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Latest emerging functions of SPP/SPPL intramembrane proteases

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ABSTRACT

Signal peptide peptidase (SPP) and the four related SPP-like (SPPL) proteases are homologues of the presenilins, which comprise the catalytic centre of the γ-secretase complex. SPP/SPPL proteases are GxGD-type aspartyl intramembrane proteases selective for substrates with a type II membrane topology. Subcellular localisations of SPP/SPPL proteases range from the early secretory pathway to the plasma membrane and the endocytic system. Similarly diverse are their functional roles at the cellular level covering the turnover of signal peptides and membrane proteins, a contribution to the ERAD pathway as well as the regulation of cellular protein glycosylation and certain signaling pathways. Much less well understood are the physiological functions of SPP/SPPL proteases in complex organisms. Whereas a major role of SPPL2a for homeostasis of B cells and dendritic cells has been documented in mice, *in vivo* functions of SPP and the other SPPLs remain largely elusive to date. SPP/SPPL proteases contribute to regulated intramembrane proteolysis (RIP), a sequential processing of single-spanning transmembrane proteins by an ectodomain sheddase and an intramembrane-cleaving protease (I-CLIP). However, recent studies reported the cleavage of tail-anchored and multi-pass membrane proteins by SPP as well as the capability of SPPL3 to accept substrates without a preceding ectodomain shedding. This revealed that the mechanistic properties within this family are more diverse than initially thought. With this review, we aim to provide an update on recent achievements in defining the function and (patho-) physiological relevance of SPP/SPPL proteases and to highlight open questions in the field.

KEYWORDS

Intramembrane Proteolysis; Signal peptide peptidase; SPPL proteases, ERAD; Proteostasis; Ectodomain Shedding

1. Introduction

Intramembrane proteases are characterized by their catalytic centres localizing to the plane of the membrane and hydrolyse their substrates either within transmembrane segments or close to membrane spanning domains. As a result, the cleavage fragments derived from substrate proteins are released from the membrane. Based on their catalytic mechanism, intramembrane-cleaving proteases (I-CLIPs) are classified as metallo-, serine, glutamyl or aspartyl I-CLIPs. Among the latter, presenilins constitute the active subunit of the γ-secretase complex (Jurisch-Yaksi et al., 2013). Identified as genes harbouring mutations causing Familial Alzheimer Disease (FAD) (Cruts et al., 1996), meanwhile more than 100 different presenilin/γ-secretase substrates, among them the amyloid precursor protein (APP) (De Strooper et al., 1998) and the Notch receptor (De Strooper et al., 1999) were described (Haapasalo and Kovacs, 2011; Jurisch-Yaksi et al., 2013). In addition, a second family of aspartyl I-CLIPs has been identified in the human genome comprising the ER-resident Signal peptide peptidase (SPP) and the four SPP-like (SPPL) proteases SPPL2a, SPPL2b, SPPL2c and SPPL3 (Friedmann et al., 2004; Ponting et al., 2002; Weihofen et al., 2002). SPP/SPPL proteases exhibit a similar catalytic center like the presenilins characterized by conserved Y/FD and GxGD motifs. However, most likely based on opposing orientations within the membrane, SPP/SPPL proteases and presenilins show selectivity for substrate proteins with a type II and type I topology, respectively. As illustrated in Fig. 1, subcellular localisations of SPP/SPPL proteases range from the ER (SPP, SPPL2c) and Golgi apparatus (SPPL3) to the plasma membrane (SPPL2b) and to late endosomes/lysosomes (SPPL2a). With the exception of SPPL2c for which proteolytic activity has not been reported yet, all other members of the SPP/SPPL family are catalytically active proteases. However, in particular recent findings on SPPL3 indicate that substrate requirements and cleavage mechanisms of different SPP/SPPL proteases may be more diverse than initially anticipated (Voss et al., 2012; Voss et al., 2014). The cellular and *in vivo* processes which are regulated by these proteases with implications

for pathophysiology and potential therapeutic strategies are similarly diverse. General aspects of SPP/SPPL proteases including their phylogeny and a comparison of their structure and topology have been recently reviewed in a comprehensive way (Voss et al., 2013) and will not be covered all-embracingly in this article. Here, we aim to provide an update by compiling recent progress in understanding the biology of SPP/SPPL intramembrane proteases. Though many questions remain to be resolved, the current state of knowledge already highlights SPP/SPPL proteases as important players at the interface of membrane protein homeostasis and signal transduction with critical importance for various cellular functions.

2. SPP

The ER-resident SPP protease was initially reported to mediate turnover of remnant signal peptides generated from nascent protein chains by Signal Peptidase (Weihofen et al., 2002; Weihofen et al., 2000). These signal peptides display a type II orientation with short luminal C-termini, thus representing prototypic I-CLIP substrates (Lemberg and Martoglio, 2002). Typically, aspartyl I-CLIPs are thought to participate in Regulated Intramembrane Proteolysis (RIP), a two-step-proteolytic event in which the removal of the ectodomain of a single-span transmembrane protein is the prerequisite for the intramembrane cleavage (Jurisch-Yaksi et al., 2013; Voss et al., 2013). In case of SPP, this step is represented by the action of Signal Peptidase which separates the signal peptide co-translationally from a nascent protein. Currently known substrates and functions of SPP are summarized in Table 1 and Fig. 2. Following its initial characterisation, SPP was demonstrated to process a broad variety of signal peptides from different proteins including Prolactin (Martoglio et al., 1997), MHC class I (Lemberg et al., 2001), Calreticulin (Lemberg and Martoglio, 2002) and Pro-calcitonin (El Hage et al., 2008). The SPP orthologue in *Plasmodium falciparum* cleaves the signal peptide of heat shock protein HSP101 (Baldwin et al., 2014). In addition to cell-intrinsic

substrates, SPP plays a role in the processing of viral proteins. This can involve the cleavage of signal peptides that have been released from newly synthesised viral proteins like HIV gp160 (Martoglio et al., 1997) and the VSVG protein (Lemberg and Martoglio, 2002). However, in some cases, SPP proteolyses internal signal peptides, thereby releasing parts of the precursor proteins from the membrane and/or critically influencing their membrane topology. Prominent examples are the hepatitis virus C core protein (McLauchlan et al., 2002) and the recently identified Bunyamwera orthobunyavirus glycoprotein precursor (BUNV Pre-Gn) (Shi et al., 2016). Thus, SPP fulfils an important function in the maturation of viral proteins.

Though SPP-mediated processing of several signal peptides has been reported (Table 1), it is still not clear if this represents a universal mechanism to clear signal peptides from the ER membrane. If so, this would require a loose cleavage specificity of SPP and support a model describing intramembrane proteases as "proteasome of the membrane" (Kopan and Ilagan, 2004). The substrate properties required for cleavage by SPP are not known in detail as discussed previously (Voss et al., 2013). In several cases, helix-destabilising and polar residues seem to play a critical role. Recently, a targeted knockout of SPP in the colorectal carcinoma cell line HCT116 was generated (Boname et al., 2014). These *SPP*$^{-/-}$ cells appeared healthy, but grew slightly slower and were less adherent than wild type cells (Boname et al., 2014). A proteomic analysis failed to detect an accumulation of uncleaved signal peptides in these cells. The loss of hydrophobic proteins and in particular peptides during sample preparation and analysis is an inherent problem in proteomic studies. Therefore, the failure to observe the hypothesized signal peptide accumulation in this study could have technical reasons (Boname et al., 2014). Approaches specifically optimised for detection of such hydrophobic peptides may need to be applied in the future to prove or disprove the postulated universal role of SPP in signal peptide turnover. In case that the SPP-mediated cleavage of

signal peptides derived from cell-intrinsic proteins does not primarily serve a degradative purpose, it may be tempting to speculate that the cleavage fragments derived from individual signal peptides have a biological function. Apart from the generation of certain HLA-E epitopes from such cleavage products that protect cells from attack by NK cells, evidence in this direction is limited (Lemberg et al., 2001). Recently, another contribution of SPP-mediated proteolysis to the generation of peptides for MHC class I (MHCI) antigen presentation was identified (Oliveira et al., 2013). However, this was independent of signal peptide processing. Instead in this case, SPP facilitated the release of a peptide from the C-terminal, type II-oriented transmembrane segment of the multi-pass membrane protein Trh4 so that presentation of this epitope did not require the peptide transporter TAP (Oliveira et al., 2013).

