

Shedding of glycan-modifying enzymes by signal peptide peptidase-like 3 (SPPL3) regulates cellular N-glycosylation

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Abstract

Protein N-glycosylation is involved in a variety of physiological and pathophysiological processes such as autoimmunity, tumour progression and metastasis. Signal peptide peptidase-like 3 (SPPL3) is an intramembrane-cleaving aspartyl protease of the GxGD type. Its physiological function, however, has remained enigmatic, since presently no physiological substrates have been identified. We demonstrate that SPPL3 alters the pattern of cellular N-glycosylation by triggering the proteolytic release of active site-containing ectodomains of glycosidases and glycosyltransferases such as N-acetylglucosaminyltransferase V, β -1,3 N-acetylglucosaminyltransferase 1 and β -1,4 galactosyltransferase 1. Cleavage of these enzymes leads to a reduction in their cellular activity. In line with that, reduced expression of SPPL3 results in a hyperglycosylation phenotype, whereas elevated SPPL3 expression causes hypoglycosylation. Thus, SPPL3 plays a central role in an evolutionary highly conserved post-translational process in eukaryotes.

Keywords glycosyltransferases; GxGD aspartyl proteases; protein glycosylation; signal peptide peptidase like-3

Subject Categories Post-translational Modification, Proteolysis & Proteomics

Introduction

Signal peptide peptidase-like 3 (SPPL3) is a multi-pass transmembrane protein that is highly conserved among multicellular eukaryotes and belongs to the family of intramembrane-cleaving GxGD proteases (Voss *et al*, 2013). Together with its mammalian paralogues, signal peptide peptidase (SPP) and the signal peptide peptidase-like (SPPL) proteases SPPL2a, SPPL2b and SPPL2c, it was first identified by database queries in 2002 (Grigorenko *et al*, 2002; Ponting *et al*, 2002; Weihofen *et al*, 2002). Notably, all members of the SPP/SPPL family share characteristic structural features and catalytic motifs with the presenilins, the catalytically active subunits of the γ -secretase complex (Voss *et al*, 2013). The two catalytic aspartate residues required for the proteolytic activity of SPP/SPPL proteases are embedded in conserved YD and GxGD amino acid motifs in transmembrane domain (TMD) 6 and TMD7, respectively (Voss *et al*, 2013). Mutagenesis of either aspartate residue inactivates the respective SPP/SPPL (Weihofen *et al*, 2002; Flührer *et al*, 2006; Friedmann *et al*, 2006; Kirkin *et al*, 2007; Voss *et al*, 2012). In contrast to presenilins, which exclusively accept type I transmembrane substrates (Kopan & Ilagan, 2004), SPP/SPPLs seem to be selective towards transmembrane substrates in type II orientation (Weihofen *et al*, 2002; Friedmann *et al*, 2004; Nyborg *et al*, 2004). While recent studies on SPP, SPPL2a and SPPL2b identified substrates of these proteases *in vitro* and *in vivo* and consequently gave a first impression of the physiological function of these family members (reviewed in Voss *et al*, 2013), the physiological function of SPPL3 has remained completely enigmatic. Highly conserved orthologues of mammalian SPPL3, however, are found in most multicellular eukaryotes pointing to a fundamental cellular function of SPPL3 (Voss *et al*, 2013). SPPL3 most likely adopts the nine TMD topology conserved

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among GxGD proteases (Friedmann *et al*, 2004), localises to the Golgi network (Friedmann *et al*, 2006) and is not glycosylated (Friedmann *et al*, 2004). Recently, we described the first SPPL3 substrate in a cell culture model system, confirming the assumption that SPPL3 indeed is proteolytically active (Voss *et al*, 2012).

In eukaryotic organisms, N-glycan synthesis is initiated in the ER, resulting in high-mannose-type glycans attached to the luminal domain of secretory and membrane proteins (Kornfeld & Kornfeld, 1985). Within the *medial*- and *trans*-Golgi network, numerous glycosyltransferase and glycosidases compete for these high-mannose-type precursor glycans converting them into higher-order, complex N-glycans (Sears & Wong, 1998; Moremen *et al*, 2012). Most glycosyltransferases are single-span type II transmembrane proteins with a short N-terminal cytoplasmic tail. Their large C-terminal ectodomain that harbours the glycosyltransferase activity faces the lumen of the Golgi (Paulson & Colley, 1989; Sears & Wong, 1998; Varki *et al*, 2009).

Secreted glycosyltransferases were observed in conditioned media of cultured cells (Elhammer & Kornfeld, 1986; Saito *et al*, 2002; El-Battari *et al*, 2003), but also in bodily fluids (Kim *et al*, 1972a,b; Elhammer & Kornfeld, 1986; Kitazume *et al*, 2009) and tissues (Weinstein *et al*, 1987). Secretion of the catalytically active glycosyltransferase domain has been discussed to negatively regulate cellular protein glycosylation, since the crucially required nucleotide- or lipid-linked sugar donor substrates that exclusively occur intracellularly are not available for secreted glycosyltransferases (Paulson & Colley, 1989; Ohtsubo & Marth, 2006; Varki *et al*, 2009). Secretion is generally believed to be due to proteolytic cleavage within the so-called stem region that tethers the catalytic glycosyltransferase ectodomain to its membrane anchor (Paulson & Colley, 1989; Ohtsubo & Marth, 2006; Varki *et al*, 2009). However, the exact nature of the protease(s) involved in this process remains controversial. The sialyltransferase ST6Gal1, for instance, was initially described to be released by a cathepsin D-like protease (Lammers & Jamieson, 1988; McCaffrey & Jamieson, 1993). Others identified the β -site amyloid precursor protein-cleaving enzyme 1 (BACE1) as protease responsible for ST6Gal1 cleavage in cultured cells (Kitazume *et al*, 2001) and, to a certain extent, *in vivo* (Kitazume *et al*, 2005). Secretion of the N-acetylglucosaminyltransferase V (GnT-V) was suggested to be mediated by the γ -secretase complex (Nakahara *et al*, 2006).

Using *Sppl3* knockout mice as well as human and murine cell lines, we identify SPPL3 as a new fundamental mediator of glycosyltransferase secretion. SPPL3 controls the proteolytic release of the ectodomain of various glycosyltransferases and glycosidases, most likely by direct cleavage, and consequently alters cellular N-glycosylation. Therefore, we define the protease responsible for proteolytic cleavage of glycan-modifying enzymes and provide the first insight into the physiological function of SPPL3.

Results

SPPL3 expression affects the glycosylation pattern of endogenous cellular glycoproteins

Analysis of cell lysates obtained from T-RexTM-293 (HEK293) cells stably expressing SPPL3 under a doxycycline-inducible promoter

revealed a markedly reduced molecular weight of mature Nicastrin, a complexly N-glycosylated γ -secretase component (Haass, 2004) (Fig 1A). In addition, the molecular weight of other endogenous glycoproteins, for instance N-cadherin, SPPL2a and Lamp2, was similarly affected in cells expressing proteolytically active SPPL3 compared to non-induced cells (Fig 1A). Notably, expression of the catalytically inactive SPPL3 D200N/D271N mutant (SPPL3 DD/NN) had no effect on the molecular weight of these glycoproteins (Fig 1A), indicating that proteolytic activity of SPPL3 is crucially required for the observed molecular weight changes. Doxycycline (Dox) treatment of non-transfected HEK293 cells had no effect on the molecular weight of the respective glycoproteins analysed (Supplementary Fig S1). In contrast to SPPL3 overexpression, siRNA-mediated SPPL3 knock-down induced a slight increase in the molecular weight of various glycoproteins compared to cells transfected with non-targeting control siRNAs or untransfected cells (Fig 1B). Importantly, solely the molecular weight of the mature, complexly glycosylated variant of Nicastrin was changed by alterations in SPPL3 expression levels, while the immature variant remained unaffected (Fig 1A and B). Moreover, non-glycosylated proteins such as the ER chaperone calnexin were not affected by alterations in SPPL3 expression levels (Fig 1A and B). We consequently hypothesised that SPPL3 interferes with cellular N-glycosylation, resulting in a reduced extent of N-glycan modifications, that is hypoglycosylation, upon SPPL3 overexpression and a more extensive N-glycan modification, that is hyperglycosylation, upon reduction of SPPL3 expression. To support this hypothesis, lysates from HEK293 cells expressing SPPL3 were subjected to enzymatic deglycosylation by peptide:N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) (Fig 1C). As expected, the hypoglycosylation phenotype induced by SPPL3 overexpression was completely abolished by treatment with PNGase F, but was still detectable after treatment with Endo H (Fig 1C). Moreover, glycoproteins in SPPL3-overexpressing cells remained Endo H resistant, indicating that SPPL3 selectively interferes with complex N-glycosylation. To verify this, we treated cells with the plant alkaloid kifunensine (Fig 1D), which is a potent inhibitor of cellular α -mannosidase I activity and, accordingly, blocks generation of both hybrid- and complex-type N-glycans (Elbein *et al*, 1990). As expected, kifunensine treatment completely eliminated generation of mature glycoproteins and SPPL3 failed to decrease the molecular weight of glycoproteins in kifunensine-treated cells (Fig 1D), demonstrating that SPPL3 interferes with complex N-glycosylation occurring in the *medial*- and *trans*-Golgi network following modification by α -mannosidase I but not with early steps of ER-based N-glycan assembly.

