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REVIEW ARTICLE

Structure, functions and regulation of CERT, a lipid-transfer protein for the delivery of ceramide at the ER–Golgi membrane contact sites

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The inter-organelle transport of lipids must be regulated to ensure appropriate lipid composition of each organelle. In mammalian cells, ceramide synthesised in the endoplasmic reticulum (ER) is transported to the *trans*-Golgi regions, where ceramide is converted to sphingomyelin (SM) with the concomitant production of diacylglycerol. Ceramide transport protein (CERT) transports ceramide from the ER to the *trans*-Golgi regions at the ER–Golgi membrane contact sites (MCS). The function of CERT is down-regulated by multisite phosphorylation of a serine-repeat motif (SRM) and up-regulated by phosphorylation of serine 315 in CERT. Multisite phosphorylation of the SRM is primed by protein kinase D, which is activated by diacylglycerol. The function of CERT is regulated by a phosphorylation-dependent feedback mechanism in response to cellular requirements of SM. CERT-dependent ceramide transport is also affected by the pool of phosphatidylinositol (PtdIns)-4-phosphate (PtdIns(4)P) in the *trans*-Golgi regions, while the PtdIns(4)P pool is regulated by PtdIns-4-kinases and oxysterol-binding protein. The ER-Golgi MCS may serve as inter-organelle communication zones, in which many factors work in concert to serve as an extensive rheostat of SM, diacylglycerol, cholesterol and PtdIns(4)P.

Keywords: CERT1; FFAT motif; functional regulation; intellectual disability; lipid-transfer protein; membrane contact sites; OSBP; phosphorylation; PKD; sphingomyelin synthase

The Golgi apparatus is a complicated organelle that sorts and transports various kinds of proteins and lipids. While transport vesicles of the Golgi apparatus have receptors to load preferable proteins into the luminal space, it does not have apparent machinery to pick up specific lipids. To compensate for this inconvenience in vesicle transport, non-vesicular lipid-transporting proteins can recognise specific lipids and transfer them to a target compartment [1]. As we observed in the vesicular transport of proteins, non-vesicle lipid transport is also regulated in response to intracellular or extracellular situations. Here, we pick up the ceramide transport (CERT) protein to describe the regulation of lipid transport.

De novo biosynthesis of sphingolipids is initiated by the condensation of L-serine and palmitoyl-CoA to produce 3-ketodihydrosphingosine [2]. Then, several

Abbreviations
25-HC, 25-hydroxycholesterol; Cer1P, ceramide-1-phosphate; Chol, cholesterol; CK1γ2, casein kinase 1γ2; DAG, diacylglycerol; ER, endoplasmic reticulum; FFAT, two phenylalanines in acidic tract; GlcCer, glucosylceramide; MCS, membrane contact sites; ORP, OSBP-related protein; OSBP, oxysterol-binding protein; PH, pleckstrin homology; PKD, protein kinase D; PPM1L, phosphatase Mg^{2+}/Mn^{2+} dependent 1L; PtdCho, phosphatidylcholine; PtdIns(4)P, phosphatidylinositol-4-monophosphate; PtdIns, phosphatidylinositol; SM, sphingomyelin; SMS, sphingomyelin synthase; SRM, serine-repeat motif; START, StAR-related lipid transfer; TGN, *trans*-Golgi network; VAP, VAMP-associated protein.

reactions in the cytosolic face of the endoplasmic reticulum (ER) membrane generate a pivotal intermediate molecular ceramide [2]. Ceramide is a precursor for numerous sphingolipids including sphingomyelin (SM), glucosylceramide (GlcCer) and ceramide-1-phosphate (Cer1P). For the next biosynthetic reaction, ceramide must be transported from the cytosolic face of the ER to various regions, where various polar head groups are attached to ceramide as described below.

Sphingomyelin synthase (SMS) transfers the phosphocholine moiety from phosphatidylcholine (PtdCho) to ceramide to generate SM and diacylglycerol (DAG). The human genome encodes two SMSs (SMS1 and SMS2). SMS1, a major SMS for *de novo* SM biosynthesis that localises to the *medial*/*trans*-Golgi cisternae, and the *trans*-Golgi network (TGN), both of which are hereafter referred to as *trans*-Golgi regions [3–5]. SMS2 is distributed to the Golgi regions and plasma membrane [3,6]. The catalytic sites of SMS1/SMS2 face to the luminal/extracellular side [3]. GlcCer synthase, which transfers glucose from UDP-glucose to ceramide to generate GlcCer, distributes largely to the *cis*/*medial*-Golgi cisternae [4,7], and its catalytic site faces to the cytosolic side [8]. Ceramide kinase, which is a member of the DAG kinase family, phosphorylates ceramide to generate Cer1P [9]. Ceramide kinase is a cytosolic protein that associates with the TGN, endosome, plasma membrane and mitochondria [10,11].

