Ceramide synthesis

As mentioned earlier, the production of Cer starts with the SPT activity catalysed by the **SPTLC** genes. The SPT activity was localized to the ER by subcellular fractionation assays, which showed that close to 98% of the SPT activity co-segregated with the ER fraction (Mandon et al., 1992). Further, catalytic activity was also shown to be restricted to the cytosolic side of the ER membrane, by protease sensitivity assays using intact ER fractions (Mandon et al., 1992). While the assays described above are robust, it is nevertheless possible that the measured activity in the ER is due to the inability of the *in vitro* assay conditions to measure SPT activity in other cellular fractions. This uncertainty was overcome by immunolocalization assays using tagged versions of the enzyme as well as by immunolocalizing endogenous proteins. Immunofluorescence staining of tagged versions of SPTLC1 localized the protein to ER in concordance with the fractionation assays, while the localization of the endogenous protein showed that the protein also localized to the nucleus and focal adhesions (Wei et al., 2009). While these observed secondary localizations could be an artefact produced by the antibody used, biochemical and functional studies that followed have shown that this may not be the case (Wei et al., 2009). Nevertheless, in the absence of appropriate controls and the fact that the tagged versions are seen only in the ER in line with the fractionation assays suggest that the most likely localization of the SPT enzymatic activity is the ER.

The 3-ketodihydrosphingosine produced by SPT is further processed sequentially by **KSR** and **CerS** whose catalytic activities were shown to reside in the isolated ER fractions. Their activities were localised to the cytosolic side of the ER membrane by assaying the protease sensitivity of the enzyme in the case of KSR and the enzyme activity in the case of CerS (Kihara and Igarashi, 2004; Pewzner-Jung et al., 2006). In addition, immunofluorescence staining has also confirmed their localization to the ER (Kihara and Igarashi, 2004; Pewzner-Jung et al., 2006). While in the case of KSR, an untagged enzyme was used to localize the protein, C-terminal epitope tagged versions of CerS (which includes 6 isoforms CERS1-6) were used for the localization studies ER (Kihara and Igarashi, 2004; Pewzner-Jung et al., 2006). The agreement between the immunolocalization and the subcellular fractionation studies strengthens the conclusion that these enzymes do localise to the ER and their biosynthetic activity takes place in this compartment.

The dihydroceramide produced by CerS activity is then desaturated

by dihydroceramide desaturases (**DES1/2**). The DES1/2 activities have been localized to the ER and specifically to its cytosolic side using the protease sensitivity assays described earlier (Michel and van Echten-Deckert, 1997). The protein has also been colocalized with cytochrome b5 an ER marker in tissue sections and it also requires the cytochrome for its activity (Enomoto et al., 2006). Thus, given the similarity in the results obtained by two very different techniques the protein localizes very likely to the ER. Altogether these data indicate that SPTLCs, KSR, DES1-2, and CERS1-6 coexist at the ER and all have their active sites oriented in a way to make the cytosolic leaflet of ER membrane a conducive environment for *de novo* Cer synthesis.

2.2. Localization of the Cer processing machinery

Most of the ceramide produced in the ER is converted to SM at the Golgi apparatus. A major portion of the SM biosynthesis depends on the non-vesicular transport of the Cer from the ER to the Golgi by the ceramide transfer protein, **CERT**. CERT has been localized to the Golgi by fluorescence microscopy (Hanada et al., 2003). It has an N-terminal pleckstrin homology (PH) domain that is necessary for its targeting to the Golgi by binding phosphatidyl inositol 4-phosphate [PtdIns(4)*P*], a FFAT (two phenylalanine in an acidic tract) motif that binds the VAP proteins in the ER and a C-terminal START domain that is involved in lipid transfer. The presence of domains to bind both ER and the Golgi suggests that the protein is likely localized to the ER - Golgi membrane contact sites that are specific regions of close membrane apposition where non-vesicular lipid exchange events take place (De Matteis and Rega, 2015). Dynamics of ceramide transfer studied using fluorescently labelled ceramide (see below) has shown the ER to Golgi transport of ceramide mediated by CERT happens in a time frame of minutes (Hanada et al., 2003).

The Cer thus transported to the Golgi is first spontaneously translocated to the Golgi lumen where it is then converted to SM by the Golgi localized **SMS**. Activity assays had localized the SMS to the cis/medial Golgi (Futerman et al., 1990; Jeckel et al., 1990) as well as to the basolateral plasma membrane. To this end, the membrane sub-fractions were analysed for their SMS activity as well as activity of the compartment markers including the Golgi enzymes mannosidase, N-acetyl glucosamine transferase (GlcNacT), galactosyl transferase and sialyl transferase. The Golgi localized fraction of the SMS activity coincided with the fraction containing Mannosidase II and GlcNacT leading to the conclusion that the enzyme is localized in the cis/medial Golgi (Futerman et al., 1990). Molecular cloning studies then revealed that the mammalian genome encodes two isoforms of the enzyme **SMS1** and **SMS2** localised to the Golgi and the plasma membrane respectively (Huitema et al., 2004). High-resolution EM studies with the tagged version of the protein showed that SMS1 was mostly concentrated in the trans-side of the Golgi with the peak observed in the penultimate cisterna on the trans side (Halter et al., 2007). This study did not explicitly discuss the localization of the SMS1 to the TGN and treatment of the transfected cells with brefeldin A was shown to redistribute the protein back to the ER suggesting that the protein mainly localized to the Golgi stacks and very little if any of the protein is found in the TGN. Of note, SMS localization also depends on the cell type used for the study with SMS activity present mostly in the Golgi stacks in fibroblasts while it is restricted to the TGN in neuronal cells (Sadeghlar et al., 2000).