Sppl3-deficient mice exhibit a hyperglycosylation phenotype

To address whether a similar function of SPPL3 is apparent under physiological conditions, we analysed glycoproteins in tissue homogenates of *Sppl3*-deficient mice and littermate controls. On a mixed C57BL/6;129S5 background *Sppl3*-deficient mice are viable and present with a rather mild phenotype that is characterised by growth retardation and haematologic abnormalities as well as sterility in male homozygous mice (Tang *et al*, 2010). Similar to our observations upon SPPL3 knock-down (Fig 1B), the molecular weight of mature Nicastrin and SPPL2a slightly increased in brain (Fig 2A), lung (Fig 2B), spleen (Fig 2C) and immortalised mouse embryonic

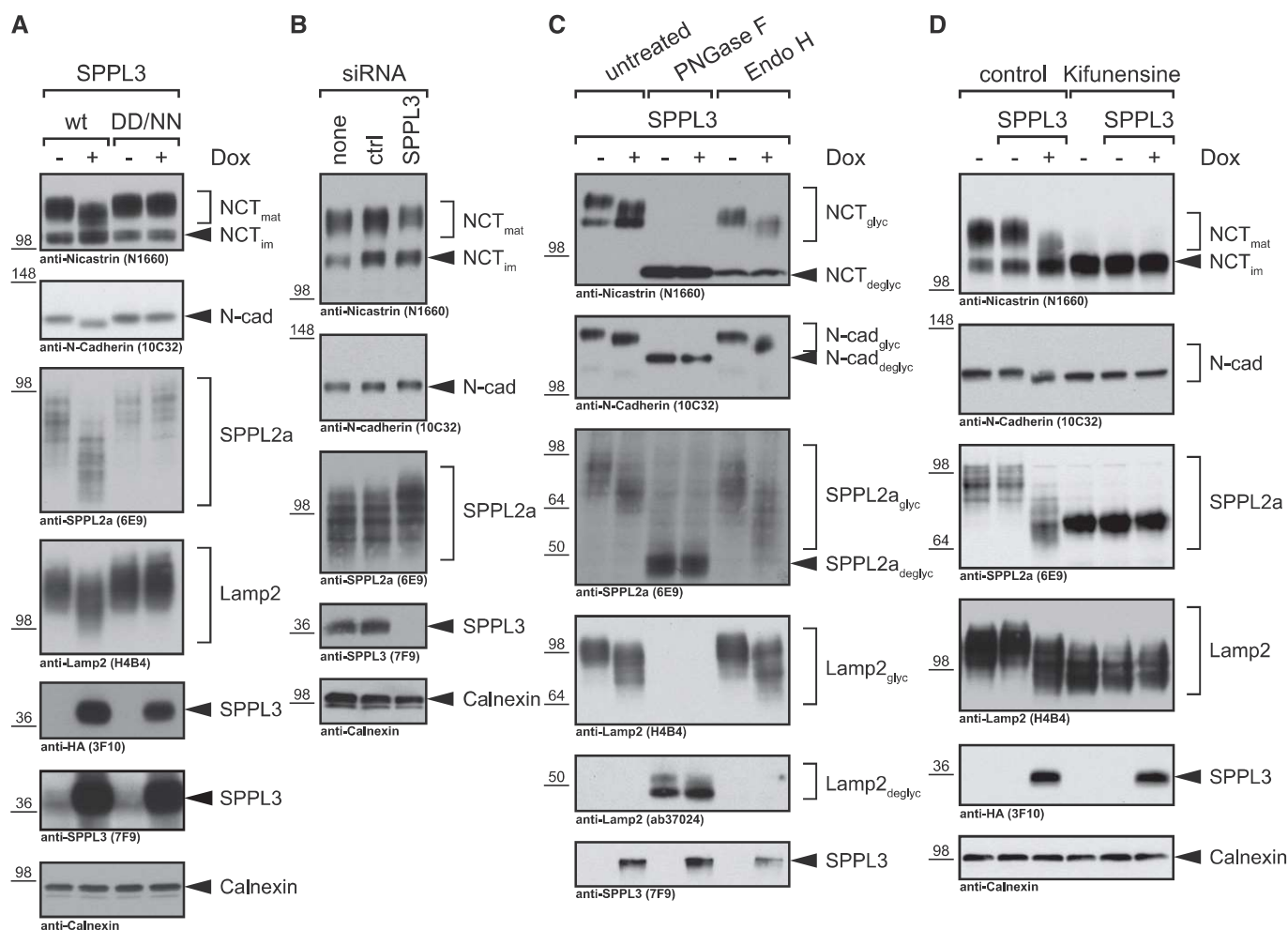


Figure 1. SPPL3 expression affects cellular N-glycosylation.

- A SPPL3 overexpression results in a reduced molecular weight of glycoproteins. Electrophoretic mobility of the endogenous glycoproteins Nicastrin (NCT), N-cadherin (N-cad), SPPL2a and Lamp2 was studied in whole-cell lysates of HEK293 cells by Western blotting following separation by SDS-PAGE. Expression of either HA-tagged catalytically active SPPL3 (WT) or an inactive SPPL3 mutant (DD/NN) was induced by doxycycline treatment (+ Dox) for 72 h. Cells not treated with doxycycline (– Dox) were used as controls. In cells expressing catalytically active SPPL3, the glycoproteins analysed displayed a reduced molecular weight. Note that only mature (NCT_{mat}) but not immature (NCT_{im}) Nicastrin is affected. Treatment of parental HEK293 cells with doxycycline had no effect on glycoprotein electrophoretic mobility (Supplementary Fig S1).
- B SPPL3 knock-down results in a slightly higher molecular weight of endogenous glycoproteins. Lysates of non-transfected (none) HEK293 cells as well as of HEK293 cells transfected with non-targeting (ctrl) or SPPL3-targeting (SPPL3) siRNA pools were analysed for electrophoretic mobility of endogenous glycoproteins as described in (A). Reduction of SPPL3 protein levels was accompanied by a slight increase in glycoprotein molecular weight.
- C Alterations in glycoprotein molecular weight induced by SPPL3 overexpression are due to altered N-glycosylation. Whole-cell lysates from SPPL3-expressing HEK293 cells (+ Dox) and non-induced control cells (– Dox) were deglycosylated using peptide:N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) prior to analysis by SDS-PAGE and immunoblotting. Non-treated cell lysates served as a control (untreated). Arrows and/or brackets indicate the respective glycosylated (glyc) and deglycosylated (deglyc) protein species. SPPL3 overexpression had no impact on the molecular weight of deglycosylated proteins. Note that anti-Lamp2 (H4B4) immunoreactivity was lost following complete N-glycosylation. N-deglycosylated Lamp2 was detected using another anti-Lamp2 antibody (ab37024).
- D Kifunensine treatment abolishes the effect of SPPL3 overexpression on cellular N-glycosylation. HEK293 cells overexpressing SPPL3 (+ Dox) were treated with solvent (control) or kifunensine for 16 h to block complex N-glycosylation. Non-transfected as well as non-induced (– Dox) cells served as a control. Electrophoretic mobility of the indicated endogenous glycoproteins was monitored as described in (A). Upon kifunensine treatment, endogenous glycoproteins were less extensively glycosylated and migrated faster. SPPL3 failed to further reduce the glycoprotein molecular weight.

Data information: In all panels, calnexin was used as a loading control. Antibodies used to visualise the respective proteins in Western blot are indicated. Source data are available online for this figure.

fibroblasts (MEFs) (Fig 2D) obtained from *Sppl3*^{-/-} mice compared to *Sppl3*^{+/+} littermate controls. In addition to these rather ubiquitously expressed glycoproteins, glycoproteins expressed in a tissue-specific manner, for instance N-cadherin in brain (Fig 2A) or

CD68 in spleen (Fig 2C), similarly displayed a hyperglycosylation phenotype in *Sppl3*^{-/-} mice, suggesting that SPPL3 has a rather global effect on cellular N-glycosylation. Similar to hypoglycosylation in SPPL3-overexpressing cells, hyperglycosylation in brain

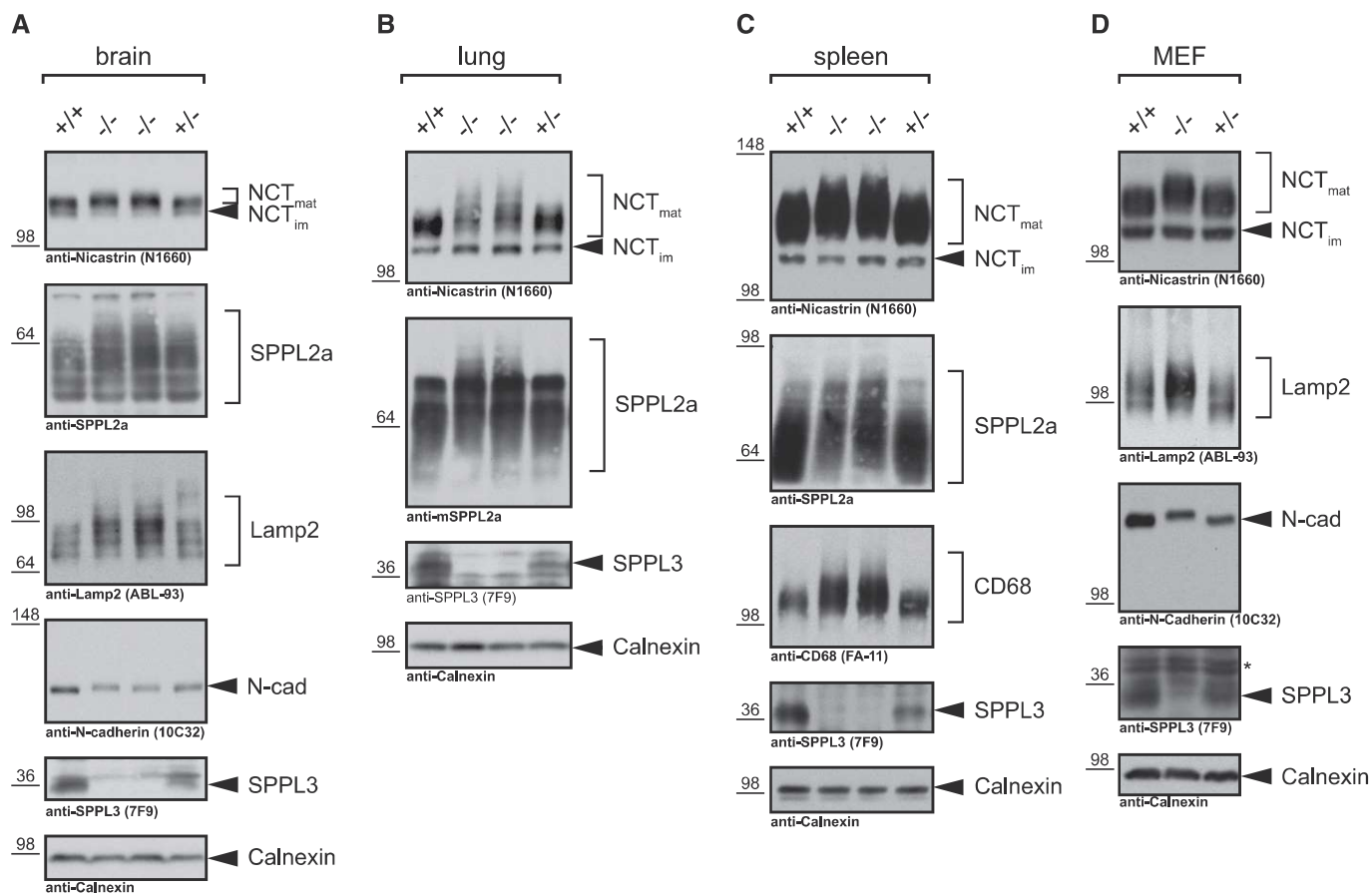


Figure 2. *Sppl3*-deficient mice exhibit a hyperglycosylation phenotype.