CERT transports ceramide from the ER to the *trans*-Golgi regions with the aid of its domains and motifs [12–14]. Loss of function of the *CERT* gene in mammalian cells reduced the content of SM, but not of glycosphingolipids, indicating that CERT delivers ceramide from the ER to the Golgi complex for the synthesis of SM [13,14]. *CERT*-disrupted (*CERT*-KO) mice die at approximately E 11.5 due to cardiovascular insufficiency. Embryonic cells of *CERT*-KO mice have a decreased level of SM in the plasma membrane and an increased level of ceramide in the ER and mitochondria [15]. CERT-dependent ceramide transport is also necessary to suppress senescence of cells [16]. CERT is reported to be partially involved in the synthesis of Cer1P [11,17].

It remains elusive how ceramide is transported from the ER to the site of its conversion to GlcCer, which has been suggested to occur the cytosolic surface of various Golgi subfractions [18]. Previous studies suggested that conventional vesicular pathway does not play a major role in the transport of ceramide from the ER to the site for GlcCer synthesis in human colon HT29 G+ cells [19] and in Chinese hamster ovary CHO-K1 cells [12], while a vesicular pathway was suggested to be involved in the conversion of ceramide to SM and GlcCer in rat C6 glioma cells [20]. Thus, a vesicle-mediated pathway(s) may also contribute to the ER-to-Golgi transport of ceramide, depending on cell types. It should also be noteworthy that very long ceramide (e.g. C24-ceramide) might be preferentially transported via a vesicular pathway to deliver glycosyl phosphatidylinositol (PtdIns)-anchored proteins from the ER to the *cis*-Golgi region [21].

The synthesis of GlcCer is independent of CERT, although localisations of SMSs and GlcCer synthase partially overlap in the Golgi cisternae. This paradox might be explained by recent findings. First, CERT may preferentially transport ceramide subspecies that are preferential substrates for SMSs [22,23]. Second, when GlcCer synthase is partitioned into the same Golgi cisterna as SMS1, the activity of the former enzyme is inhibited [5]. Third, the activity of GlcCer synthase is mildly inhibited by PtdIns-4-monophosphate (PtdIns(4)P) [24], which is enriched in CERT-recruiting Golgi regions (see also below). Notably, how ceramide is delivered from the ER to the site of GlcCer synthesis remains unknown.

The activity of CERT is likely regulated by phosphorylation in response to cellular requirements of SM and/or lipid-rafts composed of SM and cholesterol (Chol) [25,26]. CERT-dependent ceramide flow from the ER to the *trans*-Golgi regions is also affected by the pool of Golgi-resident phosphoinositide [27]. In this review, we describe the regulation of CERT and discuss its physiological and pathophysiological significance.

Domain organisation of CERT

Ceramide transport, a cytosolic 68-kDa protein, has several discrete domains and motifs suitable to exert its function in a regulatory manner [13,28]. The N-terminal ~ 120 amino acid region of CERT forms a pleckstrin homology (PH) domain, and the C-terminal ~ 230 amino acid region forms a StAR-related lipid transfer (START) domain (Fig. 1A). The ~ 250 amino acid middle region (hereafter referred to as MR) between the PH and START domains is predicted to be an intrinsically disordered region [28] (Fig. 1A).

START domain

The START domain is a globular folded domain that mediates intracellular lipid transport. The CERT START domain efficiently extracts ceramide, but not other lipid types, from membranes and transfers the bound ceramide to other membranes [13,22]. To our

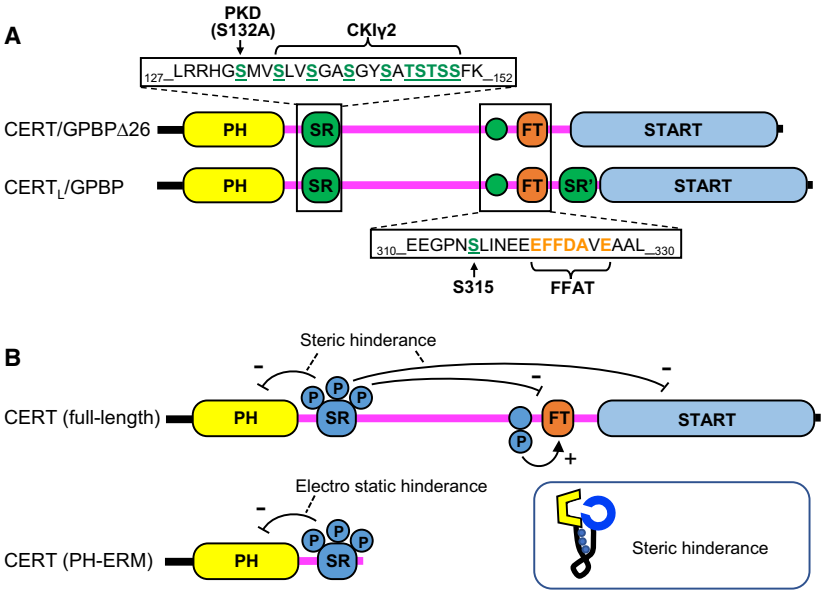

Fig. 1. (A) The domains and motifs of CERT or GPBP. The PH domain, START domain and core sequence of the FFAT motif are depicted in coloured objects, and the MR is shown in magenta bar. SR′ indicates the serine-rich region coded by the inserted exon of CERT$_L$/GPBP. The amino acid sequences around the SRM or FFAT core sequence are indicated. (B) The regulation of CERT activity by phosphorylation. The phosphorylation of SRM in the full-length CERT supresses the activity of the PH domain, FFAT motif and START domain. The PH and START domains are suppressed by mutual steric hindrance, presumably as shown in the inlet. The ERM (i.e. phosphomimetically mutated SRM) in the truncated form of CERT (PH-ERM) also suppresses the activity of the PH domain.

