Neutral sphingomyelinase (SMPD3) deficiency disrupts the Golgi secretory pathway and causes growth inhibition

Wilhelm Stoffel,1,2, Ina Hammels,1,2, Bitta Jenke,1, Erika Binczek,1, Inga Schmidt-Soltau,1, Susanne Brodesser,2, Astrid Schauss,2, Julia Etich,3, Juliane Heilig,3 and Frank Zaucke1,4

Systemic loss of neutral sphingomyelinase (SMPD3) in mice leads to a novel form of systemic, juvenile hypoplasia (dwarfism). SMPD3 deficiency in mainly two growth regulating cell types contributes to the phenotype, in chondrocytes of skeletal growth zones to skeletal malformation and chondrodysplasia, and in hypothalamic neurosecretory neurons to systemic hypothalamus–pituitary–somatotrophic hypoplasia. The unbiased smpd3−/− mouse mutant and derived smpd3+/− primary chondrocytes were instrumental in defining the enigmatic role underlying the systemic and cell autonomous role of SMPD3 in the Golgi compartment. Here we describe the unprecedented role of SMPD3. SMPD3 deficiency disrupts homeostasis of sphingomyelin (SM), ceramide (Cer) and diacylglycerol (DAG) in the Golgi SMPD3-SMS1 (SM-synthase1) cycle. Cer and DAG, two fusogenic intermediates, modify the membrane lipid bilayer for the initiation of vesicle formation and transport. Dysproteostasis, unfolded protein response, endoplasmic reticulum stress and apoptosis perturb the Golgi secretory pathway in the smpd3−/− mouse. Secretion of extracellular matrix proteins is arrested in chondrocytes and causes skeletal malformation and chondrodysplasia. Similarly, retarded secretion of proteo-hormones in hypothalamic neurosecretory neurons leads to hypothalamus induced combined pituitary hormone deficiency. SMPD3 in the regulation of the protein vesicular secretory pathway may become a diagnostic target in the etiology of unknown forms of juvenile growth and developmental inhibition.

Cell Death and Disease (2016) 7, e2488; doi:10.1038/cddis.2016.385; published online 24 November 2016

Phospholipids (PLs), sphingolipids (SLs) and cholesterol form the complex architecture of mammalian membrane lipid bilayers. In addition, PLs and SLs are substrates of membrane-associated phospholipases and phosphodiesterases, the reaction products of which act as lipid second messengers. Acid sphingomyelinase (aSMase, SMPD1) and neutral SMases (nSMases, sphingomyelin (SM) phosphodiesterases, SMPD2-5)11–14 hydrolyze SM to phosphocholine and Cer. Cers and DAG are regarded lipid second messengers in rather divergent pathways of cellular signaling in growth and development, including triggering tumor-suppressive and anti-proliferative cellular processes.5,6 However, this tenet has been challenged7–13 and Cer functions have remained enigmatic.

The null-allelic smpd1−/− (Niemann-Pick, type A)14,15 and smpd2−/−11 mouse-mutants have served as unbiased genetic tools in studies on the systemic role of mammalian SMPD3s in cellular SM metabolism and its pathophysiology. The current study elaborated the pivotal role of SMPD3 utilizing the smpd3−/− mouse, which is characterized by the retardation of systemic and skeletal growth and development. In wild-type mice, the smpd3 mRNA is ubiquitously expressed2,16. The absence of SMPD3 in hypothalamic secretory neurons inhibited the secretion of proteo-hormones, slowed down the hypothalamus–pituitary–growth axis, and triggered systemic growth retardation resulting in a novel juvenile dwarf phenotype.13 The autonomous expression of SMPD3 in chondrocytes was shown by functional reconstitution of SMPD3 in smpd3−/− chondrocytes, expressing smpd3 as transgene, driven by the chondrocyte-specific Col2a1 promoter in the smpd3−/− mutant.16

Here, we describe a novel molecular mechanism underlying the bifurcated systemic and cell autonomous SMPD3 deficiency. We first documented the dominant role of SMPD3 and defined the subcellular localization in the Golgi compartment (GC), imperative for unraveling the molecular role of SMPD3 in mammalian cells. Our finding is ad variance with the proposed plasma membrane (PLM) topology of SMPD3.18,19

We next focused our study on the cell-specific growth regulation of SMPD3 in primary chondrocytes of skeletal growth plates of p16 control and smpd3−/− mice, corresponding to approximately age 4 years in human development.20,21 Chondrocytes are competent secretory cells during the growth phase with an abundant secretion of extracellular matrix proteins (ECMs) for enchondral ossification in longitudinal growth.20 Chondrocytes in culture have proven most powerful in exploring molecular features of growth and development.22

We discovered the pivotal role of SMPD3 in Golgi vesicular protein transport. Inactivation of smpd3 stalled Golgi protein transport.
transport, disrupted proteostasis, induced ER stress and compromised chondrocyte function, leading to apoptosis and ultimately to skeletal malformation and severe chondrodysplasia.

