Mechanism, specificity, and physiology of signal peptide peptidase (SPP) and SPP-like proteases

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Highlights

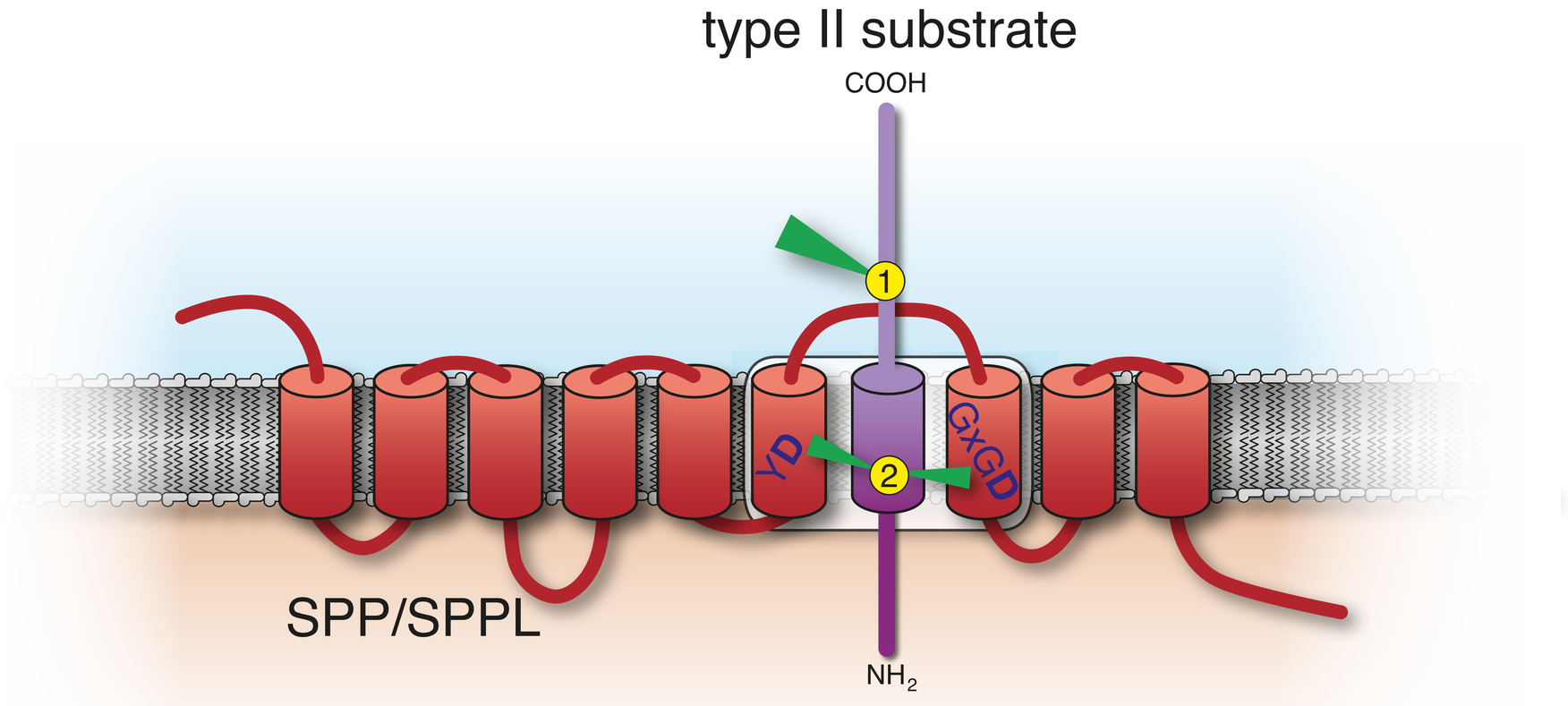
SPP and SPPL are intramembrane-cleaving proteases of the GxGD type

SPP/SPPLs are conserved among eukaryotic species and have evolved distinct functions

In humans, SPP and SPPLs are involved in diverse physiological processes

SPP/SPPLs cleave type II membrane proteins within their transmembrane domain

SPP/SPPLs cleave the transmembrane domain of their substrates in a sequential manner


type II substrate
COOH
1
YD
2
GxGD
SPP/SPPL
NH2

Mechanism, specificity, and physiology of signal peptide peptidase (SPP) and SPP-like proteases

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Abstract

Signal peptide peptidase (SPP) and the homologous SPP-like (SPPL) proteases SPPL2a, SPPL2b, SPPL2c and SPPL3 belong to the family of GxGD intramembrane proteases. SPP/SPPLs selectively cleave transmembrane domains in type II orientation and do not require additional co-factors for proteolytic activity. Orthologues of SPP and SPPLs have been identified in other vertebrates, plants, and eukaryotes. In line with their diverse subcellular localisations ranging from the ER (SPP, SPPL2c), the Golgi (SPPL3), the plasma membrane (SPPL2b) to lysosomes/late endosomes (SPPL2a), the different members of the SPP/SPPL family seem to exhibit distinct functions. Here, we review the substrates of these proteases identified to date as well as the current state of knowledge about the physiological implications of these proteolytic events as deduced from *in vivo* studies. Furthermore, the present knowledge on the structure of intramembrane proteases of the SPP/SPPL family, their cleavage mechanism and their substrate requirements are summarised.

Keywords:

Regulated intramembrane proteolysis, intramembrane-cleaving proteases, GxGD proteases, signal peptide peptidase, signal peptide peptidase-like

1. Introduction

Signal peptide peptidase (SPP) and its homologues, the signal peptide peptidase-like proteases (SPPLs) are aspartyl intramembrane-cleaving proteases (I-CLiPs). SPP and SPPLs are closely related to presenilins, which form the active subunit of the γ-secretase complex. Together with the more distantly related bacterial type IV prepilin peptidases (TFPPs) and their archaeal homologues, the pre-flagellin peptidases (PFPs), they constitute the GxGD protease family of I-CLiPs (Fig. 1). All members of this family share a conserved GxGD motif within their catalytic centre [1-4]. GxGD proteases have turned out to be part of a fundamental sequential proteolytic processing pathway of single span transmembrane proteins, termed regulated intramembrane proteolysis (RIP) [5] (Fig. 2). While a genetic link between the γ-secretase complex and familial cases of Alzheimer‘s disease is well-documented for two decades already, the SPP/SPPL family has only recently been identified [4,6,7] and their likely diverse physiological functions have not yet been completely unravelled. The aim of this review is to give an overview of the biological diversity of SPP/SPPLs and to summarise our current knowledge of the mechanistic principles applied by this fascinating protease family.

2. The SPP/SPPL family

In 2002, SPP/SPPLs were first described independently by three groups [4,6,7]. Using bioinformatic analyses Ponting *et al.* and Grigorenko *et al*. identified a family of five protein-coding genes with homology to presenilins in the human genome [6,7]. At the same time a biochemical approach sought to identify the enzyme responsible for signal peptide cleavage leading to the identification of SPP and its homologues [4]. A proteolytic activity cleaving within signal peptide sequences following their liberation from nascent proteins by signal peptidase (Fig. 2B) had been observed before *in vitro*, for instance for the prolactin signal sequence [8,9]. Subsequently, a peptide-based inhibitor, 1,3-di-(*N*-

carboxybenzoyl-L-leucyl-L-leucyl) amino acetone ((Z-LL)$_2$ ketone), was described to target this proteolytic activity and to inhibit signal peptide processing [10]. Using a photocrosslinkable derivative of (Z-LL)$_2$ ketone, Weihofen *et al.* were able to attribute this proteolytic activity to an at that time uncharacterised human multi-pass transmembrane protein. Sequence analysis revealed its relationship to the presenilins and uncovered the homologous SPPLs (Fig. 3) [4].