best knowledge, the CERT START domain is the sole human genome-encoded lipid-transfer domain that has been experimentally shown to selectively mediate ceramide transport. However, CERT seems to recognise DAG (which structurally resembles ceramide) although the activity of intermembrane transfer of DAG by CERT is far less than its activity towards ceramide [13,22]. Among the four stereochemical isomers of C16- ceramide, CERT specifically recognises the natural D-erythro isomer [22]. Despite such strict substrate specificity, CERT can transfer natural ceramide isoforms (C16-dihydroceramide and C16-phytoceramide) and various molecular species of ceramide having different acyl chain lengths [22]. The binding ratio of CERT and ceramide is estimated to be 1 : 1 [22].

The co-crystal structure of the START domain of CERT in a complex with ceramide has been solved [29]. X-ray crystallography of the CERT START domain has revealed the following: (a) all three polar groups (two hydroxyl groups and one amide group) of ceramide participate in a hydrogen-bonding network with specific amino acid residues in the amphipathic pocket of the START domain; (b) in the pocket, there is little space around the C1 hydroxyl group of the bound ceramide molecule in the co-crystals, which indicates that it is spatially impossible to accommodate sphingolipids having bulky head groups in the CERT START domain; and (c) nevertheless, different sets of amino acid residues in the amphipathic pocket participate in the hydrophobic interaction with the hydrocarbon chains of ceramide, depending on the molecular species of ceramides. These structural features could account for the strict substrate specificity to lipid classes and flexibility to the ceramide species of CERT [29]. The START domain is necessary and sufficient for the transfer of ceramide between artificial phospholipid membranes, although other regions of CERT are also important for the ER-to-Golgi trafficking of ceramide in cells.

PH domain

The PH domain is a small modular domain that binds to phosphoinositides or mediates protein–protein interactions. The CERT PH domain specifically or preferentially binds to PtdIns(4)P, which is abundant in the *trans*-Golgi regions [30], among various phosphoinositides in various types of assays [13,25,31,32].

The structural basis for the characteristics of the CERT PH domain was solved using solution NMR techniques [32]. The CERT PH domain was found to use at least two types of binding regions: a conventional phosphoinositide-binding pocket and a previously unrecognised region named a basic groove with a loop having a tryptophan pair [32]. Most amino acid residues forming the basic groove are conserved among the PtdIns(4)P-preferential PH domains, but are not in the other PH domains for PtdIns-bisphosphates or -triphosphate (PIP_2/PIP_3) [32]. The phosphoinositide-binding pocket of the CERT PH alone does not produce such a strong affinity for its target phosphoinositide, compared with the pocket for PIP_2/PIP_3, because of a less negative charge in the phosphoinositol head group [32]. Interestingly, the basic groove endows the CERT PH domain to have a mild affinity for acidic phospholipids including phosphatidylserine,

and the synergetic effect of the two regions enables the CERT PH domain to have a high affinity for PtdIns (4)P-embedded phospholipid membranes with phosphatidylserine, such as the Golgi membranes [32].

FFAT motif

The two phenylalanines in an acidic tract (FFAT) motif is a short peptide motif that interacts with the cytosolic region of VAMP-associated protein (VAP), an ER-resident type II membrane protein [33]. Mammals have two VAPs, which form a homodimer and heterodimer [34]. The FFAT motif is constituted by a core canonical consensus sequence, EFFDAxE, and surrounding acidic residues [35]. The short peptide region (321-EFFDAVE-327) of CERT matches the consensus sequence perfectly and is surrounded by acidic residues, thus it is a canonical FFAT motif. Another non-canonical FFAT-like motif in the CERT sequence was reported [33]; however, the significance of the FFAT-like motif has not been elucidated.

Ceramide transport binds to VAP-A and VAP-B in a FFAT motif-dependent manner [36]. Although the 3D structure of full size VAP in complex with the FFAT motif has not been determined, the complexes of truncated soluble VAP with the FFAT motif were determined by X-ray [37] and NMR analyses [38]. VAPs may serve as broad-spectrum adaptors for recruiting cytosolic soluble proteins onto the ER and for the association of ER membranes with other membranes.