We determined the lipidome of primary chondrocytes of control and smpd3−/− mice in the growth phase as the structural platform for the functional analysis of SMPD3 in the Golgi secretory pathway (GSP). Our studies suggest a concerted action of SMPD3 and SMS1 in the Golgi complex, which maintains SM/phosphatidylcholine and ceramide (Cer) diacylglycerol (DAG) homeostasis during remodeling of the Golgi membrane lipid bilayer for vesicular transport. This homeostasis is perturbed in SMPD3-deficient GC.

Our studies delineate a novel function of SMPD3 in the lipid-driven formation of vesicle carriers in the GSP during growth and development, and provide insight into the molecular pathology of SMPD3 deficiency leading to an unprecedented mechanism of growth inhibition and to retarded development, manifested juvenile dwarfism and osteochondrodysplasia.

**Results**

SMPD3 is the key neutral sphingomyelinase, localized in detergent-insoluble membrane domains of the Golgi complex. We first ascertained the subcellular topology, a prerequisite for exploring the mechanistic role of SMPD3. Biochemical and immunohistochemical analyses conclusively proved the absence of SMPD3 in smpd3−/− chondrocytes (Figures 1a and d). SMPD3 topology was restricted to the Golgi complex in different control cell types: in primary control chondrocytes (Figure 1c), peritoneal macrophages (Figure 1e) and C57Bl/6 EMFs (Figure 1f). Colocalization with SMS1 and Golgi-specific marker K58 ascertained the allocation to the GC (Figures 1g and h). SMPD3 and SMS1 resided in the GC of control chondrocytes and SMPD3 overexpressing HEK293 cells (Figures 1i and j).

To further substantiate the Golgi topology of SMPD3 and of SMS1, we used mouse brain, which shows highest SMPD3 expression of all tissues.2,16 The Golgi fraction of the premyelinating brain of (p14) control and smpd3−/− mice was further separated into detergent (Triton X-100)-insoluble membrane domains (DIMs) and subnatant for WB (Figures 1k-n). More than two-thirds of total SMPD3 resided in DIMS of control GC (Figure 1k), SMPD3 was absent in smpd3−/− GC (Figure 1m). Similarly, two-thirds of total SMS1 was concentrated in DIMs of control GC (Figure 1l), but was equally distributed in SMPD3-deficient DIMs and subnatant (Figure 1n).

To explore whether SMPD3 is required selectively for the canonical Golgi-mediated or non-canonical secretory pathway, we analyzed the secretion of several cytokines into the medium of control and smpd3−/− peritoneal macrophages, unstimulated (Figure 1o) and stimulated (Figure 1p) with lipopolysaccharide (LPS). The unchanged secretion of cytokines in SMPD3-deficient macrophages clearly indicated SMPD3 function to be restricted to the canonical GSP.

A valuable tool in dissecting Golgi localization, morphology and vesicle formation is exposure of chondrocytes to Brefeldin (BFA). We followed the time-resolved disintegration by IHC of the GC of SMPD3 and K58 (Supplementary Figure S2a), and Col2a and cartilage oligomeric matrix protein (COMP), in control and smpd3−/− chondrocytes (Supplementary Figure S2b). Golgi membrane stacks disintegrated, vesiculated and fused with ER membranes within 5–30 min.

We then assessed the contribution of bona fide SMPD4 (ref. 3) to cellular nSMase activity in HEK293 cell clones, stably transfected with full-length smpd4-egfp, with threefold to an eightfold overexpression, documented by qRT-PCR (Supplementary Figure S1a). SMPD4-EGFP colocalized largely with K58 in the GC (Supplementary Figure S1c). Surprisingly, the sensitive radioactive nSmase assay revealed an unchanged basal nSMase activity in the postnuclear fraction of all smpd4-egfp overexpressing cell clones (Supplementary Figure S1b). Our biochemical experiments and IHC were unable to substantiate nSMase activity of SMPD4.