SPP/SPPLs are highly conserved and can be found in eukaryotes, including fungi, protozoa, plants, and animals, highlighting their physiological importance [4,6,7]. Interestingly, the number and nature of SPP/SPPL paralogues differs among these organisms suggesting that they have evolved diverse functions. While in mammals five members (SPP, SPPL2a, SPPL2b, SPPL2c, and SPPL3) of the SPP/SPPL family have been described (Fig. 3) [11], in zebrafish, for instance, only one representative of the SPPL2 subfamily is found [12]. *Drosophila* species possess only two SPPLs, one most closely related to human SPP [13] and the other to human SPPL3 [14] and the genome of *Plasmodium falciparum* even solely encodes a single family member, an orthologue of human SPP [15]. In contrast, plants, like the monocot *Arabidopsis thaliana* or the dicot rice (*Oryza sativa*), encode a more complex set of SPP/SPPLs [16].

Phylogenetic analysis of SPP/SPPLs in various species reveals that most of them group with human SPP/SPPL family members (Fig. 4). Notable exceptions include *C. elegans* and *S. cerevisiae*, respectively. *C. elegans* encodes five SPP/SPPL family members, termed imp [17]. Imp-2 is an orthologue of human SPP [17]. Imp-1a, imp-1b and imp-1c differ only slightly and, like imp-3, are merely distantly related to mammalian and plant SPP/SPPLs. Like imp-1 and imp-3, YKL100c, the SPP/SPPL homologue in *S. cerevisiae,* does not clearly group with other SPP orthologues (Fig. 4). In line with this, no proteolytic activity on substrates based on human signal peptides was observed in yeast [4] and YKL100c deficient yeast was reported to be phenotypically normal [18]. This suggests that

the *C. elegans* and yeast SPP/SPPL homologues might have physiological functions distinct from those of the vertebrate SPP/SPPLs. Interestingly, plants share orthologues for animal SPP and SPPL3 [16,19]. In contrast, orthologues of human SPPL2a/b/c are only found in vertebrates [7], while plant SPPLs that exhibit no obvious relationship to animal SPP and SPPL3 form a distinct subfamily which is merely distantly related to vertebrate SPPL2 proteases (Fig. 4) [16].Bacteria do not possess proteins exhibiting marked homology to mammalian SPP or SPPLs. In bacteria, however, signal peptide cleavage does occur but has been attributed to proteases like SppA [20] or RseP [21] , which are both completely unrelated to SPP/SPPL GxGD proteases.

In contrast to bacteria, Archaeal genomes harbour in addition to the aforementioned PFPs other putative GxGD proteases, like *Archaeoglobus fulgidus* AF1952 [6] and many others [22], which are clearly related to presenilins and/or SPP/SPPLs. Presently, however, they cannot be clearly classified into either the SPP/SPPL or the presenilin subfamily as for instance their topological orientation requires experimental verification, Hence, it is tempting to speculate that these archaeal proteases may constitute a common ancestor of the two families of eukaryotic GxGD proteases.

3. Physiological functions of SPP/SPPLs

There is accumulating evidence that all known SPP/SPPL proteins are active intramembrane-cleaving aspartyl proteases as they are either inhibited by transition state analogue inhibitors or their activity is impaired upon mutagenesis of the catalytic aspartate residues [4,23-25]. However, non-catalytic physiological functions of these proteins cannot be excluded at the present time. In addition, for various enzyme classes inactive pseudoenzymes have been observed. Such pseudoenzymes may have evolved very important physiological functions that might rely on properties they share with their active relatives, as exemplified by the transmembrane domain (TMD)-binding capacity of inactive

rhomboid pseudoproteases (discussed in [26]). So far, however, SPP/SPPL family members lacking the intact active site motifs have not been identified.

3.1 SPP

SPP, the founding member of the family, is actively retained in the endoplasmic reticulum (ER) due to its KKXX retention signal in animals [12,24,27]. Interestingly, a splice isoform of SPP found in mice lacks the retention signal and is accordingly observed at the plasma membrane [28]. In plants, SPPs likewise localise to the ER [16,19], but *Arabidopsis* SPP lacks a conventional retention signal. Similarly, Plasmodium SPP also was observed to localise to the ER throughout the parasite life cycle [18,29]. SPP appears to have evolved very diverse functions and was initially identified due to its capability to cleave signal peptides *in vitro* and in cellular model systems. Substrates described so far are listed in Tab.1. In some cases, SPP-mediated intramembrane cleavage of signal peptides facilitates their removal from the ER membranes and subsequent degradation [30] or leads to detoxification [31]. Apart from that, signal peptides and their SPP cleavage products may possess post-targeting functions that possibly have crucial roles in diverse cellular processes [32]. In particular, SPP activity is implicated in immunosurveillance by generating peptides presented on polymorphic MHC class I [33] and non-polymorphic HLA-E molecules [34]. Interestingly, a number of antigenic MHC-presented peptides have been shown to originate from signal peptide sequences of pre-proteins [35]. In principle, intramembrane cleavage of such signal peptides within their membrane-spanning segment could lead to the release of antigenic fragments into the cytosol and/or the ER lumen, allowing for transporter associated with antigen presentation (TAP)-dependent and TAP-independent MHC class I loading, respectively (discussed in [36]). Lemberg *et al.* initially reported $(Z\text{-}LL)_2$ ketone-dependent cleavage of HLA-A*0301 signal peptides *in vitro* and showed that SPP-mediated processing of HLA-A*0301 to nonamer peptides and TAP-

dependent transport thereof was required for surface presentation by HLA-E [34]. Likewise, HLA-E surface levels could be reduced in cell culture following treatment with (Z-LL)$_2$ ketone [37]. Proper surface expression of HLA-E is thought to prevent cytotoxic action of natural killer cells, but so far the direct role of SPP in such a process has not been proven *in vivo*. In addition to MHC signal peptides, another study described a cytotoxic T cell clone obtained from tumour-infiltrating lymphocytes of a human non-small-cell lung carcinoma which specifically recognised the C-terminal portion of the calcitonin pre-protein signal peptide [33]. Generation of this peptide in the tumour was TAP-independent, yet required successive proteolytic cleavage of the pre-protein by signal peptidase and SPP [33]. Interestingly, cells of this tumour were later shown to display low levels of endogenous TAP, which is frequently observed in lung cancers, and restoration of TAP levels reduced presentation of the signal peptide-derived peptide, probably due to competition with proteasome/TAP-dependently generated peptides [38]. Similar MHC class I-presented peptides derived from signal sequences have already been observed earlier [35], suggesting that in fact successive cleavage of signal peptides by signal peptidase and SPP might constitute an alternative pathway applied to generate peptides for MHC class I presentation. We therefore anticipate that future work will identify additional immunogenic peptide fragments generated by SPP activity fromother protein substrates.