START domain, PH domain and FFAT motif are required for the function of CERT

Neither PH domain-less CERT (CERTΔPH) nor FFAT-mutated CERT (CERT(D324A)) can support the ER-to-Golgi transport of ceramide in semi-intact cells at the endogenous expression levels [36], whereas the activity of intermembrane transfer of ceramide of these CERT mutant constructs in a cell-free assay system is almost identical to that of the wild-type CERT [13,25]. Thus, targeting the organelles is a critical factor for transporting lipids between specific organelles. In addition, CERT has been suggested to simultaneously associate with both the Golgi membrane via the PH domain and the ER membrane via the FFAT motif [36]. Based on these findings, we proposed that CERT extracts ceramide from the ER and carries it to the Golgi apparatus in a non-vesicular manner, and that CERT-mediated trafficking of ceramide proceeds efficiently at membrane contact sites (MCS) between the ER and Golgi apparatus (Fig. 2). De Matteis and colleagues recently demonstrated that the knockdown of CERT in HeLa cells unexpectedly promotes ER-Golgi MCS formation [39]. However, CERT might be involved in the formation and maintenance of MCS under the specific conditions described below.

Functional regulation of CERT through its phosphorylation

The MR of CERT seems to form no globular domain but have several regions and motifs that are important for the function and regulation of CERT [28] including the FFAT motif (as described above), an oligomerization region [40], a caspase-cleavage site [41], and some phosphorylation sites [25,26,42]. The significance of oligomerization of CERT is not yet clearly determined. It has been suggested that CERT was inactivated in response to pro-apoptotic stress probably by caspase-dependent cleavage at aspartic acid 213 (D213) of CERT, which caused the reduction in SM synthesis [41]. As for phosphorylation sites, we describe below two phosphorylation sites or motifs of which the importance in the functional regulation of CERT has been elucidated.

Serine-repeat motif

A serine-repeat motif (SRM; 132-SMVSLVSGASGY-SATSTSS-150, serine/threonine residues in the motif are underlined) in proximity to the PH domain was identified as having multiple phosphorylation sites by mass spectrometric analysis of trypsin-digested peptides from purified CERT [25] (Fig. 1A). Seven to ten amino acid residues in the SRM were phosphorylated in normal cultured HeLa cells [25]. When these ten serine/threonine residues of the SRM were replaced with glutamic acid residues (which is hereafter referred to as the 10E mutant) to mimic the multisite phosphorylation in the SRM, the resultant CERT 10E mutant had reduced PtdIns(4)P-binding activity and ceramide transfer activity [25]. The repression of PtdIns(4)P-binding activity by the 10E mutation was rescued by removing the START domain, while the repression of ceramide transfer activity was rescued by removing the PH domain, suggesting an inhibitory mechanism by conformational changes to mask the PH and START domains with each other (Fig. 1B) [25]. Recently, X-ray crystallography with the purified CERT PH and START domains revealed that a basic groove in the PH domain snugly binds to the negatively charged loop of the START domain [43]. Mutations disrupting the PH/START interaction increase both PtdIns(4)P-binding activity and ceramide-transfer activity of CERT 10E, thereby supporting the inter-domain steric

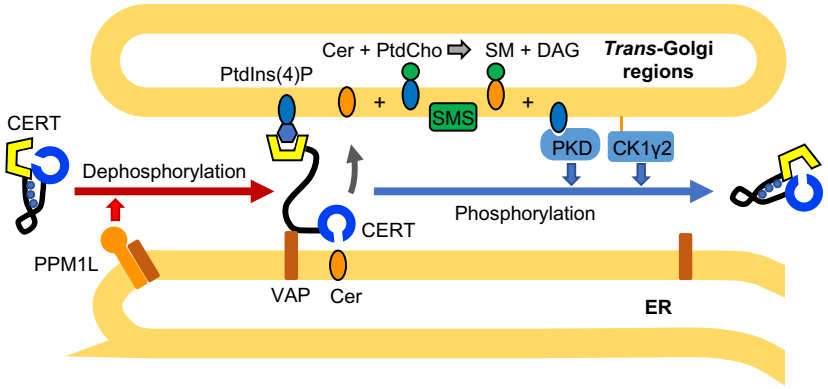

Fig. 2. The activity of CERT is regulated by phosphorylation of the SRM. CERT is inactivated by multisite phosphorylation of SRM in normal cultured cells, presumably taking a closed form as depicted on the left side of the figure. CERT is dephosphorylated by a membrane-anchored protein phosphatase PPM1L to take an open form as drawn in the middle of the figure. CERT regains the essential functions; PtdIns(4)P binding activity, VAP-binding activity and ceramide transfer activity. The activated CERT is recruited into the ER-Golgi MCS to transport ceramide from the ER to the *trans*-Golgi regions. SMS1 in the *trans*-Golgi regions generates SM and DAG from ceramide and PtdCho. PKD is recruited to the *trans*-Golgi regions by DAG and is activated. The activated PKD phosphorylates CERT S132, triggering the sequential multisite phosphorylation in the SRM by CK1γ2. CERT returns into the inactive form by the multisite phosphorylation, as depicted on the right side of the panel. The equilibrium between the phosphorylation and dephosphorylation determines the activity of CERT.

