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Regular Article

Analysis of the Effects of Brefeldin A on Deuterium-Labeled Sphinganine Metabolism Using Liquid Chromatography–Tandem Mass Spectrometry

Yasuhiro Hayashi[a] and Takashi Tanikawa*[,b]

[a] *Faculty of Agriculture, University of Miyazaki, 1–1 Gakuen-kibanadai-nishi, Miyazaki 889–2192, Japan: and*
[b] *Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan.*
* Correspondence: tanikawa@josai.ac.jp

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Ceramide (Cer) is synthesized in the endoplasmic reticulum (ER) using sphinganine as the common backbone and is then transported to the Golgi apparatus to synthesize two complex sphingolipids, sphingomyelin (SM) and glucosylceramide (GlcCer). Brefeldin A (BFA) affects the structure of the Golgi apparatus, resulting in the redistribution of the Golgi proteins into the ER. Therefore, BFA has been used to examine the ER-to-Golgi trafficking of lipids, but the detailed lipid changes in cells upon BFA treatment are not fully understood. Here, we examined the metabolism of deuterium-labeled sphinganine in HEK293T cells treated with BFA using liquid chromatography–tandem mass spectrometry. The BFA-pretreated cells were incubated in the presence of deuterium-labeled sphinganine and BFA for 5 min to 8 h, and the levels of dihydroceramide, Cer, dihydrosphingomyelin, SM, GlcCer, and phosphatidylcholine (PC) were investigated. The levels of Cer species in BFA-treated cells were lower than those in untreated cells within 2 h of incubation, but following >4 h of incubation, the levels of C14–20 Cer, encompassing C14–20 acyl chains, were similar in BFA-treated and untreated cells. Furthermore, BFA treatment increased the levels of C14–20 GlcCer but had little effect on those of C22–24 GlcCer. Moreover, after incubation for >4 h, BFA treatment decreased the levels of saturated C30–32 PC but had almost little effect on those of PC containing a monounsaturated C32–34 acyl chain. Our findings showed that BFA affected the metabolism of various lipids depending on the length and saturation of the fatty acid chain.

Key words brefeldin A, deuterium-labeled sphinganine, liquid chromatography–tandem mass spectrometry

INTRODUCTION

Sphingolipids are important components of cell membranes, and their metabolism involves a dynamic network of molecules, including important bioactive signaling molecules.[1] *De novo* biosynthesis of sphingolipids occurs in the endoplasmic reticulum (ER) and begins with the condensation of L-serine with palmitoyl-CoA to produce 3-ketodihydrosphingosine.[2] 3-Ketodihydrosphingosine is reduced to sphinganine, a common sphingolipid backbone, which is *N*-acylated to dihydroceramide (DHCer) by ceramide synthase (CerS) (Fig. 1). The specificity of the different CerSs determines the length of the *N*-acyl chain in DHCer; C14:0, C16:0, C18:0, C20:0, C24:0, and C24:1 are the major fatty acids incorporated into DHCer in mammalian cells.[3] The formation of ceramide (Cer) involves the insertion of a single double bond into DHCer by dihydroceramide Δ4-desaturase 1 (DEGS1).[4]

Cer acts as an intracellular signaling molecule and is further metabolized to form complex sphingolipids, such as glucosylceramide (GlcCer) by uridine 5′-diphosphate (UDP)-glucose ceramide glucosyltransferase (UGCG) and sphingomyelin (SM) by SM synthase (SMS), localized in the Golgi apparatus.[5] Therefore, Cer in the ER must be transported to the Golgi apparatus. These distinct pathways may be involved in the delivery of Cer for the biosynthesis of SM and GlcCer: the ceramide transport protein (CERT)-dependent pathway for SM synthesis and the CERT-independent pathway for GlcCer synthesis.[6] Different roles of Cer delivery were confirmed in CERT-deficient cells. The level of SM, but not GlcCer, was reduced in the mutant cells, indicating that CERT-transported Cer is selectively destined for SM synthesis.[6] The CERT-dependent pathway has been well studied, whereas CERT-independent pathways for GlcCer synthesis have not been well characterized.

Cer is deacetylated by ceramidase (CDase) to generate sphingosine.[7] Sphingosine and sphinganine are phosphorylated by sphingosine kinase (SKase) to produce sphingosine-1-phosphate (S1P) and sphinganine-1-phosphate (dihydrosphingosine-1-phosphate, dS1P), respectively. S1P lyase cleaves S1P (or dS1P) into a fatty aldehyde, which then enters glycerophospholipids, such as phosphatidylcholine (PC), *via* palmitoyl-CoA.[8]

The fungal metabolite brefeldin A (BFA) catalyzes the merging of Golgi and ER membranes[9,10]; therefore, it has been used to elucidate the mechanism of ER-to-Golgi transport of lipids.[11–13] Yamaji *et al.* performed metabolic labeling experiments using [^{14}C]serine in HeLa cells treated with BFA.[11] BFA treatment greatly increased the synthesis of both GlcCer and SM, but not Cer, probably because both UGCG and SMS could easily access the newly synthesized Cer without the need for intermembrane Cer transport. More