Other post-targeting functions of signal sequences are also well established (reviewed in [32]). Interestingly, however, such post-targeting functions of signal peptides not necessarily always depend on SPP activity. Fragments may be released by alternative mechanisms as exemplified by the mouse mammary tumour virus protein Rem which is released from membranes in a (Z-LL)$_2$ ketone-insensitive manner [39].

Notably, *in vitro* cleavage of SPP model substrates based on vertebrate signal peptides is not restricted to human SPP, but has also been shown for SPPs from other distinct

species, like *D. melanogaster*, suggesting that the signal peptide degrading activity of SPPs is conserved among species [13,40]. In the plant *Medicago truncatula*, SPP was observed to be co-regulated with a family of nodule-specific secretory proteins [41] suggesting that either also plant SPP could be functionally linked to signal peptide processing or that it can affect secretion by an alternative mechanism not yet understood. Further studies are required to address whether processing and turnover of signal peptides is conserved throughout species.

In addition to the processing of classical N-terminal signal peptides, SPP may also cleave within internal membrane-spanning sequences. One such example is the immunoglobulin-like protein IgSF1 which is cleaved within an internal stop transfer sequence in the ER membrane by signal peptidase and subsequently by a $(Z\text{-}LL)_2$ ketone-sensitive protease, most likely SPP [42]. Similarly, the N-terminal core domain of hepatitis C virus (HCV) and related members of the *Flaviridae* is initially proteolytically released from the respective polyprotein and cleaved by signal peptidase and SPP within a hydrophobic internal stretch [43-45]. This cleavage is required for efficient virus propagation [46-48], suggesting inhibition of SPP-mediated HCV core cleavage as a putative therapeutic approach in HCV infections. In addition, the SPP orthologue expressed in *P. falciparum* is considered a promising target for treatment of malaria, since pharmacological interference with SPP proteolytic activity using $(Z\text{-}LL)_2$ ketone, cross-reactive γ-secretase inhibitors, or newly developed potent SPP inhibitors severely inhibits *Plasmodium* growth [15,18,29,49,50]. Treatment with SPP inhibitors is also toxic to other protozoan species, suggesting that SPP could constitute a pan-protozoan drug target [18]. However, data on a potential inhibition of SPP-mediated *Plasmodium* invasiveness remains controversial [29,50] (reviewed by Sibley [51] in this issue).

While all the above-mentioned observations clearly rely on its proteolytic function, SPP has also been linked to dislocation of membrane proteins from the ER. For example, the

human cytomegalovirus (HCMV) protein US2, a viral immunoevasin that targets MHC class I molecules for proteasomal degradation by inducing their dislocation from the ER membrane, associates with SPP [52]. In addition, RNAi-mediated reduction of endogenous SPP levels led to a reduced induction of US2-dependent MHC dislocation [52]. Protein disulfide isomerase (PDI) was recently proposed to be involved in the US2 pathway of MHC dislocation and associated with SPP [53]. Similarly, the E3 ubiquitin ligase TRC8 (translocation in renal carcinoma, chromosome 8 gene) was identified in a RNAi screen as essential mediator of US2-mediated MHC translocation and was also observed to form a heteromeric complex with US2 and SPP [54]. The exact mechanism of SPP-dependent pathway of MHC class I translocation has not been fully unravelled yet. Often, viruses hijack host cellular pathways suggesting that, in fact, SPP could be an integral part of a cellular ER-associated degradation (ERAD) pathway. This notion is supported by a recent integrative study mapping the human ERAD networks [55]. In line with this, SPP was described to associate with misfolded or misassembled membrane proteins in the ER [56,57]. A crucial unresolved question is whether the role of SPP in ERAD relies on its intrinsic activity as an aspartyl protease or is mediated independently thereof. Recently, however, work on *Plasmodium*, which harbours orthologues for only a few known mammalian ERAD factors including *Plasmodium falciparum* SPP (PfSPP), revealed that PfSPP-mediated ERAD is targeted by small molecule inhibitors which interfere with its proteolytic activity leading to *Plasmodium* lethality [18], suggesting that indeed the role of SPP in ER retrotranslocation indeed relies on its proteolytic activity. In order to unravel the precise role of SPP in cellular ERAD pathways more detailed studies are required.

In addition, data from animal models suggest that SPP has an essential function during development [12,13,17]. For instance, dsRNA-induced reduction of imp-2, the SPP orthologue in *C. elegans*, led to embryonic lethality and a molting deficit. This phenotype

mimicked abnormalities observed in other loss-of-function models of genes involved in steroid homeostasis and signalling [17]. Similarly, anti-sense-mediated SPP knockdown in zebrafish resulted in developmental abnormalities and neuronal cell death [12]. Likewise, in *Drosophila*, *spp* deficient larvae had abnormal tracheae and died early [13]. The latter phenotype was rescued by exogenous addition of active SPP but not by a catalytically inactive mutant, suggesting that proteolytic activity is crucial for the SPP function in development [13]. These observations implicate that SPP activity is strictly required for eukaryotes and that SPP certainly has a very fundamental cellular function and, hence, SPP deficiency is not tolerated. Currently, however, the exact biochemical and cellular consequences of SPP dysfunction are unknown and it is unclear whether they are related to the putative roles of SPP in signal peptide processing, in ERAD pathways, in both processes, or to so far unknown functions.

3.2 SPPL2a, SPPL2b, and SPPL2c

Unlike the other members of the SPP/SPPL family, the SPPL2 subfamily members comprise a N-terminal signal sequence and a complexly glycosylated luminal/extracellular domain N-terminal of the core nine-TMD segment common to all SPP/SPPLs (Fig. 3) [58]. SPPL2a and SPPL2b are synthesized at the endoplasmic reticulum and then follow the secretory pathway. Exogenously expressed human SPPL2a was observed in the endolysosomal compartment in HEK293 cells [24]. Confirming these results, endogenous SPPL2a was recently described to reside in lysosomes/late endosomes of murine embryonic fibroblasts and lysosomal sorting of SPPL2a critically depends on a canonical C-terminal tyrosine-based sorting signal which is not present in SPPL2b [59]. Upon overexpression in HeLa cells, minor, but significant amounts of SPPL2a were present at the cell surface [59]. Further studies may be needed to systematically analyse this under endogenous conditions in different cell types and tissues and to assess any functional

relevance. In contrast, overexpressed SPPL2b was detected primarily at the cell surface [24,59], but also in the Golgi [25] and to some extent in endolysosomal vesicles [12]. To finally clarify the subcellular localisation of SPPL2b, studies on endogenous SPPL2b need to be performed.