hindrance model [43]. The CERT PH domain also electrostatically interacts with the phosphor-mimetic SRM (SRM 10E; Fig. 1B) [44]. The PH-SRM 10E protein showed less PtdIns(4)P-binding and less Golgi-targeting activity than that of its wild-type control [44]. It has been proposed that the interaction between the PH domain and phosphorylated SRM causes an initial structural change in CERT, and then the interaction between the PH and the START domain completely suppresses the activity of each other [44] (Fig. 1B). The 10E mutation also reduces FFAT-dependent CERT-VAP interaction in the full-sized CERT; although, it remains unclear how the 10E mutation affects the interaction between the FFAT motif and VAP [26]. Because of the negative impacts on the activities of PtdIns(4)P-binding, ceramide transfer, and VAP-binding, the multisite phosphorylation of the SRM down-regulates the function of CERT to deliver ceramide from the ER to the *trans*-Golgi regions. In normally cultured HeLa cells, the major population of CERT (~ 90%) is phosphorylated in the SRM and largely down-regulated [25]. Inhibition of the *de novo* synthesis of sphingolipids by myriocin (a specific inhibitor of serine palmitoyltransferase, which is the enzyme denoted in the initial step in *de novo* sphingolipid biosynthesis) causes dephosphorylation of the SRM to activate CERT [25]. Additionally, treatment of cells with the sterol adsorbent methyl-β-cyclodextrin also causes the dephosphorylation of the SRM [25]. These results suggest that the phosphorylation state of SRM is regulated by the cellular requirement of SM or by the state of SM/cholesterol-enriched lipid-rafts in cells [25].

The SRM in which serine/threonine residues are distributed at intervals of two amino acids (–S/T–X–X–) is a typical targeting sequence for phosphorylation by casein kinase 1 (CK1; Fig. 1A) [45]. Casein kinase 1γ2 (CK1γ2) was shown to phosphorylate SRM to down-regulate the function of CERT [46] (Fig. 1A). Phosphorylation by CK1 requires a negatively charged cluster or phosphoserine/phosphothreonine residue at three residues upstream from the target phosphorylation site [45]. Thus, sequential phosphorylation in the SRM by CK1 requires priming phosphorylation at S132 (the closest serine residue to the N-terminal in the motif). Protein kinase D (PKD) may be responsible for the phosphorylation of S132 in CERT [42]. Phosphorylated SRM is dephosphorylated by protein phosphatase, Mg^{2+}/ Mn^{2+}-dependent 1L (PPM1L/PP2Cε) in a VAP-dependent manner [47]. PPM1L binds to the transmembrane region of VAP-A [47].

Serine 315 in the flanking region of the FFAT motif

CERT has another phosphorylation site to impact the CERT-VAP interaction (Fig. 1A,B). The phosphoproteome analysis revealed the phosphorylation of serine 315 (S315) of CERT in an acidic flanking region of the FFAT motif [48]. It is thought that the initial electrostatic interaction between the acidic flanking region and basic electropositive face of VAP proceeds

and facilitates the binding of the core peptide with VAP in a 'lock-and-key' manner [38,49]. A phosphomimetic mutation of S315 (CERT S315E) markedly enhances the CERT-VAP interaction in a FFAT motif-dependent manner to promote ER-to-Golgi transport of ceramide [26]. In addition, CERT with phosphorylated S315 was shown to have enhanced affinity for VAP in cells [26]. The phosphomimetic mutation at S315 (S315E) does not affect PtdIns(4)P-binding activity or ceramide transfer activity. Of note, the treatment of cells with myriocin promotes S315 phosphorylation [21]. Thus, in response to the requirement of SM and/or lipid-rafts, CERT can be fully activated by dephosphorylation of the SRM and by phosphorylation of S315, although how these two events co-ordinately occur is not fully understood. This question will be briefly discussed below.

Evolutional aspects of CERT

Ceramide transport is identical to a splicing variant of the Goodpasture antigen-binding protein (GPBP) [50]. GPBP was initially identified as a serine/threonine kinase capable of binding the carboxy-terminal non-collagenous region of collagen α3 chain of collagen type IV, which is known as an antigen of autoimmune disease with progressive glomerulonephritis, Goodpasture disease [51]. To date, no discernible kinase motifs have been found in GPBP and its splicing variants [51,52]. Then, the human gene encoding GPBP (and also CERT) was initially referred to as *COL4A3BP* (standing for collagen type 4A α3 binding protein). However, because the gene became to be more widely appreciated as an element of ceramide transport, Human Genome Organization has recently revised the official symbol of the gene from *COL4A3BP* to *CERT1* (https://www.genenames.org/data/gene-symbol-report/#!/hgnc_id/HGNC:2205). *CERT1* gene is supposed to produce several transcript variants. The transcript encoding CERT/GPBPΔ26 (598 amino acid residues) is a more common splicing variant than the transcript encoding $CERT_L$/GPBP (624 amino acid residues) and is expressed widely in various tissues [52]. No *CERT1* paralogs in the human genome have been reported to date.