The SMPD3-SMS1 cycle regulates SM homeostasis and the Cer- and PLC-independent DAG pool in the GC. Current techniques preclude time and space resolution between the transfers of de novo synthesized Cer from the ER into the GC, and Cer, released locally by SMPD3 hydrolysis from SM of Golgi membrane domains. To get insight into the metabolic interrelationship of SM/Cer and PC/DAG, we focused on their analysis in control and smpd3−/− primary chondrocytes in culture. We next analyzed the PL-classes of control and smpd3−/− chondrocytes, which were separated by high-performance thin layer chromatography (HPTLC).

Basically, Cer and DAG concentrations in the lipidome are very low. In smpd3−/− chondrocytes, the molar ratios of SM and Cer were reduced to one-half and one-third, respectively (Figures 2i-k).

Mass spectrometry, using selective ion monitoring (SIM), was applied for identification and quantification of HPTLC-separated Cer bands (m/z 264 and m/z 266), respectively. In GC, the pattern of fatty acid substituents of DH-Cer of control and smpd3−/− chondrocytes represents de novo synthesized DH-Cer, which is markedly different from that of Cer. DH-Cer in control and smpd3−/− chondrocytes contained only saturated 16:0–22:0 acyl-groups, Cer species predominantly very long chain 24:0, 24:4 and 26:1-acyl-residues as substituents (Figures 2b and c).

PC, the donor in the SMS1 catalyzed transfer of the phosphoryl-choline head group for reconstitution of SM, in Golgi of control and smpd3−/− chondrocytes lacked polyunsaturated fatty acid substituted DAGs, which are closely similar to species in the pool of free DAGs. Sn1-18:0-sn2-20:4-DAG, released by PLC-specific hydrolysis of PLM-bound PIP2 (Supplementary Figure S3f) was hardly detectable in the DAG pool of the Golgi complex (Figure 2e). The hydrophobic DAG core of PS, P1 and PE in the Golgi membrane bilayer of chondrocytes of p16 control and smpd3−/− mice remained unchanged (Figures 2f-h).

SMPD3 deficiency disrupts the GSP. Primary chondrocytes of control and smpd3−/− mice (p16) in culture show an abundant ECM protein synthesis, macro-vesicular transport and secretion. We followed Golgi vesicular transport and secretion of major ECM proteins, Col2a, the main fibrillar collagen species, perifibrillar COMP, Matrilin (Matn) 3, ColIX and ColVI in primary chondrocytes of p16 control and...
smpd3−/− mice by IHC (Figures 3a-h). Control chondrocytes effectively secreted Col2a, Matn3, ColVI and ColIX and formed a high-density ECM network. Col2a secretion was stalled in smpd3−/− chondrocytes and the intercellular fibrillar network nearly absent. COMP was distributed throughout the intracellular space of control chondrocytes. Arf and β-Cop1, markers of Golgi small vesicular transport carriers, displayed indistinguishable Golgi localization in control and smpd3−/− chondrocytes (Figure 3e).

IHC of the other dominant growth regulating cell type, hypothalamic neurosecretory neurons in arcuate N. and periventricular N., using antibodies against growth hormone-releasing hormone (GHRH) and melanocyte-stimulating hormone (MSH), revealed smpd3−/− neurons heavily loaded with proteo-hormones, but low abundance in control neurons (Figures 3f and g), which confirms previous immunohistochemical results.13

Inhibited protein transport, dysproteostasis, ER stress and apoptosis in SMPD3-deficient chondrocytes. We next studied the cellular response to the stalled protein transport along the GSP. ER stress is measured by activation of unfolded protein response (UPR) and visualized by the accumulation of misfolded proteins in the lumen of the tubular ER system directly by transmission electron microscopy (EM) (for review Oslowski and Urano24,25 and Riggs et al.26).