A–C Electrophoretic mobility of selected endogenous glycoproteins in brain (A), lung (B) and spleen (C) homogenates obtained from *Sppl3*-deficient ($-/-$) mice was analysed by SDS-PAGE and subsequent immunoblotting. Age- and sex-matched WT (+/+) and heterozygous (+/-) littermates served as controls. As assessed by Western blotting (second panel from the bottom), SPPL3 protein expression was absent in homogenates of all tissues from *Sppl3* $^{-/-}$ animals and was reduced in the heterozygous mouse. Note that in all tissues analysed, glycoproteins such as mature Nicastrin (NCT_{mat}), Lamp2 and SPPL2a but also tissue-specific glycoproteins such as N-cadherin (N-cad) in brain and CD68 in spleen exhibited a higher molecular weight in *Sppl3*-deficient animals compared to control animals. Note that the molecular weight of immature Nicastrin (NCT_{im}) was not altered in *Sppl3* $^{-/-}$ tissues.

D Analysis of glycoprotein molecular weight in immortalised MEFs derived from a *Sppl3*-deficient embryo ($-/-$). MEFs derived from WT (+/+) and heterozygous (+/-) littermates served as controls. Total cell lysates were analysed as described in Fig 1A. Similar to tissue homogenates, glycoproteins displayed a higher molecular weight in cultured *Sppl3*-deficient cells compared to control cells. *, unspecific band.

Data information: In all panels, calnexin was used as a loading control. Antibodies used to visualise the respective proteins in Western blot are indicated. Source data are available online for this figure.

homogenates of *Sppl3* $^{-/-}$ mice and in *Sppl3* $^{-/-}$ MEFs was absent after treatment with PNGase F (Supplementary Fig S2A and B). Taken together, these data demonstrate that SPPL3 expression influences complex cellular N-glycosylation not only in a cellular model system but also *in vivo*.

SPPL3 mediates secretion of GnT-V

In the light of the results obtained upon kifunensine treatment (Fig 1D), we reasoned that SPPL3 affects N-glycosylation either by interfering with α -mannosidase I activity or another glycan-modifying enzyme downstream of α -mannosidase I. In addition, the molecular weight changes observed upon SPPL3 expression in HEK293 cells (Fig 1A) were rather substantial. Thus, it is likely that SPPL3 does not exclusively affect terminal glycan modifications, as a

deregulation of these would not lead to such pronounced differences in the molecular weight of glycoproteins. We rather assumed that SPPL3 interferes with N-acetylglucosaminyl (GlcNAc) branching and/or the generation of N-acetylglucosamine moieties (Fig 3A).

Accordingly, we investigated whether SPPL3 directly or indirectly affects cellular levels of glycosyltransferases implicated in these processes. We found that SPPL3 profoundly affects GnT-V (gene name: *MGAT5*), which is involved in GlcNAc branching (Fig 3A). GnT-V levels in both supernatant and lysate were strongly reduced following transfection with a *MGAT5*-specific siRNA pool (Fig 3B), demonstrating that the GnT-V signal obtained in Western blots was specific and that endogenous GnT-V is secreted in HEK293 cells. Upon siRNA-mediated knock-down of SPPL3, secretion of endogenous GnT-V was markedly reduced and the protein strongly accumulated intracellularly (Fig 3B). Moreover, cellular GnT-V, which

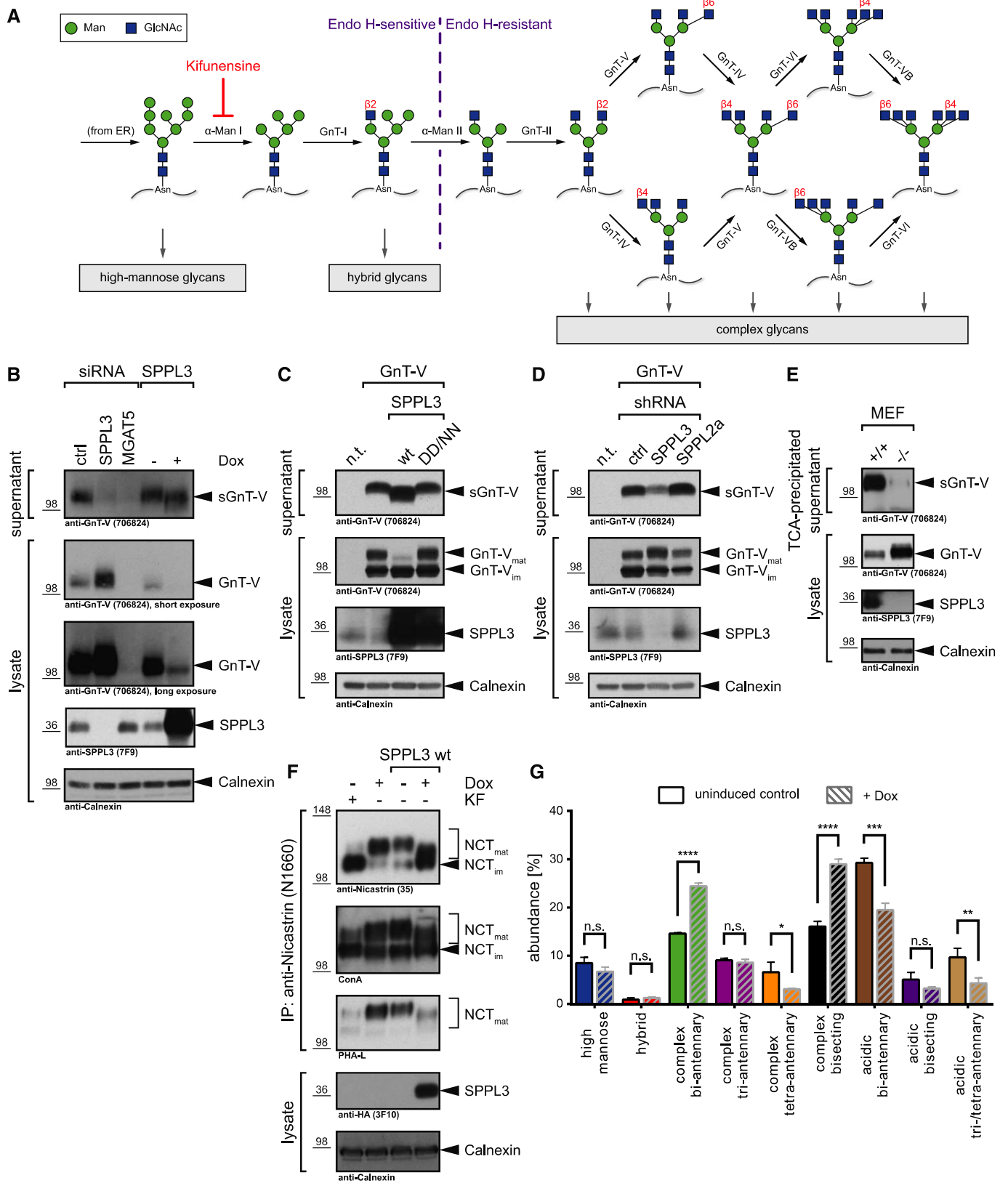


Figure 3.

itself is known to be complexly glycosylated (Kamar *et al.*, 2004), was found to shift to a slightly higher molecular weight, indicating that SPPL3 knock-down also induces GnT-V hyperglycosylation (Fig 3B). In contrast, doxycycline-induced SPPL3 overexpression, yet not doxycycline treatment *per se*, caused GnT-V hypoglycosylation accompanied by a strong reduction of cellular GnT-V levels (Fig 3B and Supplementary Fig S3). Similar results were obtained from HEK293 cells co-expressing exogenous GnT-V and either SPPL3 WT (Fig 3C) or a *SPPL3*-specific shRNA (Fig 3D). In HEK293 cells overexpressing GnT-V, we observed two distinct GnT-V species, immature, EndoH-sensitive GnT-V and complex glycosylated, mature GnT-V (Fig 3C and D, and Supplementary Fig S4). Exogenous co-expression of active SPPL3 and GnT-V resulted in slightly increased GnT-V secretion and reduction of intracellular mature GnT-V, while immature GnT-V remained unaffected (Fig 3C). Moreover, SPPL3 overexpression induced hyperglycosylation of secreted GnT-V but did not affect the molecular weight of immature GnT-V (Fig 3C). Catalytically inactive SPPL3 did not affect levels of secreted and intracellular GnT-V (Fig 3C). In contrast, shRNA-mediated knock-down of SPPL3 slightly reduced secretion of exogenous GnT-V and induced hyperglycosylation exclusively of the mature intracellular GnT-V species (Fig 3D). This received additional support from pulse-chase experiments performed in cells expressing epitope-tagged GnT-V that were either co-expressing catalytically active SPPL3 (Supplementary Fig S5A) or were transfected with *SPPL3*-specific siRNA (Supplementary Fig S5B). Taken together, this suggests that SPPL3 acts on GnT-V most likely in the Golgi, where both GnT-V (Chen *et al.*, 1995) and SPPL3 (Friedmann *et al.*, 2006) may co-localise. Next, we examined cellular GnT-V levels and GnT-V secretion in *Spp13*^{-/-} MEFs (Fig 3E). As expected, GnT-V was more abundant in lysates from *Spp13*^{-/-} MEFs than in wild-type control

cells and its secretion was almost completely abolished in *Spp13*-deficient cells (Fig 3E).

