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

Matthias Voss¹, Ulrike Künzel¹, Fabian Higeli², Peer-Hendrik Kuhn³,⁴, Alessio Colombo³, Akio Fukumori³, Martina Haug-Kröper¹, Bärbel Klier³, Gudula Grammer³, Andreas Seidl², Bernd Schröder⁵, Reinhard Obst⁶, Harald Steiner¹,³, Stefan F Lichtenthaler³,⁷,⁸, Christian Haass¹,³,⁷ & Regina Fluhrer¹,³,*

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-acetylgalactosaminyltransferase 1 and β-1,4 galactosyltransferase 1. Cleavage of these enzymes leads to a reduction in their cellular activity. In line with this, reduced expression of SPPL3 results in a hyperglycosylation phenotype, whereas elevated SPPL3 expression causes hypoglycosylation. Thus, SPPL3 plays a central role in an evolutionary 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

DOI 10.15252/emboj.2014488175 | Received 3 March 2014 | Revised 19 September 2014 | Accepted 22 September 2014 | Published online 29 October 2014

The EMBO Journal (2014) 33: 2890–2905

See also: S Urban (December 2014)

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 (SPL) 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; Fluhrer 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
Among G×G 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 catalytically 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-acetylgalactosaminyltransferase 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-Rex™-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, complex 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 N-acetylgalactosaminidase I (PNGase F) and 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 N-acetylgalactosaminidase I activity and, accordingly, blocks generation of both hybrid- and complex-type N-glycans (Elbein 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 catalytically 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-acetylgalactosaminyltransferase 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.

**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...
SPPL3 regulates cellular N-glycosylation  Matthias Voss et al

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 (NCTmat) but not immature (NCTim) 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 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 (14B4) 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
A-B: brain and lung homogenates of Sppl3+/− mice and in Sppl3−/− MEFs was absent after treatment with PNGase F (Supplementary Fig S2A and B).

C-D: spleen and MEF homogenates. N-cadherin and Calnexin were used as loading controls. Antibodies used to visualise the respective proteins in Western blot are indicated.

**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 (+/+; lower band) and heterozygous (+/−; upper band) littersmates 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 (NCTmat), 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 (NCTim) was not altered in Sppl3−/− tissues.

- D: Analysis of glycoprotein molecular weight in immortalised MEFs derived from Sppl3−/− animals and 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.

**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 β-mannosidase I activity or another glycan-modifying enzyme downstream of β-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-acetyllactosamine 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 and/or the generation of N-acetyllactosamine moieties (Fig 3A).
Figure 3.

The EMBO Journal Vol 33 | No 24 | 2014
itself is known to be complex 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 accomplished 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 Sppl3−/− MEFs (Fig 3E). As expected, GnT-V was more abundant in lysates from Sppl3−/− MEFs than in wild-type control cells and its secretion was almost completely abolished in Sppl3-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 GlcNAcβ1,6Man 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 glycose-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-acetylgalactosaminyltransferase 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-acetylgalactosaminyltransferases (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 MCA75-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-Vm) and immature GnT-V (GnTVi) (see Supplementary Fig S4), n.t., not transfected.

E GnT-V secretion is impaired in Sppl3-deficient MEF cells. GnT-V levels were monitored in whole-cell lysates and in TCA-precipitated conditioned supernatants of Sppl3-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 (NCTm) 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–blocked 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 S3 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 ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 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 $\gamma$-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 $\gamma$-secretase inhibitors (GSIs) L-685,458 (Shearman et al, 2000) or N-[3,5-difluorophenacetyl]-L-alanyl-S-phenylglycine t-butylerester (DAPT) (Dovey et al, 2001). Both GSIs efficiently interfered with $\gamma$-secretase activity, as demonstrated by the drastically impaired secretion of endogenous A$\beta$, a well-established $\gamma$-secretase cleavage product (Haass, 2004), and the intracellular accumulation of APP C-terminal fragments, the corresponding $\gamma$-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 $\gamma$-secretase subunits (Fig 4D) as observed in $\text{Sppl}^{3/-/-}$ 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 $\text{Sppl}^{3/-/-}$ cells (Fig 3E). In sum, this clearly demonstrates that alterations in cellular N-glycosylation observed before (Figs 1–4) are not linked to $\gamma$-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
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 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 MGA75-specific siRNA pools (20 nM each), and HEK293 cells overexpressing SPPL3 (+ Dox) were analysed for electrophoretic mobility of endogenous mature Nicastrin (NCT_m). 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 MGA75-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-acetylgalactosaminyltransferase 1 (β3Gnt1). HEK293 cells were transfected with β3Gnt1-specific, non-targeting (ctrl) or SPPL3-specific siRNA pools as in (A). β3Gnt1 levels were analysed by immunoblotting in conditioned supernatants (β3Gnt1) and cell lysates (β3Gnt1). β3Gnt1 strongly accumulated intracellularly following siRNA-mediated SPPL3 knock-down in HEK293 cells, and β3Gnt1 secretion was significantly decreased (see also quantification in Supplementary Fig S8A). Overexpression of SPPL3 in doxycycline-induced cells (+ Dox) resulted in a reduction of β3Gnt1 in cellular lysates. Antibody staining was specific as β3Gnt1 as well as β3Gnt1 levels were reduced in cells transfected with a β3Gnt1-specific siRNA pool.