EM of p16 control chondrocytes revealed normal rough endoplasmic reticulum (rER) tubular network (Figures 4a-c), but dilated and giant ER cisternae in smpd3−/− chondrocytes, the cytoplasm filled with macro-vesicular structures, displacing and disrupting the rER (Figures 4d-f). Enhanced expression of ER stress sensor/transducer activating transcription factor 6 (ATF6), following stress-induced proteolysis indicated activation of UPR in smpd3−/− chondrocytes, but UPR responder protein pIRE remained unchanged in WB (Figures 4g and h). Annexin V staining of smpd3−/− chondrocytes (Figure 4j), FACS analysis of single-cell control and smpd3−/− p16 brain. In all, 100 μg aliquots of total protein were applied to each lane. Signals were evaluated by densitometry and normalized to SMPD3 and SMS1 present in the Golgi fraction, N = 4. The non-canonical secretory pathway was assayed within a multi-cytokine assay quantifying secretion of cytokines into the growth-medium of peritoneal macrophages, (o) unstimulated and (p) stimulated with LPS.
malformation, paradigmatically documented in sections of decalcified femoral epiphysis of smpd3−/− mice (Figure 4u).

**Smpd3−/− gene expression in primary chondrocytes.** Next, we studied steady-state gene expression in chondrocytes of p16 control and smpd3−/− littermates by real-time PCR of (a) key enzymes of SM metabolism (Figure 5a), of ceramide synthases (cerS) and fatty acid elongases (elovl) (Figure 5b), (b) growth and transcription factors regulating chondrocyte differentiation, (c) ECM proteins and (d) sec23, essential for protein transport (Figure 5c). Expression of comp, bone morphogenetic protein 1 (bmp1), a procollagenase, and growth factor bmp4, required for cartilage formation was downregulated (Figure 5c).

**Figure 2** Molar ratios of SM and Cer are reduced in Golgi-lipidome of smpd3−/− chondrocytes. MS/MS profiling of (a-h) species of SM (a), dihydroceramide (DH-Cer) (b), Cer (c), PC (d), DAG (e), phosphatidylinositol (PS) (f), PI (g) and phosphatidylethanolamine (PE) (h) of Golgi fraction of control and smpd3−/− chondrocytes. (i) Molar ratio of main PL classes (PC, PI and PE) and SM, Cer, C and DAG. (j and k) Thin layer chromatographic separation of lipid extract of control and smpd3−/− chondrocytes. Solvent system: cyclohexane/ethylacetate 3:2 v/v (j); two runs in chloroform/ethanol/water/triethylamine 30:35:7:35 v/v/v (k). N=3
Complementary WB analysis of structural proteins Col2a, COMP, Golgi-transport regulatory proteins β-Cop1 and Arf, Igf-1, IgfR-1 and SMS1 (Figures 5d-j). SMS1 expression was increased nearly threefold (Figure 5j), matching the increased SMS1 enzyme activity in the lysate of $smpd3^{-/-}$ chondrocytes (Figure 5k).

Equal amounts of lipid total extracts of enzyme assays were separated by HPTLC and visualized by charring lipid bands (Figure 5l), different from enzyme assay of kidney, which shows equal amounts of de novo synthesized fluorescent SM (Figures 5m and n).

**Discussion**

This study is focused on the mechanism underlying SMPD3 deficiency, which causes systemic and cell-specific growth inhibition, a novel form of juvenile dwarfism. We used the unbiased $smpd3^{-/-}$ mouse model. In this study, primary chondrocytes in culture of control and $smpd3^{-/-}$ mice – as compelling in vitro system – were instrumental in unraveling the molecular mechanism underlying the crucial function of SMPD3 in the GSP. The absence of SMPD3 suppressed ECM protein transport and secretion, disrupted proteostasis, activated UPR and ER stress and finally apoptosis, which is reflected phenotypically in skeletal growth inhibition and joint malformation. Translation of this mechanism into disruption of the GSP in hypothalamic proteo-hormones secreting neurons provides a molecular interpretation of the previously described hypothalamus induced combined pituitary hormone deficiency, underlying the systemic hypoplasia of the $smpd3^{-/-}$ mutant.\(^1\^3\)

