



# Functions of neutral ceramidase in the Golgi apparatus

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**Abstract** Ceramidases hydrolyze ceramides into sphingosine and fatty acids, with sphingosine being further metabolized into sphingosine-1-phosphate (S1P); thus, ceramidases control the levels of these bioactive sphingolipids in cells and tissues. Neutral ceramidase (nCDase) is highly expressed in colorectal tissues, and a recent report showed that nCDase activity is involved in Wnt/ $\beta$ -catenin signaling. In addition, the inhibition of nCDase decreases the development and progression of colorectal tumor growth. Here, to determine the action of nCDase in colorectal cancer cells, we focused on the subcellular localization and metabolic functions of this enzyme in HCT116 cells. nCDase was found to be located in both the plasma membrane and in the Golgi apparatus, but it had minimal effects on basal levels of ceramide, sphingosine, or S1P. Cells overexpressing nCDase were protected from the cell death and Golgi fragmentation induced by C6-ceramide, and they showed reduced levels of C6-ceramide and higher levels of S1P and sphingosine. Furthermore, compartment-specific metabolic functions of the enzyme were probed using C6-ceramide and Golgi-targeted bacterial SMase (bSMase) and bacterial ceramidase (bCDase). The results showed that Golgi-specific bCDase also demonstrated resistance against the cell death stimulated by C6-ceramide, and it catalyzed the metabolism of ceramides and produced sphingosine in the Golgi. Targeting bSMase to the Golgi resulted in increased levels of ceramide that were attenuated by the expression of nCDase, also supporting its ability to metabolize Golgi-generated ceramide. **These results are critical in understanding the functions of nCDase actions in colorectal cancer cells as well as the compartmentalized pathways of sphingolipid metabolism.**—Sakamoto, W., N. Coant, D. Canals, L. M. Obeid, and Y. A. Hannun. **Functions of neutral ceramidase in the Golgi apparatus.** *J. Lipid Res.* 2018. 59: 2116–2125.

**Supplementary key words** neutral ceramidase • sphingolipids • Golgi • apoptosis • colon cancer

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Ceramide and sphingosine-1-phosphate (S1P) are bioactive sphingolipids that are involved in multiple cellular functions such as apoptosis, cell death, migration, and autophagy (1, 2). Ceramide is a key regulatory factor for apoptosis and acts as an intracellular mediator for extracellular stimuli, including chemical agents, irradiation, growth factors, and some stresses. On the other hand, S1P regulates cell proliferation, motility, and survival (3).

Regulating sphingolipid metabolism helps maintain homeostasis, and targeting these pathways could improve diseases affected by defects in their pathways (4). Fingolimod (FTY720) is a first-in-class orally active S1P receptor modulator that has been approved as a new therapeutic drug for multiple sclerosis (5). Recently, ceramide nanoliposomes for the improved treatment of cancer have been developed and approved for clinical human trials by the Food and Drug Administration.

Ceramidases are responsible for hydrolyzing ceramide into sphingosine and free fatty acids. There are three types of ceramidases, acid, neutral, and alkaline, based on their pH required for optimal activity (6, 7). Acid ceramidase (aCDase) is located within lysosomes, and a genetic deficiency of aCDase causes the lysosomal lipid storage disorder known as Farber's disease (8). Alkaline ceramidases (ACERs) 1, 2, and 3 are primarily located in the endoplasmic reticulum (ER) and Golgi complexes.

Neutral ceramidase (nCDase), which is encoded by *N*-acylsphingosine amidohydrolase 2, has been identified not only from human and mammalian organisms but also from certain bacteria (9). nCDase is expressed in some tissues, including the kidney, liver, brain, lung, and heart, and is particularly highly expressed in the small intestine and colon (10). The localization of nCDase in the plasma membrane has been reported in previous studies (11), and

Abbreviations: aCDase, acid ceramidase; ACER, alkaline ceramidase; bCDase, bacterial ceramidase; bSMase, bacterial sphingomyelinase; CHX, cycloheximide; ER, endoplasmic reticulum; MEF, mouse embryo fibroblast; MTT, methylthiazolyl-diphenyl-tetrazolium bromide; nCDase, neutral ceramidase; S1P, sphingosine-1-phosphate.

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the enzyme has also been shown to be expressed in the ER/Golgi complex and mitochondria, the latter especially in the liver (12, 13). nCDase is known to be regulated by stress stimuli, including UV-B irradiation, nitric oxide, and nutrient deprivation (14–18). Furthermore, treatment with gemcitabine, a chemotherapeutic drug, reduces nCDase protein levels and causes accumulation of ceramides, leading to suppression of cell growth (19). In addition, in a previous study we found that the inhibition of nCDase results in a loss of  $\beta$ -catenin, which is important for colon cancer development (20). In a xenograft model, tumor growth remarkably declined by inhibiting nCDase. In addition, nCDase knockout mice treated with azoxymethane, an inducer of carcinogenesis, were protected from tumor formation. Therefore, this enzyme has a crucial role in the tumor progression of colon cancer.

nCDase may also be involved in the metabolism and action of C6-ceramide, which is known to sensitize multiple cancer cell lines to apoptosis-inducing agents such as doxorubicin (21). In fact, C6-ceramide is now emerging as a novel cancer therapeutic (in nanoliposome formulation) in clinical trials (Keystone Nano, Inc., ClinicalTrials.gov Identifier: NCT02834611).

Here, we report new findings on where and how the enzyme functions in colorectal cancer cell lines. At first, a stable cell line overexpressing nCDase was generated, and the localization of nCDase and the sphingolipids levels were analyzed. Second, the gain-of-function effects of nCDase were determined, and its ability to interfere with cell death in response to C6-ceramide was evaluated. The results showed that the upregulation of nCDase activity imparts tolerance to apoptosis and cell death induced by C6-ceramide (22). Therefore, the inhibition of nCDase may lead to increased sensitivity to chemotherapeutic drugs, and this enzyme may emerge as an attractive target for drug development. In addition, validating the detection of intracellular nCDase activity is developed in this study. These results disclose that nCDase is active in both the plasma membrane and in the Golgi apparatus. The implications of these results are discussed.