Of the rest of the Cer that is not converted to SM, most is converted to glycosphingolipids (GSLs). The GSL biosynthetic machinery is mainly localized to the Golgi apparatus. An exception is the GalCer Synthase (**GalCerS**) that is localized to the ER as evidenced by immunofluorescence analysis of epitope tagged version of the enzyme (Sprong et al., 1998). The activity assays on isolated subcellular fractions had localized the enzymes to several compartments including ER, Golgi and the plasma membrane. A later study had showed that the anomalous localization of the GalCerS activity in Golgi was due to the activity of GlcCer synthase (GCS) which can also accept UDP-Galactose as a substrate in the absence of glucose (Sprong et al., 1998). The cloned GalCerS was found to be a type I membrane protein with its catalytic portion in the lumen of the ER as evidenced by protease sensitivity assays (Sprong et al., 1998). The localization of the enzyme to ER and the presence of its catalytic activity on the luminal side imply that the ceramide synthesized in the cytosolic side of the membrane translocates across the membrane in order to accesses the catalytic domain. Further, studies have shown that GalCerS interacts with galactose transporter UGT1 to retain the transporter partially in the ER that in turn provides the UDP-galactose necessary for the reaction (Sprong et al., 2003).

Unlike GalCerS which is a type I membrane protein of the ER, most other ceramide processing machinery are localized to the Golgi and are either type II membrane proteins or multi-transmembrane proteins like GCS and SMS1. Among the GSL biosynthetic pathway, the first enzyme, the **GCS** has been localized to the Golgi (Coste et al., 1985). Experiments on the localization of GCS enzymatic activity have suggested two different localizations. One study localized the activity to the *cis*-side of the Golgi and a pre-Golgi compartment (Futerman and Pagano, 1991), while another study showed a predominant localization only to the Golgi apparatus but an equal distribution between the *cis*/medial and *trans*-Golgi (Jeckel et al., 1992). Localization of the endogenous GCS in *Drosophila* localized the protein both to the Golgi apparatus and the ER (Kohyama-Koganeya et al., 2004). Expression of a tagged version of the enzyme localized it to the Golgi and a quantitative immuno-EM of this protein showed that it is distributed across the Golgi stack with a peak in the trans-side, but unlike SMS1 a substantial portion of the enzyme was also present in the *cis*-side of the Golgi (Halter et al., 2007). Thus, both the activity based and the immunolocalization based assays (albeit with a tagged construct) have shown that the protein is localized mostly to the Golgi, where it is distributed across the stack with a slight preference for the cis-medial portion of the stack.

GCS active site is oriented towards the cytosolic membrane leaflet (Jeckel et al., 1992) while LacCer synthesis and subsequent GSL synthetic reactions are confined to the luminal membrane side of the Golgi complex (Burger et al., 1996). Moreover, differently from Cer, GlcCer shows a remarkably low rate of spontaneous transbilayer movement in model membranes, which implies the existence of an active mechanism for the translocation of GlcCer from the cytosolic to the luminal membrane for its processing (Buton et al., 2002). Both ER and Golgi membranes have been found to rapidly translocate GlcCer to their lumen in *in vitro* reconstitution assays in a saturable and protein-mediated process (Buton et al., 2002; Chalat et al., 2012). The identity of the GlcCer translocating protein is still largely elusive and the possibility that multiple factors contribute to GlcCer translocation at different cellular locations with different metabolic outcomes has been proposed (De Rosa et al., 2004).

The next enzyme in the GSL biosynthetic pathway is the **LCS**. The LCS activity had been localized to the lumen of the Golgi apparatus using protease sensitivity assays coupled to sub-cellular fractionation, though its sub-Golgi localization was not worked out (Lannert et al., 1994). Molecular cloning studies had shown that there are at least two enzymes encoding the LCS activity B4GALT5 and B4GALT6. Studies using mice and cell lines demonstrated that B4GALT5 is the main LCS in most tissues (Tokuda et al., 2013) while B4GALT6 contributes 40% of LacCer production in the brain (Yoshihara et al., 2018). The localization of the B4GALT5 using quantitative cryo-immunoEM demonstrated that the protein is localized all over the Golgi with a predominant concentration in the trans-side (Halter et al., 2007). Though this study had not examined its localization to the TGN, another study using semi-quantitative immuno EM methods had demonstrated the localization of B4GALT5 also the TGN (D'Angelo et al., 2013b).