Although the impact of SPP-deficiency on general signal peptide turnover may require further clarification, the respective proteomic approach (Boname et al., 2014) has provided important insights in substrate acceptance by SPP, which is more versatile than initially anticipated. Based on an accumulation in *SPP*$^{-/-}$ cells, SPP was discovered to be responsible for the turnover of ER-resident, type II-oriented tail-anchored (TA) proteins, which exhibit a transmembrane segment immediately at or very close to the C-terminus. TA proteins that were identified to be substrates of SPP include Cytochrome B5A (CYB5A), Ribosome-associated membrane protein 4 (RAMP4) and Heme oxygenase-1 (HO-1) (Boname et al., 2014; Hsu et al., 2015).

HO-1 catalyses the rate-limiting degradation of heme to biliverdin and is upregulated in several cancers (Liu et al., 2004; Schmidt et al., 2004). SPP-mediated cleavage of HO-1 was dependent on its short luminal domain, reflecting the properties of a classical RIP substrate after ectodomain shedding (Boname et al., 2014). Furthermore, mutagenesis of certain residues close to the cleavage site within its transmembrane segment significantly

reduced the cleavability of HO-1 (Hsu et al., 2015). Under certain conditions, especially in cancer cells, the HO-1 cleavage product exhibits certain stability and fulfils specific biological roles (Hsu et al., 2015). SPP controls these functions by enabling the nuclear translocation of the HO-1 fragment which depends on its proteolytic liberation from the membrane (Hsu et al., 2015). Nuclear levels of this HO-1 fragment stimulated proliferation and migration of HeLa cells thereby increasing the tumorigenic potential of this cell line upon injection in immune-incompetent mice (Hsu et al., 2015). These effects were independent of the HO-1 catalytic activity and the specific functions of this protein in the nucleus remain to be determined. In any case, the proteolytic activity of SPP represents the decisive molecular switch to allow nuclear entry of HO-1 (Hsu et al., 2015). However, the released HO-1 fragment also undergoes proteasomal degradation (Boname et al., 2014). Thus, SPP-mediated intramembrane proteolysis of HO-1 also controls its ER levels and turnover by subjecting it to ER-associated degradation (ERAD) (Boname et al., 2014). Even though ERAD was initially reported to control elimination of misfolded proteins from the ER, it also enables degradation of intact ER-resident molecules (Avci and Lemberg, 2015). In case of HO-1, cleavage by SPP precedes TRC8-mediated ubiquitination which then initiates its proteasomal degradation (Boname et al., 2014; Lin et al., 2013).

In agreement with these findings, SPP evolved to play a central role in ERAD of selected substrates. One of the first indications was provided by the observation that SPP may be critically involved in the dislocation and degradation of MHC class I complexes upon infection with the Human Cytomegaly Virus (HCMV), which is mediated by the viral protein US2 (Loureiro et al., 2006). However, in the above mentioned SPP-deficient HCT116 cells this process was not compromised (Boname et al., 2014). Nonetheless, SPP can associate with ERAD substrates and form high molecular-weight complexes (Schrul et al., 2010). Since an inactive mutant of SPP was also part of these complexes, it seems conceivable that SPP may

also contribute protease-independent functions to the ERAD process. However, in two recently identified examples the proteolytic activity of SPP is critically involved. SPP directly modulates ERAD by processing the unspliced type II transmembrane protein X-box binding protein 1 (XBP1u) (Chen et al., 2014), which inhibits the unfolded protein response (UPR)-inducing functions of its spliced isoform XBP1s by heterodimerization (Yoshida et al., 2006). Interestingly, SPP-mediated XBP1u cleavage does not require the proteolytic removal of the XBP1u luminal domain comprising 59 amino acid residues (Chen et al., 2014). This ectodomain size was previously reported to only allow restricted intramembrane proteolysis of a luminally elongated Integral membrane protein 2 B (ITM2B; Bri2) N-terminal fragment by SPPL2b (Martin et al., 2009). Mechanistically, XBP1u cleavage might be enabled by binding of its luminal domain to the SPP-interacting protein Derlin-1. This interaction could mask the substrate's rather long ectodomain thereby removing the steric hindrance and allowing SPP to bind and cleave its substrate (Chen et al., 2014). Cleavage by SPP initiates ERAD of XBP1u which is dependent on the pseudoprotease Derlin-1 as well as the ubiquitin ligase TRC8 (Chen et al., 2014).

In addition to the emerging functions of SPP in ERAD by cleavage of the type II transmembrane protein XBP1u (Chen et al., 2014), Ypf1, the yeast orthologue of SPP, also participates in the regulation of multi-pass membrane protein stability (Avci et al., 2014). Under high zinc-conditions, Ypf1 cleaves the zinc transporter Zrt1 in one of its eight transmembrane domains thereby initiating its removal from ER-membranes by the ERAD pathway. Comparable to the proteolysis of XBP1u, Ypf1-induced Zrt1 destabilisation required the yeast Derlin-1 homolog Dfm-1 and the E3 ubiquitin ligase Doa10 suggesting a conserved function of the Derlin-1/SPP/E3-ligase axis in ERAD (Avci et al., 2014). Interestingly, Ypf1-mediated destabilization of Zrt1 is strictly dependent on the availability of extracellular zinc presenting a highly dynamic nutrient sensing system based on

intramembrane proteolysis. Moreover, this novel pathway that was termed ERAD regulatory (ERAD-R), seems to be of general relevance, since genetic ablation of Ypf1 in yeast cells elevated surface expression of several amino acid, ion and metabolite transporters (Avci et al., 2014). To what extent these functions of Ypf1 in ERAD-mediated regulation or nutrient transport are conserved in mammalian cells and whether SPP has an equivalent role in this context remains to be determined.

It should be mentioned that our current understanding of SPP functions originates mostly from cell-based experimental systems and that so far very little *in vivo* data is available. Plasmodium parasites, the malaria-causing pathogen, critically depend on the activity of SPP (Harbut et al., 2012; Voss et al., 2013). Pharmacological inhibition of plasmodium SPP was found to impair parasite growth, whereas conflicting data were reported whether also the process of erythrocyte invasion is affected (Marapana, Traffic 2012). Mechanistically, this is caused by an impairment of the ERAD pathway which sensitizes the parasites to ER stress (Harbut et al., 2012). In C. elegans by RNAi-mediated depletion of imp-2, the orthologue of SPP, leads to embryonic lethality and molting defects. The observed phenotype resembled changes induced by deprivation of exogenous dietary cholesterol as well as disruption of lrp1 (lipoprotein receptor-related protein 1) (Grigorenko et al., 2004). However, a putative molecular connection is currently not clear. Similarly, deficiency of SPP in Drosophila resulted in lethal defects upon larval development (Casso et al., 2005). A knockdown of SPP in zebrafish resulted in a cell death phenotype within the nervous system (Krawitz et al., 2005). A constitutive knockout of SPP in mice leads to embryonic lethality after day 13.5 d clearly highlighting the importance of this protein (Aizawa et al., 2016). *SPP*$^{-/-}$ embryos were smaller than those from control mice, but exhibited no obvious abnormalities upon histological analysis (Aizawa et al., 2016). Future studies will be required to define SPPs *in vivo* function in more detail.