C β3Gnt1 accumulates in SPPL3-deficient MEFs. β3Gnt1 levels were monitored by Western blotting in whole lysates (β3Gnt1) obtained from Sppl3-deficient (−/−) or WT control (+/+) MEFs. In Sppl3−/− MEFs, β3Gnt1 strongly accumulated intracellularly.

D SPPL3 affects secretion of β-1,4 galactosyltransferase 1 (β4GalT1). The experiment was conducted as in (B), yet a siRNA pool targeting β4GalT1 was used to control for antibody specificity. Intracellular β4GalT1 levels and levels of secreted β4GalT1 were monitored by Western blotting using a specific antibody. Note that SPPL3 knock-down reduced β4GalT1 secretion while intracellular β4GalT1 accumulated intracellularly. SPPL3 overexpression reduced intracellular β4GalT1 levels.

E β4GalT1 secretion is impaired in Sppl3-deficient MEFs. β4GalT1 and sβ4GalT1 levels were monitored in lysates and TCA-precipitated conditioned supernatants obtained from Sppl3-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 (sα-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.
in vitro glycosyltransferases retain their catalytic activity when they are overexpressed in cell culture. Various in vivo studies have shown that SPPL3 alters N-glycosylation in multicellular organisms, including plants, arthropods and mammals. The authors tested whether SPPL3 is involved in the secretion of a number of distinct glycosyltransferases (GnT-V and 4GalT1) and found that SPPL3 knock-down leads to decreased secretion of these enzymes. Additionally, they investigated whether inhibition of SPPL3 affects the secretion of other enzymes and found that SPPL3 inhibition leads to decreased secretion of other glycosyltransferases. These results suggest that SPPL3 is 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, the authors 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 essential for multicellularity (Ohtsubo & Marth, 2006). While defects in Golgi N-glycosylation are often well tolerated and are typical of unusual phenotypic abnormalities in murine genetic models but also in inherited human diseases (Lowé & Marth, 2003). Based on our findings, we assume that SPPL3 orchestrates cellular N-glycosylation in multicellular organisms, but we cannot currently explain the phenotype observed in Sppl3-deficient mice.
and cannot exclude that SPPL3 fulfills 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 β1,6GlcNAc 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-galactin lattice (Dennis et al., 2009). Therefore, it is particularly interesting that we identified GnT-V, β3GnT1 and β4GalT1, 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 β3GnT1 (Sasaki et al., 1997), together with β4GalT1 (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

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 turnover and proteolytic liberation, 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 endoproteolyzed. Hence, active glycosyltransferases accumulate in their destined compartment, resulting in more extensive glycan modification (depicted here: only triantennary N-glycans) and a hyperglycosylation phenotype.
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-Aβ antibodies have been described elsewhere (Steiner et al., 2000; Yamasaki et al., 2006; Shiriotani 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, ah37024, 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-C6/6 (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-cizinexin (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-B4GALNT1 (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).o

Molecular cloning and cDNA constructs

The cDNA that encodes SPPL3 is based on Genbank RefSeq NM_1390154. 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 cloned 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 (GKPIPNPLLGLDST) 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 μg/ml zeocine (Invitrogen). To induce expression of SPPL3, cells were incubated with 1 μg/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), B4GALNT1 (#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 μm 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 μg/ml for 48 h. To detect secretion of endogenous Aβ, 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 with 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 genetrap mouse line C57BL/6;129S5-Sppl3Gt(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 then subjected to SDS–PAGE and Western blotting. 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 CaCl₂, 0.08% (w/v) NaN₃)) 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).

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 and transferred into ultrafiltration vials 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 resin (Lexicon Pharmaceuticals, Inc. and was obtained through the MMRRC at the University of California at Davis, CA, USA) supplemented 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 reconstituted 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://embj.embopress.org

Acknowledgements

The Sppl3 knockout mouse line was kindly provided by Genentech, Inc., and Lexicon Pharmaceuticals, Inc. and was obtained through the MMRRC at the University of California at Davis, CA, USA) supplemented with 1% (v/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 ECL TM or ECL TM Prime chemistry (GE Healthcare, Chalfont St Giles, UK) and X-ray films.

N-glycan analysis by nano-LCMS

One day after plating of cells, SPP3 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 resin (Lexicon Pharmaceuticals, Inc. and was obtained through the MMRRC at the University of California at Davis, CA, USA) supplemented 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 reconstituted 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.).
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 Spl3Δ 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.

References
enzyme, is responsible for cleavage secretion of a Golgi-resident sialytransferase. \textit{Proc Natl Acad Sci USA} 98: 13554 – 13559


Ujita M, McKullife J, Hindsgaul O, Sasai K, Fukuda MN, Fukuda M (1999) Poly-N-acetyllactosamine synthesis in branched N-glycans is controlled by...
complemental branch specificity of I-extension enzyme and beta1,4-galactosyltransferase I. J Biol Chem 274: 16717–16726
Poly-N-acetyllactosamine extension in N-glycans and core 2- and core 4-branched O-glycans is differentially controlled by i-extension enzyme and different members of the beta 1,4-galactosyltransferase gene family. J Biol Chem 275: 15868–15875