## MATERIALS AND METHODS

### Reagents

Cell culture medium and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). nCDase antibody (Ab174) was a kind gift from Richard Proia (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Anti-GAPDH was obtained from Cell Signaling (Beverly, MA), anti-V5 tag antibody was from Invitrogen, anti-Giantin antibody was from Covance (Berkeley, CA), and anti-GM130 antibody was from BD Biosciences (San Jose, CA). The ECL kit was from Thermo Fisher Scientific (Rockford, IL). C6-ceramide and SIP were purchased from Avanti Polar Lipids (Alabaster, AL), and C6-urea-ceramide was provided by the Medical University of South Carolina.

### Cell culture

Colon cancer HCT116 cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum. Cell lines were grown in a 5% CO<sub>2</sub> incubator at 37°C.

### Generation of nCDase-overexpressing cells and cells transfected with the Golgi-targeted bacterial ceramidase and sphingomyelinase

For the generation of nCDase-overexpressing cells, the human nCDase gene in pLenti6.3/TO/V5 was generated in two steps. Using a 5'-caccatggccaacgcacctctctaac-3' primer containing 24 nucleotides of the 5'-translated region starting with ATG and a 3'-aatagttacaactcaaaagccggggaag-5' primer containing the last 29 nucleotides minus the stop codon, the amplified human nCDase was directionally subcloned into intermediate vector pENTR-D/TOPO (Life Technologies, Carlsbad, CA). Using Gateway LR Clonase II enzyme mix (Life Technologies cat#11791-020), the intermediate was recombined with the destination cloning vector pLenti6.3/TO/V5-DEST (Life Technologies cat# K531520). The orientation of the insert was verified by restriction mapping and sequencing. An inactive nCDase (S354A) was also developed similarly (23). The Golgi-targeted bacterial ceramidase (bCDase) and bacterial SMase (bSMase) genes were directionally subcloned into pCMV and pEF6 vectors tagged with V5 or Flag. All constructs were verified by restriction mapping and sequencing. The Golgi-targeting signal is a 249 bp fragment of the human  $\beta$ -1,4-galactosyltransferase gene that contains a cytoplasmic tail, membrane-spanning region, and partial stem structure. The Golgi-targeting sequence is on the N terminus of the bCDase and bSMase genes, targeting them to the luminal side of the Golgi. Cells were seeded at 10<sup>5</sup> per 60 mm dish and transfected with each construct using X-tremeGENE 9 DNA Transfection Reagent in accordance with the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). After transfection for 48 h, transformed cells were selected by adding 50  $\mu$ g/ml blasticidin and replacing the medium containing blasticidin every 3–4 days. After 10 days of selection, stably transfected cells were generated.

### Western blot analysis

Protein extracts from cells were obtained after harvesting or homogenization with RIPA buffer (with PMSF, orthovanadate, and protease inhibitor cocktail; Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentration was measured using a Pierce BCA Protein Assay Kit from Thermo Fisher Scientific (#23225). Proteins were separated by SDS-PAGE using the Criterion system (Bio-Rad Laboratories, Hercules, CA) and immunoblotted as described previously (24).

### Measurement of nCDase activity

N-[12-[(7-Nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]D-erythro-sphingosine (C12-NBD ceramide) as a substrate was purchased from Avanti Polar Lipids. Cell lysates and micellar C12-NBD ceramide solution were mixed and incubated in 100 mM Tris buffer (pH 7.5) with 0.3% (w/v) Triton X-100. After incubation at 37°C for 3 h in the dark, quench solution (1:1 chloroform-methanol) was added and vortexed. After centrifugation at 3,000 rpm for 10 min, supernatants were dried under nitrogen gas. Samples were placed in 100  $\mu$ l methanol, and NBD-dodecanoic acid generated by the action of the enzyme form C12-NBD ceramide was detected by HPLC (25).

### Assessment of cell viability and apoptosis

Cell viability was assessed by methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, #M5655). Cells were treated with MTT (1 mg/ml) for 30 min, and supernatants were discarded. DMSO was added, and the absorbance was measured at 560 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). Apoptosis was determined by the Caspase-3

Fluorometric Assay Kit ((#K105-100,BioVision, Milpitas, CA) as indicated by the manufacturer (26).

### Confocal microscopy

Cells were seeded in poly-D-lysine-coated confocal dishes (Mat-Tek Corp., Ashland, MA) and treated and fixed in 4.0% paraformaldehyde solution for 20 min and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with 2% human serum for 20 min, cells were incubated with primary and secondary antibodies overnight at 4°C. Cells were observed using an LSM510 META confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany) and a Leica SP8 confocal microscopy system (Leica Microsystems, Wetzlar, Germany) (27). The Golgi fragmentation was visualized using anti-GM130 antibody in the green channel. RGB images were split and the green channel was selected, a morphological transformation was used to remove the background, and the number of signal pixels was quantified per each image (Adobe Photoshop CS4 software).

### Measurement of lipids

Lipid analysis was performed as previously described (28). Briefly, media and cells were collected and sonicated, and sphingolipid species were identified on a Thermo Finnigan TSQ700 triple quadrupole mass spectrometer. Sphingolipids from cellular extracts were normalized to total lipid phosphates present in the cells after a Bligh and Dyer extraction.

### Statistical analysis

Results are expressed as means  $\pm$  SDs. For statistical analysis, the *t*-test for paired sample means was performed using GraphPad Prism 5 software.

## RESULTS

### Subcellular localization of nCDase in HCT116 human colon cancer cells

To examine the subcellular localization of nCDase in colon cancer cells, a plasmid vector containing V5 tagged at the C terminus of the human nCDase gene was transfected into HCT116 human colon cancer cells. Cells with a stable overexpression of nCDase showed an increase of nCDase activity (Fig. 1A, B). On the other hand, the nCDase activity of cells expressing inactive nCDase (S354A) was not increased. Furthermore, cells were fixed and stained with anti-V5 antibody. Confocal microscopy revealed that the V5-nCDase protein was localized in the plasma membrane (Fig. 1Ce–h) as well as in intracellular structures. Costaining with an anti-giantin antibody (Golgi marker) revealed that the protein colocalized with giantin. On the other hand, LacZ on V5 vector-transfected cells showed that they were stained diffusely with V5 antibody (Fig. 1Ca–d).