3. The SPPL2 subfamily (SPPL2a, SPPL2b, SPPL2c)

In contrast to SPP, intramembrane proteolysis by the closely related SPPL2a and SPPL2b proteases was so far exclusively demonstrated for classical RIP substrates including Tumor Necrosis Factor α (TNFα) (Fluhrer et al., 2006; Friedmann et al., 2006), FasL (Kirkin et al., 2007), Integral membrane protein 2 B (ITM2B; Bri2) (Martin et al., 2008), Cluster of differentiation 74 (CD74) (Beisner et al., 2013; Bergmann et al., 2013; Schneppenheim et al., 2013), and Transferrin Receptor 1 (TfR1) (Zahn et al., 2013). A compilation of the currently known SPPL2 substrates is shown in Table 1.

For all these type II-oriented single-span transmembrane substrates, SPPL2-mediated intramembrane proteolysis follows the ectodomain release either performed by the metalloproteinases A disintegrin and metalloproteinase (ADAM) 10 or 17 or lysosomal proteases (Voss et al., 2013). Recently, the Transmembrane protein 106b (TMEM106b) has been added to the substrate list of SPPL2a (Brady et al., 2014). TMEM106b localizes to the lysosomal compartment and requires ectodomain trimming by pH-sensitive lysosomal cysteine and serine proteases prior to SPPL2a-mediated intramembrane proteolysis as shown in cell-based overexpression systems (Brady et al., 2014). TMEM106b was originally identified as a genetic risk factor for the development of frontotemporal lobar degeneration with TDP-43 positive inclusions (FTLD-TDP) (Finch et al., 2011; Van Deerlin et al., 2010; van der Zee et al., 2011) and plays a critical role for organisation of the spatial distribution of lysosomes in neurons (Schwenk et al., 2014). However, it is currently unclear how SPPL2a-mediated TMEM106b proteolysis influences the physiological functions of this type II transmembrane protein especially since SPPL2a was demonstrated to be expressed at rather low levels in murine brain (Schneppenheim et al., 2014b).

An important aspect is that type II-oriented membrane-bound protein fragments, which are cleaved by SPPL2a or SPPL2b, can also derive from proteins spanning the membrane twice, thus exhibiting a hairpin-like structure with cytosolic N- and C-termini. Two such SPPL2a/b substrates have been reported. One is the Foamy virus envelope protein (FVenv), which is processed by furin or SPPL3, thereby producing a type II oriented membrane stub that can undergo further proteolysis by SPPL2a/b (Voss et al., 2012). The other recently identified, cell-intrinsic SPPL2 hairpin substrate is Neuregulin 1 type III (NRG1 type III) (Fleck et al., 2016). This protein is predominantly expressed in the central and peripheral nervous system where it acts as a key mediator of oligodendrocyte- and Schwann cell-mediated myelination (Brinkmann et al., 2008; Chen et al., 2006; Lemke, 2006; Nave and Salzer, 2006; Willem et al., 2006). This myelination-inducing function of NRG1 type III requires proteolytic processing by BACE1 or ADAM10/17 in the extracellular loop in order to expose the Epidermal growth factor (EGF)-like domain that facilitates activation of ErbB-receptors (Luo et al., 2011; Willem et al., 2006). This processing results in the formation of a type I- and a type II-oriented membrane-bound NRG1 type III fragment. While the NRGI type III CTF is cleaved by γ-Secretase, the luminal domain of the corresponding NTF is shortened by an unknown protease and then cut by SPPL2a and SPPL2b within the transmembrane domain as demonstrated in a HEK cell-based model system (Fleck et al., 2016). Even though still no functional role could be assigned to the cleavage fragments released by the intramembrane proteases, these findings establish NRG1 type III as the first protein that is processed by three different I-CLIPs (Fleck et al., 2016).

The observation that SPPL2a/b substrates can be derived from more complex membrane proteins significantly expands the theoretical substrate spectrum of these proteases, which was initially considered to be limited to type II, single-span membrane proteins. In principle also multi-pass proteins with more than two transmembrane domains could give rise

to single-spanning fragments with the required orientation when suitable loops on either side of the membrane are proteolytically cleaved. So far, however, no example for this has been identified.

SPPL2c, the least characterized member of the SPPL2 family, still remains an orphan protease. Despite the presence of conserved catalytic aspartyl residues in transmembrane domain 6 and 7, so far no proteolytic activity could be attributed to this ER-resident protein (Friedmann et al., 2004). Due to the unconventional gene structure of SPPL2c lacking any introns, it might even represent a pseudogene without functional expression. However, microarray analysis revealed mRNA expression of SPPL2c in human testis, brain, skin and heart (Friedmann et al., 2004), but evidence for the expression of this putative protease at the protein level is currently pending.

Substrate analysis of SPPL2a and SPPL2b in many cases relied on cell-based experimental systems employing overexpression of the substrates. The so far only *in vivo*-validated substrate is CD74, the invariant chain of the Major Histocompatibility Complex II (MHCII) (Beisner et al., 2013; Bergmann et al., 2013; Schneppenheim et al., 2013). Besides its potential function as surface receptor for the Macrophage migration inhibitory Factor (MIF), CD74 acts as a chaperone for MHCII molecules preventing them from premature binding of antigenic peptides (Schröder, 2016). In MHCII compartments, the type II-oriented CD74 molecule is sequentially processed by several serine and cysteine proteases generating a stable CD74 NTF (Nakagawa and Rudensky, 1999). As shown by three independent laboratories using different mouse models, this fragment requires SPPL2a for its subsequent degradation (Beisner et al., 2013; Bergmann et al., 2013; Schneppenheim et al., 2013). Accumulation of the CD74 NTF results in a developmental defect of B cells at the splenic T1 stage as well as in reduced total numbers of splenic dendritic cells (Schneppenheim et al., 2014b) thereby strongly impacting the adaptive immune system of affected mice.

While these findings established SPPL2a as an important factor for murine B cell homeostasis, the role of this protease in human B cells was only recently validated using B cells of two patients bearing a microdeletion in chromosome 15q21.2 (Moreno-De-Luca et al., 2011) that also includes the first exon of SPPL2a and therefore abolishes expression of this protease (Schneppenheim et al., 2014a). EBV-immortalized B cells from these patients accumulated significant amounts of the CD74 NTF equivalent to the situation in mice. This confirms that the role of SPPL2a in CD74 NTF degradation is conserved (Schneppenheim et al., 2014a). Since no information about the immune status of the respective patients was available, the phenotypic consequences of a CD74 NTF accumulation on B cell and dendritic cell homeostasis in humans currently remain elusive.

Mechanistically, the B cell developmental arrest observed upon genetic SPPL2a-ablation in mice could be correlated with an impaired tonic B cell receptor (BCR) signaling (Hüttl et al., 2015). The resulting accumulation of the CD74 NTF severely compromises activation of the PI3K/Akt signaling pathway downstream of this receptor thereby increasing expression of the pro-apoptotic genes p21, p27 and Bim in $B220^{+}$ B cells (Hüttl et al., 2015). In addition to the PI3K/Akt pathway, also signaling by the BCR-associated Splenic tyrosine kinase (Syk) is negatively impacted by the accumulating CD74 NTF, possibly involving a direct interaction of this kinase with the CD74 fragment as shown by proximity ligation assays (Hüttl et al., 2015).