To finally investigate whether SPPL3 also modulates cellular GnT-V activity, we performed lectin blots (Fig 3F). Both mature and immature Nicastrin immunoprecipitated from HEK293 cells expressing active SPPL3 were detected by concanavalin A, which has a broad reactivity towards mannose-containing glycans (Cummings & Etzler, 2009). Strikingly, reactivity towards PHA-L, a leucoagglutinin that predominantly binds N-glycans that harbour the GlcNAc β 1,6Man motif generated by GnT-V activity (Cummings & Kornfeld, 1982), was strongly reduced in cells overexpressing SPPL3 (Fig 3F, lane 4) compared to cells with endogenous SPPL3 levels (Fig 3F, lane 2). To control for specificity of lectin binding, cells were treated with kifunensine, which resulted in impaired complex glycosylation of mature Nicastrin. As expected, immature Nicastrin was only detected by ConA but not by PHA-L. In contrast, mature Nicastrin from non-treated cells was readily detected by both lectins (Fig 3F). This indicates that, upon SPPL3 expression, glycans on mature Nicastrin are less extensively modified by GnT-V. To investigate whether this generally applies, we performed a glycome-wide analysis of N-glycans by liquid chromatography–mass spectrometry (LCMS). To this end, we isolated secreted glycoproteins from cells overexpressing SPPL3 and from control cells. N-glycans decorating these glycoproteins were released enzymatically using PNGaseF, purified and subsequently labelled with anthranilic acid (2-AA) via reductive amination. 2-AA-conjugated N-glycans of SPPL3-overexpressing cells and control cells were analysed individually by nano-LCMS and identified using their respective retention time as well as their MS and MS/MS profiles. The relative abundance of individual glycans was determined by integration of the MS signal (Fig 3G, Supplementary Fig S6 and Supplementary Table S1). Differences between cells ectopically expressing active SPPL3

Figure 3. SPPL3 affects secretion of the N-acetylglucosaminyltransferase GnT-V.

- A Schematic overview of Golgi asparagine (Asn)-linked N-glycan processing and GlcNAc branching resulting in the generation of high-mannose, hybrid and complex N-glycans (grey boxes). The latter types of glycans are subsequently subjected to terminal modification (not depicted). Kifunensine interferes with N-glycosylation by blocking α -mannosidase I (α -Man I) activity. After modification by α -mannosidase II (α -Man II) glycoproteins gain Endo H resistance. Mannose (Man) residues are depicted as green circles. N-acetylglucosaminyltransferases (GnTs) attach N-acetylglucosamine (GlcNAc) residues (blue squares) to the core mannose residues in specific stereochemical configurations (red). Modified after (Sears & Wong, 1998) and (Stanley *et al.*, 2009).
- B SPPL3 affects secretion of endogenous GnT-V. HEK293 cells were transfected with non-targeting (ctrl), *SPPL3*-specific or *MGAT5*-specific siRNA pools (20 nM each). SPPL3 overexpression was induced by doxycycline (+ Dox). GnT-V levels were examined by SDS-PAGE and Western blotting in conditioned supernatants (sGnT-V) and whole-cell lysates (GnT-V). Cellular SPPL3 levels were similarly analysed.
- C, D SPPL3 affects secretion of overexpressed GnT-V. GnT-V was transiently transfected (C) into HEK293 cells stably overexpressing catalytically active SPPL3 (WT) or the inactive SPPL3 D200N/D271N mutant (DD/NN) or (D) into cells that stably expressed non-targeting (ctrl), *SPPL2a*- or *SPPL3*-specific shRNAs. GnT-V levels were analysed in conditioned supernatants (sGnT-V) and lysates (GnT-V) as described in (B). Note that under overexpression conditions, two GnT-V species were detected, mature GnT-V (GnT-V_{mat}) and immature GnT-V (GnT-V_{imm}) (see Supplementary Fig S4). n.t., not transfected.
- E GnT-V secretion is impaired in *Spp13*-deficient MEF cells. GnT-V levels were monitored in whole-cell lysates and in TCA-precipitated conditioned supernatants of *Spp13*-deficient (-/-) MEFs and WT (+/+) controls as described in (B).
- F SPPL3 overexpression reduces lectin binding. Nicastrin (NCT) was immunoprecipitated from HEK293 cells overexpressing catalytically active SPPL3 following doxycycline induction (+ Dox) and from non-induced control cells (- Dox). Immunoprecipitates were visualised either with an anti-Nicastrin mAb or the biotinylated lectins, concanavalin A (ConA) and PHA-L, respectively. Note that PHA-L only detected mature Nicastrin (NCT_{mat}) and that PHA-L reactivity was reduced in cells overexpressing SPPL3. Kifunensine-treated (+ KF) cells were used as a control and lacked PHA-L-reactive N-glycans.
- G SPPL3 overexpression reduces GnT-V-mediated glycan branching. N-glycans on glycoproteins secreted from cells overexpressing SPPL3 following doxycycline induction (+ Dox, grey hatched bars) or from non-induced control cells (black-bordered bars) were analysed by LCMS. N-glycans were assigned on the basis of their LC retention time and their (tandem) mass spectrometry signatures (see Supplementary Table S1 for a list) and their relative abundance was determined by integration of mass spectrometry signals. Individual glycan species assigned to the individual N-glycan classes are listed in Supplementary Table S1 and these species and the respective bar fillings are colour-coded throughout this study. Error bars represent SD ($n = 3$ for non-induced controls, $n = 4$ for doxycycline-induced SPPL3 overexpression). P -values were determined using Student's unpaired t -test (two-tailed). n.s., not significant ($P > 0.05$), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Data information: In panels (B–F), calnexin was used as a loading control. Antibodies and lectins used to visualise the respective proteins in Western blots are indicated. Source data are available online for this figure.

and control cells became already apparent in LC chromatograms (Supplementary Fig S6A). Subsequent analysis of N-glycan species by mass spectrometry revealed that, as expected from previous experiments (Fig 1D), SPPL3 overexpression was not associated with significant changes in high-mannose or hybrid N-glycans (Fig 3G and Supplementary Table S1). It did, however, significantly affect the relative abundance of a number of complex N-glycan species. Cells overexpressing SPPL3 were found to secrete glycoproteins more extensively modified with complex bi-antennary as well as bisected complex bi-antennary N-glycans. These N-glycan species are expected to accumulate when GnT-V activity is impaired (Fig 3A) (Lee *et al.*, 2012). Moreover, under these conditions, tetra-antennary N-glycans as well as acidic tri- and tetra-antennary N-glycans were less abundant on secreted glycoproteins. Since GnT-V activity contributes to the generation of tri-antennary and tetra-antennary N-glycans (Fig 3A), this further demonstrates that reduction in intracellular GnT-V levels observed following SPPL3 overexpression (Fig 3A and B) is also associated with impaired cellular GnT-V activity. At the same time, however, additional changes observed in these experiments suggested that SPPL3 does not only affect GnT-V activity but may also affect the cellular activity of other glycan-modifying enzymes.

GnT-V secretion results from proteolytic cleavage

In order to confirm that GnT-V secretion is due to proteolytic cleavage (Fig 4A), we co-expressed N-terminally Flag- and C-terminally V5-tagged GnT-V and SPPL3 WT (Fig 4B). The secreted variant of the epitope-tagged GnT-V retained its anti-V5 immunoreactivity, yet was not anti-Flag reactive, demonstrating that it had been N-terminally truncated (Fig 4B). In addition, we determined the cleavage site within GnT-V using a GnT-V expression construct that lacks the bulk of the GnT-V ectodomain and allows for purification and mass spectrometric analysis of secreted, GnT-V-derived peptides (Supplementary Fig S7). In conditioned supernatants from cells expressing this construct, we detected five individual peptides with N-termini between residues L29 and T33 at the luminal

membrane interface. As demonstrated before (Nakahara *et al.*, 2006), one of the most abundant peptides originates from proteolytic cleavage between L30 and H31. Since it was previously suggested that γ -secretase is responsible for the release of GnT-V in a pancreas carcinoma cell line (Nakahara *et al.*, 2006), we treated HEK293 cells with the potent γ -secretase inhibitors (GSIs) L-685,458 (Shearman *et al.*, 2000) or *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) (Dovey *et al.*, 2001). Both GSIs efficiently interfered with γ -secretase activity, as demonstrated by the drastically impaired secretion of endogenous A β , a well-established γ -secretase cleavage product (Haass, 2004), and the intracellular accumulation of APP C-terminal fragments, the corresponding γ -secretase substrates (Haass, 2004), in a concentration-dependent fashion (Fig 4C). We failed, however, to detect an inhibitor concentration-dependent reduction of GnT-V secretion and a concomitant intracellular GnT-V accumulation (Fig 4C). Moreover, endogenous glycoproteins were not hyperglycosylated in PS1/PS2 double-deficient MEFs that lack the catalytically active γ -secretase subunits (Fig 4D) as observed in *Sppl3*^{-/-} cells (Figs 2D and 4D). Instead, we observed a mild hypoglycosylation on endogenous glycoproteins in these MEFs compared to control cells that could be due to a slightly higher SPPL3 expression in the PS1/PS2 double-deficient MEFs (Fig 4D). Finally, we did not observe a reduction of GnT-V secretion in PS1/PS2 double-deficient MEFs (Fig 4E), while GnT-V secretion was clearly impaired in *Sppl3*^{-/-} cells (Fig 3E). In sum, this clearly demonstrates that alterations in cellular N-glycosylation observed before (Figs 1–4) are not linked to γ -secretase activity. Instead, our data suggest that the secretion of GnT-V is due to proteolytic cleavage of GnT-V by SPPL3.

SPPL3 mediates secretion of other glycan-modifying enzymes

Assuming that SPPL3 overexpression in HEK293 cells results in hypoglycosylation solely due to the loss of intracellular GnT-V activity, we expected that SPPL3 overexpression would phenocopy a siRNA-mediated knock-down of GnT-V. As expected, both

Figure 4. GnT-V secretion in HEK293 cells occurs via a SPPL3-dependent proteolytic mechanism.