The LacCer synthesized by LCS action can undergo at least 3 mutually exclusive pathways of processing. The initiating enzymes of all three pathways have been localized to the Golgi apparatus by activity-based assays (Lannert et al., 1998). The first is the globoside pathway,

where Gb3 synthase (**Gb3S**) adds a galactose to initiate the production of the globo series of GSLs. The biosynthesis of Gb3 by Gb3S is promoted by the non-vesicular transport of GlcCer by **FAPP2** (D'Angelo et al., 2007) to a likely complex containing Gb3S and LCS. FAPP2 has a glycolipid transfer domain through which it picks up GlcCer from the cytosolic side of the Golgi apparatus and transfers it to the TGN where the LCS and Gb3S are localized. Immunoelectron microscopy with a tagged version of Gb3S showed that is predominantly present in the TGN and as expected from a TGN localized protein the synthesis of Gb3 is sensitive to BFA (D'Angelo et al., 2013b). FAPP2 recognizes the trans-Golgi through its PH domain that binds PtdIns(4)*P*. Immunoelectron microscopy-based localization studies have shown that at steady state FAPP2 localizes mainly to *trans*-side of the Golgi and TGN (D'Angelo et al., 2013b). Its association with the *cis*-side of the Golgi to bind GlcCer for delivery to the trans-Golgi probably depends on the ability of the protein to bind ARF through its PH domain (D'Angelo et al., 2013b).

The second arm of the GSL biosynthetic pathway is the lacto/neolacto series of GSLs. Lc3 synthase (**Lc3S**) adds a N-acetylgalactosamine to start this series of GSLs (Henion et al., 2001). While the Lc3S is expected to be localized to the *trans*-side of the Golgi its precise intra-Golgi localization is yet be to be explored in detail. The final arm of the GSL biosynthetic pathway is the ganglioside series characterized by the presence of sialic acid residues. GM3 synthase (**GM3S**) adds a sialic acid to start the ganglio series of GSLs. While GM3S is localized to the Golgi apparatus it was not found in the TGN but showed a predominant localization to the trans-side of the Golgi apparatus (D'Angelo et al., 2013b) and GM3 production is not sensitive to BFA (D'Angelo et al., 2013b). GM3 thus produced is processed further by other enzymes including GM2/GD2 synthase and others that are mainly localized to the TGN (Giraudo et al., 1999).

A minor fraction of the ceramide is also phosphorylated by Cer Kinase (**CERK**) to form Cer-1-phosphate (Cer1P) that has important roles including the regulation of proliferation and apoptosis (Gomez-Munoz, 2006). CERK localization has been controversial with published reports that support its localization in the plasma membrane, endosome like structures as well as Golgi apparatus (Lamour et al., 2007; Rovina et al., 2009; Van Overloop et al., 2006). All of these studies had used tagged versions of the protein and different cell lines for these studies. Whether the localization depends on the cell lines used for the study or the presence of the tag that might differentially affect localization is not clear. Localization of the endogenous protein may help resolve this issue. Further, the Cer1P produced by CERK is transported to the plasma membrane by the Cer1P transfer protein (**CPTP**) whose action reduces the Cer1P levels in the Golgi where cytosolic phospholipase A2 alpha acts on it to initiate the eicasonoid biosynthetic pathway (Simanshu et al., 2013). The CPTP was localized to the TGN and endosomes by immunofluorescence analysis (Simanshu et al., 2013) and the reduction in CPTP levels leads to an accumulation of Cer1P in TGN suggesting that Cer1P is likely to be produced by the Golgi localized CERK.

Finally, a fraction of Cer produced by the CerSes localized to the ER can also be converted to acyl ceramide and stored in the lipid droplets. This reaction is catalyzed by the diacylglycerol acyltransferase 2 (**DGAT2**) and the acyl chain for the reaction is supplied by fattyacyl CoA synthase 5. These two enzymes interact with CerS in the ER and are also found in the lipid droplets as evidenced by IF microscopy (Senkal et al., 2017). The data about the localization of the SL biosynthetic system discussed here is depicted as a scheme in Fig. 2.