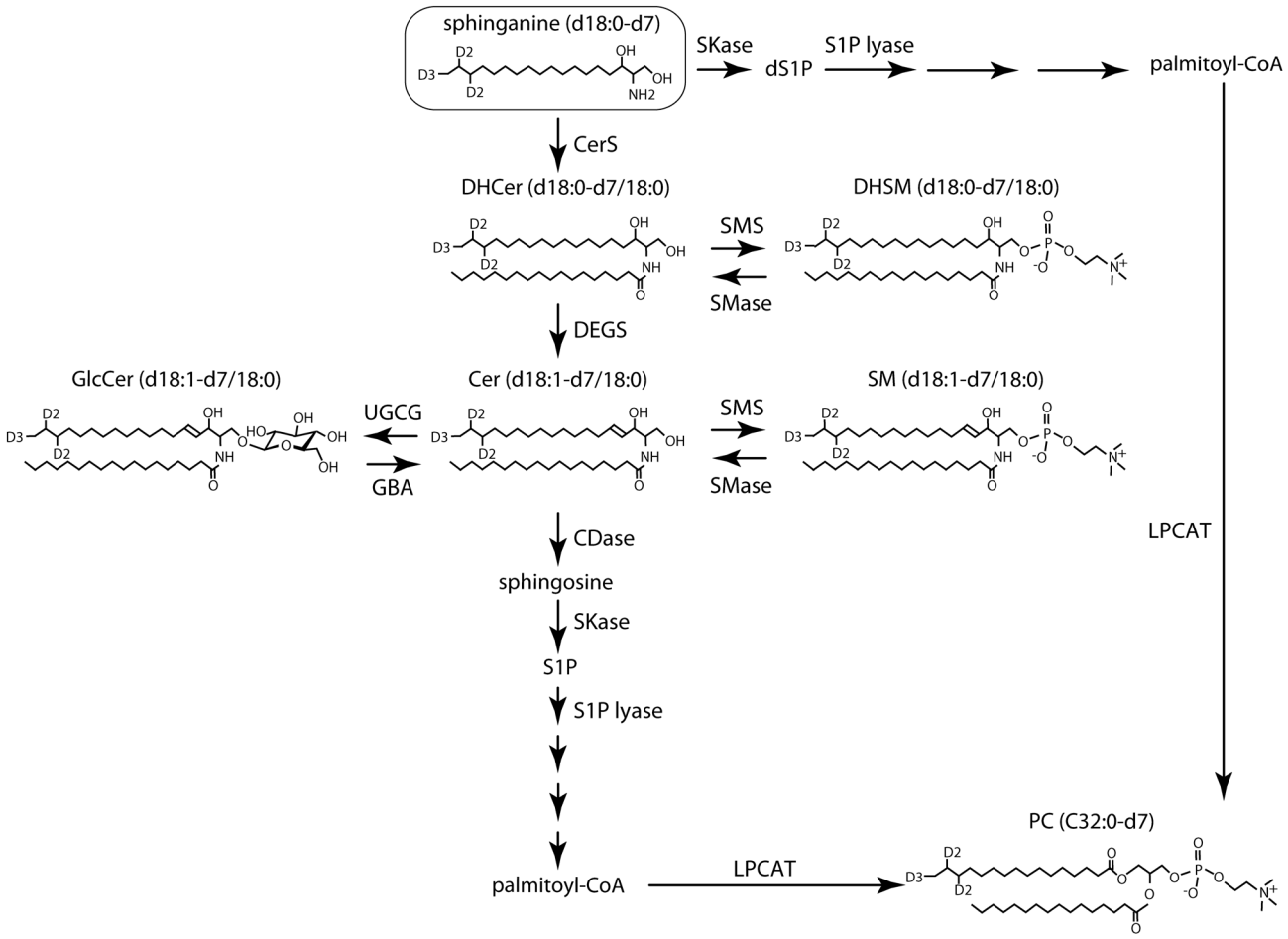

Fig. 1. Lipid Metabolic Pathway Using Sphinganine as a Precursor in Mammalian Cells

The structural formulas of the deuterium-labeled sphinganine used as a precursor and the analyzed lipids by LC–MS/MS are shown. DHCer, Cer, DHSM, SM, and GlcCer are composed of a saturated sphingoid base backbone sphinganine and a fatty acid of varying lengths. PC is composed of two fatty acids varying in length.

interestingly, in HeLa cells with genome-edited Cer synthase 2 gene, BFA decreased the proportion of GlcCer containing a C20–26 acyl chain length, possibly because of the higher accessibility of UGCG to C16-Cer, suggesting that the existence of a yet-to-be-identified CERT-independent mechanism renders GlcCer containing a C20–26 acyl chain length more accessible than C16-Cer to UGCG. Although this report is very interesting, the method of separating the metabolism of radioisotope-labeled lipids by TLC does not allow the detailed identification of changes in the *N*-acyl chain length of sphingolipids.

Snider *et al.* developed a method in which intracellular sphingolipid metabolism of odd-chain 17 deuterated sphinganine in MCF-7 cells was comprehensively analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS).[12] BFA treatment considerably increased the synthesis of SM, but not hexosylCer (HexCer), which may consist of both GlcCer and galactosylceramide (GalCer). Although this method is very useful, sphingosine, containing 17 carbons, is a minor sphingoid base *in vivo*, and only sphingolipids containing C16:0, C24:0, and C24:1 were analyzed.

BFA has been used to examine the ER-to-Golgi trafficking of lipids, but the detailed lipid changes in cells upon BFA treatment are not fully understood. Here, we examined the metabolism of deuterium-labeled sphinganine in HEK293T cells treated with BFA using LC–MS/MS. In this study, we used 18-carbon sphinganine, the main sphingoid base in mammalian cells, to investigate the lipid metabolism in BFA-treated HEK293T cells.

MATERIALS AND METHODS

Cells The HEK293T cells were obtained from Abcam (Cambridge, U.K.). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL of penicillin, and 100 μg/mL of streptomycin.

Chemicals The BFA was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). *D-Erythro*-sphinganine-d7 (d7-sphinganine), *N*-palmitoyl-d31-*D-erythro*-sphingosine (d31-Cer), *N*-palmitoyl-d31-*D-erythro*-sphingosylphosphorylcholine (d31-SM), and 1-palmitoyl-d31-2-oleoyl-*sn*-glycero-3-phosphocholine (d31-PC) were obtained from Avanti Polar Lipids (AL, U.S.A.), and *N*-omega-d3-hexadecanoyl-glucopsychosine (d3-GlcCer) was obtained from Cayman Chemical (MI, U.S.A.).