- Similar to most Golgi glycosyltransferases, GnT-V is a type II membrane protein. It comprises a short cytosolic N-terminus, a TMD, a luminal stem region and the luminal catalytic ectodomain. Its secretion is likely mediated by proteolytic cleavage (scissors) at the TMD/stem region boundary.
- GnT-V secretion occurs via a proteolytic mechanism. HEK293 cells were stably transfected with N-terminally Flag-tagged and C-terminally V5-tagged GnT-V (Flag-GnT-V-V5). In lysates, mature (GnT-V_{mat}) and immature (GnT-V_{im}) species of Flag-GnT-V-V5 were detected by Western blotting. Intracellular GnT-V_{mat} was reduced and soluble GnT-V (sGnT-V) enriched in conditioned supernatants of cells co-expressing SPPL3 (+ Dox). Note that sGnT-V was detected using the ectodomain-specific mAb and an anti-V5 antibody but was not reactive with antibodies directed against the N-terminal Flag tag, suggesting that sGnT-V lacks an intact Flag tag. n.t., not transfected; *, unspecific band.
- GnT-V secretion is not affected by GSI treatment. HEK293 cells were treated for 16 h with the GSIs DAPT and L-685,458 at the concentrations indicated. Vehicle-treated cells (DMSO) served as a control. GnT-V levels were monitored by Western blotting in conditioned supernatants (sGnT-V) and in cell lysates (GnT-V). To control for γ -secretase inhibition, β -amyloid precursor protein (APP) processing by γ -secretase was monitored. To this end, total secreted amyloid- β peptide (A β) was immunoprecipitated (IP) from conditioned supernatants. Immunoprecipitates were analysed by Western blotting. Levels of full-length APP and APP C-terminal fragments (APP CTF) were likewise monitored in cell lysates. GSI treatment led to a concentration-dependent reduction in A β secretion and a concomitant intracellular accumulation of APP CTF, indicating that APP processing by γ -secretase was blocked. Note, however, that GnT-V secretion was altered following GSI treatment. The increase in secreted A β at low GSI concentrations has been observed before (Shen & Kelleher, 2007).
- Loss of γ -secretase activity does not induce hyperglycosylation. Glycoproteins were analysed in PS1/PS2-deficient (-/-) and control (+/+) MEFs and compared to *Sppl3*-deficient (-/-) MEFs as described in Fig 1A. Note that PS1/PS2^{-/-} MEFs display a mild hypoglycosylation phenotype which could be explained by the slightly higher SPPL3 expression compared to the respective control.
- GnT-V levels were analysed in lysates (GnT-V) and TCA-precipitated conditioned supernatants (sGnT-V) of PS1/PS2^{-/-} MEFs and of control cells as described in Fig 3E. Note that GnT-V secretion was not abrogated by loss of γ -secretase activity, whereas loss of SPPL3 was associated with a marked reduction in secretion of GnT-V.

Data information: Antibodies used to visualise the respective proteins in Western blots are indicated.

SPPL3 overexpression and GnT-V knock-down resulted in hypoglycosylation of endogenous glycoproteins, but the extent of glycoprotein molecular weight changes were more drastic upon

SPPL3 overexpression (Fig 5A). This observation suggested that GnT-V is not the only glycan-modifying enzyme affected by SPPL3. Indeed, we found that β -1,3 N-acetylglucosaminyltransferase 1

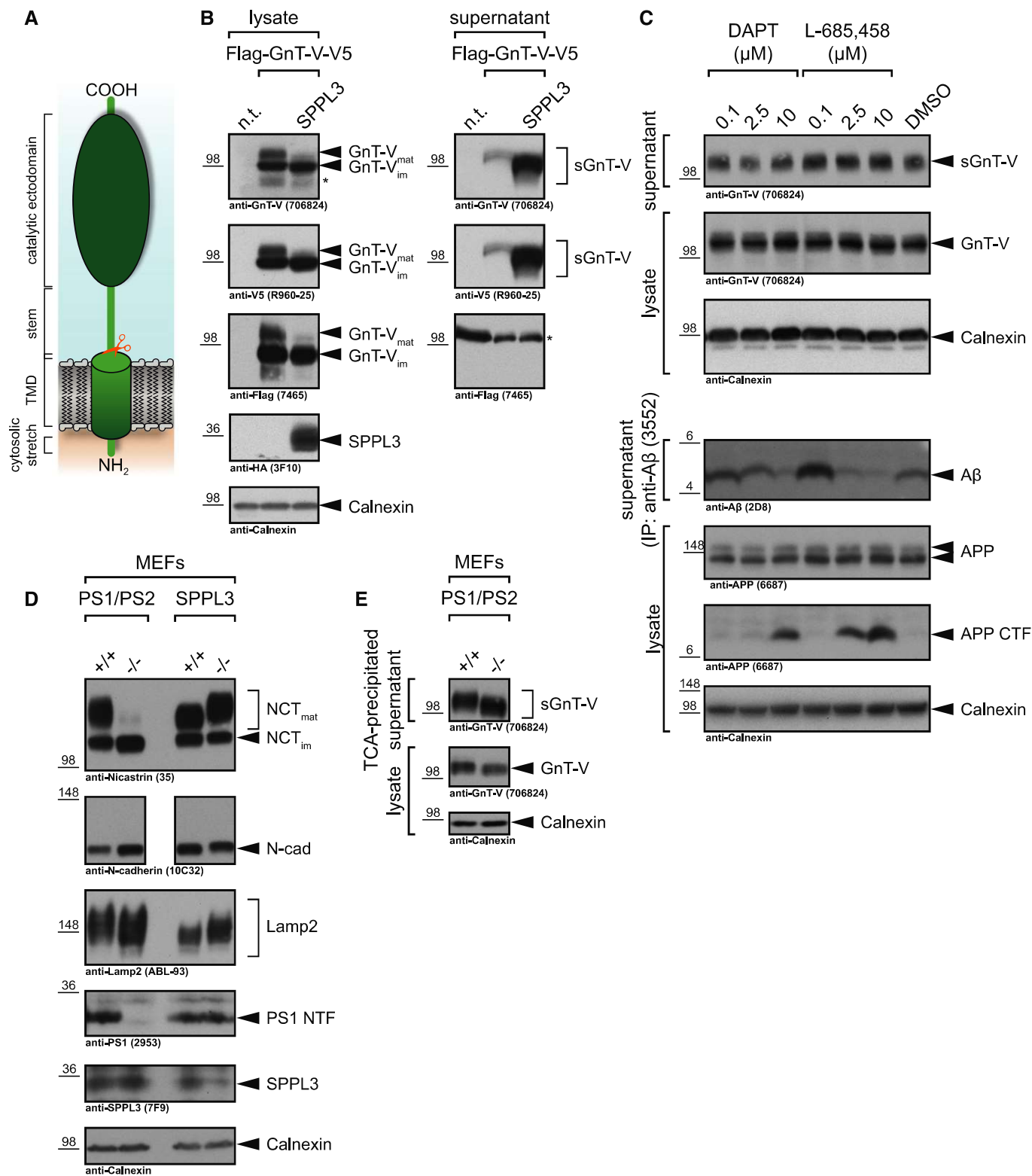


Figure 4.

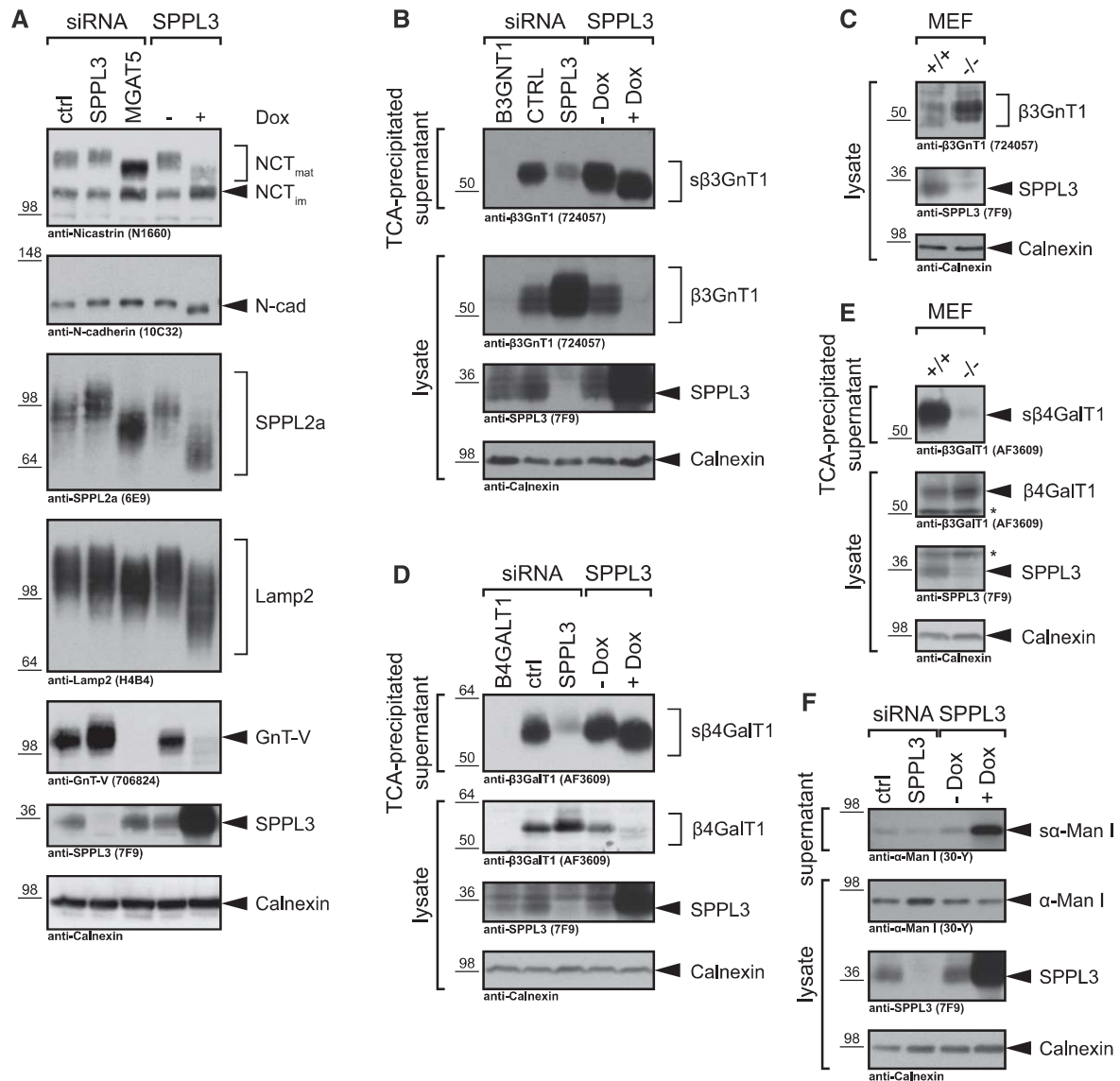


Figure 5. SPPL3 similarly affects other glycosyltransferases and glycosidases.