Besides its importance for the regulation of cellular CD74 NTF levels (Beisner et al., 2013; Bergmann et al., 2013; Hüttl et al., 2015; Schneppenheim et al., 2013; Schneppenheim et al., 2014a), SPPL2a also seems to control immune cell function by the proteolytic liberation of the CD74 intracellular domain (ICD). In line with previous studies (Becker-Herman et al., 2005; Matza et al., 2002), SPPL2a-dependent nuclear translocation of the CD74 ICD could be demonstrated with a novel β-galactosidase enzyme fragment complementation (βGEFC)

translocation assay (Mentrup et al., 2015). As compared to other reporter assays which have been employed to measure γ-secretase activity, the βGEFC system requires only a comparably short tag (~50 amino acids) to be fused to the cytosolic portion of the substrate protein (Mentrup et al., 2015). This is a major advantage for the analysis of type II transmembrane proteins where large N-terminal tags can significantly interfere with subcellular targeting (Mentrup et al., 2015). The liberated CD74 ICD was reported to influence nuclear transcription in a $TAF_{II}105$-dependent manner by activating the NFκB subunit p65 (Becker-Herman et al., 2005; Matza et al., 2002; Matza et al., 2001). Even though these findings were not recapitulated in a microarray system using CD74 ICD-expressing HEK cells, this set-up identified secreted Frizzled-related protein 2 (SFRP2) as a target gene which is regulated by this proteolytically released fragment at the transcriptional level. In line with this finding, a down-regulation of SFRP2 in bone marrow-derived dendritic cells deficient for SPPL2a or CD74 was observed (Mentrup et al., 2015). Since this soluble regulator of Wnt-signaling was shown to be upregulated in multiple myeloma cells (Oshima et al., 2005) that also show increased expression of CD74 (Burton et al., 2004), it could be interesting to analyse the influence of the potential CD74 ICD/SFRP2-axis in the context of tumorigenesis.

While these findings clearly elucidated the role of SPPL2a for the immune system, the physiological functions of SPPL2b remain much less understood. In principle, both I-CLIPs demand more or less similar conditions for turnover of C-terminally truncated type II transmembrane proteins. Comparable to observations made for related I-CLIPs (Lemberg and Martoglio, 2002; Struhl and Adachi, 2000; Voss et al., 2013), mutational studies using the SPPL2b substrate ITM2B (Martin et al., 2009) and the SPPL2a substrate CD74 (Hüttl et al., 2016) suggested that certain glycine residues, potentially those that induce a destabilisation of the helical transmembrane domain structure may be critical, but not absolutely required for intramembrane proteolysis by SPPL2a/b. SPPL2a-mediated intramembrane proteolysis was

not altered upon exchanging the intracellular domain of CD74 with those of the lysosomal membrane proteins DIRC2 or TMEM192 (Hüttl et al., 2016). Systematic mutagenesis of the CD74 transmembrane segment and the luminal domain of the CD74 NTF did not reveal any sequence motifs that are absolutely required for cleavage by SPPL2a (Hüttl et al., 2016). However, in addition to the glycines within the transmembrane segment, two residues within the luminal membrane-proximal domain were found to support intramembrane proteolysis of CD74 by SPPL2a (Hüttl et al., 2016). Altogether, our understanding how SPPL2a and SPPL2b recognize substrates and discriminate substrates from non-substrates is currently incomplete. It seems likely that rather yet to be recognized structural motifs in the transmembrane and membrane-adjacent domains than the amino acid sequence itself are the critical determinants.

Regarding their mechanistic properties, SPPL2a and SPPL2b appear to be very similar. This is supported by the observation that in cell-based systems which rely on co-expression of substrates and proteases, many substrates, including CD74 are cleaved with comparable efficiency by both enzymes (Schneppenheim et al., 2014b). However, *in vivo* CD74 is not a physiological substrate of SPPL2b (Schneppenheim et al., 2014b). SPPL2b-deficient B cells as well as dendritic cells do not accumulate significant amounts of the CD74 NTF and therefore do also not present the immune cell defects observed in *Sppl2a*$^{-/-}$ mice. This might be dependent on the divergent subcellular localisation of these I-CLIPs with SPPL2a localizing to the endolysosomal compartment and SPPL2b residing at the plasma membrane (Behnke et al., 2011; Friedmann et al., 2004; Schneppenheim et al., 2014b). Even though both proteases are present in lymphoid organs, SPPL2a displays a more ubiquitous expression profile while SPPL2b is primarily expressed in lymphoid organs, bone marrow and the central nervous system (Schneppenheim et al., 2014b). However, these data clearly indicate non-redundant functions of SPPL2 proteases highlighting the urgent need for *in vivo*-

validation of the known substrates. Further work will especially be needed to define the physiological functions of SPPL2b.

4. SPPL3

SPPL3 is the smallest and the only non-glycosylated member of the SPP/SPPL family. FVenv was the first SPPL3 substrate identified to be cleaved in a cellular model system (Voss et al., 2012). FVenv is a hairpin protein comprising two transmembrane domains of which the N-terminal transmembrane domain adopts a type II orientation and is part of an unusually long leader peptide (LP18) (Lindemann and Rethwilm, 2011). Other than SPPL2a/b, SPPL3 cleaves FVenv without requiring a preceding processing step that generates a short substrate ectodomain (Voss et al., 2012). Based on this finding, a mass spectrometric screen for physiological substrates was performed (Kuhn et al., 2015). Using the proteomics 'secretome protein enrichment with click sugars (SPECS)' method (Kuhn et al., 2012), changes in secreted membrane protein ectodomains were analyzed in cells deficient for or overexpressing SPPL3 (Kuhn et al., 2015). This confirmed that SPPL3 acts as a type II membrane protein specific sheddase that cleaves its substrates within the very C-terminal part of their transmembrane domain independent of their ectodomain length (Kuhn et al., 2015). Most of the candidate substrates identified in this screen localize to the Golgi and are implicated in modification of N- and O-linked glycans as well as in glycosaminoglycan biosynthesis. Verification of selected candidate substrates in cell culture and in tissues of SPPL3 knockout mice confirmed SPPL3 as one of the major proteases responsible for releasing the luminal domain of various glycosyltransferases and glycosidases into the extracellular space (Kuhn et al., 2015; Voss et al., 2014).

Since the catalytic center of glycan-modifying enzymes is localised within their ectodomain, shedding of these enzymes reduces their catalytic activity in the Golgi.

Consequently, expression levels of SPPL3 impact on the glycosylation status of proteins in the secretory pathway (Voss et al., 2014). Increased SPPL3-expression results in hypoglycosylation of many, if not all, secretory and membrane proteins, while reduced levels of SPPL3 produce hyperglycosylated proteins (Voss et al., 2014). In a physiological context this enables a cell to rapidly change the glycan pattern of many proteins within the secretory pathway by adapting the expression level of one protease, thus allowing a pronounced reaction to, for instance, changes in environmental conditions like growth factor or nutrient concentrations. In line with this, constitutive overexpression and knock-down of SPPL3 in certain cell lines like HEK293 or Hela induces cell death and only inducible overexpression or transient knockdown are tolerated for a short period of time (Fluhrer, unpublished observation). Thus, SPPL3 emerges as a crucial player of Golgi function and cellular growth control. However, so far it remains unclear what kind of signals and cellular mechanisms regulate endogenous SPPL3 expression. Ongoing and future experiments will help to clarify this.