Treatment of HEK293T Cells with BFA HEK293T cells were seeded in 6-well plates (3×10^5 cells/well). The following day, cells were treated with DMEM containing 1% Nutridoma SP and 1 μg/mL BFA. After incubating at 37 °C for 30 min, the cellular medium was exchanged with DMEM containing 1% Nutridoma SP, 1 μg/mL BFA, and 0.5 μM deuterated

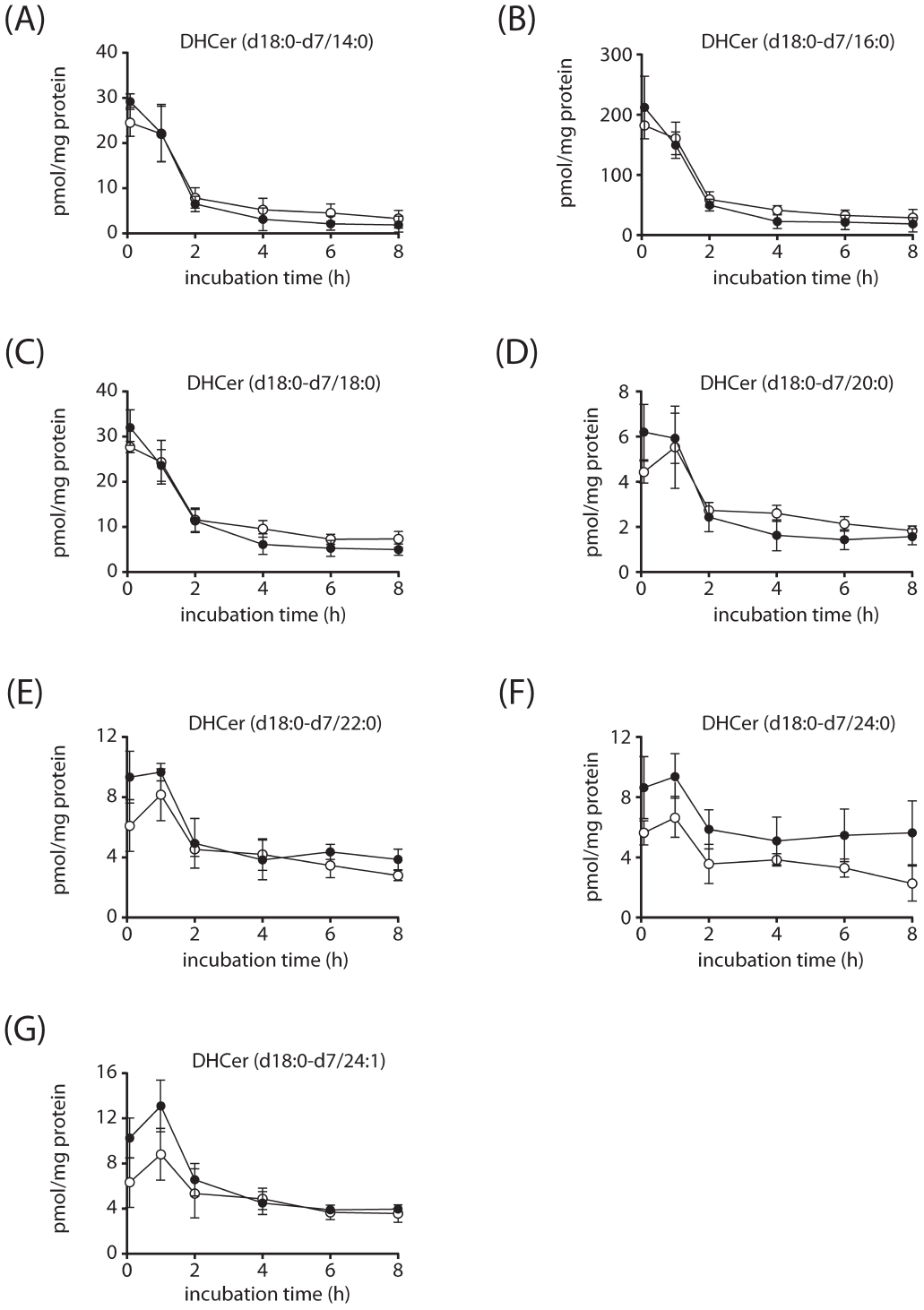

Fig. 2. Effect of BFA on Cellular DHCer Species in HEK293T Cells

After the cells were treated with 1 μg/mL BFA for 30 min, the cellular medium was exchanged with DMEM containing 1 μg/mL BFA and 0.5 μM deuterated d7-sphinganine, and the cells were incubated for 5 min to 8 h. Cellular levels of DHCer species with distinct acyl chains were quantified using LC–MS/MS. The BFA-treated and untreated cells are shown as white and black circles, respectively. Student's *t*-tests were used for comparisons. The results are shown as the mean ± S.D.

d7-sphinganine, and the cells were incubated for 5 min to 8 h. The cells were washed once with cold phosphate-buffered saline (PBS) and harvested by centrifugation.

Lipid Extraction and Quantification of Sphingolipids by LC–MS/MS Lipid extraction and sphingolipid quantification by LC–MS/MS were performed as previously described.[14] The harvested cells were resuspended in 100 μL of cold PBS and then homogenized using sonication. A portion of the sample (5 μL) was used in the bicinchoninic acid protein assay to determine the amount of protein. Total lipids were extracted by adding 375 μL of chloroform:methanol (1 : 2, v/v) containing 40 pmol of each component, specifically d31-Cer, d31-SM, d3-GlcCer, and d31-PC. Deuterated d31-Cer, d31-SM, d3-GlcCer, and d31-PC were used as internal standards for Cer and DHCer, SM and DHSM, GlcCer, and PC, respectively. For sphingolipids analysis, 100 μL of chloroform : methanol : 5N