- A** Loss of GnT-V expression does not phenocopy SPPL3 overexpression. HEK293 cells transiently transfected with non-targeting (ctrl), SPPL3-specific or MGAT5-specific siRNA pools (20 nM each), and HEK293 cells overexpressing SPPL3 (+ Dox) were analysed for electrophoretic mobility of endogenous mature Nicastrin (NCT_{mat}), N-cadherin (N-cad), SPPL2a and Lamp2 as described in Fig 1A. Note that hypoglycosylation induced by SPPL3 overexpression is more pronounced compared to transfection with MGAT5-specific siRNAs, suggesting that GnT-V is not the only SPPL3 substrate contributing to SPPL3 effects on N-glycosylation.
- B** SPPL3 affects secretion of β -1,3 N-acetylglucosaminyltransferase 1 (β 3GnT1). HEK293 cells were transfected with B3GNT1-specific, non-targeting (ctrl) or SPPL3-specific siRNA pools as in (A). β 3GnT1 levels were analysed by immunoblotting in conditioned supernatants (s β 3GnT1) and cell lysates (β 3GnT1). β 3GnT1 strongly accumulated intracellularly following siRNA-mediated SPPL3 knock-down in HEK293 cells, and s β 3GnT1 secretion was significantly decreased (see also quantification in Supplementary Fig S8A). Overexpression of SPPL3 in doxycycline-induced cells (+ Dox) resulted in a reduction of β 3GnT1 in cellular lysates. Antibody staining was specific as β 3GnT1 as well as s β 3GnT1 levels were reduced in cells transfected with a B3GNT1-specific siRNA pool.
- C** β 3GnT1 accumulates in *Spp13*-deficient MEFs. β 3GnT1 levels were monitored by Western blotting in whole lysates (β 3GnT1) obtained from *Spp13*-deficient ($-/-$) or WT control ($+/+$) MEFs. In *Spp13*^{-/-} MEFs, β 3GnT1 strongly accumulated intracellularly.
- D** SPPL3 affects secretion of β -1,4 galactosyltransferase 1 (β 4GalT1). The experiment was conducted as in (B), yet a siRNA pool targeting B4GALT1 was used to control for antibody specificity. Intracellular β 4GalT1 levels and levels of secreted β 4GalT1 (s β 4GalT1) were monitored by Western blotting using a specific antibody. Note that SPPL3 knock-down reduced β 4GalT1 secretion while intracellular β 4GalT1 accumulated intracellularly. SPPL3 overexpression reduced intracellular β 4GalT1 levels.
- E** β 4GalT1 secretion is impaired in *Spp13*-deficient MEFs. β 4GalT1 and s β 4GalT1 levels were monitored in lysates and TCA-precipitated conditioned supernatants obtained from *Spp13*-deficient ($-/-$) or WT control ($+/+$) MEFs by Western blotting. *, unspecific band.
- F** SPPL3 affects secretion of ER α -mannosidase I (α -Man I). In a setting similar to (B), intracellular (α -Man I) and secreted (s α -Man I) levels of α -Man I were analysed in lysates and conditioned supernatants, respectively. Note that SPPL3 knock-down led to a mild intracellular accumulation of α -Man I and soluble α -Man I strongly accumulated in the supernatant of cells overexpressing SPPL3 (see also quantification in Supplementary Fig S8B and C).

Data information: Antibodies used to visualise the respective proteins in Western blots are indicated.
Source data are available online for this figure.

(β 3GnT1, gene name: *B3GNT1*) and β -1,4 galactosyltransferase 1 (β 4GalT1, gene name: *B4GALT1*) were affected by SPPL3 in a manner resembling its effect on GnT-V. SPPL3 overexpression caused hypoglycosylation of secreted β 3GnT1 (Fig 5B and Supplementary Fig S8A) and β 4GalT1 (Fig 5D) accompanied by a reduction of cellular β 3GnT1 (Fig 5B and Supplementary Fig S8A) or β 4GalT1 (Fig 5D) levels, respectively. Upon SPPL3 knock-down (Fig 5B and D, and Supplementary Fig S8A), as well as in lysates from *Sppl3*^{-/-} MEFs (Fig 5C and E), intracellular β 3GnT1 and β 4GalT1 accumulated and secretion of β 4GalT1 was strongly reduced (Fig 5B, D and E, and Supplementary Fig S8A). In addition, SPPL3 overexpression resulted in an statistically significant increase in secretion of α -Man I (gene name: *MAN1B1*) (Fig 5F and Supplementary Fig S8B).

Taken together, our results demonstrate that SPPL3 alters cellular N-glycosylation, most likely by directly cleaving several glycosyltransferases and glycosidases to release their active site-containing ectodomain and thus reducing their respective intracellular activity.

Discussion

Proteolytic secretion of a number of distinct glycosyltransferases is a well-established concept (Paulson & Colley, 1989; Ohtsubo & Marth, 2006; Varki *et al*, 2009) and has been previously observed in cell culture as well as *in vivo* (Kim *et al*, 1972a,b; Elhammer & Kornfeld, 1986; Weinstein *et al*, 1987; Saito *et al*, 2002; El-Battari *et al*, 2003). However, the protease(s) involved in this process have not been unambiguously identified, and the physiological implications of proteolytic glycosyltransferase secretion are unclear.

In the present study, we identify Golgi-resident type II-oriented glycan-modifying enzymes as a novel substrate class for SPPL3. Recently, we reported that SPPL3, unlike γ -secretase (Struhl & Adachi, 2000), SPP (Lemberg & Martoglio, 2002), and SPPL2b (Martin *et al*, 2009), can cleave a viral full-length membrane protein substrate without previous shortening of its ectodomain (i.e. shedding) and thus is, in contrast to all other members of the GxGD protease family, capable of acting like a sheddase. Since most glycosyltransferases and glycosidases are type II transmembrane proteins, which are the preferred substrates for proteolytic cleavage by SPP/SPPL proteases (reviewed in Voss *et al*, 2013), it is very likely that SPPL3 may directly mediate, shedding of glycosyltransferases and glycosidases. This notion, however, requires further experimental validation, ideally in an *in vitro* setting that is devoid of any other cellular factors than SPPL3 and the respective substrate.

Another unresolved question is to what extent proteolytic cleavage affects glycosyltransferase activity *in vivo*. Isolated soluble glycosyltransferases retain their catalytic activity *in vitro* when reactions are supplemented with nucleotide-conjugated monosaccharides (Elhammer & Kornfeld, 1986), but *in vivo* nucleotide-conjugated monosaccharides are only present intracellularly. Accordingly, secretion of the active site-containing glycosyltransferase domain is discussed as a potential mechanism to regulate the extent of cellular glycan modifications (Paulson & Colley, 1989; Ohtsubo & Marth, 2006; Varki *et al*, 2009). Here, we demonstrate that SPPL3 alters N-glycosylation in a cell culture model but also

in living animals by mediating the proteolytic release of glycosyltransferases and glycosidases. While, under physiological conditions, glycosyltransferase secretion is in equilibrium with membrane-bound glycosyltransferase, ensuring a particular pattern of glycan modifications (Fig 6A), overexpression of SPPL3 reduces the amount of catalytically active glycosyltransferases within the Golgi compartment causing hypoglycosylation of cellular glycoproteins (Fig 6B). In case of α -Man I and overexpressed GnT-V this reduction of active enzyme in cell lysates and the concomitant effect of hypoglycosylation is accompanied by a strong increase of enzyme secretion. Secretion of endogenous GnT-V, β 4GalT1 and β 3GnT1, however, was only marginally increased under these conditions, indicating that a certain amount of glycosyltransferases released from Golgi compartments may rather be subjected to intracellular degradation than being secreted. In contrast, reduced levels of endogenous SPPL3 strongly diminish glycosyltransferase secretion and catalytically active glycosyltransferases accumulate within the Golgi compartment resulting in more extensive modification of cellular N-glycans, that is hyperglycosylation (Fig 6C). In line with this, using LCMS, we were able to show that SPPL3 overexpression was accompanied by reduction in tri-antennary and tetra-antennary N-glycans. Consequently, SPPL3 functions as a mediator of cellular N-glycosylation, providing the first insight into one possible physiological function of this GxGD intramembrane protease.

Using a truncated GnT-V model substrate, we mapped the cleavage site of SPPL3 within a peptide stretch predicted to localise to the membrane border of GnT-V. One of the major cleavage sites (L30-H31) had previously been identified by N-terminal sequence analysis of purified secreted GnT-V, but was attributed to a cleavage by the γ -secretase complex (Nakahara *et al*, 2006). γ -secretase is generally considered to selectively cleave type I transmembrane protein substrates with a rather short ectodomain (Hemming *et al*, 2008; Steiner *et al*, 2008; Haapasalo & Kovacs, 2011). Nonetheless, we tested whether inhibition of γ -secretase affects secretion of endogenous GnT-V. Two potent GSIs, which are not cross-reactive with SPPL3 (Voss *et al*, 2012), however, did not impact on GnT-V secretion. Moreover, while we observed neither impaired GnT-V secretion nor alterations in N-glycosylation in γ -secretase-deficient MEFs, both phenotypic abnormalities were apparent in *Sppl3*^{-/-} MEF cells. Since secretion of GnT-V was not completely abolished in *Sppl3*^{-/-} MEFs, it is, however, possible that other proteases also marginally contribute to the secretion of GnT-V.