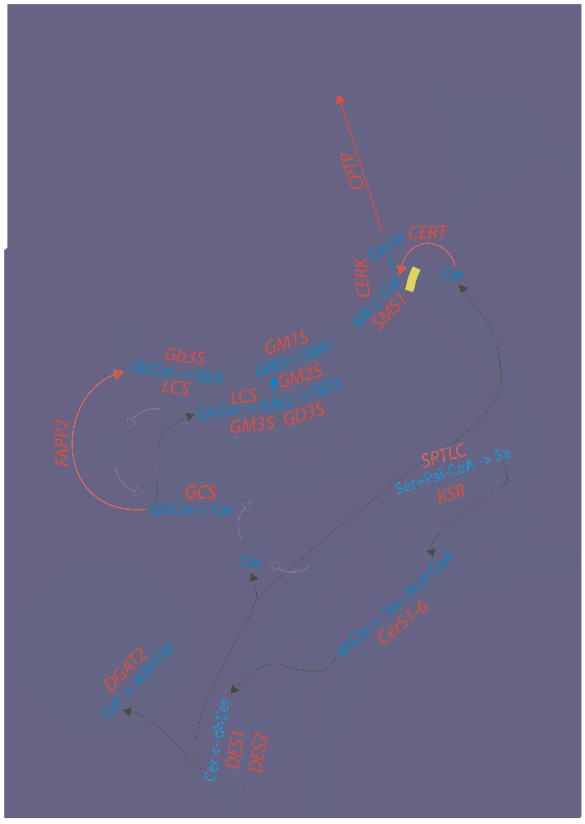

Fig. 2. Schematic representation of the intracellular distribution of the SL biosynthetic system.
SL metabolites (in cyan) and SL synthetic enzymes/ tranfer proteins (in red) are represented according to their reported subcellular distribution. En, endosomal system; GC, Golgi complex; ER, endoplasmic reticulum; LD, lipid droplet. The yellow shaded area represents ER-Golgi membrane contact sites.

3. Dynamics of the SL biosynthetic machinery

An important aspect in the study of SL biosynthesis relates to the dynamics of the system. Protein dynamics are usually analysed by transfecting protein of interest tagged with a fluorescent protein followed by video microscopy (Lippincott-Schwartz et al., 2001). Fluorescence bleaching or photoconversion (change in fluorescence excitation/emission properties of a fluorophore) obtained by the use of high power laser sources allow the precise assessment of the dynamic behaviours of the molecules of interest. For instance, the movement of molecules within one compartment or among different compartments can be approached by Fluorescence recovery after photobleaching (**FRAP**). In this method, a high intensity laser is focused on a small area of the cell or an organelle of interest so as to bleach the fluorophores present in the area. The recovery of fluorescence in this area due to the movement of the fluorophores from other regions of the cell is monitored by video microscopy. The dynamics of the GSL biosynthetic enzymes (mostly involved in ganglioside synthesis) in the Golgi apparatus and their cycling between different compartment of the secretory pathway was analysed by FRAP (Giraudo and Maccioni, 2003b). These studies have shown that these enzymes do recycle through the ER with kinetics comparable to that of the other glycosylation enzymes examined (Giraudo and Maccioni, 2003a). Further studies are needed to examine their transport across the secretory pathway, the dynamics of their recycling and role of the transport machinery in this process.

The FRAP analysis is easy to perform, but the ensuing analysis of kinetics is complicated by the fact the measured fluorescence recovery is a combined effect of both fluorophore entry into as well as its exit out of the bleached area. While in most cases involving a homogenous compartment, the rates are the same, in specific cases it may not be so. In these cases, to obtain the precise measure of the rates, FRAP is usually combined with another technique called fluorescence loss in photobleaching (**FLIP**). In this analysis, all regions of the cell except the region of interest is continuously bleached while monitoring the fluorescence levels in the region of interest. This method provides the precise description of the kinetics of fluorescence loss from the region of

interest or a measure of the kinetics of the fluorophore exit from the compartment or region of interest (McNally, 2008).

To date there have been limited attempts to study on the dynamics of the SL biosynthetic system. This kind of approach would likely produce interesting information especially if applied to the study of the two lipid transfer proteins – CERT and FAPP2.

As discussed above CERT and FAPP2 pick-up their lipid cargoes from the cytosolic leaflet of ER and *cis*-Golgi membranes respectively subtracting them from the membrane trafficking stream to deliver them directly to distal Golgi thus bypassing the intervening sub-compartments (D'Angelo et al., 2013a; Hannun and Obeid, 2018). Both CERT and FAPP2 mediated non-vesicular lipid transfer activities have been proposed to convey SL substrates to an alternative metabolic fate to that favoured by vesicular transport (D'Angelo et al., 2013b; Hanada et al., 2003). Thus CERT by delivering Cer to the *trans*-Golgi makes it available to SMS1 and CERK for SM and Cer1P syntheses while FAPP2 delivers GlcCer to the TGN to favour the production of globo-series GSLs that is initiated by the TGN localized Gb3S enzyme, while bypassing the medial Golgi compartment where GM3S, the first enzyme involved in ganglio-series GSL synthesis resides (D'Angelo et al., 2013b). In agreement with this model changes in the lipid transfer activities of CERT and FAPP2 result in changes in the relative amounts of the different SL produced along the biosynthetic pathway. Moreover, the static localization of CERT is influenced by phosphorylation (Yamaji et al., 2008) and that of FAPP2 by its apo or lipid bound states (D'Angelo et al., 2013b) suggesting that these parameters control their dynamical association with cell membranes (Fig. 3).

Information is available on the dynamics of the related cholesterol transfer protein OSBP (oxysterol binding protein). OSBP dynamics as measured by FRAP assays indicated that the protein is highly mobile (with a complete turnover observed within a minute and almost 80% of the protein being mobile) in the absence of cholesterol, while the presence of cholesterol decreases the fraction of mobile protein (Mesmin et al., 2013).