Next, to assess whether the effect of overexpression might cause the Golgi localization because the Golgi nCDase can represent a pool of newly synthesized protein en route to the cell surface, the cells were treated with cycloheximide (CHX), an inhibitor of protein synthesis, and the protein level and localization of V5-nCDase were observed. Protein levels of nCDase decreased time-dependently (0–6 h) (Fig. 2A), indicating a half-life of the protein of approximately 4 h. However, under these conditions, nCDase still

localized to the Golgi (Fig. 2B). Therefore, the results indicate that the expression of nCDase in the Golgi does not represent newly synthesized protein. Taken together, nCDase is localized in both the plasma membrane and Golgi apparatus in HCT116 cells.

### nCDase-overexpressing cells show resistance against cell death induced by C6-ceramide

nCDase overexpression did not show significant effects on cell proliferation (data not shown). Therefore, we evaluated the effects of nCDase on modulating cell growth in response to ceramide.

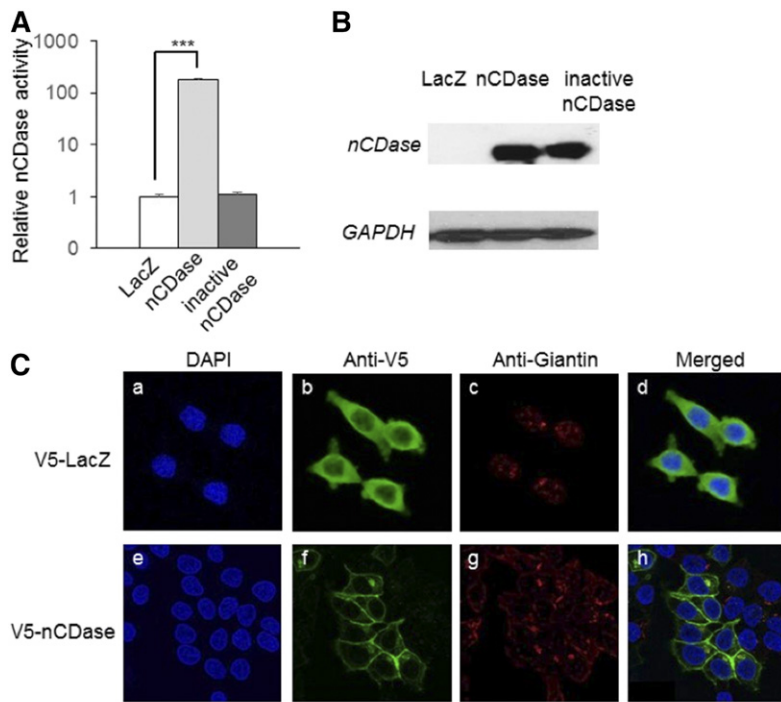
C6-ceramide plays roles in inhibiting tumor proliferation and is emerging as a novel cancer therapeutic. To assess whether the nCDase activity is involved in cell functions mediated by C6-ceramide, nCDase-overexpressing cells were treated with C6-ceramide, and the viable cell number, MTT values, and caspase-3 activity were measured. As shown in Fig. 3A and B, nCDase-overexpressing cells were protected from the growth suppression induced by C6-ceramide (at 5  $\mu$ M for 48 h). Likewise, caspase-3 activity was increased 1.5-fold in mock cells after treatment with C6-ceramide at 10  $\mu$ M for 12 h (Fig. 3C). In cells overexpressing nCDase, C6-ceramide caused only a 1.2-fold increase of caspase-3 activity. On the other hand, the effects of C6-ceramide on MTT and caspase-3 activity in cells overexpressing inactive nCDase were similar to the results of mock-transfected cells. Therefore, these data suggest that the increase of nCDase activity protects cells from C6-ceramide-induced growth suppression.

### The effect of Golgi-specific ceramidase on C6-ceramide-induced Golgi fragmentation

C6-ceramide has been shown to induce Golgi fragmentation (29). To investigate the ability of nCDase to modulate Golgi fragmentation, HCT116 cells treated with C6-ceramide at 5  $\mu$ M for 12 h were stained with antibody against GM130 and observed by confocal microscopy. Treatment with C6-ceramide caused significant Golgi fragmentation in mock cells (Fig. 4A, B). In contrast, cells overexpressing nCDase did not show Golgi fragmentation (Fig. 4E, F). We then investigated whether the activity of nCDase was required to inhibit C6-ceramide-induced Golgi fragmentation. To that end, we employed C6-urea-ceramide, an nCDase inhibitor developed based on structure-activity analysis (30). C6-urea-ceramide on its own did not induce any detectable Golgi disruption (Fig. 4C, G). On the other hand, pretreatment with C6-urea-ceramide reversed the protection from Golgi fragmentation by nCDase in cells treated with C6-ceramide (Fig. 4D, H). In addition, Fig. 4I shows the area in the Golgi stained with GM130 for quantification of the Golgi fragmentation, and it was found that the Golgi apparatus was sustained in HCT116 cells overexpressing nCDase treated with C6-ceramide.

Next, we prepared the bCDase gene fused with the V5 tag and subcloned it into a vector containing the sequence that targets the fusion protein to the Golgi apparatus (Golgi-bCDase) or cytosolic-targeted bCDase to focus on functions of the enzyme in the Golgi. It should be noted





**Fig. 1.** Subcellular localization of nCDase in nCDase stable overexpressed HCT116 cells. A plasmid vector containing the V5-tagged nCDase or inactive nCDase (S354A) gene was transfected into HCT116 cells. A: Cells were harvested, and homogenates were incubated at 37° for 3 h using C12-NBD-ceramide as a substrate, and nCDase activity was measured by quantitating the levels of NBD-dodecanoic acid by HPLC. B: The expression of nCDase was detected by Western blotting. C: LacZ vector (a–d) and nCDase vector (e–h) overexpressing HCT116 cells were stained with DAPI (blue), anti-V5 antibody (green), and anti-giantin antibody (Golgi marker, red). Cells were observed by confocal immune fluorescence microscopy. Scale bars: 25  $\mu$ m. Results are representative of three experiments. \*\*\* $P < 0.0005$ .