Overexpression or RNAi-mediated knock down of either SPPL2a or SPPL2b in cell culture models demonstrates that both proteases are able to cleave selected type II membrane proteins like for instance TNFα [23,24] (Tab. 1 lists all SPPL2a and/or SPPL2b substrates identified so far). Under the same conditions, however, Fas ligand (FasL/CD95L), another member of the TNF superfamily, was reported to be cleaved exclusively by SPPL2a [60]. Recently, the invariant chain (Ii, CD74) of the MHC II complex was identified as a novel substrate of murine SPPL2a [61-63]. It has not been investigated yet, if SPPL2b is also able to proteolyse CD74. For all substrates of SPPL2a/b reported to date (Tab. 1), the fate and putative biological function of the resulting cleavage products is a central unresolved question. SPPL2a-mediated intramembrane proteolysis of FasL liberates a FasL intracellular domain (ICD), which bears SH3 domain binding sites and was suggested to translocate to the nucleus [60]. There, it was shown to inhibit lymphoid-enhancer binding factor-1 (Lef-1)-dependent transactivation of transcription [64]. Using a knock-in mouse model lacking the FasL ICD, the authors conclude that FasL ICD signalling negatively regulates activation-induced proliferation of B and T cells by diminishing phosphorylation of PLCγ, PKC and ERK1/2 [64]. However, in order to define the precise role of the FasL intramembrane cleavage in initiating or terminating these signalling pathways additional studies may be required. Similarly, SPPL2a/b-mediated intramembrane proteolysis of TNFα in bone marrow-derived dendritic cells was claimed to up-regulate transcription and secretion of IL-12, a Th1 cytokine [24]. Whether TNFα ICD translocates to the nucleus and directly activates transcription of the IL-12 gene or whether this is due an indirect effect remains to be shown. Furthermore, the *in vivo* relevance of this process is far from clear. It

may be anticipated that SPPL2a/b single and double deficient animals will help to clarify whether the IL-12 induction observed in bone marrow-derived dendritic cells has a fundamental role during the initiation of immune responses.

Recently, unambiguous *in vivo* relevance was demonstrated for the SPPL2a-mediated intramembrane cleavage of CD74 [61-63]. The main function of CD74 is to mediate assembly and subcellular targeting of MHC class II complexes. In the peptide-loading compartments, the luminal domain of CD74 is degraded by endosomal proteases [65]. In B lymphocytes of SPPL2a-deficient mice, the remaining membrane-bound N-terminal fragment (NTF) of CD74 accumulates [61-63]. This clearly highlights the absolute requirement of SPPL2a intramembrane cleavage for the turnover of this NTF. At the subcellular level, the apparently unphysiological amounts of this fragment significantly disturb membrane trafficking within the endocytic system as documented by massive ultrastructural changes [61]. Phenotypically, this manifests as a distinct developmental arrest of splenic B cell maturation at the transitional stage T1 leading to a deficiency of mature B cells in these mice in combination with significantly impaired functionality of the residual B cells [61-63]. A significant amelioration of these phenotypes at the cellular and subcellular level was observed by additional ablation of CD74 in *SPPL2a*$^{-/-}$ *CD74*$^{-/-}$ mice [61,62]. This clearly identifies a causative role of the accumulating CD74 fragment and reveals the important role of SPPL2a for controlling the levels of this fragment by initiating its degradation. Based on these findings, pharmacological inhibition of SPPL2a may represent a promising therapeutic approach for depleting and/or modulating B cells for treating autoimmune disorders. In addition, decreased numbers of CD8^{-} dendritic cells were observed in SPPL2a deficient animals and were similarly restored in *SPPL2a*$^{-/-}$ *CD74*$^{-/-}$ mice [62,63]. Interestingly, *in vivo* SPPL2a-mediated intramembrane proteolysis also appears to be critical for tooth enamel formation [66] but the exact molecular mechanisms underlying this observed phenotype are presently unclear.

In addition, a genome-wide association study linked a single nucleotide polymorphism on chromosome 15 close to the SPPL2a locus to the inflammatory disorders psoriasis and psoriatic arthritis [67]. In line with this, the authors also observed profound epidermal SPPL2a expression in sections of lesional and unaffected skin, yet a detailed characterisation of SPPLa's role in such skin disorders in currently missing.

Taken together, this highlights the physiological role of SPPL2a and SPPL2b within the hematopoietic system and for the regulation of inflammatory responses. Nonetheless, based on its ubiquitous expression [58], the list of hitherto identified SPPL2a/b substrates is certainly not yet complete. Therefore, the role of these proteases in other tissues needs to be addressed in future studies that certainly will lead to the identification of yet unknown substrates.

To date, the physiological function of SPPL2c is completely unknown. In fact, given its highly polymorphic and intron-less structure, the *SPPL2C* gene was discussed to be a recently evolved pseudogene [68]. Moreover, SPPL2c fails to cleave all known SPPL2a/b substrates when co-expressed in cellular model systems (Fluhrer *et al.* unpublished data) and to date still no substrates have been described that are selectively proteolysed by SPPL2c. Thus, although the active site motives of GxGD aspartyl proteases are conserved within SPPL2c [6,58], the final proof that SPPL2c is indeed a catalytically active member of the SPP/SPPL family is still missing.

3.3 SPPL3

SPPL3 is the smallest member of the SPPL family, is not glycosylated and localises to the Golgi network [24]. Initial studies in cellular model systems and *in vitro* described proteolytic activity of human SPPL3 towards a reporter construct [15]. The construct was based on the membrane-spanning stretch of HCMV gpUL40 which only after mutagenesis becomes a substrate for SPP [69]. In addition, the SPPL3 orthologue from *D.*

melanogaster was shown to cleave a synthetic peptide *in vitro* [40]. This peptide, in turn, was based on the bovine pre-prolactin sequence, another known SPP substrate [10,69]. Based on these observations, the high sequence homology, a very close evolutionary relationship of SPP and SPPL3 (Fig. 4) and the ER localisation of both proteases observed in an early study [12], it was suggested that SPP and SPPL3 have a redundant physiological function in signal peptide cleavage/degradation [12,15]. This notion, however, was recently heavily challenged, since catalytically inactive SPPL3 – in contrast to the respective SPP mutant – failed to associate with signal peptide substrates in a cellular model system [57]. Moreover, genomic ablation of both *D. melanogaster spp* and the respective SPPL3 orthologue, *sppL*, did not exacerbate the phenotype observed before in flies solely deficient for *spp* [14]. Recently, foamy virus envelope glycoprotein (FVenv) was shown to be endoproteolysed by human SPPL3 in a cell culture model system (Fig. 2C) [70].

Given its high evolutionary conservation in diverse organisms (see Fig. 4) it is nonetheless tempting to assume that SPPL3 has an important physiological function throughout these species, which still remains enigmatic. Unexpectedly, however, *in vivo* studies with SPPL3-deficient *Drosophila* have so far not observed an overt phenotype [14]. Merely in the developing zebrafish gripNA-mediated knock-down of SPPL3 led to neuronal cell death [12]. In addition, the *D. melanogaster* SPPL3 orthologue is widely expressed during early developmental stages, while at later stages mRNA levels are reduced [14], suggesting an important role during embryonic development.