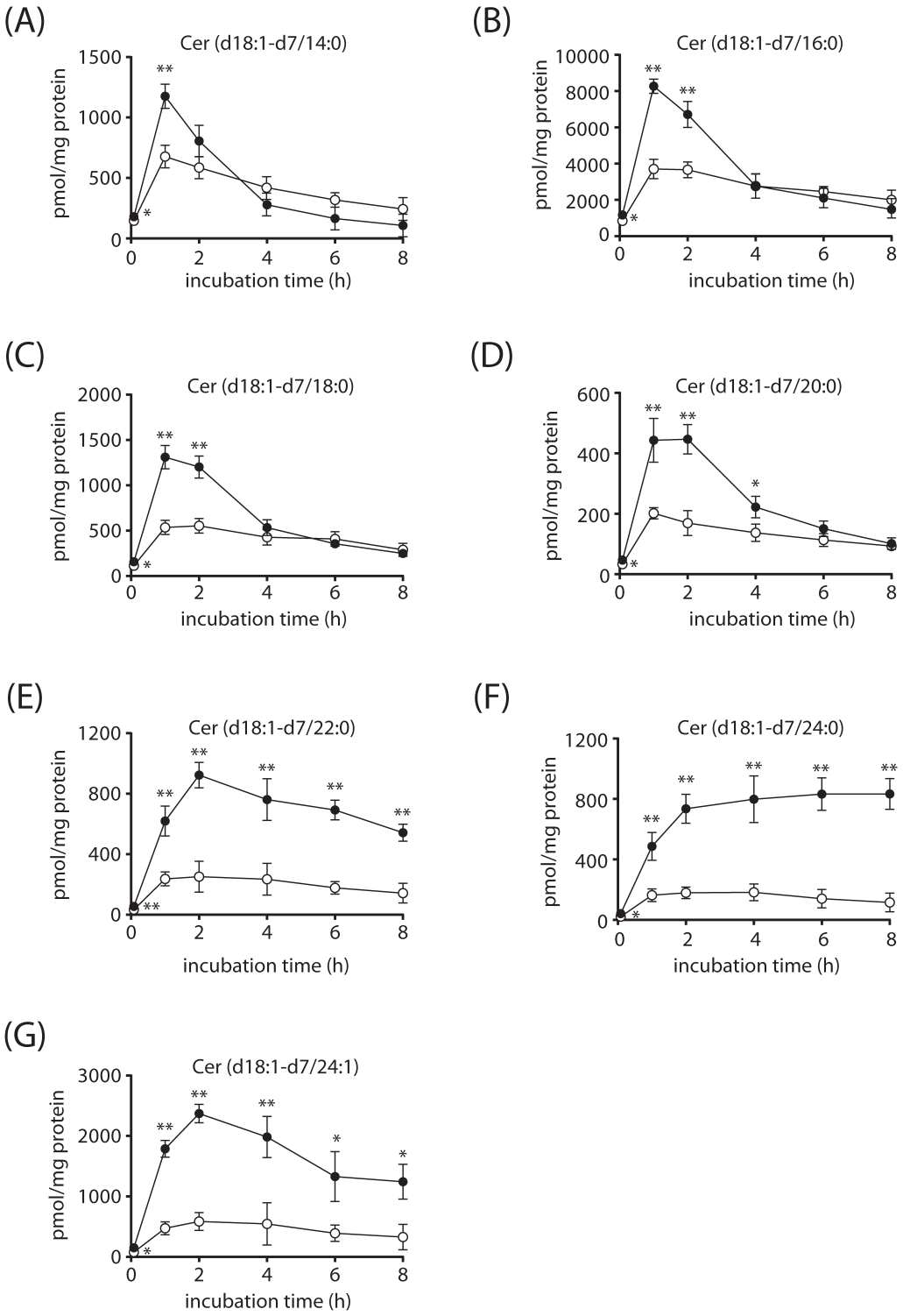

Fig. 3. Effect of BFA on Cellular Cer Species in HEK293T Cells

The levels of d7-sphinganine-derived Cer species in BFA-treated cells were quantified using LC–MS/MS. The BFA-treated and untreated cells are shown as white and black circles, respectively. Student's t-tests were used for comparisons. The results are shown as the mean ± S.D. Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$.

NaOH (1 : 2 : 0.8, v/v/v) was added, and the solution was incubated for 1 h at 37 °C, followed by neutralization with acetic acid. For PC analysis, 100 μL of chloroform : methanol : water (1 : 2 : 0.8, v/v/v) was added without alkaline treatment.

Subsequently, 158 μL of chloroform and 158 μL of water were added, and the mixture was vigorously vortexed, before centrifuging for 1 min at 13000 $\times g$ at 4 °C. The lower phase was withdrawn, dried, and resuspended in acetonitrile:methanol (1 : 1, v/v), sonicated for 10 s, and centrifuged at 14000 $\times g$ for 5 min, and the supernatant was transferred to a vial. The concentrations of DHCer, Cer, DHSM, SM, GlcCer, and PC were analyzed using a QTRAP4500 instrument (SCIEX, Framingham, MA, U.S.A.). Lipids with d7-sphinganine as a precursor were detected using the

multiple reaction monitoring (MRM) method (Supplementary Table).

Statistical Analysis All data are expressed as mean ± standard deviation (S.D.). Student's *t*-test was used for comparisons between the two groups. A *p*-value of <0.05 was considered statistically significant. All statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS AND DISCUSSION

Effects of Deuterium-Labeled DHCer and Cer Levels on BFA Treatment We examined the metabolism of deuterium-labeled sphinganine in HEK293T cells treated with BFA. HEK293T cells pretreated with BFA for 30 min were incubated in the presence of deuterium-labeled sphinganine and BFA for 5 min to 8 h. Confocal microscopy showed that the *cis*-Golgi marker GM130 was localized in the perinuclear region without BFA treatment (Supplementary Fig. S1). HEK293T cells pretreated with BFA for 30 min caused Golgi disruption, and GM130 partially colocalized with the ER marker protein. GM130 localization in the presence of deuterium-labeled sphinganine and BFA for 5 min to 8 h did not differ from that in BFA-pretreated cells; GM130 was partially colocalized with ER marker proteins.

Sphinganine is *N*-acylated *via* CerS to produce DHCer. There are six CerS isoforms in mammalian cells, each of which synthesizes DHCer using acyl-CoA with different chain lengths as the substrate.[15] The levels of DHCer species in BFA-treated cells were not significantly different from those in BFA-untreated cells (Fig. 2). Because all CerS isoforms are mainly localized in the ER,[15] BFA treatment did not affect DHCer production. Furthermore, the levels of DHCer declined during the 2-h incubation period.