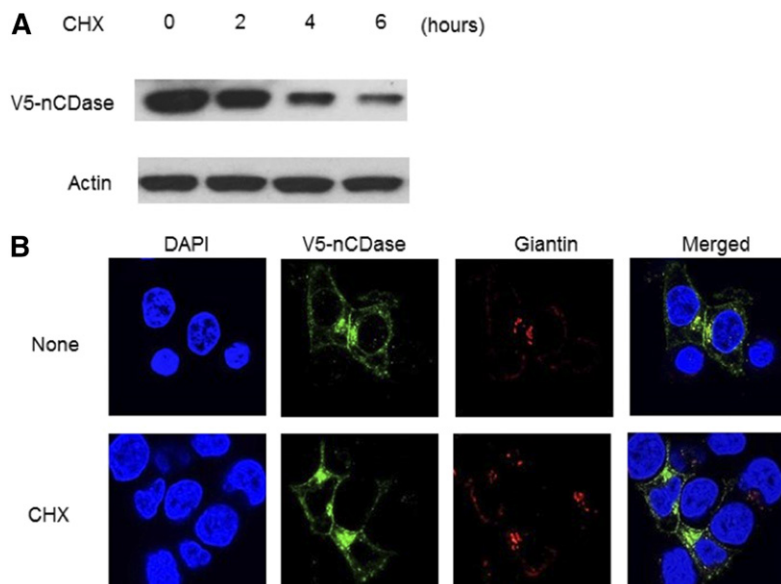
that this approach targets the bCDase to the lumen of the Golgi, similar to the topology of nCDase, which is a type-II transmembrane protein whose catalytic site faces the extracytoplasmic area (in the plasma membrane and Golgi). As shown in **Fig. 5** in red, it was confirmed that both Golgi-bCDase and cytosolic-targeted bCDase were expressed in the appropriate organelle. HCT116 cells transfected with empty vector showed Golgi fragmentation (shown in green) in response to C6-ceramide (**Fig. 5A, B**). In contrast, the transfection of Golgi-bCDase in HCT116 cells inhibited the Golgi fragmentation induced by C6-ceramide (**Fig. 5C, D**). On the other hand, cells transfected with cytosolically targeted bCDase showed Golgi fragmentation induced by C6-ceramide (**Fig. 5E, F**). These effects

were quantified (**Fig. 5G**), and, thus, the results suggest that bCDase functions in the Golgi to counteract the effects of C6-ceramide.

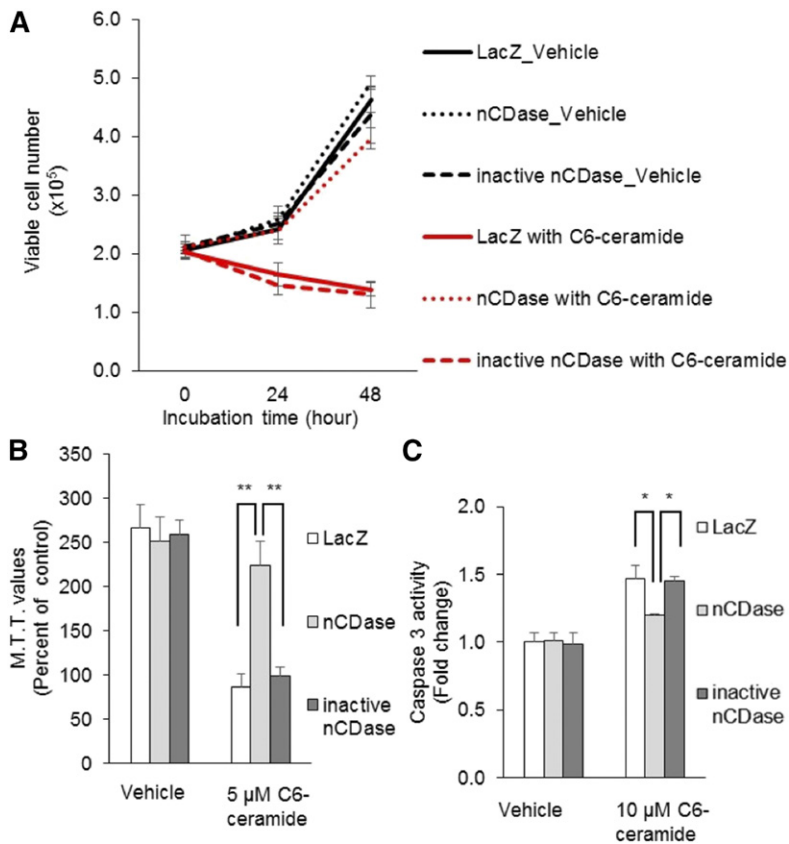
Taken together, the results demonstrate a clear cellular function of nCDase in that it prevents the ability of C6-ceramide from leading to Golgi fragmentation.

#### Involvement of nCDase in sphingolipid metabolism

Sphingolipids regulate many cellular functions, including apoptosis, cell cycle, migration, and proliferation. To investigate the involvement of nCDase in sphingolipid metabolism, the levels of ceramide, sphingosine, and SIP were measured by HPLC-ESI-MS. Interestingly, their basal levels in nCDase-overexpressing cells did not show significant



**Fig. 2.** Localization of nCDase in HCT116 cells after treatment with CHX. nCDase-overexpressing HCT116 cells were treated with 100  $\mu$ g/ml CHX for the indicated time (A) or for 4 h (B). A: Extracted proteins were analyzed by Western blotting with anti-nCDase and anti-actin antibodies. B: Cells treated with CHX were fixed and stained with anti-V5 (green) and anti-giantin (red) antibodies. Cells were observed by confocal microscopy.