$CERT_L$/GPBP is 26 amino acid residue protein longer than CERT/GPBPΔ26. The additional 26 amino acid sequences in $CERT_L$/GPBP (371-PYSRSSSMSSIDLVSASDDVHRFSSQ-396) is enriched in serine (Fig. 1A). The region was reported to enable $CERT_L$/GPBP to secrete from cells [52,53], even though $CERT_L$/GPBP has no typical transmembrane signal sequences. Moreover, another transcript of *CERT1* may produce longer isoform (752 amino acid residues) although the protein product has not been clearly identified. Notably, $CERT_L$/GPBP and its longer isoform have full-sizes of the PH and START domains and FFAT motifs, and $CERT_L$/GPBP was demonstrated to mediate intracellular ceramide transport to a similar extent of CERT/GPBPΔ26 [13].

Phylogenetic analysis suggested that all multicellular animals have clear homologues of CERT/GPBPΔ26, but that $CERT_L$/GPBP has appeared from vertebrates in the evolution of life [28,54]. No clear homologue of CERT has been found in plants, protists (or protozoan), fungi or bacteria. Thus, it is most likely that CERT appeared to exert its intracellular ceramide trafficking functions earlier than $CERT_L$/GPBP, which gained the additional sequences in front of the START domain to exert yet-to-elucidated intracellular or extracellular functions. Organisms with CERT are almost consistent with those with SM and its close relatives (e.g. phosphosphingolipids having mono- or di-methyl ethanolamine in place of choline), suggesting co-evolution of CERT with these SM cognates [27]. Interestingly, the SRM and serine residues corresponding to the S315 of human CERT are well-conserved among multicellular animals, except for nematodes, suggesting that phosphor-regulation of CERT is crucial for the life of animals.

Various factors involved in the regulation of CERT

Recent studies revealed that more factors, except for the regulation of CERT activity, are relevant to the CERT-dependent synthesis of SM in the Golgi regions. SM synthesis in the *trans*-Golgi regions by SMS1 co-produces DAG, which recruits PKD to the Golgi membranes [55]. DAG also serves as an activator of TGN-associated protein kinase Cη, which phosphorylates and activates PKD [56]. The activated PKD phosphorylates S132 of CERT, which is followed by sequential phosphorylation of SRM by CK1γ2. Thus, CERT-mediated ceramide transport from ER to *trans*-Golgi regions is alleviated by excessive synthesis of SM. In line with this model, the phosphorylation states of the CERT SRM are co-related to the level of the SM synthesis in cells: the CERT SRM tends to be dephosphorylated when cells are treated with myriocin, and its phosphorylation states are reversed by the addition of sphingosine, even in the presence of myriocin [25].

Diacylglycerol also serves as a direct-feedback inhibitor of SMS [57]. Although the level of DAG in the Golgi complex should be controlled, the fate of DAG

co-produced with SM is unclear. It may be converted to PtdCho by Golgi apparatus-localising phosphocholine transferase [58] or might be transported to the ER by a possible ceramide/DAG-co-exchange by CERT [22,29] and metabolised to triacylglycerol [59] by DAG acyltransferase or to PtdCho by phosphocholine/phosphoethanolamine transferase in the ER [1,58].

PtdIns(4)P in the Golgi complex is a crucial factor for CERT to associate with this organelle. It is likely that CERT-mediated ceramide transport is also regulated by the pool of PtdIns(4)P in the Golgi complex. Among the four phosphatidylinositol-4-kinases (PtdIns 4-kinase IIα, PtdIns 4-kinase IIβ, PtdIns 4-kinase IIIα and PtdIns 4-kinase IIIβ), knockdown of PtdIns 4-kinase IIIβ and treatment with the PtdIns 4-kinase IIIβ-specific inhibitor PIK93 significantly reduces ceramide transport from the ER to the Golgi region [60], which strongly suggests that PtdIns 4-kinase IIIβ is the primary kinase to produce PtdIns(4)P for the function of CERT. CERT-dependent ceramide transport and oxysterol-binding protein (OSBP)-dependent Chol transport influence each other. The molecular mechanism of mutual influence between CERT and OSBP-dependent transport was an enigma until the bidirectional transport of the OSBP-related domain (ORD) was reported [61,62]. Antonny, Drin, and colleagues elegantly revealed that OSBP ORD can transport Chol from the ER to the *trans*-Golgi regions, followed by back transport of PtdIns(4)P to the ER, in which PtdIns(4)P is converted to PtdIns by the ER-resident phosphoinositide-phosphatase SAC1 (Fig. 3) [62]. Note that PtdIns is transported from the ER to the Golgi regions presumably by the PtdIns-transfer protein Nir2, and it is converted to PtdIns(4)P by Golgi-associated PtdIns 4-kinase(s) [1,63]. Therefore, one ATP is consumed during one cycle of OSBP-dependent cholesterol/PtdIns(4)P exchange between the ER and Golgi in cells. The overall ATP-consuming system likely sustains the apparent up-hill transport of Chol from the ER to the Golgi regions against its concentration gradient (Fig. 3). D'Angelo *et al*. recently showed that the sustained sphingolipid inflow into the *trans*-Golgi regions reduces the level of PtdIns(4)P at the *trans*-Golgi regions, which causes the down-regulation of Golgi-targeting of CERT and consequently represses SM biosynthesis [64]. The reduction in PtdIns(4)P level is dependent on SMS, PKD, OSBP and SAC1 [64]. When a water-miscible short-chain ceramide was exogenously added to cultured cells, it was converted to short-chain SM in the Golgi complex by SMS with an equimolar generation of DAG in a CERT-independent manner. The increased DAG activates PKD that phosphorylates OSBP at serine 240 (human OSBP), which modulates affinity of OSBP for the ER and TGN [65]. Phosphorylated OSBP enhances PtdIns(4)P degradation by promoting retrograde transport of PtdIns(4)P.