DHCer is then converted into Cer *via* a desaturation reaction, yielding a 4,5-*trans*-double bond. This process is catalyzed by DEGS1, which is mainly localized in the ER.[16] Consistent with the results obtained in a previous report using TLC analysis,[11] BFA treatment decreased the levels of Cer species within 2 h of precursor incubation, except for C14 Cer at 2 h of incubation (Fig. 3). This is thought to be because the fusion of the ER and Golgi apparatus made it easier for SMS and UGCG localized in the Golgi apparatus to access *de novo* synthesized Cer in the ER, as proposed by Yamaji *et al.*[11] The levels of Cer species in BFA-treated cells were lower than those in untreated cells within 2 h of incubation, but following >4 h of incubation, the levels of C14–20 Cer, encompassing C14–20 acyl chains, were similar in BFA-treated and untreated cells. These results reveal differences in the metabolic mechanisms of Cer containing C14–20 acyl chains and C22–24 acyl chains. The reason for the difference in the acyl chain length during Cer metabolism is unknown; however, it may be influenced by the substrate specificity of CDase, a Cer-degrading enzyme. Cer can be generated *via* the salvage pathway through the actions of sphingomyelinase (SMase) and *β*-glucosylceramidase (GBA).[17] During a relatively long incubation time, the salvage pathway, not only the *de novo* pathway, can affect the level of Cer and its metabolites. Furthermore, as the level of DHCer decreased during the 2-h incubation period, the levels of Cer increased. This may be because an increase in DHCer activates DEGS1, as proposed by Snider *et al.*[12]

Effects of Deuterium-Labeled DHSM, SM, and GlcCer Levels on BFA Treatment Cer, and to a lesser extent DHCer, is further metabolized to form complex sphingolipids, such as sphingomyelins (SM and DHSM) and glucosylceramides (GlcCer and DHGlcCer), in the Golgi apparatus. Under our experimental conditions, only C16–18 DHSM could be detected by LC–MS/MS, whereas DHGlcCer could not. The BFA treatment increased the levels of DHSM and SM species within 4 h of incubation (Figs. 4, 5). Again, this is thought to be because the fusion of the ER and Golgi apparatus makes it easier for SMS localized in the Golgi apparatus to access the newly synthesized Cer within the ER. Following >6 h of incubation, C18 DHSM and C18–20 SM remained high in BFA-treated cells, whereas the levels of other DHSM and SM were comparable, except for C24:0 SM at 6 h of incubation (Figs. 4, 5).

Before analyzing HexCer levels in BFA-treated cells, we determined whether HexCer in HEK293T cells was GlcCer or GalCer. We established UGCG-knockout HEK293T cells using the CRISPR/Cas9 gene-editing system described previously,[5] and the levels of Cer, SM, and HexCer were examined (Supplementary Figs. S2–S4). Because the level of HexCer was greatly reduced in UGCG-knockout HEK293T cells, we found that HexCer in HEK293T cells was mainly GlcCer and not GalCer (Supplementary Fig. S4). Although Cer was a substrate for UGCG, Cer did not increase in UGCG knockout cells compared to wild-type (WT) cells (Supplementary Fig. S2). The levels of C16, C18, and C24:1 Cer in UGCG-knockout

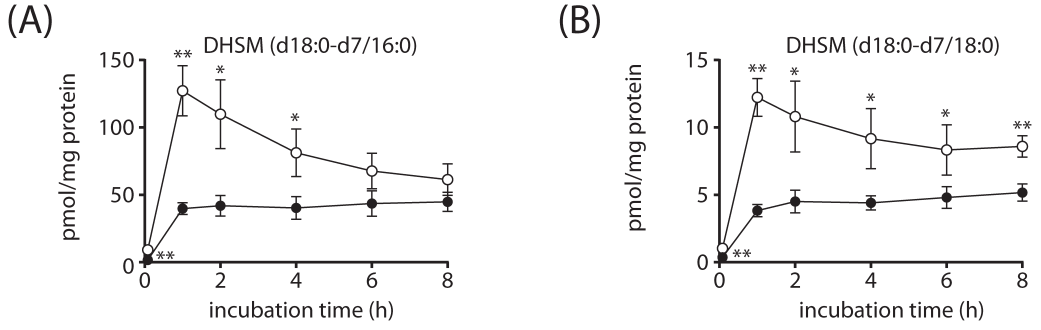

Fig. 4. Effect of BFA on Cellular DHSM Species in HEK293T Cells

The levels of d7-sphinganine-derived DHSM species in BFA-treated cells were quantified using LC–MS/MS. The BFA-treated and untreated cells are shown as white and black circles, respectively. Student's *t*-tests were used for comparisons. The results are shown as the mean ± S.D. Statistical significance is indicated as follows: $*p<0.05$, $**p<0.01$.

cells were lower than those in WT cells, but the levels of C20, C22, C24 Cer in UGCG-knockout were similar with those in WT cells. The levels of SM species in UGCG-knockout cells were higher than those in WT cells, except for C18-SM (Supplementary Fig. S3). We hypothesized that the UGCG knockout cell clone in this study had an enhanced ability to synthesize SM, which prevented Cer accumulation, resulting in decreased C16 and C24:1 Cer and increased C16 and C24:1 SM. Further investigation is needed to elucidate the mechanisms underlying the differences in changes in each species of Cer and SM in UGCG knockout cells.