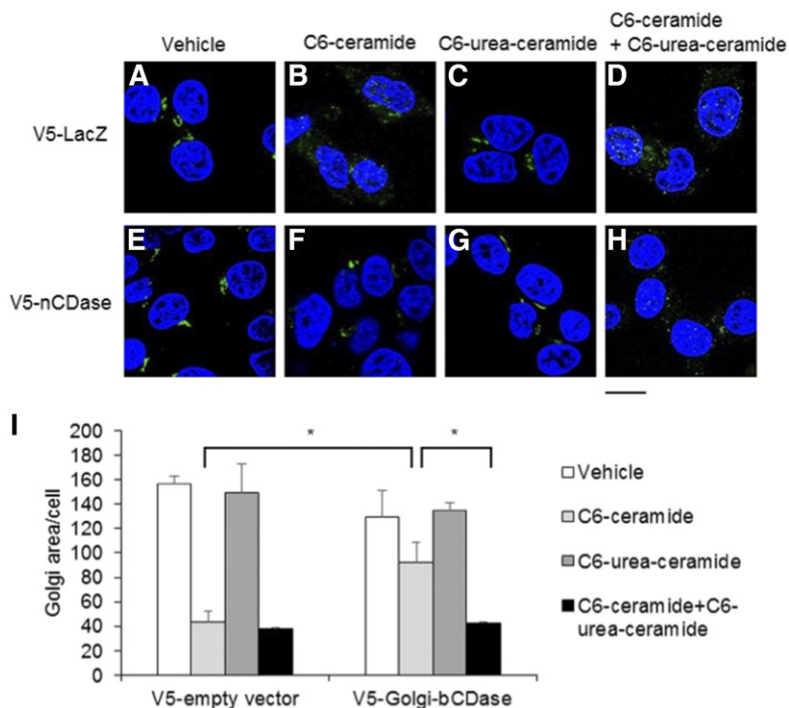


**Fig. 3.** Effects of C6-ceramide on cell growth and apoptosis in HCT116 cells overexpressing nCDase. A, B: nCDase-overexpressing HCT116 cells were treated with 5  $\mu$ M C6-ceramide for 48 h, and then cell growth was measured by the viable cell count and MTT assay. C: Caspase-3 activity was measured by fluorescent assay. Results are representative of three experiments. \*\* $P < 0.005$  and \* $P < 0.05$ .

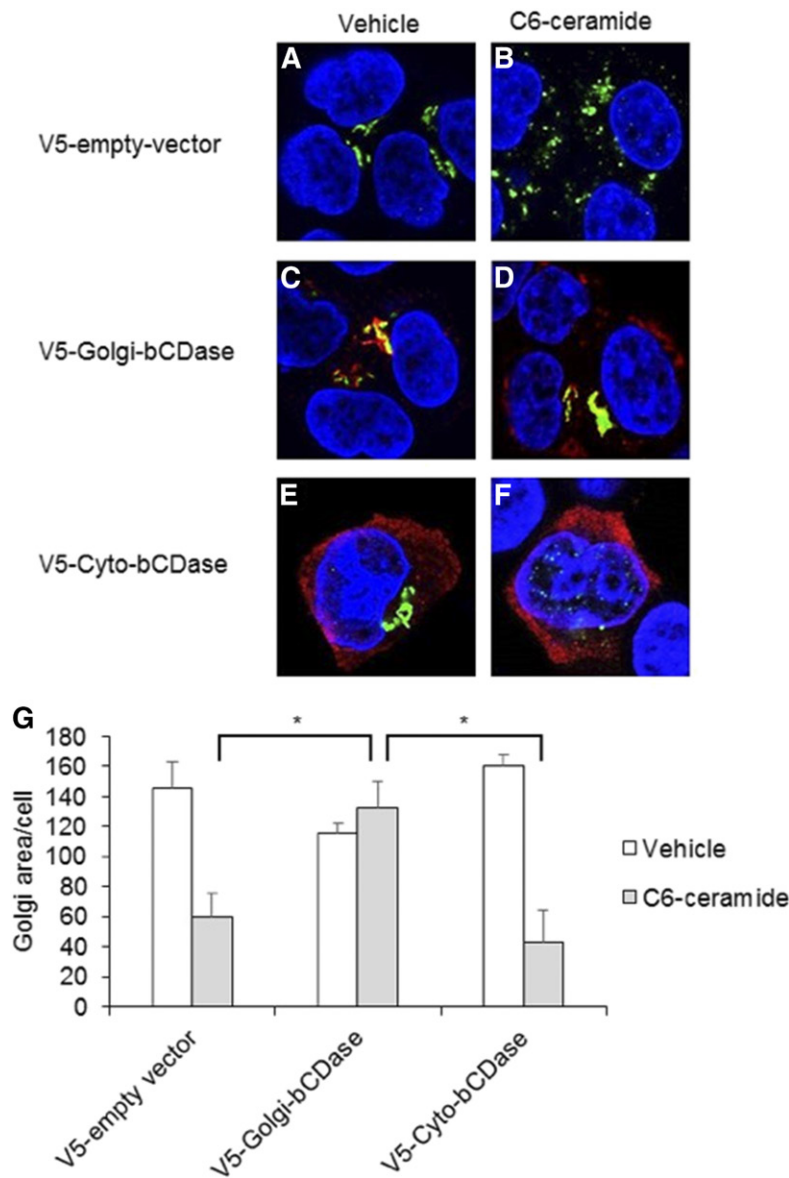
changes compared with results in mock cells (Fig. 6). These results suggest that nCDase may regulate ceramide metabolism under specialized conditions and not constitutively or that specific effects could not be evaluated due to compensatory changes in sphingolipid metabolism.

C6-ceramide is known to change sphingolipid metabolism and upregulate intracellular ceramide levels through

deacylation/reacylation (31). Therefore, we investigated the effects of nCDase on changes in sphingolipid metabolism induced by C6-ceramide. Treatment with C6-ceramide induced an increase in sphingosine, and nCDase-overexpressing cells showed larger increases of sphingosine by 2.3-fold compared with mock cells (Fig. 6A). As for S1P levels, the results were similar to those seen with sphingosine such



**Fig. 4.** Fragmentation of the Golgi complex is induced by C6-ceramide and prevented by the overexpression of nCDase. nCDase-overexpressing HCT116 cells were treated with 5  $\mu$ M C6-ceramide for 12 h and stained with anti-GM130 antibody (Golgi marker, green) and DAPI (blue). Cells were observed by confocal immune fluorescence microscopy. Cells were untreated (A, E) or treated with C6-ceramide (B, F), C6-urea-ceramide (nCDase inhibitor; C, G), or their combination (D, H). Scale bars: 10  $\mu$ m. I: The intensity of the Golgi area stained with GM130 in green was quantified at three different spots. \* $P < 0.05$ .