4. Mechanism of SPP/SPPL-mediated intramembrane proteolysis

4.1 Structure and topology of SPP/SPPLs

All SPP/SPPL family members are predicted to be multi-pass membrane proteins with nine TMDs (Fig. 3). The nine TMD topology has been anticipated from the homology to the presenilin family and the luminal and cytosolic orientation of the N- and C-termini, respectively, as well as the luminal orientation of the hydrophilic loop between TMD 6 and TMD 7 were confirmed experimentally [58]. Strikingly, the overall topology of SPP/SPPLs is inverted compared to presenilins (Fig. 1) [4,58,71]. Another distinction between SPP/SPPLs and presenilins is that upon incorporation into the high molecular weight complex presenilin is autoproteolytically cleaved [72] and thus activated [73]. In contrast, no autoproteolytic cleavage required for SPP/SPPL function has been observed so far. Due to the inverted topology the corresponding loop domain in SPP/SPPLs is exposed to the luminal/extracellular site [58,71]. In addition, it is comparably short and lacks the hydrophobic amino acid sequence conserved in presenilins. Therefore it is likely, that the loop between TMD 6 and TMD 7 of SPP/SPPLs does not interfere with the catalytic centre and thus activation of SPP/SPPLs through autoproteolysis is not necessary. As in all known aspartyl ICLiPs, the two catalytic aspartic residues required for the proteolytic activity of SPP/SPPL proteases, are embedded in conserved (Y/F)**D** and G(L/I/F)G**D** amino acid motifs in TMD6 and TMD7, respectively (Fig. 3). Mutation of either aspartyl residue inactivates the proteolytic activity of the respective SPP/SPPL [4,23,24,60,70] but, in contrast to presenilins, exogenous expression of such SPP/SPPL mutants does not affect the levels of the respective endogenous proteases [25,73]. In addition to the amino acid motifs in TMD 6 and TMD7 all SPP/SPPLs comprise a conserved QPALLY motif in the N-terminal part of TMD 9 [4,6,7]. By the use of cysteine scanning methods, a similar motif in TMD 9 of presenilin was shown to be located close to the active site aspartic residue in TMD 6 [74,75]. Moreover, mutation of the proline, alanine or leucine in either SPP or presenilin significantly affected the catalytic activity of the respective enzyme [76-78], suggesting that this conserved motif is of crucial importance for catalysis, substrate

interaction, or active site architecture. Yet the formal proof whether the QPALLY motif in TMD 9 of all other SPP/SPPL family members also contributes to their catalytic activity is missing.

While presenilins strictly require three other proteins (anterior pharynx defective 1(Aph-1), presenilin enhancer 2 (Pen-2), and nicastrin) to form the proteolytically active γ-secretase complex [73,79], exogenous expression of SPP/SPPLs in cellular model systems strongly increases the processing of the respective substrates [23,25,57,60,70]. This suggests either that SPP/SPPLs are proteolytically active as monomers or homomeric dimers/multimers or that potential additional cellular co-factors needed for proteolytic activity of SPP/SPPLs are highly abundant. Endogenous SPP from human cell lysates and brain tissue has been detected as a SDS-resistant but heat-sensitive dimer [12,80,81], which was specifically labeled by an active site-directed photoaffinity probe, supporting the hypothesis that the SPP dimer is catalytically active [80]. Fluorescence lifetime imaging microscopy of intact cells expressing two distinctly fluorophore-labelled species of human SPP also provides evidence that human SPP forms homodimers [82]. These homodimers, however, were predominantly observed close to the plasma membrane, whereas, under physiological conditions, SPP is localized to the ER via a KKXX retention signal (discussed above). More recently, however, analysis of SPP and SPPL2b on native gels revealed species with a significantly higher molecular weight than expected for the respective dimers [57,81]. The molecular weight of these complexes suggests the formation of homotetrameric complexes of SPP [81]. Interestingly, also the archaeal PSH was crystallised as a tetrameric protein [83]. Such SPP homotetramers were also observed when human SPP was expressed in insect cells. By affinity purification of these tetramers recently the first structural data on SPP at a resolution of 22 Å following 3D reconstitution of electron microscopic images of negatively stained SPP particles were obtained [81]. According to this, the SPP complex has four low density interior regions as well as four

concave regions which, the authors hypothesize, could correspond to putative active and substrate entry site, respectively [81]. Interestingly, it is claimed that the C-terminus (i.e. TMD 6 to 9) of SPP harbours both the active as well as the initial substrate binding site, since a recombinantly expressed C-terminal fragment of SPP was sufficient to cleave a model substrate in DDM-solubilised *E. coli* membranes [40]. In line with this, the corresponding N-terminal SPP fragment could neither be targeted with helical peptides, known to bind the substrate "docking site", nor with transition state analogue inhibitors, known to bind to the active site [81]. Nonetheless, the same N-terminal SPP fragment was able to disrupt the assembly of the homotetrameric complex, implying that the N-terminal region of SPP acts as internal scaffold to ensure homotetrameric complex assembly and proteolytic activity of the C-terminal core domain [81]. In addition, high molecular weight complexes other than a putative homotetrameric SPP complex have been described [57]. These complexes with a molecular weight two to three times larger than that of the homotetrameric complex seem to differ in regard to substrate binding and binding of misfolded/unfolded proteins [57]. Recently, in a proteomic analysis of SPP binding partners, vigilin, a cytoplasmic protein involved in RNA binding and protein translation control, was found to interact with SPP in a high molecular weight complex, yet it did not alter or affect proteolytic activity of SPP [84]. This may indicate that SPP (and possibly SPPLs) forms high molecular weight complexes with additional binding partners that, though they do not to affect proteolytic activity, contribute to possible non-proteolytic functions of SPP/SPPLs. Whether these interactions are dynamic and potentially regulated and whether they are cell type-specific or dependent on special cellular conditions remains to be investigated.

In order to obtain an in-depth biochemical and spatial understanding of the catalytic mechanisms applied by SPP/SPPLs the atomic structures of the protease ideally with a substrate in a lipid environment is ultimately required. As this is a technically highly

demanding goal, atomic structures have neither been described for vertebrate SPP/SPPLs nor for the obviously more complex multi-subunit γ-secretase (reviewed by Wolfe [85] in this issue). Recently, however, the first atomic structures of archaeal GxGD proteases have been reported [83,86]: Hu *et al*. obtained a crystal structure of the PFP Flak (Fig. 1D) from *Methanococcus maripaludis* at 3.6 Å [86]. FlaK bears one aspartyl residue essential for catalytic activity in TMD 1, a GxGD motif close to the helical segment of TMD 4, and a proline-tipped, kinked TMD 6 which together form the three transmembrane segments of the active site [86] suggesting they can be compared to TMD 6, TMD 7, and TMD 9 which contribute to the formation of the active site in presenilins (see the review by by Wolfe [85] in this issue). Since the primary structure of FlaK has only a vague resemblance to SPP/SPPLs and presenilin it is questionable at this point whether the results of this study can be fully transferred to these proteases. More recently, in a landmark paper Li *et al*. published the first crystal structure of the *Methanoculleus marisnigri* intramembrane protease JR1 [83]. Judged by its sequence homology this protease can clearly be attributed to the presenilin/SPP/SPPL type of GxGD proteases (see section 1) [22] and is targeted by the same type of active-site directed inhibitors [22,83]. Hence, it can be considered a prototypic protease of this class. Like presenilins and SPP/SPPLs it harbours nine transmembrane helices with TMD 6, TMD 7, and TMD 9 containing the YD, GxGD, and the proline motif, respectively, forming the putative active site [83]. Of note, however, JR1 was crystallised in an obviously inactive conformation, as the distance observed between the active site aspartates would not support catalysis. Therefore, the structure reported might not fully resemble the active protease in a substrate-bound state. Given its evolutionary relationship to presenilins and SPP/SPPLs (compare Fig. 4) and that the structure obtained is mostly in line with biochemical data for presenilins in the γ-secretase complex we can, however, assume that SPP and SPPLs will structurally likely also adopt

the presenilin fold reported by Li *et al*. [83]. Nonetheless, structural studies on vertebrate SPP/SPPLs are ultimately required to confirm this notion.