SPPL3-deficient mice are viable on a mixed C57BL/6;129S5 background and present with a rather mild phenotype characterized by growth retardation, haematologic abnormalities and sterility in male homozygous mice (Tang et al., 2010). Targeted deletion of SPPL3 in the mouse hematopoietic system or solely in NK cells suggests a key role of SPPL3 in cell autonomous control of the NK cell maturation process documented by decreased numbers of $CD27^{+}CD11b^{+}$ and $CD27^{-}CD11b^{+}$ NK cells in these mice and reduced clearance of MHC class I-deficient tumors (Hamblet et al., 2016). In addition to the maturation defect, *SPPL3*$^{-/-}$ NK cells showed impaired cytotoxicity against tumor cells *in vitro* indicating an functional deficit of the residual NK cells in these mice (Hamblet et al., 2016). Phenotypes were copied by knock in mice that express a catalytically inactive variant of SPPL3 demonstrating the requirement for proteolytically active SPPL3 in NK cell maturation and function (Hamblet et

al., 2016). However, the substrates cleaved by SPPL3 to regulate NK cell maturation and thus the underlying cellular mechanisms remain currently elusive. NK cells are critically required to prevent tumor growth (Vivier et al., 2008) and exome sequencing recently identified SNPs in the coding region of SPPL3 as a potential risk for developing breast cancer (Noh et al., 2015), indicating that SPPL3 may have a key role in surveillance of tumor growth. Using a haploid cell line and a genetic screening approach, SPPL3 was also found to be involved in the maturation process of the GPI-anchored CD59, which is a key regulator of complement-mediated cell lysis (Davis et al., 2015). Interestingly, SPPL3 did not affect maturation of the Prion Protein (PrP) arguing against a general role of this protease in the maturation of GPI-anchored proteins (Davis et al., 2015).

In addition to its proteolytic function, also a non-proteolytic function has been attributed to SPPL3. SPPL3 interacts with stromal interaction molecule 1 (STIM1) and Orai1 and serves as an enhancer of the T cell receptor signal to trigger maximal Ca^{2+} influx and NFAT activation necessary for lymphocyte signaling (Makowski et al., 2015). These findings, together with our knowledge about the physiological function of SPPL2a, further outline the specific role of SPP/SPPL proteases in the immune system and most likely in tumor development.

5. Open questions and Conclusions:

Though sharing a similar catalytic center with the presenilins, a major difference between SPP/SPPL proteases and the γ-secretase complex is their requirement for co-factors. So far and despite several attempts, no accessory subunits required for catalytic activity of SPP/SPPL proteases could be identified. According to a recently proposed model, the γ-secretase subunit nicastrin prevents access of type I membrane proteins with large

ectodomains to the catalytic site of presenilins based on steric hindrance (Bolduc et al., 2016). Consequently, only type I membrane proteins that undergo shedding of their ectodomains or those with a naturally short ectodomain, like the B cell maturation antigen (BCMA) (Laurent et al., 2015), have been identified as γ-secretase substrates. In case of SPP, the association with Derlin-1 and TRC8 influences substrate access. However, instead of excluding proteins with long ectodomains, Derlin-1 seems to specifically facilitate cleavage of such substrates by masking their ectodomain and resolving the steric hindrance (Chen et al., 2014). For SPPL2a/b no co-factors in context of substrate recognition are currently known.

Beyond several common properties of SPP/SPPL proteases, recent identifications of novel SPP and SPPL3 substrates suggest a greater functional and mechanistic heterogeneity within the SPP/SPPL family as anticipated. To what extent this may point to unknown roles of SPPL2a/b/c is not clear yet. Similar to γ-secretase, most of the identified SPP and SPPL2a/b substrates also suggest a requirement of a short substrate ectodomain that is generated by a preceding proteolytic processing. The recently reported processing of HO-1 by SPP (Boname et al., 2014) has highlighted TA proteins as substrate candidates not only of SPP, but potentially also of other members of this protease family. TA proteins exhibit a transmembrane segment in type II topology and an extremely short C-terminal extracellular/luminal domain, usually just comprising a few residues. Therefore, these proteins fulfil the basic requirements to be naturally short SPP/SPPL substrates which could be subjected directly to the intramembrane cleavage without any preceding proteolysis. Bioinformatic analysis has predicted altogether more than 400 TA proteins in the human genome (Kalbfleisch et al., 2007). Though the ERAD pathway and the proteasome have been implicated in the turnover of selected TA proteins (Walter et al., 2001), the mechanisms how homeostasis of these proteins is maintained, in particular in the late secretory and endocytic pathway, is currently poorly understood. Based on the reported SPP-mediated cleavage of

HO-1 as well as CYB5A, RAMP4 and RAMP4-2, it is tempting to speculate that SPP/SPPL proteases could play a significant role in this process. However, beyond these substrates, which may be regarded as a proof-of-principle (Boname et al., 2014; Hsu et al., 2015), experimental evidence that other SPP/SPPL family members than SPP can cleave TA proteins is currently pending. Future studies will be required to evaluate this interesting hypothesis.

Another unresolved question is how intramembrane cleavage of TA proteins like HO-1 is regulated. Since the proteolytic release of HO-1 from the membrane can critically influence cellular proliferation, a tight regulation can be expected. However, currently, no protease-intrinsic mechanisms influencing the activity of SPP/SPPL proteases are known. For a long time, the paradigm in the RIP field has been that regulatory mechanisms primarily target those proteases, e.g. of the ADAM family, which mediate the ectodomain shedding, thus, the first step of the RIP cascade. The subsequent intramembrane cleavage of the remaining membrane-bound stub was then assumed to occur constitutively. It is unclear how, for instance, a constitutively active SPP protease could titrate levels of a TA substrate protein, which resides in the same cellular compartment, without initiating uncontrolled degradation. Therefore, it seems likely that yet unknown mechanisms either directly modulate the activity or expression levels of certain SPP/SPPL proteases, or control the interaction of individual substrates with these enzymes in a more selective way. In this context, it is of particular interest that such a regulatory mechanism was recently identified for the *Drosophila* serine protease rhomboid-4 (Baker and Urban, 2015). Calcium binding to cytosolic loops of this enzyme promotes substrate gating and thereby enhances proteolysis (Baker and Urban, 2015).

Similar considerations apply to the function of SPPL3 which was surprisingly found to directly cleave type II-oriented substrate proteins with large ectodomains (Kuhn et al., 2015; Voss et al., 2014). It is unclear what imparts this property on SPPL3, which is based on current knowledge not shared by other family members, in particular SPPL2b. However, it

cannot be entirely excluded that this capability of SPPL2a/b has not been recognized yet because it is limited to selected, currently unknown substrates or a certain physiological context. Mechanistically, it is not clear why SPPL3 is capable of accepting substrates with long bulky ectodomains. Comparing the topology of SPPL3, SPPL2a and SPPL2b it is obvious that SPPL2a/b exhibit a large luminal/extracellular N-terminal domain, which in SPPL3 comprises less than 10 amino acids. In a very simplistic model, the "bulky" N-terminus in SPPL2a/b could act as a gatekeeper like nicastrin in the γ-secretase complex and block entry of substrate proteins with large ectodomains. Detailed structure-function analysis, including the generation of chimeric proteases will be required to evaluate this concept in detail.