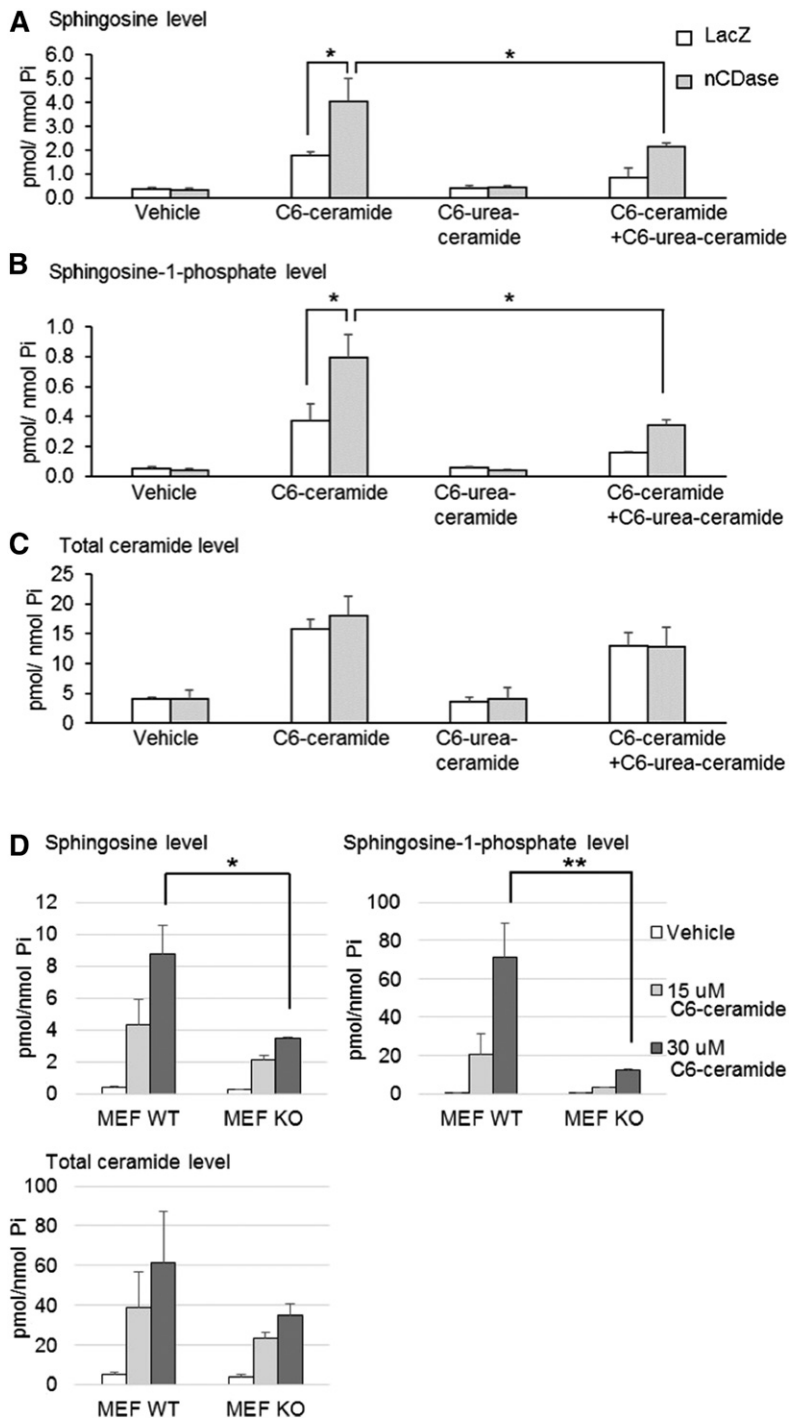
**Fig. 5.** The effects of Golgi-bCDase on the fragmentation of the Golgi complex. HCT116 cells overexpressing the Golgi and cytosolic-targeted bCDase tagged with V5 were treated with 5  $\mu$ M C6-ceramide for 12 h and stained with anti-GM130 antibody (Golgi marker, green), anti-V5 antibody (bCDase expression, red), and DAPI (nucleus marker, blue). Cells were treated with vehicle (A, C, E) or C6-ceramide (B, D, F). G: The Golgi area at the three different spots was analyzed similar to Fig. 4I. \* $P < 0.05$ .

that the levels of SIP were higher in cells overexpressing nCDase than those observed in mock cells (Fig. 6B). The levels of endogenous ceramides were also increased by C6-ceramide stimulation, but they did not change significantly between mock and nCDase-overexpressed cells (Fig. 6C). These results suggest that nCDase can accelerate the metabolism of C6-ceramide into sphingosine and subsequently SIP.

To further evaluate the effects of nCDase on sphingolipid metabolisms, cells were treated with C6-ceramide in combination with C6-urea-ceramide, which is an nCDase inhibitor (32). C6-urea-ceramide on its own at 5  $\mu$ M did not affect the levels of sphingolipids after 12 h of treatment (Fig. 6). In contrast, pretreatment with C6-urea-ceramide decreased both sphingosine and SIP levels in response to treatment with C6-ceramide. Ceramide levels were not affected by C6-urea-ceramide. These data demonstrate the need for activity of the overexpressed nCDase in metabolizing C6 ceramide. They also indicate that endogenous

nCDase is involved in the metabolism of C6-ceramide to sphingosine and subsequently to SIP and possibly ceramide.