Interestingly, BFA treatment increased the levels of C14–20 GlcCer but had little effect on C22–24 GlcCer, except for C24:0 GlcCer, at 8 h of incubation (Fig. 6). These results indicate that there are differences in the metabolic mechanisms of C14–20 and C22–24 GlcCer. Although UGCG is mainly distributed in the Golgi apparatus,[18–20] it has also been shown to localize to the ER.[21] Therefore, C14–20 and C22–24 GlcCer

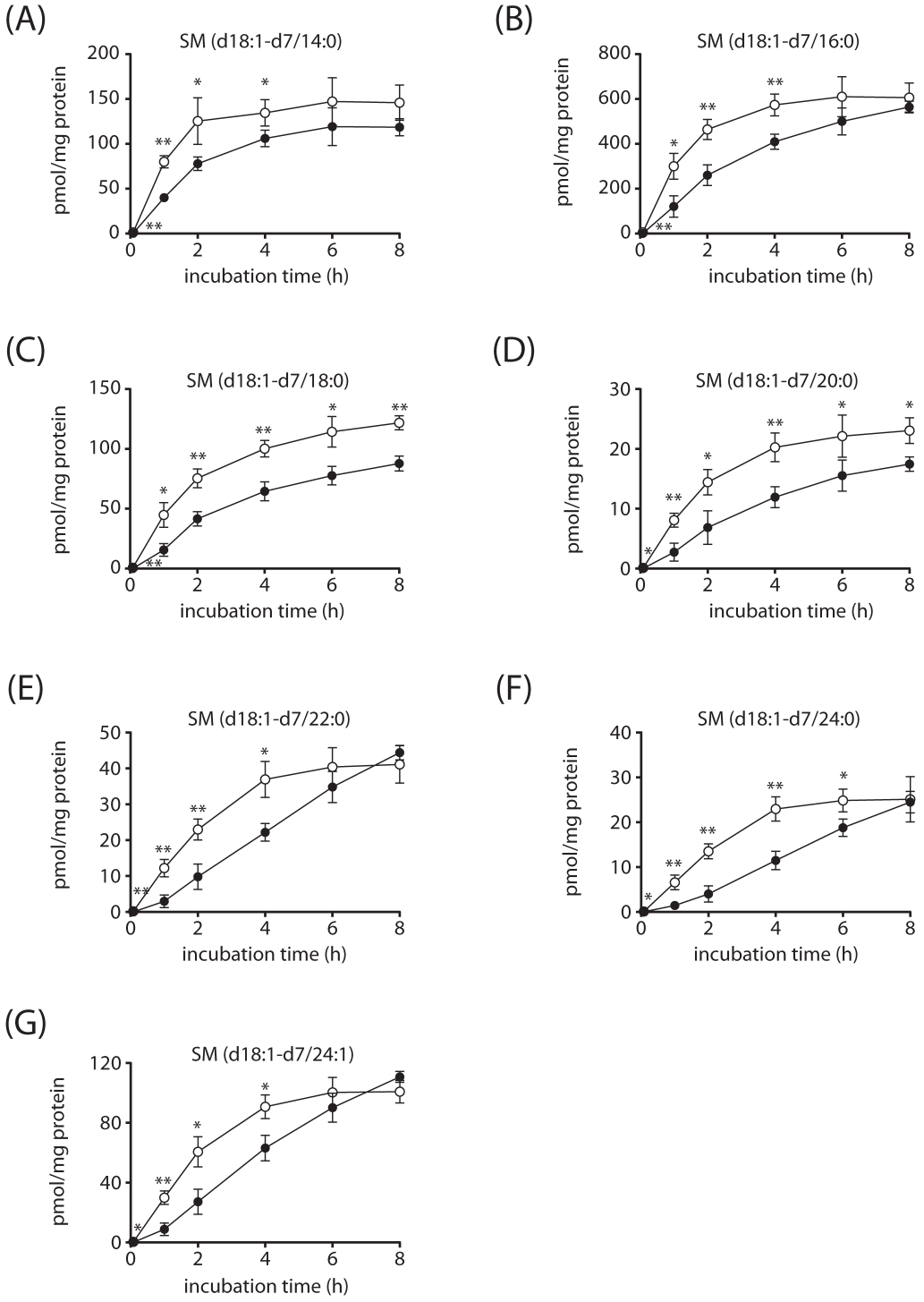

Fig. 5. Effect of BFA on Cellular SM Species in HEK293T Cells

Levels of d7-sphinganine-derived SM species in cells treated with BFA were quantified using LC–MS/MS. The BFA-treated and untreated cells are shown as white and black circles, respectively. Student's *t*-tests were used for comparisons. The results are shown as the mean ± S.D. Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$.

might be synthesized in the Golgi apparatus and ER, respectively.

Snider *et al.* revealed that there were no significant changes in HexCer levels in MCF-7 cells treated with BFA for below 60 min.[12] The reason for the discrepancy in these results is unknown, but it may be due to the different cells used or because we analyzed a wide range of GlcCer species rather than only the main species.

Effects of Deuterium-Labeled PC Levels on BFA Treatment S1P lyase, which cleaves S1P or dS1P into a fatty aldehyde, can then enter glycerophospholipids such as PC *via* palmitoyl-CoA.[8] Therefore, we examined the effects of BFA on sphingolipid-mediated PC synthesis. Under our experimental conditions, deuterium-labeled C30:0-, C32:0-, C32:1-, and C34:1-PC were detected by LC–MS/MS (Fig. 7), and fatty acid patterns were identified by LC–MS/MS/MS (Supplementary