SPPL3 is found in multicellular organisms, including plants, arthropods and mammals (Voss *et al*, 2013). In addition, its degree of conservation is extraordinarily high (e.g. human and murine SPPL3 proteins are identical), suggesting it was evolved to fulfil a fundamentally important physiological function in multicellular organisms. N-glycosylation is such a fundamental physiological process. Complex N-glycans are critically involved in various physiological processes including, for instance, the regulation of cell-to-cell interactions and cell signalling—processes that are essentially required for multicellularity (Ohtsubo & Marth, 2006). While defects in Golgi N-glycosylation are well tolerated and often phenotypically unapparent in cultured cells, they have often been associated with overt phenotypic abnormalities in murine genetic models but also in inherited human diseases (Lowe & Marth, 2003). Based on our findings, we assume that SPPL3 orchestrates cellular N-glycosylation in multicellular organisms, but we can presently not explain the phenotype observed in *Sppl3*-deficient mice

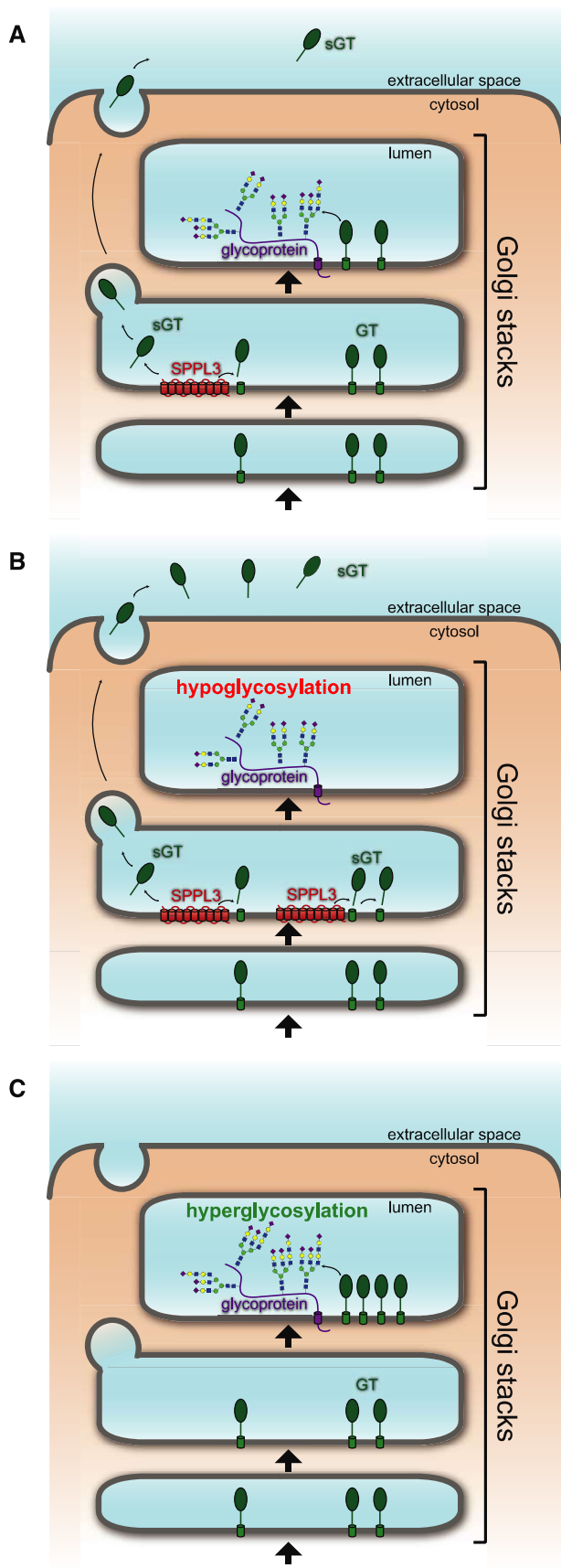


Figure 6. Model depicting the effect of SPPL3 on glycosyltransferase secretion and cellular N-glycosylation.

- A** Membrane-tethered glycosyltransferases (GT, green) mature in the Golgi stacks (bold arrows) and are destined to catalyse protein glycosylation in particular subcompartments (depicted here: generation of a tri-antennary N-glycan, e.g. by GnT-V activity), leading to a specific glycosylation profile on a particular glycoprotein (purple). Under physiological conditions, the endogenous SPPL3 pool (red) facilitates proteolytic liberation of a certain fraction of the Golgi glycosyltransferases from their membrane anchor, resulting in their subsequent secretion (soluble glycosyltransferases, sGT).
- B** More abundant SPPL3 protein in the Golgi, for example upon overexpression, results in a substantially larger fraction of glycosyltransferase turned over proteolytically, leading to enhanced glycosyltransferase secretion and loss of intracellular glycosyltransferase activity. Consequently and as proteolytic cleavage occurs prematurely, glycans are not modified by the given glycosyltransferase (depicted here: no tri-antennary N-glycans) resulting in a hypoglycosylation phenotype.
- C** In cells lacking SPPL3, glycosyltransferase secretion is impaired as glycosyltransferases are not endoproteolysed. Hence, active glycosyltransferases accumulate in their destined compartment, resulting in more extensive glycan modification (depicted here: only triantennary N-glycans) and a hyperglycosylation phenotype.

and cannot exclude that SPPL3 fulfils additional biological functions that could likewise lead to the observed phenotypic abnormalities. On a mixed C57BL/6;129S5 background, *Sppl3*-deficient animals feature a rather mild phenotype (Tang *et al*, 2010), though they are not obtained at expected Mendelian ratios pointing to problems during embryonic or early post-natal development.

Moreover, glycosylation plays a fundamental role in the pathophysiology of human diseases (Ohtsubo & Marth, 2006; Dennis *et al*, 2009), for instance in cancer progression and metastasis (Dennis *et al*, 1999). GlcNAc branching and cellular hexosamine metabolism in particular have emerged as important cellular processes recently, and deregulation has been linked to human developmental defects or other disorders (Lau & Dennis, 2008; Dennis *et al*, 2009; Grigorian *et al*, 2009). GnT-V expression, for instance, is up-regulated in tumours and, consequently, PHA-L reactivity is higher in such tumours (reviewed in Dennis *et al*, 1999). Moreover, high GnT-V expression levels correlate with the metastatic potential and poor prognosis in colorectal carcinoma (Murata *et al*, 2000). On the other hand, loss of β 1,6GlcNAc branching results in hyper-reactive T cells and autoimmune disorders in mice (Demetriou *et al*, 2001). Importantly, all these observations are thought to be intricately linked to the generation of poly-N-acetyllactosamine binding motifs that allow recruitment of a given glycoprotein into the glycoprotein-galectin lattice (Dennis *et al*, 2009). Therefore, it is particularly interesting that we identified GnT-V, β 3GnT1 and β 4GalT1, which are all implicated in the generation of poly-N-acetyllactosamine-carrying N-glycans, as SPPL3 substrates. GnT-V generates N-glycans preferably modified by poly-N-acetyllactosamines (van den Eijnden *et al*, 1988; Ujita *et al*, 1999) and β 3GnT1 (Sasaki *et al*, 1997), together with β 4GalT1 (Ujita *et al*, 2000), elongates these sugar chains.

In summary, we identify a family of biological substrates of SPPL3, providing a first insight into the physiological function of this GxGD intramembrane protease. SPPL3 emerges as mediator of cellular N-glycosylation in mammals, as it appears to control the cellular availability of glycosyltransferases and glycosidases for

N-glycan elaboration by facilitating their proteolytic liberation. Therefore, it is tempting to speculate that other Golgi glycosyltransferases may similarly be subject to endoproteolysis mediated by SPPL3 and that SPPL3 may interfere with disease processes in which cellular glycosylation is dysregulated.

Materials and Methods

Antibodies, lectins and other reagents

Monoclonal antibodies directed against human SPPL3 (clone 7F9) and SPPL2a (clone 6E9) as well as a polyclonal antibody directed against murine SPPL2a were described previously (Behnke *et al.*, 2011; Voss *et al.*, 2012; Schneppenheim *et al.*, 2013). The monoclonal (2D8) and polyclonal (3552 & 6687) anti-A β antibodies have been described elsewhere (Steiner *et al.*, 2000; Yamasaki *et al.*, 2006; Shirovani *et al.*, 2007) as well as the polyclonal anti-PS1 antibody (rabbit pAb, 2953) (Walter *et al.*, 1997). Other antibodies used in this study were obtained from commercial sources as follows: anti-Nicastrin (rabbit pAb, N1660, Sigma-Aldrich, St. Louis, USA), anti-Nicastrin (mouse mAb, clone 35, BD Biosciences, Franklin Lanes, USA), anti-human Lamp2 (mouse mAb, clone H4B4, Developmental Studies Hybridoma Bank, University of Iowa, USA), anti-human Lamp2 (rabbit pAb, ab37024, Abcam, Cambridge, UK), anti-mouse Lamp2 (mouse mAb, clone ABL-93, Developmental Studies Hybridoma Bank), anti-N-cadherin (mouse mAb, clone 10C32, BD Biosciences), anti-GnT-V (mouse mAb, clone 706824, R&D Systems, McKinley Place, USA), anti-CD68 (rat mAb, clone FA-11, AbD serotec, Düsseldorf, Germany), anti-V5 (mouse mAb, clone R960-25, Life Technologies, Carlsbad, USA), anti-Flag (rabbit mAb, F7425, Sigma), anti-calnexin (rabbit pAb, Enzo Life Sciences, Farmingdale, USA), anti-B3GNT1 (mouse mAb, clone 724057, R&D Systems), anti-MAN1B1 (mouse mAb, clone 30-Y, Santa Cruz Biotechnology (Dallas, USA), anti-B4GALT1 (goat pAb, AF3609, R&D Systems). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Promega (Madison, USA) and Dianova (Hamburg, Germany). For immunoblotting, all antibodies were diluted in phosphate-buffered saline (PBS) containing 0.5% (v/v) Tween-20 (PBS-T) and supplemented with 2% (w/v) Tropix I-BLOCK™ blocking reagent (Applied Biosystems, Life Technologies, Carlsbad, USA). The HRP-conjugated anti-HA antibody 3F10 was from Roche Diagnostics (Rotkreuz, Switzerland). Biotinylated lectins and HRP-conjugated streptavidin were obtained from Vector Laboratories (Burlingame, USA). Kifunensine was purchased from Santa Cruz Biotechnology and was dissolved in double-distilled water. The γ -secretase inhibitors, DAPT and L-685,458, were from Enzo Life Sciences and Calbiochem (Darmstadt, Germany), respectively. All protease inhibitors were dissolved in DMSO.