4. Supra-molecular complexes

A further layer of organization in the SL synthetic system deals with the establishment and functioning of multi-enzymatic complexes. Complex formation involving the SL synthetic enzymes promotes substrate channelling and makes SL processing reactions more efficient (Spessott et al., 2012). Protein-protein interactions are often studied using biochemical methods including co-purification, size exclusion chromatography, surface plasmon resonance, or acrylamide gel electrophoresis (Phizicky and Fields, 1995). More recently imaging-based methods have been developed to study protein complexes formation that rely on fluorescence measurements (Ciruela, 2008). Here, the interaction between two proteins is translated into a fluorescence signal that can be monitored by microscopy that provides the additional information about the location where the interaction takes place.

Bimolecular interactions are studied by **split-fluorescent protein technique** where in the fluorescent protein molecule is divided into

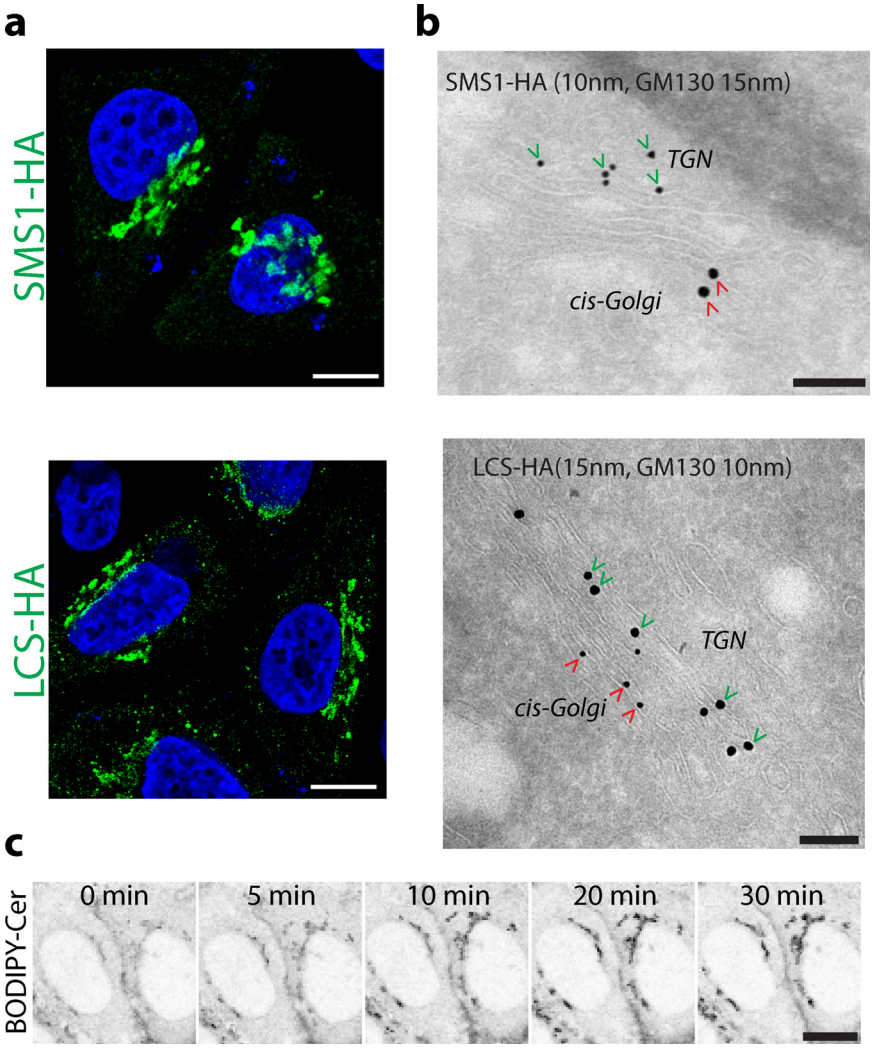

Fig. 3. Localization of SL biosynthetic machinery and dynamics of ceramide transport.
a. Immunofluorescence staining of hemagglutinin peptide (HA) -tagged SMS1 and HA-tagged LCS using anti-HA antibodies (green) demonstrates their localization to a perinuclear structure (nucleus stained with DAPI in blue) typical of Golgi apparatus. **b.** Immunogold labelling of SMS1-HA and LCS-HA using colloidal gold tagged probes. The HA-tagged proteins are indicated by green arrow heads (10 nm gold for SMS1-HA and 15 nm gold for LCS-HA) while a cis-Golgi marker GM130 is indicated with red arrows (either 10 nm or 15 nm gold). As can be clearly seen here both SMS1-HA and LCS-HA are localized to the pole opposite GM130 suggesting trans-Golgi localization of these proteins. **c.** HeLa cells were incubated with BODIPY-labelled ceramide at 4 °C when it accumulates in the ER as indicated by a diffuse staining and exclusion from the nuclear area (0 min). The shift to 37 °C leads to very quick transport of ceramide from the ER and concentration in the peri-nuclear structure (Golgi apparatus). The imaging of the transport of this fluorescently labelled ceramide was done by video microscopy and selected frames from this movie are shown with corresponding time after the shift to 37°C.

two halves and these halves are tagged to two proteins that are being tested for their interaction. For instance, the yellow fluorescent protein (YFP) molecule can be divided into the N-terminal half containing the residues 1–173 and the C-terminal half containing the residues 155-238. If the proteins to which they are tagged interact the two halves of the YFP protein are brought together restoring a functional fluorescent protein that can be imaged (Kodama and Hu, 2013).