4.2 Substrate requirements of SPP/SPPLs

While presenilins exclusively accept type I transmembrane substrates [87], SPP/SPPLs seem to be selective towards transmembrane substrates in type II orientation [4,58,71]. It is very likely, that the inverted topology of the two protease families (Fig. 1A + B) causes this strict specificity for either class of substrates [4,58,71]. The formal proof for this hypothesis is, however, missing.

SPP/SPPLs are implicated in a two-step proteolytic process, termed RIP (Fig. 2), which either liberates bioactive protein fragments or domains from transmembrane proteins or facilitates the clearance of protein fragments from cellular membranes and initiates their subsequent degradation [11,87]. In an initial step the substrate is cleaved within its luminal/extracellular domain to liberate the ectodomain from single-pass transmembrane proteins or to cut a hairpin loop between two TMDs of a multi-pass transmembrane protein. This proteolytic process is termed “shedding” and generates an integral membrane protein fragment with a rather short ectodomain (Fig. 2A). These protein fragments are subsequently endoproteolysed within the lipid bilayer by the respective intramembrane protease, causing the release of ICDs into the cytosol and of C-peptides into the lumen/ extracellular space (Fig. 2A) [5].

Substrate shedding is a prerequisite for the subsequent intramembrane proteolysis and consequently the rate-limiting, regulative step initiating this whole proteolytic cascade. In line with this, endoproteolysis of signal peptides by SPP within their membrane-spanning stretch strictly requires them to be removed from the nascent protein by signal peptidase cleavage (Fig. 2B) [69]. Moreover, efficient intramembrane cleavage by SPPL2b requires substrates which have undergone ectodomain shedding [70,88]. Consistently, TNFα

[23,24], Bri2 [25], and FasL [60] require membrane-proximal ectodomain shedding which is mediated by the family of A Disintegrin and Metalloproteases (ADAMs) in order to be subsequently cleaved within their membrane-spanning domains by SPPL2a/b. Similar requirements have been known for substrates of presenilin [89]. The γ-secretase complex component nicastrin, which is a single-span transmembrane protein with a large and highly glycosylated ectodomain, was claimed to be responsible for the selectivity of presenilin towards transmembrane proteins with a short ectodomain [90] though this notion has meanwhile been challenged [91]. Since SPP/SPPLs seem to lack nicastrin-like co-factors [4] they must have developed a protease-intrinsic mechanism to selectively bind substrates that match the criteria described above. So far, however, domains within SPP/SPPLs that mediate specific binding of type II transmembrane substrates with an ectodomain shorter than 60 amino acids, have not been identified.

Unlike SPP, SPPL2a, and SPPL2b, which, according to the data published so far, are strictly selective towards substrates with a short ectodomain, SPPL3 was recently described to efficiently cleave its substrate FVenv carrying a mutation that prevents pro-protein convertase-mediated shedding of this viral protein [70]. Moreover, cleavage of full-length FVenv by SPPL3 results in a membrane protein fragment with a short ectodomain that is consecutively processed by SPPL2a or SPPL2b (Fig. 2C) [70]. Hence, in this context SPPL3 acts as sheddase initiating an alternative RIP cascade of FVenv [70]. The ability of I-CLiPs to cleave intact single-pass transmembrane proteins was so far only attributed to members of the rhomboid family, which belongs to the class of serine intramembrane proteases [92]. Since no cellular substrates of SPPL3 have been described, it is not yet known whether SPPL3 also cleaves full-length substrates in a physiological context. Nonetheless, it is tempting to speculate that SPPL3 applies a different mechanism of substrate recognition than SPP, SPPL2a, and SPPL2b and that SPPL3 may be functionally more similar to rhomboid proteases.

In contrast to many conventional aspartic proteases, so far no consensus cleavage site based on primary sequence elements within the substrate has been described for GxGD aspartyl proteases. TMDs of membrane proteins preferentially adopt an α-helical confirmation in which their peptide bonds are hardly accessible to proteases [93]. In order to make TMDs susceptible for intramembrane proteolysis it was therefore postulated that their α-helical content needs to be reduced by helix destabilizing amino acids [94]. Consistent with this hypothesis, various signal peptides have been shown to contain helix destabilizing amino acids within their h-region which critically influence their proteolytic processing by SPP [44,47,69,95]. In addition, polar residues within the h-region of signal peptides may influence cleavage by SPP, as for instance serine and cysteine residues within the signal peptide of various HCV strains are critical for SPP cleavage [44,47,95]. Whether these polar residues also simply affect the helical content of the signal peptides or the hydroxyl or sulfhydryl group in particular is required to trigger cleavage by SPP is not yet fully understood. Similarly, cleavage of the Bri2 TMD by SPPL2b is significantly increased when the α-helical content of the Bri2 TMD is reduced [96]. Interestingly, only one amino acid residue out of four residues with a putative helix destabilizing potency significantly reduced the α-helical content of the Bri2 TMD in a phospholipid-based environment [96]. This suggests that destabilisation of an α-helical TMD is not simply caused by certain amino acid residues but that rather context and position of these amino acids determine their helix destabilizing potential and thus the accessibility of TMDs to intramembrane proteolysis by SPP/SPPLs.

Although the length of a substrates ectodomain and the α-helical content of its TMD are important determinants for the efficiency of processing by SPP/SPPLs, these two properties of a substrate alone are not sufficient to trigger its processing. For instance, Bri3, which is a close homologue of the SPPL2a/b substrate Bri2, does not undergo intramembrane cleavage by SPPL2b, even if the ectodomain is artificially shortened and

its TMD is replaced by that of Bri2 [88]. Bri2/Bri3 domain swapping analysis revealed that further determinants within the juxtamembrane domains N- and C-terminal to the TMD are required in addition to allow efficient intramembrane proteolysis by SPPL2b [88]. In line with this charged residues in the juxtamembrane domain C-terminal to the TMD of SPP substrates have been shown to interfere with substrate processing [69].

Similar substrate determinants have been described earlier also for the processing of γ-secretase substrates [97-99]. But in addition it has been discussed that substrate dimerization via GxxxG motifs influences substrate recognition and cleavage by γ-secretase [100]. Since a GxxxG motif within the Bri2 TMD does not affect its processing by SPPL2b [96], this may suggest either that SPP/SPPLs and γ-secretase differ fundamentally in this regard or that substrate dimerization only influences the processing in certain enzyme-substrate combinations.

To finally judge which substrate determinants are unique to individual members or common to all members of the GxGD aspartyl protease family and whether certain enzyme-substrate combinations are subject to a special regulation, further studies on newly identified substrates, for instance on CD74, FasL, and FVenv as well as on so far uncharacterized enzymes like SPPL3 or SPPL2c will be essential.

Nonetheless, one important conclusion that must be drawn from the present results is that SPP/SPPLs have considerably higher substrate specificity than initially thought [87] and are probably involved in regulation of specific cellular pathways as well as in the degradation of selected membrane proteins.

4.3 Cleavage mechanism

Presently, only the cleavage mechanism applied by SPP and SPPL2b has been investigated in more detail. SPPL2b utilizes multiple cleavages within the TMD of TNFα to efficiently release TNFα ICD and TNFα C-peptide (Fig. 5) from the cellular membrane [23].