An even less well explored area is the putative capability of SPP/SPPL proteases to proteolyse polytopic transmembrane proteins. Though it does not seem obvious how a transmembrane segment which is part of a multi-pass membrane protein could access the catalytic center of an intramembrane protease, the glutamate receptor subunit 3 (GR3) (Meyer et al., 2003) and polycystin-1 (Merrick et al., 2012), both multi-pass transmembrane proteins, were reported to be cleaved by γ-secretase. In both cases distinct processing events generate defined cleavage fragments. The catalytic activity of Ypf1, the yeast orthologue of SPP, is critical for the ERAD-mediated turnover of the zinc transporter Zrt1, a protein with eight transmembrane segments, as well as several other nutrient transporters (Avci et al., 2014; Avci and Lemberg, 2015). This observation was interpreted as a direct proteolytic cleavage of the polytopic substrate protein by the intramembrane protease Ypf1, which rather unselectively produces several cleavage products and degradation intermediates. According to the proposed model, multiple cleavage events by intramembrane proteases, possibly in a concerted action by different I-CLIPs, could represent a general cellular mechanism to facilitate the removal and extraction of membrane proteins from the ER membrane during the

ERAD process (Avci and Lemberg, 2015). In yeast as well as mammalian cells, membrane protein degradation does not only take place in the ER but also at the plasma membrane and within the endocytic system where the abundance of many G protein-coupled receptors and nutrient transporters is controlled by degradative pathways (Babst and Odorizzi, 2013; Cottrell, 2013; Hanyaloglu and von Zastrow, 2008; MacGurn et al., 2012). This is accomplished by lysosomal degradation following sorting of these proteins into intraluminal vesicles by the sequential action of the Endosomal Sorting Complexes Required for Transport (ESCRT) (Piper and Katzmann, 2007). In case that SPPL2a and SPPL2b would exhibit an activity on polytopic membrane proteins similar to Ypf1, these proteases could contribute significantly to membrane protein turnover in the endocytic system, complementing the role of the ESCRT pathway. However, this remains to be investigated. The SPP-mediated liberation of a peptide from the C-terminal transmembrane segment of the ceramide synthase Trh4 (Oliveira et al., 2013) may be the first indication that also mammalian SPP is capable of accepting multi-pass substrate proteins. The physiological relevance of such cleavage events in mammalian cells as compared to other components of the ERAD pathway needs to be determined.

Proving direct proteolytic cleavage of a substrate by a certain protease in a cellular context is inherently difficult. Thus, indirect effects of yeast SPP on ERAD components and/or proteases which then control and mediate the proteolytic degradation of the Zrt1 transporter may not be fully excluded. It also seems conceivable that proteolytic cuts in the loops of a multi-pass membrane protein may be needed to initiate the attack of the intramembrane proteases by providing single-spanning fragments. Unambiguous evidence for the particular role of Ypf1 can only be provided by reconstituting proteolysis *in vitro* with purified proteases and substrates. Though challenging due to the special requirements of intramembrane proteases, this has been achieved for γ-secretase and also rhomboid proteases,

which are intramembrane serine proteases (Edbauer et al., 2003; Urban and Wolfe, 2005). Furthermore, detergent-solubilised SPP from yeast microsomes has been found to be proteolytically active (Weihofen et al., 2002) and expression and purification of functional SPP from E.coli has been reported (Narayanan et al., 2007). However, for none of the SPPL proteases an *in vitro* assay has been established yet. This is currently a major limitation in the field which needs to be approached. Beyond clarifying a direct action of SPPLs on selected substrates like multi-pass membrane proteins, this would also be a valuable tool to further analyse substrate requirements of SPPL proteases, in particular for those family members that are not localized in the ER. Mutagenesis of substrate proteins or proteases often interferes with their subcellular targeting resulting in an ER retention of the mutants (Hüttl et al., 2016) which then *per se* prevents proteolysis. A set-up where proteases and substrates are brought together *in vitro* would uncouple this and allow to directly assess substrate cleavability independent of substrate trafficking. Furthermore, also the testing and evaluation of novel inhibitory compounds would be greatly facilitated. Currently, this also relies on cellular reporter assay systems, for instance based on β-galactosidase enzyme fragment complementation that was used to detect SPPL2a/b-mediated proteolysis (Mentrup et al., 2015). Assay systems utilising a Gal4/VP16 reporter have been successfully employed for SPP substrates (Dev et al., 2006), but were not applicable to monitor cleavage by SPPL2a/b due to the size of the tags (Mentrup et al., 2015).

Despite some progress has been made in unravelling the substrate spectra of SPP/SPPL proteases, the current list (Table 1) is most likely incomplete. With regards to SPPL2a, the still rather short collection of known substrates, many of them with specialized functions in immune cells, does not sufficiently explain why this protease is so ubiquitously expressed (Schneppenheim et al., 2014b). Similarly, the molecular function of SPPL2b in the central nervous system, where it exhibits by far the highest abundance in mice

(Schneppenheim et al., 2014b), is not readily disclosed by the current substrate repertoire. Altogether this strongly advocates that major efforts for search and identification of further substrates are needed. In particular, with regard to novel protein classes like TA proteins or multi-pass membrane proteins, some interesting discoveries may be anticipated. In addition to defining the physiological roles of SPP/SPPL proteases, this will also clarify on a broader basis how pronounced the mechanistic differences between the individual SPP/SPPL protease are.

In conclusion, the emerging picture clearly demonstrates that SPP/SPPL proteases are more than just a functional counterpart of γ-secretase with an inverted topology. The functional diversity within this family is much greater than initially anticipated and we are just beginning to fully appreciate the important regulatory functions of these proteases.

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TABLES

Table 1 List of currently known substrates of SPP/SPPL intramembrane proteases

I-CLIP	Substrate type	Substrates	Reference
SPP	Signal Peptide	Prolactin (bovine)	(Martoglio et al., 1997; Weihofen et al., 2000)
		HIV gp160 (viral)	(Martoglio et al., 1997; Weihofen et al., 2000)
		MHC class I (HLA-A*0301)	(Lemberg et al., 2001)
		Calreticulin (rat)	(Lemberg and Martoglio, 2002)
		VSVG (viral)	(Lemberg and Martoglio, 2002)
		Crumbs (*D. melanogaster*)	(Kilic et al., 2010)
		Pro-calcitonin	(El Hage et al., 2008)
		Eosinophil cationic protein	(Wu and Chang, 2004)
		BUNV Pre-Gn (viral)	(Shi et al., 2016)
		HSP101 (*P. falciparum*)	(Baldwin et al., 2014)
	Type II protein	Xbp1u	(Chen et al., 2014)
		SrbA (*A. nidulans*)	(Bat-Ochir et al., 2016)
	Multi-pass	IgSF1	(Robakis et al., 2008)
		TRH4	(Oliveira et al., 2013)
		HCV polyprotein (viral)	(McLauchlan et al., 2002)
		Zrt1 (*S. cerevisiae*)	(Avci et al., 2014)
	Tail-anchored	Heme oxygenase 1	(Boname et al., 2014; Hsu et al., 2015)
		CYB5A	(Boname et al., 2014)
		RAMP4	(Boname et al., 2014)
		RAMP4-2	(Boname et al., 2014)
	Retrotranslocation	MHC class I	(Loureiro et al., 2006)
SPPL2a	Type II protein	TNFα	(Fluhrer et al., 2006; Friedmann et al., 2006)
		ITM2B	(Martin et al., 2008)
		FasL	(Kirkin et al., 2007)
		CD74	(Beisner et al., 2013; Bergmann et al., 2013; Schneppenheim et al., 2013; Schneppenheim et al., 2014a)
		TMEM106B	(Brady et al., 2014)
		NRG1 type III	(Fleck et al., 2016)
	Type III protein	FVenv (viral)	(Voss et al., 2012)
SPPL2b	Type II protein	TNFα	(Fluhrer et al., 2006; Friedmann et al., 2006)
		ITM2B	(Martin et al., 2008)
		Transferrin Receptor 1	(Zahn et al., 2013)
		NRG1 type III	(Fleck et al., 2016)