While this method is qualitative and at best semi-quantitative, a quantitative measure of protein-protein interaction can be obtained using fluorescence resonance energy transfer (FRET). **FRET** involves non-radiative energy transfer between two fluorophores by dipole-dipole coupling and this transfer is inversely proportional to the distance between the fluorophores (as sixth power of the distance, so it rapidly reduces!). In simpler terms, when two appropriate fluorophores are brought together, the activation of the donor fluorophore leads to the non-radiative energy transfer to the acceptor fluorophore such that it becomes excited. The emission from the acceptor fluorophore can be quantitated as a measure of protein-protein interaction (Pietraszewska-Bogiel and Gadella, 2011; Shimozono and Miyawaki, 2008).

The FRET technique was used to study complex formation between glycosphingolipid biosynthetic enzymes in the Golgi (Bieberich et al., 2002; Ferrari et al., 2012). Studies based on split GFP and FRET measurements have shown that the enzymes involved in consecutive reactions in the SL synthetic pathway at the Golgi complex physically interact to form multi-enzymatic complexes able to direct SL fluxes towards specific metabolic directions. Thus LCS, GM3S and GD3S form a complex at medial trans Golgi able to mediate the direct conversion of GlcCer to GD3 (Spessott et al., 2012); GM2S can interact with GM1S at the TGN leading to GM3 conversion to GM1 (Giraudo et al., 2001) or with GD3S leading to the conversion of GM3 to GD2 (Bieberich et al., 2002) and similarly, LCS and Gb3S synthase have been suggested to interact to yield Gb3 production from GlcCer. Importantly the composition of such multi-enzymatic complexes has been suggested to influence their sub-Golgi localization thus demonstrating an inherent capacity of the biosynthetic system to adapt itself to cellular needs and intercept different substrate fluxes (Uliana et al., 2006).

4.1. Visualizing the SLs and their dynamics

While studying the biosynthetic machinery is important to understand the processes involved in biosynthesis, the direct visualization of the substrates and products of these enzymatic reactions provides insights into how the metabolites are handled by these machineries. Visualizing SLs has been a challenge since any addition of fluorophores to these lipids has a high probability of changing their physic/chemical nature and hence the results from these studies have to considered with certain reservation. Nevertheless, several chemical SL probes have been developed that have helped visualize the transport of these lipids and their metabolism. These chemical SL probes rely on the use of chemically modified SLs where they are functionalized with a label that can be detected by imaging techniques.

Fluorescently labelled SLs have been produced since the early 1980s that can be incorporated in cell membranes and followed by conventional and live microscopy (or, following photo-conversion by electron microscopy) along the endocytic and biosynthetic pathways (Koval and Pagano, 1989, 1990; Lipsky and Pagano, 1983). Fluorescent SLs are obtained by substituting the naturally occurring fatty acid with a short fluorescent one. A first generation of fluorescent SL probes was based on 7-nitrobenz-2-oxa-1, 3-diazole **(NBD)-labelled** fatty acids (Lipsky and Pagano, 1983, 1985a; Lipsky and Pagano, 1985b) and their use allowed to reveal the compartmentalized nature of SL biosynthetic pathway (Pagano and Sleight, 1985) i.e. the NBD labelled ceramides first label the ER, from where they are transported actively to the Golgi apparatus and then to the plasma membrane.

Nonetheless the low fluorescence yield and the relatively high hydrophilic nature of NBD that makes it to loop back to the membrane/water interface, which translates into a short residence time of NBD-SLs in lipid bilayers and this has cast doubts about their ability to recapitulate the behaviour of their non-labelled counterparts. In the 1990s, a second generation of fluorescent SL probes based on boron dipyrromethene difluoride (**BODIPY**) was obtained (Pagano et al., 1991, 2000) where their properties were more similar to that of natural SLs. BODIPY-SLs have been instrumental in the understanding of non-vesicular SL transport to and through the Golgi and for the study of membrane microdomains due to the property of BODIPY to undergo a concentration-dependent monomer-excimer fluorescence shift (Marks et al., 2008). Dynamics of Cer transport to the Golgi complex have been studied by the use of fluorescently labelled Cer analogues and were found to be fast and dependent on the presence of CERT (Hanada et al., 2003). A newer development in the production of SL probes for cellular imaging is the use of click chemistry. Click chemistry allows the detection of compounds containing azido groups or terminal alkynes that can be visualized by microscopy after a chemical reaction with a fluorophore. Clickable SL analogues have been used recently for the super resolution imaging of SLs in live cells (Erdmann et al., 2014).

A parallel approach to SL imaging is that involving affinity probes that have been developed for the visualisation of endogenous SL. These probes consist either of proteins or protein domains with high binding affinity and specificity for a given SL or of antibodies recognizing specific SL epitopes. Two main classes of affinity probes have been employed for SL imaging: these are toxins and anti-SL antibodies.