In a complementary approach, nCDase knockout mouse embryo fibroblasts (MEFs) were analyzed to determine whether the increase of sphingosine levels induced by C6-ceramide was dependent on nCDase activity (Fig. 6D). In nCDase KO MEFs, the increase of sphingosine levels following the addition of C6-ceramide was reduced compared with those in WT MEFs. The resultant SIP levels were also decreased after nCDase deletion. The effects of nCDase activity on changes of ceramide levels induced by C6-ceramide in MEFs were not significantly changed. These data demonstrate that nCDase produces sphingosine from C6-ceramide; however, because there was residual formation of sphingosine, the results suggest the involvement of additional ceramidases in the metabolism of C6-ceramide. Practically, these results demonstrate the utility of C6-ceramide as a tool for detecting the nCDase activity in cells.



**Fig. 6.** Effects of nCDase overexpression on sphingolipid metabolism. HCT116 cells overexpressing nCDase were treated with C6-ceramide for 12 h (or vehicle) with or without pretreatment with C6-urea-ceramide (5  $\mu$ M) for 1 h. Cells were harvested, and lipids were extracted and then subjected to HPLC-ESI-MS analysis for quantification of sphingosine (A), S1P (B), and total ceramides (C). D: Changes of sphingolipids in nCDase knockout MEFs treated with 15 and 30  $\mu$ M C6-ceramide for 12 h were measured. Results are representative of three experiments. \*\* $P < 0.005$  and \* $P < 0.05$ .

#### Fate of C6-ceramide levels in media and cells

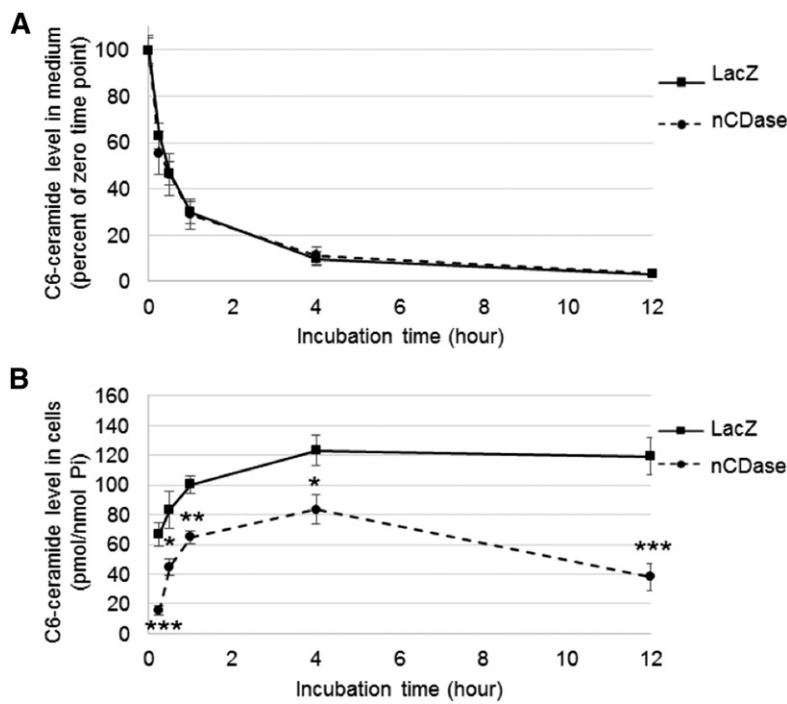
To further define the effects of nCDase on metabolism and uptake of C6-ceramide, C6-ceramide levels were measured by HPLC-ESI-MS. C6-ceramide levels in media decreased in a time-dependent manner, accompanied by an increase in cellular levels of C6-ceramide that continued to increase up to 4 h (Fig. 7). It is important to note that nCDase overexpression did not affect the loss of C6-ceramide from the media, suggesting little activity on C6 ceramide extracellularly and no effects on uptake (Fig. 7A). On the other hand, nCDase overexpression lowered levels of

C6-ceramide in cells (Fig. 7B). These data suggest that nCDase does not affect the uptake of C6-ceramide but promotes the metabolism of C6-ceramide, consistent with the results from Fig. 6.

#### Effects of Golgi-targeted bSMase on sphingolipids in nCDase-overexpressing cells

To focus on functions of nCDase in the Golgi, we prepared the bSMase gene fused with the V5 tag and subcloned it into a vector containing the sequence that targets the fusion protein to the Golgi apparatus (Golgi-bSMase),





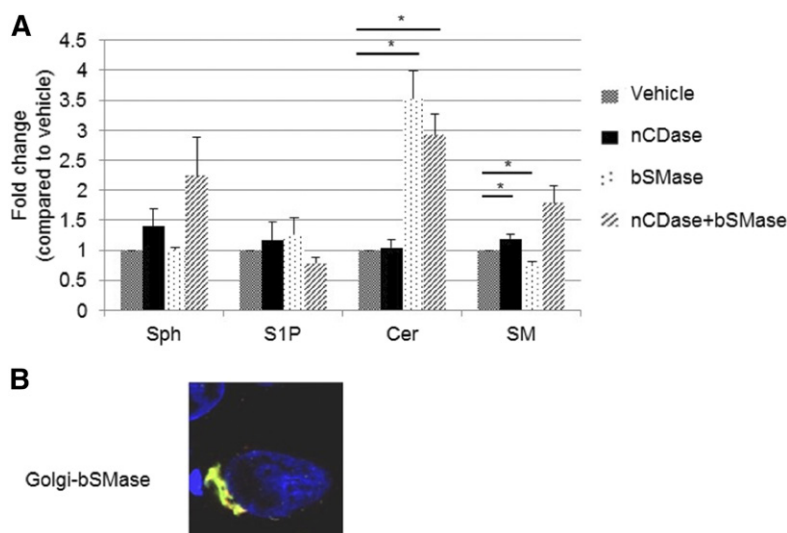
**Fig. 7.** Levels of C6-ceramide in media and cells. HCT116 cells overexpressing nCDase were treated with C6-ceramide for the indicated times (0–12 h). Lipids were extracted from the media (A) or cell pellets (B) and measured. Results are representative of three experiments. \*\*\* $P < 0.0005$ , \*\* $P < 0.005$ , and \* $P < 0.05$ .