---

**Figure 3** SMPD3-deficient primary chondrocytes display inhibited secretion of ECM proteins. Fluorescence images of p16 control and $smpd3^{-/-}$ primary chondrocytes, using following antibodies for triple staining: (a) Col2a (green), COMP (red), and Matn3 (yellow), and double staining: merged images of (b) Col2a (green), COMP (red), (c) Col2a (green), ColIX (red), (d) Col2a (green), ColVI (red), and (e) Arf (green), β-Cop1 (red) double stained primary chondrocytes in culture. Inhibited GSP in hypothalamic secretory neurons of control and $smpd3^{-/-}$ mice: fluorescence images of (f) GHRH secreting neurons in control, storage of GHRH in $smpd3^{-/-}$ neurons using anti-GHRH, (g) MSH in control and storage of MSH in proopiomelanocortin (POMC) expressing $smpd3^{-/-}$ neurons. N = 3
Figure 4  Perturbed proteostasis, UPR, ER stress and apoptosis in primary smpd3−/− chondrocytes. (a-c) EM displays regular rER in control chondrocytes and (d-f) dilated and giant ER cisternae in smpd3−/− chondrocytes, N = 3. (g and h) WB of control and smpd3−/− chondrocytes of ATF6 and pIRE, N = 3. (i and j) Annexin V staining of control and smpd3−/− chondrocytes. Flow cytometry of single-cell primary chondrocytes from control (upper panel) and smpd3−/− (lower panel) mice, using AnxA5-Dye490 and SYTOX Blue Dead Cell Stain. Representative dot plots are shown. (k) Cell debris were excluded according to size (FSC) and granularity (SSC), followed by gating of single cells (data not shown). (l) AnxA5−/Sytox Blue− viable, AnxA5−/Sytox Blue+ apoptotic and AnxA5+/Sytox Blue+ dead cells were detected. (m) Mean percentage of AnxA5−/Sytox Blue− viable, AnxA5−/Sytox Blue− apoptotic and AnxA5−/Sytox Blue+ dead cells ± S.D. is given for control (+/+) and smpd3−/− animals, N = 4. TUNEL assay in sections of radial epiphysis. (n and q) negative control, (o and r) positive control and (p and s) assay of control and smpd3−/− chondrocytes, N = 4. (t and u) Light microscopy of HE-stained paraffin sections (5 μm) of decalcified tibial epiphysis of control and smpd3−/− mice (4mo), N = 4.
Exploring the function of SMPD3 in the non-canonical GSP, we quantified cytokine secretion in the medium of control and smpd3−/− peritoneal macrophages. The unaltered secretion indicated the restriction of SMPD3 function to the canonical GSP (Figures 1o and p).

SMPD3 is the dominant among the four mammalian nSMases (SMPD2-5), contributing >90% of total cellular nSMase activity in all mouse tissues, followed by SMPD2. Gene expression, protein expression and nSMase enzyme activity studies on SMPD4 overexpressing HEK293 cells, reported here, preclude SMPD4 as nSMase (Supplementary Figure S1).

Conclusive data on the subcellular topology are a prerequisite for functional studies, as this issue has been controversially discussed and the PLM proposed as the scaffold of SMPD3. The immune-histochemical and biochemical studies, reported here, further suggested the localization and association of SMPD3 and SMS1 in the Golgi complex.

SMPD3 and SMS1 are concentrated in DIMs of the GC of control chondrocytes. In the absence of SMPD3, SMS1 segregates from DIM domains, which disturbed structure of DIMs and deregulate SM synthesis in the GC (Figure 1n). SMS1 protein concentration and enzyme activity were increased in lysates of smpd3−/− chondrocytes (Figures 5k and l). SM and Cer concentrations were reduced (Figure 2i).

Free DAG species resembled that of the DAG core of PC, the donor substrate in the SMS1 reaction. SMPD3 deficiency caused no SM storage and the phospholipidome in all tissues of smpd3−/− mice remained unimpaired, unlike the fatal lysosomal SM storage in the smpd1−/− mouse. A mimicry of human Niemann–Pick disease (Figures 2a-h).

EM convincingly unveiled the morphological changes, reflecting these processes: the dense coherent rER tubular system in control chondrocytes (Figures 4a-c) is contrasted by inflated cisternae of the rER network, loaded with polymorphic macro-vesicular, fibrillar and granular structures dispersed in the cytoplasm of smpd3−/− chondrocytes (Figures 4d-f). This finally leads to skeletal growth retardation, malformation and chondrodysplasia (Figures 4i-t).