4.1.1. SL binding toxins

Being components of the luminal leaflet of the eukaryotic plasma membranes some SLs are exposed to the extracellular environment and several pathogens have evolved strategies to either infect or intoxicate cells by using plasma membrane SLs as their targets. Specifically, some SL-targeting toxins are equipped with sufficient affinity and specificity for individual SLs to be used as affinity probes for SL visualization. These include bacterial toxins such as Shiga and Cholera toxins (Heyningen, 1974; Jacewicz et al., 1986) or toxins derived from metazoa such as Lysenin and Equinatoxin II (Bonev et al., 2003; Yamaji et al., 1998).

Lysenin (Yamaji et al., 1998) is a 33-kDa (297 aa) protein produced by the earthworm *Eisenia foetida* that strongly binds SM and induces cell death by forming pores upon oligomerisation at the plasma membrane (Bokori-Brown et al., 2016; Yilmaz et al., 2018). Each lysenin monomer binds five to six SM molecules preferentially when present in clusters in the membranes. The C-terminus of lysenin is essential for binding to SM while its N-terminal part is required for oligomerisation, thus a non-toxic C-terminal lysenin fragment has been isolated that maintains SM binding activity and is referred to as NT-lysenin. Both natural and NT-lysenin have been employed to visualize SM by fluorescence and electron microscopy (Kishimoto et al., 2016) while NT-lysenin, due to its lack of toxicity, has been used to follow SM dynamics in living cells by video microscopy (Carquin et al., 2014).

Also **Equinatoxin II** (EqtII), a 179-aa pore-forming toxin produced by sea anemones, (Kishimoto et al., 2016) binds specifically to SM. Nonetheless, EqtII preferentially decorates liquid disordered domains in monolayers containing SM suggesting that differently from lysenin, EqtII recognizes SM when in a 'dispersed' conformation (Kishimoto et al., 2016). GFP-tagged EqtII (EqtII-GFP) has been used as an SM probe both added exogenously to intact or permeabilised cells or as an intracellularly (and luminally) expressed protein (Deng et al., 2016). Depending on the mode of use EqtII-GFP has been found to decorate specific PM domains, the endosomal compartment and the Golgi complex (Kishimoto et al., 2016). Due to their different affinities to packed and dispersed SM, lysenin and EqtII-GFP decorate mutually exclusive SM pools in model membranes and in cells thus their combined use is required to reveal the overall cellular SM distribution (Kishimoto et al., 2016).

Shiga toxin is a bacterial toxin produced by *Shighella dysenteriae*

and by some serotypes of *Escherichia coli* that causes bloody diarrhoea in humans (Melton-Celsa, 2014). Shiga toxin is an AB_5 toxin consisting of a single A subunit associated with a pentamer of identical B subunits. While the A subunit is responsible for cell toxicity by blocking protein synthesis, the B pentamer binds to the GSL Gb3 in host cell membranes (Johannes, 2017; Melton-Celsa, 2014) to mediate Shiga toxin endocytosis. Each Shiga toxin B subunit intercepts 2–3 Gb3 molecules resulting in the high affinity binding of the pentamer to Gb3 rich membranes (Melton-Celsa, 2014). Shiga toxin B has been used to visualize Gb3 distribution in tissues by fluorescence and histochemistry approaches and on cells to evaluate Gb3 levels on cell membranes (D'Angelo et al., 2013b).

Also, **Cholera toxin** produced by *Vibrio cholerae* that causes massive secretory diarrhoea in humans, is a AB_5 toxin (Wernick et al., 2010). As in the case of Shiga toxin, the A subunit is responsible for toxicity as it ADP-ribosylates the Gs alpha subunit of trimeric G proteins and forces it to a constitutively GTP-bound activated form (Wernick et al., 2010). The pentameric B subunit of Cholera toxin is instead responsible for the binding to cell membranes and for toxin endocytosis thanks to its binding to the GSL GM1. Due to its affinity for GM1, Cholera toxin B subunit has been used in a variety of imaging approaches to visualize GM1 on cell membranes similar to Shiga toxin B subunit (D'Angelo et al., 2013b).

4.1.2. Anti-SL antibodies

Antibodies recognizing different SLs have been produced over the years, some of which have been used for lipid immuno-localization. These include anti-Cer (Cowart et al., 2002), anti-GlcCer (Brade et al., 2000), anti LacCer (Iwabuchi et al., 2015; Symington et al., 1987), anti-GM3 (Kotani et al., 1992), anti-Gb3 (Kotani et al., 1992), and have been used for immunofluorescence and immunoEM studies on cells and tissues and in cytofluorimetric studies. Nevertheless, while affinity probes have provided valuable information about the tissue and cellular distribution of SLs they have limitations since their ability to bind SLs is often influenced by the accessibility of SLs in membranes and that most of them require elaborate fixation procedures to preserve the SL localization prior to detection.