Ridgway and colleagues showed that CERT-dependent SM synthesis is enhanced by the treatment of cells with 25-hydroxycholesterol (25-HC) in an OSBP- and VAP-dependent manner [66]. 25-HC is a competitive ligand to Chol in OSBP. It has been proposed that 25-HC blocks OSBP-dependent exchange of Chol and PtdIns(4)P between the ER and Golgi complex and thus, stabilises the Golgi complex-associated PtdIns(4)P pool, thereby enhancing the association of CERT with the Golgi regions [27]. Notably, 25-HC-dependent CERT-activation requires a sterol-sensitive PtdIns 4-kinase IIα, while the production of PtdIns(4)P for CERT recruitment to the Golgi regions in drug-free control cells is largely attributable to PtdIns 4-kinase IIIβ [67].

A hypothetical rheostat model of SM, DAG, cholesterol and PtdIns(4)P at ER-Golgi membrane contact sites

As summarised above, the synthesis and degradation of SM, DAG, cholesterol and PtdIns(4)P in the Golgi complex are connected directly (e.g. co-production of SM and DAG) or indirectly (e.g. cholesterol/PtdIns(4)P-exchange by OSBP affects CERT-dependent synthesis of SM; Fig. 3). In addition, the reactions involved in the connections likely occur at the ER-Golgi membrane contacts, in which reactions occurring in the different organelles can be spatially connected in a simple way. Cumulatively, previous studies may provide the hypothetical model that ER-Golgi MCS serve as inter-organelle communication zones, in which many factors work in concert to serve as an extensive rheostat of SM, DAG, cholesterol and PtdIns(4)P in the Golgi complex (Fig. 3).

Conclusions and possible future perspectives

Ceramide transport from the ER to the *trans*-Golgi regions is regulated by two factors: (a) regulation of activity of CERT by phosphorylation; and (b) regulation of the PtdIns(4)P pool in the Golgi. However, there are various questions to be resolved. For instance, kinase responsible for the phosphorylation of CERT S315 has not been identified. Is the rheostat model described above really working in living cells? To validate the model, multidisciplinary approaches,