While the major cleavage event leading to the release of TNFα C-peptide takes place in the C-terminal part of the TNFα TMD between Cys49 and Leu50, the C-termini of the prevailing TNFα ICD species are Ser34, Leu39 and Arg28 (Fig. 5) [23]. Neither ICDs with C-termini at Cys49 nor C-peptides with longer N-termini have been detected ([23] and Fluhrer *et al*., unpublished data), suggesting that SPPL2b cleaves one and the same TNFα molecule multiple times in a sequential manner starting at Leu50 and terminating at Arg29. In addition to C-peptides starting at Leu50, a minor fraction of C-peptides starting at His52, Val55 or R60 have been identified ([23] and Fluhrer *et al*., unpublished data). This may indicate that substrate processing by SPPL2b does not commence at one defined position but rather in a certain region within the substrates TMD. Given that sequential intramembrane proteolysis occurs periodically, the different starting points of intramembrane proteolysis result in different product lines. In line with this, minor amounts of TNFα ICDs terminating at Ser37 and Cys30 have been detected using MALDI-TOF MS (Fluhrer *et al*., unpublished data). Similar observations have been made for substrate processing by γ-secretase [101-103]. In particular for γ-secretase processing of βAPP two independent product lines have been discussed, which result in Aβ species with variable neurotoxicity [101,103]. Detergent-solubilized SPP cleaves synthetic peptides based on the prolactin signal sequence mainly between Leu23 and Leu24, resulting in ICD and C-peptide species with C- and N-termini, respectively, that exactly match [104]. In addition a minor amount of shorter ICD species, but interestingly no other C-peptide species have been detected [104]. Whether these additional ICD species originate from a sequential cleavage catalysed by SPP or artificially result from the detergent solubilisation remains to be elucidated.

Recently, palmitoylation of TNFα has been implicated in the stability of its N-terminal membrane bound fragment and is therefore suggested to be necessary for an efficient

intramembrane cleavage by SPPL2b [105]. The palmitoylation of Cys30 within the cytosolic juxtamembrane of TNFα is discussed to be responsible for targeting of TNFα to lipid rafts [106,107]. Since SPPL2b was also found to be enriched in lipid rafts [15], co-localisation of substrate and enzyme might favour intramembrane proteolysis in this particular lipid microdomains [105]. So far it remains however unclear, whether substrate acylation is a prerequisite for efficient intramembrane proteolysis by SPP/SPPL proteases in general or solely improves processing of individual substrates by selected members of the SPP/SPPL family.

Substrate processing catalysed by SPP or SPPL2b is altered by mutations within the conserved active site motifs [72,104,108]. While mutation of the glycine residue next to the catalytically active aspartate within the GxGD motif of SPP only affected the overall protease activity [104], a similar mutation in SPPL2b additionally slowed the sequential processing of TNFα and caused a relative increase of the amounts of longer TNFα ICD species without significantly affecting the secretion of the respective TNFα C-peptide [108]. A similar mutation within presenilin has been linked to familial Alzheimer's Disease [109], since it increases the secretion of neurotoxic Aβ42 relative to the less neurotoxic Aβ40 [2,110]. In this case the relative increase of Aβ42 is intensified by a reduction of Aβ40 secretion [108], which corresponds to the reduced ICD production from mutated SPP and SPPL2b. To date only two more mutations known to increase Aβ42 in presenilin [111,112] have been transferred to SPP [104]. One of these mutations reduced the processing of the synthetic prolactin signal peptide *in vitro*, while the other mutation had no effect [104]. Therefore, in order to clarify whether mutations within in conserved regions of GxGD proteases similarly affect the cleavage mechanism of all family members significantly more mutations in different proteases need to be investigated. Moreover, it

cannot be excluded that the same mutations within one SPP/SPPL protease differentially affect individual substrates.

SPP activity was shown to be inhibited by $(Z\text{-}LL)_2$ ketone [10]. $(Z\text{-}LL)_2$ ketone mimics the leucine-rich hydrophobic core of signal peptides cleaved by SPP [10] and a derivative thereof was used to isolate SPP from detergent solubilized ER membranes [4]. Based on this, it is likely that $(Z\text{-}LL)_2$ ketone directly targets the active site of SPP or interacts with a potential substrate binding site in SPP. However, the formal proof for this assumption is still missing. While in addition to SPP also SPPL2a and SPPL2b are inhibited by $(Z\text{-}LL)_2$ ketone treatment [23-25,60], γ-secretase activity is not blocked at similar concentrations [113]. This is particularly interesting, as γ-secretase inhibitors (GSIs), like L-685,458, which are known to target the active site of γ-secretase [114,115], also reduce SPP and SPPL2a/b activity [23,24,113]. This may imply that $(Z\text{-}LL)_2$ ketone targets a site within the active center of SPP and SPPL2a/b which is structurally different from that of presenilins while L-685,458 and other peptide-based γ-secretase inhibitors seem to bind a structure in the active center common to γ-secretase, SPP and SPPL2a/b. Another γ-secretase inhibitor, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester (DAPT) [116], which specifically targets the C-terminal fragment of presenilin when incorporated within the high molecular weight γ-secretase complex [117], only inhibits γ-secretase but fails to block SPP and SPPL2b activity [25,113]. This indicates that the DAPT binding site is unique to γ-secretase and not conserved within SPP and SPPL2b.

Besides GSIs which abolish the liberation of both cleavage products, so called γ-secretase modulators (GSM) have been shown to selectively shift the sequential cleavage of γ-secretase substrates, resulting in a changed pattern of secreted peptides for example of different Aβ species without alteration of the respective ICD species (reviewed in [118]).

First generation GSMs were based on the structure of certain non-steroidal anti-inflammatory drugs and shifted the sequential cleavage of γ-secretase substrates in N-terminal direction [119]. Two such GSMs, sulindac sulfide and indomethacin, were also shown to alter the cleavage site specificity of SPP within the prolactin signal peptide *in vitro*, but only at high concentrations [104]. But, in contrast to γ-secretase, the cleavage was shifted in C-terminal direction [104]. Yet whether GSMs also influence the cleavage site specificity of SPP in cellular model systems and *in vivo* and whether SPPL proteases are affected similarly remains to be elucidated.

Helical peptides that mimic SPP substrates and thus potentially target the initial substrate binding site ("docking site") within SPP, inhibit both SPP and γ-secretase [120]. Photoaffinity labeling of SPP by these helical peptides revealed a binding site distinct from that of $(Z\text{-LL})_2$ ketone [120], suggesting that, as observed previously for γ-secretase [121], the site of initial substrate binding to SPP is distinct from the site of final proteolysis. Moreover, binding of GSMs to SPP did neither overlap with the binding of the helical peptides nor with $(Z\text{-LL})_2$ ketone [120].