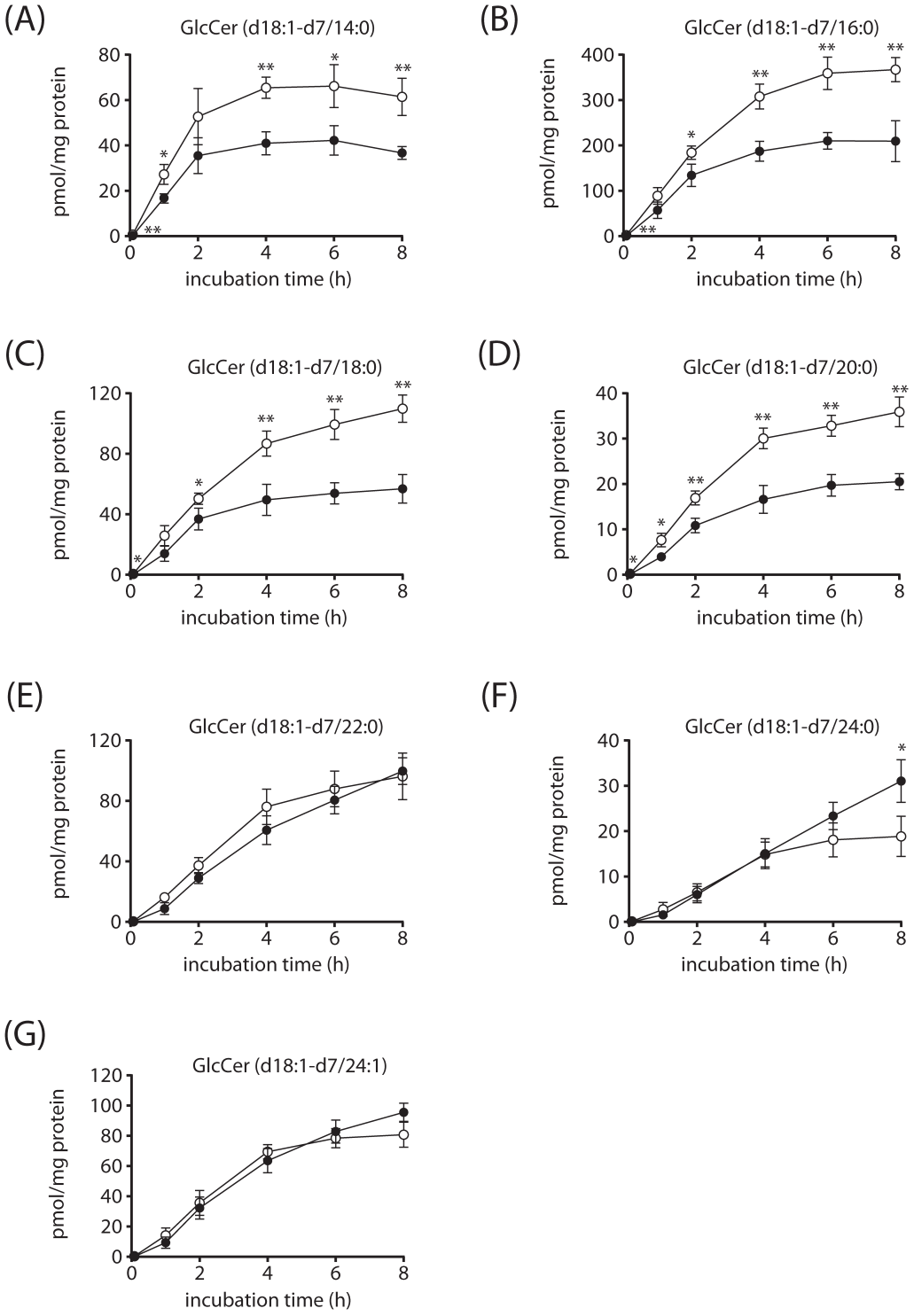

Fig. 6. Effect of BFA on Cellular GlcCer Species in HEK293T Cells

The levels of d7-sphinganine-derived GlcCer species in BFA-treated cells were quantified by LC–MS/MS. The BFA-treated and untreated cells are shown as white and black circles, respectively. Student's *t*-tests were used for comparisons. The results are shown as the mean ± S.D. Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$.

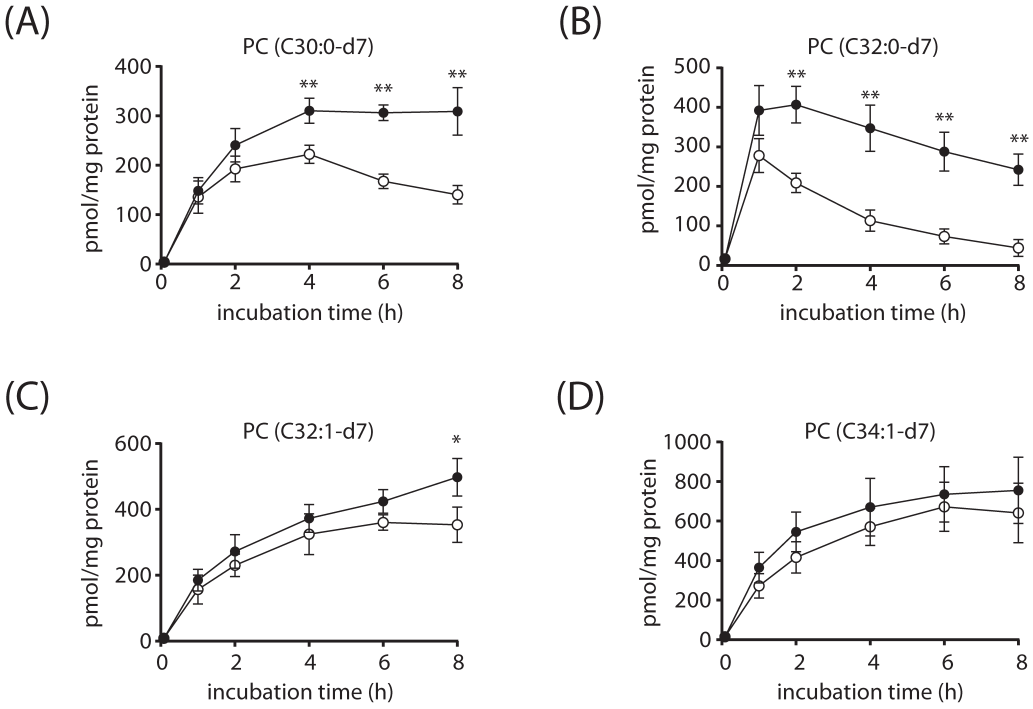

Fig. 7. Effect of BFA on Cellular PC Species in HEK293T Cells

Levels of d7-sphinganine-derived PC species in cells treated with BFA were quantified using LC–MS/MS. The BFA-treated and untreated cells are shown as white and black circles, respectively. Student's *t*-tests were used for comparisons. The results are shown as the mean ± S.D. Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$.