Molecular cloning and cDNA constructs

The cDNA that encodes SPPL3 is based on Genbank RefSeq NM_139015.4. Mutations to generate SPPL3 D200N/D271N were introduced by site-directed mutagenesis (primer sequences are available upon request) as described earlier (Voss *et al.*, 2012). SPPL3 D200N/D271N containing a C-terminal HA-tag (YPYDVPDYA) was subcloned into the EcoRI and XhoI sites of pcDNA 4/TO A (Invitrogen). Human *MGAT5* coding sequences

were purchased from Source BioScience (Nottingham, UK), PCR-amplified and subcloned using XhoI and KpnI restriction enzymes into pcDNA3.1hygro(+) (Life Technologies). To obtain tagged full-length GnT-V, an N-terminal Flag (DYKDDDDK) epitope tag after the initiating methionine residue and a C-terminal in-frame V5 (GKPIPPLLGLDST) epitope tag, respectively, were introduced using PCR amplification. Tagged GnT-V was subcloned into pcDNA3.1hygro(+) using XhoI and KpnI. All primer sequences are available upon request, and all cloned expression constructs were sequence-verified prior to experimental use.

Cell lines and cell culture experiments

All cells were cultured in DMEM GlutaMAX™ media (Life Technologies) supplemented with L-glutamine (Life Technologies), 10% (v/v) foetal calf serum (Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (Life Technologies). Stably transfected HEK293 cells expressing SPPL3 under the control of a doxycycline-inducible promoter have been described before (Martin *et al.*, 2008). To obtain stable cell lines expressing SPPL3 D200N/D271N, T-Rex™-293 (Invitrogen) were transfected with the respective plasmid DNA as described above. Transfection of cells was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and single cell clones were generated by selection in 200 μ g/ml zeocine (Invitrogen). To induce expression of SPPL3, cells were incubated with 1 μ g/ml doxycycline (BD Biosciences) added to the cell culture medium for at least 48 h. T-Rex™-293-derived clonal cell lines that express plasmids encoding *SPPL3*-, *SPPL2a*- and non-targeting shRNAs (Sigma-Aldrich), respectively, or epitope-tagged Flag-GnT-V-V5 were generated as described before (Martin *et al.*, 2008). PS1/PS2 double-deficient MEFs that lack active γ -secretase were described elsewhere (Herreman *et al.*, 2003) and were kindly provided by Dr. Bart de Strooper. Transient transfections with plasmid DNA were carried out using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. To achieve transient knock-downs, T-Rex™-293 cells were reversely transfected with siGENOME SMARTpools (Thermo Scientific, Waltham, USA) targeting human *SPPL3* (#M-006042-02), *B3GNT1* (#M-012307-01), *B4GALT1* (#M-012965-00) or *MGAT5* (#M-011334-01) (all dissolved in RNase-free water (Qiagen, Hilden, Germany)) at a final concentration of 20 nM using Lipofectamine RNAiMAX™ (Life Technologies) according to the manufacturer's instructions. To collect conditioned supernatants, the respective culture media were removed from cells after 24 h or 48 h and cleared from detached cells by centrifugation or 0.2 μ m filtration. Protease inhibitor treatment was performed for 16 h at the concentrations indicated. To inhibit complex- and hybrid-type N-glycosylation, cultured cells were treated with kifunensine at a final concentration of 4 μ g/ml for 48 h. To detect secretion of endogenous A β , cells were incubated in serum-free Opti-MEM™ + GlutaMAX™ (Life Technologies) overnight. To enrich for secreted factors by trichloroacetic acid (TCA) precipitation, cells were washed twice and then incubated in pre-warmed Opti-MEM™ + GlutaMAX™. Following harvest, 1/5 volume 100% (w/v) TCA was added, and samples were mixed and incubated on ice for 1 h. Following centrifugation (17,000 g, 4°C, 30 min), supernatants were discarded and pellets washed twice with cold acetone. Dried pellets were re-suspended in sample buffer and normalised to lysate protein content prior to loading.

Mice, tissue homogenates and MEFs

The genetrapp mouse line C57BL/6;129S5-Spp13^{Gt(OST279815)}Lex[/]Mmucd was obtained from the Mutant Mouse Regional Resource Center (MMRRC) at the University of California at Davis, CA, USA. These mice were described earlier (Tang *et al.*, 2010) and have generously been made available to the research community by Genentech, Inc., and Lexicon Pharmaceuticals, Inc. These mice were inter-crossed to maintain the C57BL/6;129S5 hybrid background. Age- and sex-matched animals were sacrificed, and indicated organs were removed and snap-frozen on dry ice. Thawed organs were cut into pieces and homogenised in ice-cold buffer (5 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM EGTA, supplemented with protease inhibitor mix (1:500, Sigma)), first using a Wheaton tissue grinder (Thermo Scientific), then 23G needles. Homogenates were centrifuged for 5 min at 5,000 rpm in a tabletop centrifuge. The supernatants were again subjected to centrifugation (17,000 g, 1 h, 4°C). Pellets were re-suspended in homogenisation buffer containing 2% (v/v) Triton X-100 and incubated on ice for 30 min. Lysates were cleared from cell debris by centrifugation (17,000 g, 1 h, 4°C) and subjected to SDS-PAGE and Western blotting. MEFs were obtained from mice that had been backcrossed to the C57BL/6 background for five generations. Heterozygous mice were mated, and embryos were removed at E13.5. Cells were taken into culture and immortalised as described earlier (Xu, 2005). All animals and derived cells were genotyped by PCR (primer sequences are available upon request).

Immunoblotting, lectin blotting, enzymatic deglycosylation and immunoprecipitation

Cells were harvested on ice and lysed in ice-cold STE buffer (150 mM NaCl, 50 mM Tris (pH 7.6), 2 mM EDTA) supplemented with 1% (v/v) NP-40 and 1% (v/v) Triton X-100 and protease inhibitor mix (1:500). Protein concentrations in lysates were determined using the BCA assay (Interchim, Montluçon, France) and, prior to loading on gels, samples were normalised accordingly. For lectin blotting, membranes were first incubated in 5% (w/v) BSA in PBS-T overnight. Biotin and streptavidin blocking was performed using a commercial kit (Vector Laboratories) according to the manufacturer's instructions. Membranes were incubated with the respective biotinylated lectin (2 µg/ml in lectin buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1 mM Ca²⁺, 0.08% (w/v) Na₃N)) for 1 h at room temperature. Following three washing steps (10 min each with PBS-T), blots were incubated with a streptavidin-HRP conjugate (2 µg/ml, diluted in PBS-T) at room temperature for 30 min. After three additional washing steps, blots were developed using conventional ECL chemistry (GE Healthcare, Chalfont St Giles, UK). For enzymatic deglycosylation, proteins in cell lysates were denatured in glycoprotein denaturation buffer (New England Biolabs, Ipswich, USA) supplemented with 1% (w/v) SDS by incubation at 95°C for 10 min. Samples were diluted tenfold with deglycosylation buffer (50 mM Na₂HPO₄, pH 7.2, 12 mM EDTA, 0.4% (v/v) NP-40) and digested with endoglycosidase H (Endo H) (Roche) or peptide: N-glycosidase F (PNGase F) (Roche) overnight. PNGase F removes all N-glycans irrespective of their exact structure and composition (Maley *et al.*, 1989), while Endo H only removes high-mannose and hybrid but not complex glycan structures from glycoproteins (Maley *et al.*, 1989). Immunoprecipitations, gel electrophoresis and

immunoblotting were carried out as described previously (Krawitz *et al.*, 2005; Fluhrer *et al.*, 2006). Immunoblots and lectin blots were developed using ECLTM or ECLTM Prime chemistry (GE Healthcare, Chalfont St Giles, UK) and X-ray films.

N-glycan analysis by nano-LC/MS

One day after plating of cells, SPPL3 overexpression was induced by supplementing culture medium with doxycycline as described earlier. 48 h later cells were washed twice with pre-warmed, serum-free DMEM and incubated in serum-free DMEM supplemented with doxycycline for another 72 h to collect secreted glycoproteins. Conditioned supernatants were harvested, and detached cells were removed by centrifugation. Supernatants were concentrated roughly 40-fold by centrifugation (5,000 g at 4°C) in an ultrafiltration unit with a molecular weight cut-off of 5,000 Da (Vivaspin, Sartorius, Göttingen, Germany). Concentrated proteins were collected by TCA precipitation as detailed earlier. Dried pellets were resuspended in PBS and transferred into ultrafiltration vials with a molecular weight cut-off of 10,000 Da (Vivaspin, Sartorius, Göttingen, Germany). Samples were washed, and buffer was exchanged to denaturation solution (6 M guanidine hydrochloride, 50 mM Tris-HCl and 5 mM EDTA) by centrifugation. Samples were reduced (16 mM dithiothreitol, DTT) for 15 min at 37°C followed by alkylation (30 mM iodoacetamide) for 1 h at 37°C in the ultrafiltration units. Denaturation solution was subsequently removed by centrifugation, and samples were diluted in PBS. N-glycans were released by digestion with PNGaseF, which was conducted for 17 h at 37°C. Released N-glycans were eluted by centrifugation and desalted using 96-well PGC (porous graphitised carbon) plates (Thermo Scientific, Waltham, USA). PGC resin was conditioned with 70% (v/v) acetonitrile (ACN), 0.1% (v/v) trifluoroacetic acid (TFA) and equilibrated with 0.1% (v/v) TFA in water. After sample application, resin was washed with 0.1% (v/v) TFA in water. N-glycans were eluted with 40% (v/v) ACN, 0.1% (v/v) TFA and dried using vacuum centrifugation. Dried N-glycans were resuspended in water (10 µl) and 2-anthranilic acid (2-AA) labelling solution (15 µl; 50 mg/ml 2-AA, 80 mg/ml picoline borane in 70:30 DMSO: acetic acid (v/v)). Reductive amination was performed at 37°C for 17 h. Excess label was removed by gel filtration using Sephadex G-10 columns (GE Healthcare). Nano-LC/MS analysis of 2-AA-conjugated N-glycans was performed as described earlier (Higel *et al.*, 2014), and statistical evaluation was conducted with GraphPad PRISM (v6, GraphPad Software Inc.).

Supplementary information for this article is available online: <http://emboj.embopress.org>

Acknowledgements

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Author contributions

MV and RF conceived the experiments. UK performed GnT-V overexpression experiments. FH and AS conducted mass spectrometric analysis of the N-glycome. PHK, SFL, AF and HS performed mass spectrometric analysis of the GnT-V cleavage site. AC, SFL and BS assisted with the generation of MEF cells. MHK, BK and GG provided technical assistance. RO supervised work with the *Sppl3*^{-/-} mice. MV performed all other experiments. RF and CH supervised the project. MV and RF wrote the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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