Gene and protein expression of representative ECM proteins Col2a and COMP, Golgi-micro vesicular transport proteins Arf and β-Cop1 and of growth factor Igf1 and IgfR were inconspicuous (Figures 5d-i). Expression of growth factors, bmp1, bmp4 and ECM protein comp, respectively, is downregulated (Figure 5c). Secreted BMP1 acts as Ca2+ and Zn2+-dependent metalloproteinase processing procollagen I for ECM organization. SMPD3 is selectively required for the canonical Golgi-mediated secretory pathway. Evidence for unimpaired secretion of non-canonical secretory proteins was provided by multi-cytokine assay, quantifying of 23 cytokines, secreted into the growth-medium of unstimulated and LPS-stimulated peritoneal macrophages of control and smpd3−/− mice, which revealed closely similar concentrations, presented in Figures 1o and p.

SMPD3 transiently modifies the Golgi membrane for vesicular transport. The Golgi complex is a budding organelle with domain formation by lateral diffusion. Coatomer complex COP1 and II drive budding, fission and fusion in the formation of small vesicular transport carriers. The function of the SMPD3-SMS1 cycle in the formation of large pleomorphic carriers for intracellular transport and secretion of ECM proteins in the GSP of chondrocytes, reported here, is a novel facet in studies addressing this enigmatic process.

The central role of SMPD3 and its concerted activity with SMS1 in the SMPD3-SMS1 cycle, which maintains homeostasis of SM metabolism in the GC, is delineated in Figure 6. The current methodology precludes space- and time-resolved analysis of Golgi membrane domains undergoing remodeling during Golgi vesicular transport. Backed by biochemical, cellular and morphological finding, we conclude that the SMPD3-SMS1 cycle generates a PLC-independent DAG pool in the GC. It is of note that the species of this DAG pool largely reflect those of the major PC species, donor substrates in the...
SMS1 catalyzed transfer of the phosphoryl-choline head group to Cer (Figures 2d and e), unlike the cellular PI pool of control and smpd3−/− chondrocytes, which contained 18:0/20:4-DAG core as major species (Supplementary Figure S3),30 the well-known activator of protein kinases (PKC)31 in the reversible recruitment of DAG-responsive proteins with C1 subdomains to PLM.30–32 Potential intricate regulatory functions of Cer and DAG in the Golgi complex await further investigations.

SM/C enriched DIMS of GC membranes are the scaffold of SMPD3 and SMS1 proteins (Figures 1i-k). Sophisticated atomic force microscopy and fluorescence correlation spectroscopy have been applied to phase-separated lipid bilayer model systems, consisting of ternary SM/PC/C domains, embedded in a liquid disordered PC phase, to investigate the effect of Cer on liquid-ordered membrane domains.33–34 Treatment with bacterial nSMase released Cer from SM of the SM/C complex and simultaneously displaced cholesterol.
SMPD3 regulates Golgi secretory pathway
W Stoffel et al

Figure 6  Proposed mechanism of the SMPD3-SMS1 cycle in the Golgi complex.  Proposed mechanism of maintenance of homeostasis and regulation of SM, PC, Cer, and DAG pools in the SMPD3-SMS1-cycle of the Golgi complex.

stoichiometrically at the rim of ordered nanoscale domain structures. It is tempting to correlate results obtained from these model systems with those of this study on the role of SMPD3 in liquid-ordered DIMS of the Golgi membrane complex.

Complementary and in support of our results are the observations that protein trafficking to the cell surface is retarded in response to downregulation of SMS1, 36 and the observations that protein trafficking to the cell surface is complex.

domain structures. It is tempting to correlate results obtained stoichiometrically at the rim of ordered nanoscale

Proposed mechanism of the SMPD3-SMS1 cycle in the Golgi complex.

Figure 6  Proposed mechanism of maintenance of homeostasis and regulation of SM, PC, Cer, and DAG pools in the SMPD3-SMS1-cycle of the Golgi complex.

Materials and Methods

Mouse experiments. All experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Cologne, Cologne, Germany.

Generation and genetic characterization of smpd3−/− mice (C57Bl/6 x 129 background) have been described. 13 They were backcrossed ten times into the C57BL/6 background.

Egfp-N2 fusion constructs. Full-length smpd3 cDNA 2 inserted into the pcDNA 3.1 myc/his vector, was amplified with primers NSM2 5′-Xhol-sense (5′-CTCGAGAGTTTTGTACGACACCCCTTTTCT-3′) and NSM2 3′KpnI as (5′-GGTACCAACGCTCTCCTCTCCTGACACACA-3′) and ligated into the Xhol-KpnI restricted pEGFP-N2 vector.