	Type III protein	FVenv	(Voss et al., 2012)
SPPL3	Type II protein	GnT-V	(Voss et al., 2014)
		β3GnT1	(Voss et al., 2014)
		β4GalT1	(Voss et al., 2014)
		α-Man I	(Voss et al., 2014)
		XYLT2	(Kuhn et al., 2015)
		HS6ST1	(Kuhn et al., 2015)
		HS6ST2	(Kuhn et al., 2015)
		B3GALT6	(Kuhn et al., 2015)
		SGK196	(Kuhn et al., 2015)
		EXTL3	(Kuhn et al., 2015)
		Cant1	(Kuhn et al., 2015)
		GalNacT10	(Kuhn et al., 2015)
		OGFOD3	(Kuhn et al., 2015)
		ASPH	(Kuhn et al., 2015)
		TOR1AIP1	(Kuhn et al., 2015)
	Type III protein	FVenv	(Voss et al., 2012)

FIGURE LEGENDS

Fig. 1. *Intracellular distribution of the different mammalian SPP/SPPL proteases.* The predominant subcellular localisations of Signal peptide peptidase (SPP) and the four homologous SPP-like (SPPL) proteases SPPL2a, SPPL2b, SPPL2c and SPPL3 are depicted.

Fig. 2. *Functions of Signal Peptide Peptidase (SPP).* Initially identified as an enzyme processing signal peptides after their release from nascent proteins, SPP is also involved in the ERAD pathway and the processing of viral proteins. The identification of Heme oxygenase-1 (HO-1) as SPP substrate has demonstrated the capability of SPP to cleave tail-anchored proteins and proteolytic activity of Ypf 1, the yeast SPP orthologue, was found to be critical for the turnover of the zinc transporter Zrt1, a multi-pass transmembrane protein.

Fig. 3. *Cleavage mechanisms within the SPP/SPPL family.*

All currently identified substrates of SPPL2a/b undergo a Regulated Intramembrane Proteolysis (RIP) as depicted here for the processing of TNFα. This involves cleavage of the C-terminal ecto-/ luminal domain, which is mediated by either ADAM metalloproteases at the cell surface thereby leading to the secretion of the ectodomain or, by endosomal/lysosomal proteases in intracellular compartments. The remaining N-terminal fragment (NTF) is membrane-bound and represents the actual substrate for the intramembrane cleavage by SPPL2a/b. Thereby, an intracellular domain (ICD) is released into the cytosol. In contrast, SPPL3 directly mediates shedding of type II transmembrane proteins like glycosyltransferases. No preceding ectodomain trimming is required prior to the intramembrane cleavage.

Figure 1

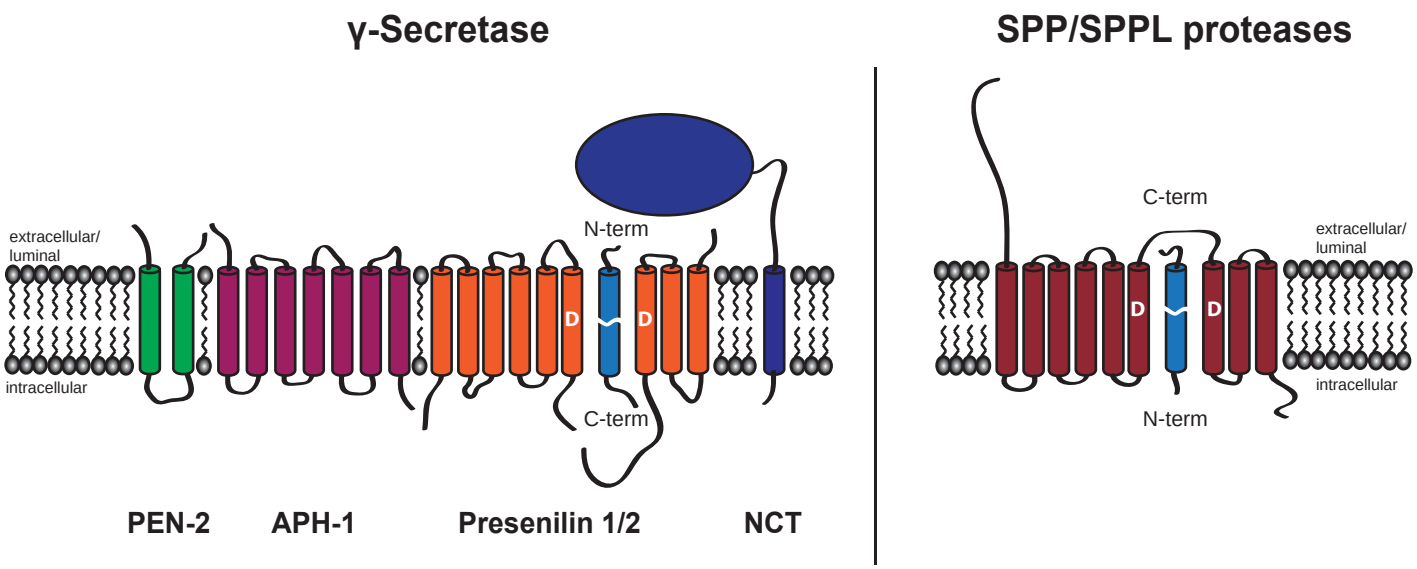
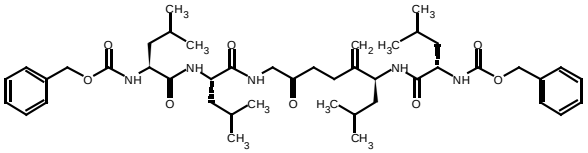

γ-Secretase
SPP/SPPL proteases
extracellular/
luminal
intracellular
N-term
C-term
D
D
PEN-2
APH-1
Presenilin 1/2
NCT
C-term
N-term
extracellular/
luminal
intracellular
D
D

Figure 2

$(Z\text{-}LL)_2$-ketone



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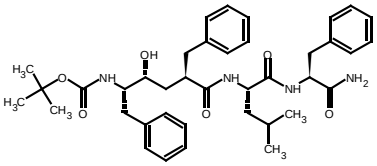

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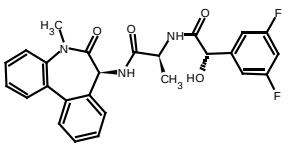
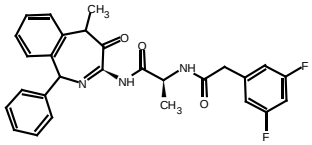
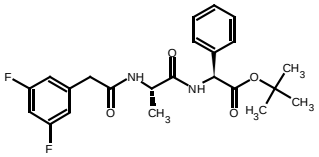


Compound E



DAPT



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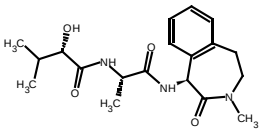