Fig. S5). LC–MS/MS/MS revealed that C30:0-PC contained deuterium-labeled C16:0 and natural C14:0; C32:0-PC contained deuterium-labeled C16:0 and natural C16:0; C32:1-PC contained deuterium-labeled C16:0 and natural C16:1 or deuterium-labeled C16:1 and natural C16:0; and C34:1-PC contained deuterium-labeled C16:0 and natural C18:1 (Supplementary Fig. S5). Although BFA treatment had little effect on the levels of all PC species within 1 h of precursor incubation, it decreased the levels of C30:0-PC after 4 h of incubation and C32:0-PC after 2 h of incubation (Fig. 7). In contrast, the levels of C32:1-PC and C34:1-PC remained comparable in the BFA-treated and untreated cells, except for C32:1-PC at 8 h of incubation. The reason for the different effects of BFA treatment on PC molecular species is unknown, and further studies are required to clarify which PC pathway, synthesized *via* S1P or dS1P, is affected by BFA treatment.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

REFERENCES

1) Iessi E, Marconi M, Manganelli V, Sorice M, Malorni W, Garofalo T, Matarrese P. On the role of sphingolipids in cell survival and death. *Int. Rev. Cell Mol. Biol.*, **351**, 149–195 (2020).
2) Hanada K. Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids*, **1632**, 16–30 (2003).
3) Shaner RL, Allegood JC, Park H, Wang E, Kelly S, Haynes CA, Sullards MC, Merrill AH Jr. Quantitative analysis of sphingolipids for lipidomics using triple quadrupole and quadrupole linear ion trap mass spectrometers. *J. Lipid Res.*, **50**, 1692–1707 (2009).
4) Ternes P, Franke S, Zähringer U, Sperling P, Heinz E. Identification and characterization of a sphingolipid delta 4-desaturase family. *J. Biol. Chem.*, **277**, 25512–25518 (2002).
5) Hayashi Y, Nemoto-Sasaki Y, Matsumoto N, Hama K, Tanikawa T, Oka S, Saeki T, Kumasaka T, Koizumi T, Arai S, Wada I, Yokoyama K, Sugiura T, Yamashita A. Complex formation of sphingomyelin synthase 1 with glucosylceramide synthase increases sphingomyelin and decreases glucosylceramide levels. *J. Biol. Chem.*, **293**, 17505–17522 (2018).
6) Hanada K, Kumagai K, Yasuda S, Miura Y, Kawano M, Fukasawa M, Nishijima M. Molecular machinery for non-vesicular trafficking of ceramide. *Nature*, **426**, 803–809 (2003).
7) Coant N, Sakamoto W, Mao C, Hannun YA. Ceramidases, roles in sphingolipid metabolism and in health and disease. *Adv. Biol. Regul.*, **63**, 122–131 (2017).
8) Kihara A. Sphingosine 1-phosphate is a key metabolite linking sphingolipids to glycerophospholipids. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids*, **1841**, 766–772 (2014).
9) Wood SA, Park JE, Brown WJ. Brefeldin A causes a microtubule-mediated fusion of the *trans*-Golgi network and early endosomes. *Cell*, **67**, 591–600 (1991).
10) Sciaky N, Presley J, Smith C, Zaal KJ, Cole N, Moreira JE, Terasaki M, Siggia E, Lippincott-Schwartz J. Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J. Cell Biol.*, **139**, 1137–1155 (1997).
11) Yamaji T, Horie A, Tachida Y, Sakuma C, Suzuki Y, Kushi Y, Hanada K. Role of intracellular lipid logistics in the preferential usage of very long chain-ceramides in glucosylceramide. *Int. J. Mol. Sci.*, **17**, 1761 (2016).
12) Snider JM, Snider AJ, Obeid LM, Luberto C, Hannun YA. Probing *de novo* sphingolipid metabolism in mammalian cells utilizing mass

spectrometry. *J. Lipid Res.*, **59**, 1046–1057 (2018).
13) Capasso S, Sticco L, Rizzo R, Pirozzi M, Russo D, Dathan NA, Campelo F, van Galen J, Hölttä-Vuori M, Turacchio G, Hausser A, Malhotra V, Riezman I, Riezman H, Ikonen E, Luberto C, Parashuraman S, Luini A, D'Angelo G. Sphingolipid metabolic flow controls phosphoinositide turnover at the *trans*-Golgi network. *EMBO J.*, **36**, 1736–1754 (2017).
14) Hayashi Y, Tsuchiya K, Yamamoto M, Nemoto-Sasaki Y, Tanigawa K, Hama K, Ueda Y, Tanikawa T, Gohda J, Maeda K, Inoue JI, Yamashita A. *N*-(4-hydroxyphenyl) retinamide suppresses SARS-CoV-2 spike protein-mediated cell–cell fusion by a dihydroceramide Δ4-desaturase 1-independent mechanism. *J. Virol.*, **95**, e0080721 (2021).
15) Levy M, Futerman AH. Mammalian ceramide synthases. *IUBMB Life*, **62**, 347–356 (2010).
16) Karsai G, Kraft F, Haag N, Korenke GC, Hänisch B, Othman A, Suriyanarayanan S, Steiner R, Knopp C, Mull M, Bergmann M, Schröder JM, Weis J, Elbracht M, Begemann M, Hornemann T, Kurth I. DEGS1-associated aberrant sphingolipid metabolism impairs nervous system function in humans. *J. Clin. Invest.*, **129**, 1229–1239 (2019).
17) Kitatani K, Idkowiak-Baldys J, Hannun YA. The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell. Signal.*, **20**, 1010–1018 (2008).
18) Halter D, Neumann S, van Dijk SM, Wolthoorn J, de Mazière AM, Vieira OV, Mattjus P, Klumperman J, van Meer G, Sprong H. Pre- and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. *J. Cell Biol.*, **179**, 101–115 (2007).
19) Sprong H, Degroote S, Claessens T, van Drunen J, Oorschot V, Westerink BH, Hirabayashi Y, Klumperman J, van der Sluijs P, van Meer G. Glycosphingolipids are required for sorting melanosomal proteins in the Golgi complex. *J. Cell Biol.*, **155**, 369–380 (2001).
20) Jeckel D, Karrenbauer A, Burger KN, van Meer G, Wieland F. Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J. Cell Biol.*, **117**, 259–267 (1992).
21) Kohyama-Koganeya A, Sasamura T, Oshima E, Suzuki E, Nishihara S, Ueda R, Hirabayashi Y. Drosophila glucosylceramide synthase: a negative regulator of cell death mediated by proapoptotic factors. *J. Biol. Chem.*, **279**, 35995–36002 (2004).