Full-length smpd4 RNA was amplified by RT-PCR using primers smpd4sXhol 5′-CCCTGAGGTCTGCTATGGGCTCTCCCATCAC-3′ and smpd4 as BamHI 5′-ATGGCCACAGCCTCGATCGTGGTGCAGCTTCT-3′. Smpd4 cDNA was ligated into the PCRII.1 Vector (Invitrogen, Karlsruhe, Germany), isolated as BamHI-Xhol fragment and ligated into the pEGFP-N2 vector.

Transfection. C57Bl/6 embryonic fibroblasts (EMFs) and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum, and transfected by electroporation with expression vectors smpd2-EGFP-N2 and smpd4-EGFP-N2.

Real-time PCR. RNA was isolated for RT-PCR from p16 control and smpd3−/− chondrocytes using Trizol (Invitrogen), reverse-transcribed using a Transcriptase kit (Life Technologies Inc., Darmstadt, Germany). Quantitative PCR reactions were performed with the ABI Prism 7900HT using a 96-well format, the Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA), following the manufacturer’s protocol. Data analysis was performed using the 2-ΔΔCT method.

Acid and neutral sphingomyelinase assays. Total aSMase- and Mg2+-dependent nSMase activity were determined as described 38

SM synthase assay. SMS1 activity in cell lysates was determined following an established procedure. 43

Cell fractionation. Cell fractionation and isolation of Golgi fractions and Triton X-100 insoluble DIMs were performed by established procedures 30–41

Cytokine analysis. Cytokines, secreted by peritoneal macrophages via the non-Golgi-secretory pathway, were quantitated in the medium using the Bio-PlexPro mouse cytokine 23-plex assay (Bio-Rad, Bioplex #M600009RDPD, Hercules, CA, USA) following the manufacturer’s protocol. Macrophages were isolated from adult (4m) male control and smpd3−/− mice 72 h after intraperitoneal injection of 1.5 ml 4% thioglycolate, washed with PBS. In all, 1.5 x 10^6 macrophages unsaturated and stimulated with 100 μg LPS/ml medium for 48 h were used. The supernatant was centrifuged for the cytokine assay.

Cell culture. EMFs (3–6 passage) derived from e14 C57Bl/6, C57Bl/6 x 129 smpd3−/− (ref.33) and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma, Taufkirchen, Germany), supplemented with 10% fetal calf serum (Life Technologies Inc.), 2 mmol glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C in a 5% CO2 atmosphere. Primary chondrocytes from p16 control and smpd3−/− of femur epiphyseal cartilage and rib cages were cultured following established procedures. 44

Lipidomic analysis. Isolation, separation, identification and quantification of phospho classes and SL classes were analyzed by MSMS using an Applied Biosystems QTrap analyzer (Darmstadt, Germany). 41 In brief, total lipids were isolated following the method of Bligh and Dyer. 45 Phospho and SLs were separated by HPTLC in solvent system chloroform/ethanol/tertbutylamine/water 60/70/70/14 (v/v/v/v). Bands were identified by staining with 10% CuSO4·5 H2O, 8% H3PO4 at 180 °C for 5 min and quantified using the CAMAG Scanner and software (CAMAG, Muttenz, Switzerland).

Lipid bands were visualized with primuline for analysis by MS/MS using an Applied Biosystems QTrap analyzer. 41