Figure 3

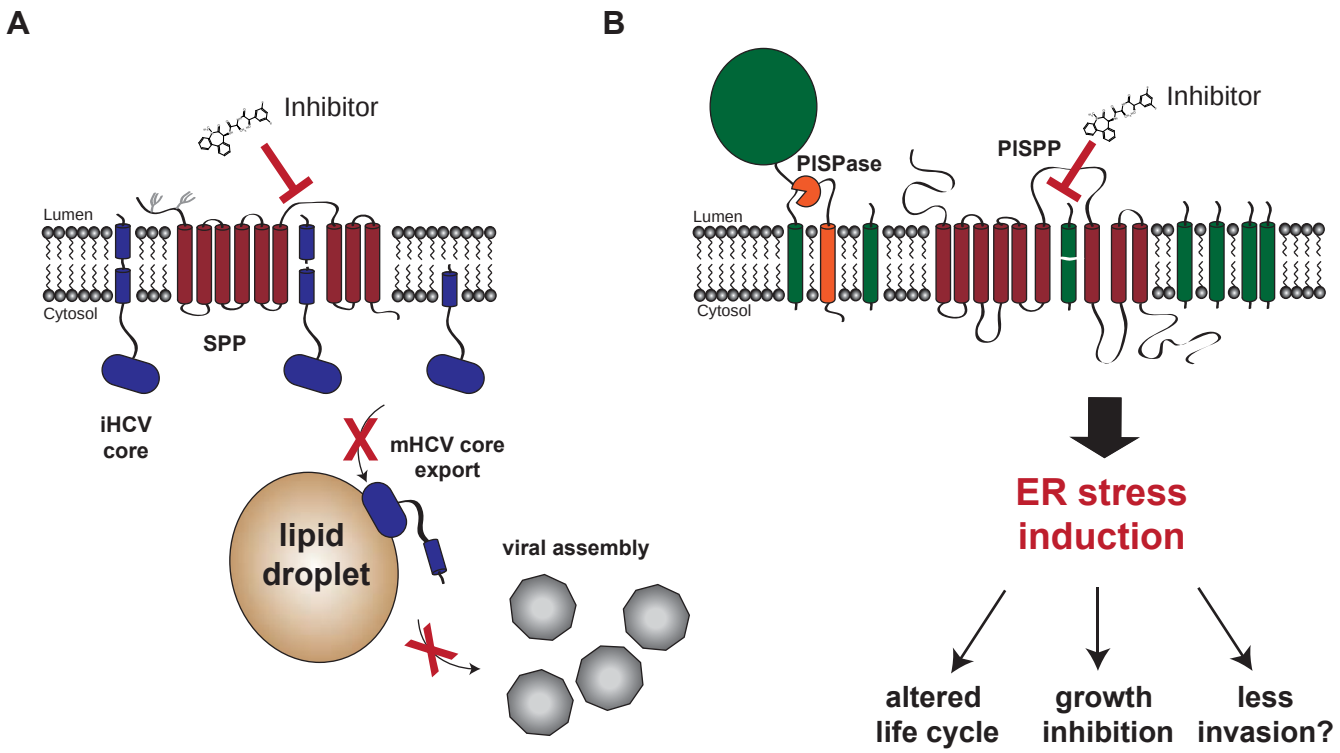
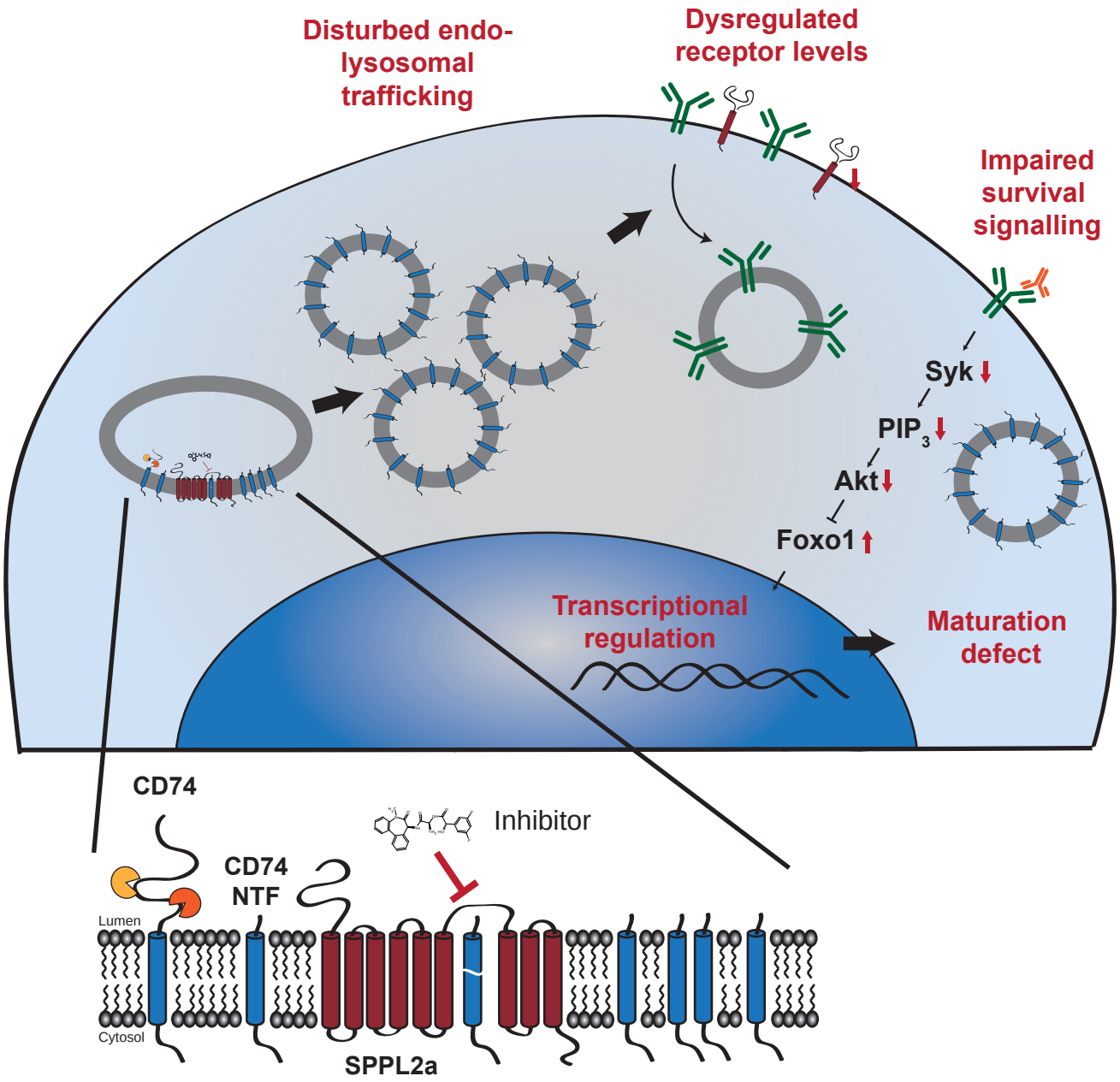

A
Inhibitor
Lumen
Cytosol
SPP
iHCV
core
mHCV core
export
lipid
droplet
viral assembly
B
PISPase
PISPP
Inhibitor
Lumen
Cytosol
ER stress
induction
altered
life cycle
growth
inhibition
less
invasion?

Figure 4


Disturbed endo-lysosomal trafficking
Dysregulated receptor levels
Impaired survival signalling
Syk
PIP3
Akt
Foxo1
Transcriptional regulation
Maturation defect
CD74
Inhibitor
CD74 NTF
Lumen
Cytosol
SPPL2a