A recent advance in visualization of the SLs is the development of label free methods based on mass spectrometry. Both the use of affinity and chemical probes for the visualisation of SLs have limitations due to the fact that SLs when chemically modified or engaged by toxins or antibodies behave differently than their natural counterparts. This has stimulated interest in the development of label-free (or minimal labelling) imaging strategies for SLs. One promising technology in this respect is **Imaging Mass Spectrometry** (IMS). IMS is a MS-based technique that enables the detection metabolites in a space resolved fashion and the generation distribution maps of a wide range of molecules, in the same specimen, without the need for invasive labelling and without altering the morphology of the tissue (Sugiura and Setou, 2010). Among the different IMS methods available, two have found application in SL research - nanoscale secondary ion mass spectrometry [nanoSIMS] and Matrix-assisted laser desorption/ ionization [MALDI]-IMS.

NanoSIMS enables the visualization of metabolically incorporated stable-isotope labelled SLs in membranes with a space resolution < 50 nm (Klitzing et al., 2013). In a nanoSIMS experiment a cesium or oxygen ion beam is scanned over the sample surface, molecules within the beam's focal volume are fragmented and the charged secondary ions ejected from the sample surface are analysed by a mass spectrometer (Nunez et al., 2017). NanoSIMS allows the assessment of the nanoscopic distribution of the different isotope-containing ions based on their precise mass-to-charge ratios (Nunez et al., 2017). A key advantage of nanoSIMS imaging is that it does not require using SLs modified with bulky fluorescent tags. Stable isotopes have indeed little or no effect on the biological properties of SL and a large variety of stable isotope–labeled lipids are available from commercial vendors. The main disadvantages of nanoSIMS lay in the facts that (i) it requires some preconceived knowledge of the sample and allows the assessment of only the labelled compounds and (ii) it requires invasive sample manipulations including fixation and dehydration and analysis under high vacuum.

Some of these limitations are overcome by **MALDI-IMS**. In a typical MALDI-IMS experiment, a frozen tissue sample is sectioned and mounted on a target plate. Subsequently, the tissue is coated with a homogenous layer of an organic matrix solution, placed in an ionization chamber and analysed. There, a laser beam 'scans' a specific area of the sample and collects a mass spectrum for each coordinate. The final distribution image of a specific ion is then generated by dedicated software that plots the intensity of that ion against the x-y coordinate. MALDI-IMS is completely label-free and it has been optimized to work at atmospheric pressure with minimal sample manipulation (Yalcin and de la Monte, 2015). The main disadvantage of MALDI-IMS is in its low spatial resolution (> 1 μm) that does not yet allow subcellular studies of lipid distribution.

5. Future directions

A significant body of knowledge has been accumulated on the localization and dynamics of both the SL biosynthetic machinery and the substrates/products themselves. Nevertheless, there are some glaring lacunae that need to be filled and also recent advances in molecular biology and imaging that need to be incorporated in the future studies to understand the system better. Some of these future directions are summarized below:

a As mentioned earlier, the **dynamics of the sphingolipid** machinery is an almost untouched area. Study of dynamics of glycosylation enzymes started nearly 20 years ago and has yielded several insights into the cell biology of the Golgi organization (Cole et al., 1996). We can predict that the study of dynamics of the SL biosynthetic enzymes and products would provide insights into their organization, the method of transport of the metabolites and quantitative test of present models of sphingolipid biosynthesis based on the study dynamics of the biosynthetic machinery.

b Most of the SL biosynthetic enzymes are expressed **in low copy numbers** in the cell and the need to study their dynamics and localization necessarily involves transfecting exogenous proteins that sometimes far exceed the endogenous levels. This casts doubts on the observed results. The recent development of CRISPR/Cas9 system that allows for genome editing so as to tag the endogenous proteins provides the opportunity to study the localization and dynamics of the proteins expressed at the low endogenous levels and under the regulatory controls that they are expected to have.

c The recent development of the *Retention using selective hooks* (RUSH) system (Boncompain et al., 2012) provides an easy way to synchronize the transport of several proteins along the secretory pathway. Application of this system to the GSL biosynthetic pathway will provide insights into the dynamics of the **transport of GSL biosynthetic enzymes** and help in the identification of the molecular machinery involved in this process.

d The development of **novel chemical probes to visualize the sphingolipids** is an active area of research and new developments in the future can be expected to lead to probes that are easily detectable and have physical-chemical properties similar to the endogenous ones.

e The SL biosynthetic pathway, in most cases, is studied in isolation, while it interacts extensively with the **other lipid biosynthetic pathways**. A prominent example is the PtdIns(4)*P* dependent regulation of both cholesterol and ceramide transport at the ER-Golgi contact sites. Thus, to study SL biosynthesis in the context of larger lipid biosynthesis would provide insights into communication channels between them and how they might be co-regulated to maintain cellular homeostasis.

f The accumulated knowledge on the localization of the different segments of SL synthetic machinery allows the drawing of a coherent model for the distributed SL biosynthesis under static conditions. Whether and how this topological orgaziation is altered under physiological or pathological conditions is currently not known. Learning about instances where changes in enzymes localization or SL trafficking impact on SL metabolism will probably reveal new important details in the organization of the SL synthetic system.

Conflicts of interest

The authors declare no conflicts of interest.

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