and we set up a system that can detect Golgi-specific changes in sphingolipid levels. HCT116 cells overexpressing nCDase were transfected with Golgi-bSMase vector for 24 h, and then their sphingolipid levels, including sphingosine, SIP, ceramides, and SM were analyzed (Fig. 8A). This resulted in a significant increase of ceramide species and a decrease of SM with little changes in sphingosine or SIP in HCT116 cells. Next, the results revealed that the increase of ceramide levels induced by bSMase was decreased, and sphingosine levels were increased markedly in cells with overexpressed nCDase, demonstrating that the increased ceramides were metabolized into sphingosine by nCDase. It is unclear why SM levels in HCT116 cells overexpressing nCDase were higher than those in mock cells, but the pathway of SM synthesis might be affected by the overexpression of nCDase and bSMase. In addition, it was confirmed

that this construct was located in the appropriate organelle (Fig. 8B). Taken together, these results demonstrate that nCDase exerts action on bSMase-induced Golgi ceramide, again attesting to the Golgi site of action of this enzyme.

## DISCUSSION

This study demonstrates that nCDase is located in the Golgi in HCT116 cells in addition to its location in the plasma membrane. The results also demonstrate the ability of nCDase in hydrolyzing the lipid in the Golgi complex and protecting cells from apoptosis induced by C6-ceramide. This role of nCDase in the Golgi was also validated by using C6-ceramide as well as compartmentally targeted SMases and CDases. The results add to the ability to define the



**Fig. 8.** Changes of sphingolipids in HCT116 cells overexpressing nCDase transfected with Golgi-targeted bSMase vector. A: HCT116 cells overexpressing nCDase were transfected with Golgi-targeted bSMase and incubated for 24 h. Cells were harvested, and lipids were extracted and then subjected to HPLC-ESI-MS analysis for quantification of the levels of sphingosine, SIP, total ceramides, and total SMs; all were normalized to total phospholipid phosphate. B: Golgi-targeted bSMase-overexpressing cells were stained and observed with anti-GM130 antibody (Golgi marker, green), anti-V5 antibody (bSMase expression, red), and DAPI (nucleus marker, blue). Results are representative of three experiments. \* $P < 0.05$ .



subcellular biochemistry of nCDase and provide further insight into the subcellular regulation of sphingolipid metabolism and action.

These results showed that nCDase is involved in the sphingolipid metabolic pathway in the Golgi apparatus. To detect the changes in sphingolipid levels via nCDase action, it was productive to add an exogenous perturbation of sphingolipid metabolism in the form of either bSMase or C6-ceramide. Although HCT116 cells overexpressing nCDase did not show changes in basal levels of sphingosine, SIP, or ceramide, they did demonstrate significant increases of sphingosine and SIP levels after stimulation with C6-ceramide. Exogenous SIP did not counteract the effects of C6-ceramide on the cell growth and Golgi fragmentation (see supplemental Fig. S1). Because short-chain ceramides such as NBD-C6-ceramide are known to concentrate in the Golgi (29), these results suggest a possible Golgi site of action of nCDase on C6-ceramide, especially because nCDase did not affect the extracellular levels of C6-ceramide. Moreover, these results show that the addition of C6-ceramide resulted in significant Golgi fragmentation. This was prevented by nCDase activity. In further support of a Golgi site of action, nCDase metabolized ceramides that were produced by the action of Golgi-targeted bSMase.


The Golgi apparatus is a major organelle that plays a role in sphingolipid metabolism, especially in the de novo synthesis of SMs and glycosphingolipids (33–35). Synthesized ceramides are transferred by the ceramide transfer protein from the ER (the major site of de novo synthesis of ceramide) (36, 37). However, much less is known about hydrolytic pathways in the Golgi. Neutral SMs, Acer2, and Acer3 have also been shown to reside in the Golgi (38–40). These studies disclose that nCDase can hydrolyze ceramides to sphingosine and fatty acid in the Golgi. In addition, nCDase appears to play a role in maintaining the morphology and functions of the Golgi apparatus to protect it from exposing stimuli that induce intracellular ceramides.

Likewise, it has been reported that nCDase can act on ceramide generated in the plasma membrane by the action of exogenous bSMase (12). Taken together, these results disclose a significant duality in the existence of nCDase and its biochemical activity in two distinct compartments in the plasma membrane and in the Golgi.

In terms of nCDase biological functions, the upregulation of nCDase activity protected cells from cell death induced by C6-ceramide. Interestingly, this function is similar to that observed with aCDase, where aCDase inhibition and the addition of C6-ceramide induces a synergistic decrease in breast cancer cells (41). It has also been reported that autophagy is increased in prostate cancer cells overexpressing aCDase, which enhances resistance to C6-ceramide (22). These functions of nCDase are also consistent with prior results on the roles of this enzyme in cells and in vivo. Thus, the overexpression of nCDase in hepatocytes inhibited the TNF- $\alpha$ -induced increase of C16-ceramide and apoptosis and protected against cytokine-induced death, and the effect was also seen in vivo (42). Moreover,

gemcitabine, a chemotherapeutic agent, decreases the nCDase activity in cells and arrests cell growth (43).

These results also carry therapeutic implications. Thus, the high expression of nCDase in some cancer cells might impart resistance to the action of C6-ceramide, which is being developed as a novel chemotherapeutic, especially as a nanoliposomal formulation (44). High levels of nCDase could also provide resistance to the action of death ligands and anticancer drugs. Reciprocally, the inhibition of nCDase may affect the sensitivity for chemotherapy and lead to the induction of apoptosis and cell death by the accumulation of intracellular ceramides. Our previous (20) and ongoing studies also disclose a role for nCDase as a therapeutic target for colorectal cancer. Thus, nCDase may emerge as a new target for cancer and other sphingolipid metabolism-related diseases, and nCDase inhibitors will also develop the sensitivity in combination with anticancer drugs.

In summary, this study supports that nCDase in the Golgi apparatus has a new function involved in the resistance to the action of C6-ceramide, which is becoming a novel human therapeutic. Further studies are needed to understand how nCDase activity is regulated in cells and tissues and how it functions to regulate sphingolipid metabolism. 

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