Protein analysis by western blotting. Protein aliquots were analyzed by WB, using the following antibodies: anti-SMS1, anti-IgFR and anti-Ifg (Santa Cruz Biotechnology, Heidelberg, Germany), anti-MSH (Dianova, Hamburg, Germany), anti-CoLa (Millipore, Darmstadt, Germany), anti-COMP, anti-CoLi, anti-CoLX (kindly provided by M Paulsson, Center for Biochemistry, University of Cologne, Cologne, Germany), anti-Arf and anti-Anexinin V-Cy3 Apotosis Detection Kit (Alexis Biochemicals, San Diego, CA, USA), anti-GHRH (Inserm, Paris, France), anti-pIre and anti-ASTG (Abcam, Cambridge, UK), anti-Caveolin (BD Transduction Laboratories, Heidelberg, Germany), anti-K58 and anti-p-j-Cop (Sigma, Taufkirchen, Germany), affinity purified anti-SMPD2 and SMPD3 (Ser310 − Ala655) polyclonal antibodies, 1 mouse anti-matrilin 3 (Matn3) antibodies (kindly provided by R. Wagener, 44 Center for Biochemistry, Cologne, Germany). Quantification was carried out by densitometry using the IMAGE J2X program (Rarow Software, Informer Tech Inc).
Immunofluorescence microscopy. Primary chondrocytes of p10 control and homozygous female smpd3−/− mice were grown to semi-confluency on cover slips or on Aclar membranes, respectively, fixed for processing for light and immunofluorescence microscopy with 4% paraformaldehyde in PBS and permeabilized with PBS/0.5% Triton X-100, 4 °C. After blocking with 3% BSA/PBS, cells were treated with respective antibodies in TBS supplemented with 5% non-fat dry milk at 4 °C over-night. After washing with PBS/0.5% Triton X-100, cells were incubated with Cy3-conjugated second IgG antibody (Jackson Immuno Research, Baltimore, PA, USA) for 1 h at 37 °C, washed with PBS/0.5% Triton X-100, and analyzed by epifluorescence using a Zeiss Axiosplan Imager M1 (Oberkochen, Germany) or Leica confocal microscope (Wetzlar, Germany).

FACS analysis of single-cell chondrocytes. For cell survival experiments, cultured primary chondrocytes were analyzed by flow cytometry as previously described. Brieﬂy, chondrocytes were isolated by collagenase type 2 treatment (Worthington Biochemical Corporation, Lakewood, NJ, USA) and subsequently stained with ﬂuorescently labeled annexin A5 (AnxA5-Dye493) and Sytox Blue Dead Cell Stain (Thermo Fisher Scientiﬁc, Schwerte, Germany) in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) followed by ﬂow cytometry analysis (FACSCantoll, Becton Dickinson, Heidelberg, Germany). Using the FlowJo 7.6 software (LLC Ashland, OR, USA), cell debris were excluded and by comparing the area and width of the FSC and SSC signals only single cells were considered for further analysis. Viable (AnxA5+/Sytox Blue−), apoptotic (AnxA5+/Sytox Blue+) and dead cells (AnxA5+/Sytox Blue+) were quantiﬁed and data represented as mean ± S.D. of n = 4 individual mice with three technical replicates each. Statistical analysis was performed with Student’s t-Test using the unpaired two tails method.

Electron microscopy. Primary chondrocyte were grown on Aclar-membranes (Fa. TED Pella Inc., Redding, CA, USA) for 6 days, washed 3x with PBS, ﬁxed for 1 h at 4 °C with 2% glutaraldehyde, 2% PFA, 0.2% picric acid in 0.1 M cacodylate buffer, pH 7.35 post-ﬁxed in 1% OsO4 solution for 1 h, and stained in 1% uranyl acetate for 1 h at 4 °C with 2% glutaraldehyde, 2% PFA, 0.2% picric acid in 0.1 M cacodylate buffer, pH 7.35. Fixation buffer was removed and cells washed 3x with 0.1 M cacodylate buffer, pH 7.35 post-ﬁxed in 1% OsO4 solution for 1 h, and stained with 1% uranyl acetate for 1 h at room temperature. After dehydration, specimens were embedded in Araldite (Serva, Heidelberg, Germany). Ultra-thin sections (about 70 nm) were stained with uranyl acetate and lead citrate and were examined by EM (Zeiss 902A, Zeiss, Oberkochen, Germany). The semi-thin sections (1 μm) were stained with methylene blue for light microscopy.

Statistical analyses. Results are expressed as mean ± S.D. Statistical signiﬁcance of differences between two groups was determined by a two-tailed Student’s t-Test using GraphPad QuickCalcs (La Jolla, CA, USA); Hest calculator. A P-value of < 0.05, * < 0.01, ** < 0.001 was considered signiﬁcant. Sizes of animal cohorts are listed under respective ﬁgures.

Conflict of Interest
The authors declare no conﬂict of interest.

Acknowledgements. We thank M. Paulsson and B. Brachvogel, Center of Biochemistry, University of Cologne, for valuable discussion. We gratefully acknowledge the support of this work by the Center of Molecular Medicine, University of Cologne, CECAD (Cluster of Excellence, Cellular Stress Response in Aging-Related Diseases), University of Cologne and Deutsche Forschungsgemeinschaft (St32/38-2). JE. KOLN FORTUNE (136/2013, 120/2014) and FZ SFB829, project B11.
Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)