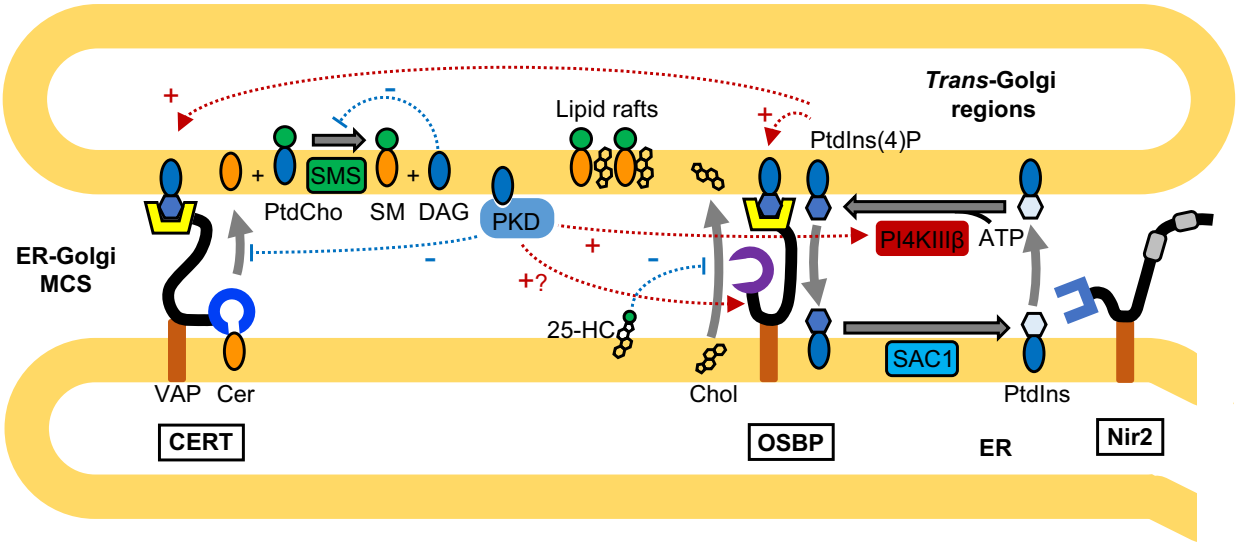

Fig. 3. CERT-mediated ceramide transport is affected by the PtdIns(4)P pool. The pool of PtdIns(4)P required for the function of CERT is primarily generated by PtdIns 4-kinase IIIβ (shown as PI4KIIIβ in this figure) in the *trans*-Golgi regions. The precursor PtdIns is transported from the ER to the Golgi apparatus presumably by PtdIns-transfer proteins, such as Nir2. The OSBP-mediated Chol transport from the ER to the *trans*-Golgi regions decreases the PtdIns(4)P pool by the coupled reverse-transport of PtdIns(4)P from the Golgi to the ER. PtdIns(4)P is converted to PtdIns by the ER-resident phosphoinositide-phosphatase SAC1 in the ER membrane. The cycle with these reactions and transports determines the PtdIns(4)P pool in the *trans*-Golgi regions, as depicted on the right side of the panel. PKD may serve as a key kinase that controls the cycle. 25-HC inhibits OSBP-mediated exchange of ER Chol and Golgi PtdIns(4)P, thereby stabilising the Golgi pool of PtdIns(4)P. CERT-mediated ceramide transport is affected by these factors.

LTPs	Sequences of the canonical FFAT motif
CERT	EGPN**S**LINEEEFFDAVEAALDRQDKIE
OSBP	GDM**S**DEDDENEFFDAPEIITMPENLGH
ORP1	**S**PPASILSEDEFYDAL**S**DSESERSLSR
ORP2	MNGEEEFFDAVTGFDSDN**SS**GE
ORP3	**S**RLSITDSLSEFFDAQEVLLSPSSSEN
ORP4	GED**S**EEDEDTEYFDAMEDSTSFITVIT
ORP6	SRLSMSESVSEFFDAQEVLLSASSSEN
ORP7	SILSLADSHTEFFDACEVLLSASSSEN
ORP9	PEF**S**YSSSEDEFYDADEFHQSG**S**SPKR
NIR1	LSDSVE**SS**DDEFFDAREEMAEGKNAIL
NIR2	ARD**S**EN**SS**EEEFFDAHEGFSDSEEVFP
NIR3	ARD**S**DES**S**DDEFFDAHEDLSDTEEMFP

Fig. 4. Phosphorylation sites of the flanking region of various canonical FFAT motifs in human lipid-transfer proteins (LTPs). Orange letters indicate the core sequence of the FFAT motif (EFFDAxE). Red bold letters indicate phosphorylation sites based on previous studies; CERT [26,48,78], OSBP [48,78–81], OSBP-related protein 1 (ORP1) [82], ORP2 [83], ORP3 [81], ORP4 [82], ORP9 [83,84], NIR1 [79], NIR2 [85], NIR3 [86]. ORP5 and ORP8 do not have FFAT motif but have a C-terminal transmembrane region. The canonical FFAT motif is not found in ORP10 and ORP11, but the FFAT-like motif is.

including conventional biochemical and genetic analyses, and computational simulation analysis should be encouraged. Simulation studies on CERT have been recently published [68,69].

Searching for the phosphorylation sites around the canonical FFAT motif of lipid-transfer proteins, we can find several phosphorylation sites in the flanking region of the FFAT motif (Fig. 4). Levine and colleagues demonstrated that a phosphomimetic substitution of the core sequence of a FFAT-like motif modulates the affinity to VAP [49]. The phosphorylation of serine/threonine residues in the acidic flanking region of a canonical FFAT motif may also be involved in the common modulation of interactions between VAP and various lipid-transfer proteins with FFAT motifs.

When cells are infected with *Chlamydia trachomatis*, an obligate intracellular bacterium, CERT is hijacked by the bacterial protein IncD to deliver the host-derived ceramide to the pathogen [70,71]. PtdIns 4-kinase IIα is also hijacked by *C. trachomatis* to modulate host trafficking by generating a PtdIns(4) pool in the inclusion membrane. The hepatitis C virus down-regulates the activity of PKD, and knockdown of PKD expression by exogenous siRNA enhances HCV secretion [72]. The suppressive effect of PKD is

subverted by the ectopic expression of CERT S132A [72]. CERT is a critical factor for some pathogens; thus, it may be beneficial to human health to understand the mechanism of CERT-hijacking or development of CERT inhibitors [73].

A large-scale, unbiased, genotype-driven approach for children with developmental disorders and intellectual disabilities revealed that a non-synonym mutation in *CERT/COL4A3BP* gene producing the S132L substitution in CERT is associated with these phenotypes [74]. The substitution of the priming serine in the SRM (Figs 1A and 2) is expected to render CERT constitutively active. More recently, mutations causing S138C and G243R substitutions in CERT were found in patients with severe intellectual disabilities [75,76]. The precise mechanism underlying how these specific mutations in the *CERT* gene cause developmental disorders and intellectual disabilities are likely to be difficult to elucidate. However, if these mutations deregulate CERT to be constitutively active, repression of the activated CERT to the normal level by inhibitors might ameliorate the disease. It is noteworthy that two types of CERT inhibitors have been developed: one is structurally ceramide-mimetic [77] and the other is nonmimetic [73].

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