Based on the current knowledge about the homology of SPP/SPPL proteases and the $(Z\text{-LL})_2$ ketone sensitivity of SPP and SPPL2a/b it was expected that SPPL3 proteolytic activity is also blocked by $(Z\text{-LL})_2$ ketone. Surprisingly, however, cleavage of FVenv by SPPL3 is insensitive to active site-directed inhibitors such as $(Z\text{-LL})_2$ ketone and to L-685,458 [70]. This finding may point to marked spatial differences between the active site architecture of and of other SPP/SPPL family members. However, two studies analysing the cleavage of synthetic peptides based on the prolactin signal sequence by recombinantly expressed *D. melanogaster* SPPL3 [40] as well as the SPPL3-mediated cleavage of a model substrate optimized for SPP cleavage in a cell-based context [15], concluded that SPPL3 is sensitive to such inhibitors. Contrary to these observations, a catalytically inactive mutant of SPPL3 was not able to interact with those signal peptide-

based model substrates in a cellular context, while the corresponding SPP mutant co-precipitated these substrates [57]. These discrepancies may indicate that the effect of certain inhibitors on SPP/SPPL proteases is dependent on the context applied in the respective study. In line with this, a reporter construct based on a signal sequence which is not cleaved by SPP *in vitro* [69] is cleaved by SPP in a cellular context [71]. Therefore, additional studies using various enzyme-substrate combinations and experimental conditions will be required to finally answer the question whether inhibitors and modulators affect the cleavage mechanism of all GxGD proteases in a similar manner.

5. Outlook

Despite recent advances, we are just at the beginning of understanding the biochemical diversity and the biological significance of SPP/SPPL intramembrane proteases. A central issue will be to extend and define the substrate spectrum of each individual protease. Based on this, it can be anticipated that a more precise characterisation of the substrate requirements of SPP/SPPLs as well as the molecular mechanisms for substrate recognition and cleavage will be achieved. For each of the already known or yet to be identified substrates, the biological relevance of SPP/SPPL-mediated intramembrane proteolysis may need to be evaluated individually, specifically related to the question if their cleavage products fulfill any signaling function after their liberation from the membrane. In this context the suggested contribution of SPP/SPPL proteases to generalized membrane protein turnover may be further assessed. In order to validate biochemical *in vitro* findings and scrutinize the impact of these proteolytic events in a complex organism, significant advances can be expected from *in vivo* studies using knockout mice. In particular, these will be instrumental to further corroborate the specific role of SPPL2a/b in immune regulation and inflammation and to evaluate the suitability of

certain SPP/SPPL proteases as therapeutic targets for the treatment of malaria infections, the depletion of B cells in autoimmune disorders or not yet recognised indications.

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Figure Legends

Fig. 1: Overview of the GxGD protease family. Members of the GxGD protease family include the presenilins (exemplified by human PS1), (**A**), SPP/SPPLs (exemplified by human SPP), (**B**), the bacterial type 4 prepilin peptidases (exemplified by *Vibrio cholerae* TcpJ), (**C**), and archael preflagellin peptidases (exemplified by *Methanococcus maripaludis* FlaK), (**D**). Active site motifs are shown in violet, catalytic Asp residues in bold. Presenilins are autocatalytically endoproteolysed within one of their cytosolic loops to generate an N-terminal fragment (NTF) and C-terminal fragment (CTF) and associate with Aph-1, Nicastrin, and Pen-2 to form the active γ-secretase complex (shaded box). The luminal/extracellular space is shaded in blue, while cytosol is shown in orange.

Fig. 2: Regulated intramembrane proteolysis (RIP) by SPP/SPPLs. (**A**) TNFα as prototypic type II RIP substrate. In an initial step, the ectodomain moiety of the substrate is liberated by membrane-proximal shedding which is usually mediated by members of the ADAM family. This generates a N-terminal fragment (NTF). Intramembrane cleavage of the NTF by SPPL2a and/or SPPL2b releases secreted C-peptides and ICDs. (**B**) Cleavage of signal peptides by SPP in the ER membrane. Following synthesis at the ER membrane, nascent secretory proteins are translocated into the ER lumen through the translocon (yellow) and signal peptides are removed by signal peptidase. Subsequently, signal peptides are cleaved by SPP within the lipid bilayer and the respective signal peptide fragments are released from the ER membrane. (**C**) Intramembrane cleavage of type III membrane proteins by SPPLs, exemplified by foamy virus envelope protein (FVenv). The type III membrane protein is initially cleaved by SPPL3 or other proteases (e.g. proprotein convertases, not depicted) to generate a NTF and the membrane anchored counterpart.

The NTF is subsequently cleaved by SPPL2a/b to liberate C-Peptides and ICDs. The luminal/extracellular space is shaded in blue, while cytosol is shown in orange.

Fig. 3: Overview of human SPP/SPPL family members. In humans the SPP/SPPL family includes SPP (**A**), SPPL3 (**B**), SPPL2a (**C**), SPPL2b (**D**), and SPPL2c (**E**). Members are shown schematically and their respective YD, GxGD, and PAL motifs are highlighted (violet). The catalytically active aspartyl residues are highlighted in bold and their respective position is indicated. SPPL2a, SPPL2b, and SPPL2c are synthesised as pre-proteins and their signal sequences (yellow) are removed, presumably by signal peptidase. Putative signal peptidase cleavage sites (black arrowhead) are indicated according to predictions in Uniprot. Consensus N-glycosylation sites for human SPP (N10, N20), human SPPL2a (N58, N66, N74, N116, N126, N149, N155), human SPPL2b (N97, N129, N403), and human SPPL2c (N100) are indicated. Two SPPL2b isoforms have been documented in mammals that differ in the length of their C-terminal cytosolic tails (aa 512 or 592, shaded). Signals ensuring proper subcellular localisation to the ER and the endosomal/lysosomal compartment for SPP and SPPL2a, respectively are indicated. The luminal/extracellular space is shaded in blue, while cytosol is shown in orange.

Fig. 4: Phylogenetic relationship of SPP/SPPL. Protein sequences were obtained from Genbank (accession numbers given in parentheses). The tree was obtained using the software package MEGA5 [124] and the neighbour-joining method following multiple sequence alignment using ClustalW2.1. Family members are grouped (coloured boxes) according to their relationship to human SPP/SPPL orthologues. At, *Arabidopsis thaliana*, Os, *Oryza sativa* Japonica group, Ce, *Caenorhabditis elegans*, Hs, *Homo sapiens*, Mm,

Mus musculus, Dr, *Danio rerio*, Dm, *Drosophila melanogaster*, Pf, *Plasmodium falciparum*, Sc, *Saccharomyces cerevisiae*, Ap, *Archaeoglobus fulgidus*.

Fig. 5: Cleavage sites identified in SPP/SPPLs substrates. So far, cleavage sites were only identified for SPP-mediated intramembrane proteolysis of the bovine prolactin (Prl) signal peptide and for SPPL2b-mediated cleavage of TNFα. Membrane-spanning amino acid sequences are shown in one letter code, predicted TMD (TMHMM2.0) and signal peptide (Uniprot) regions are shown in italics. Black arrowheads, C-peptide species observed, grey arrowheads, ICD species identified, white arrowheads, signal peptidase cleavage sites, *, C30 in TNFα is palmitoylated [106]. Large arrowheads indicate major cleavage products, small arrowheads indicate minor cleavage products. The luminal/extracellular space is shaded in blue, while cytosol is shown in orange.

Table 1: Substrates identified for SPP/SPPL proteases. Substrates identified for the respective SPP family *in vitro* or in a cellular setting. For SPPL2c no substrates have been reported so far. For non-human substrate proteins the respective species is given in parentheses. *, FVenv is synthesized as a type III transmembrane protein precursor and its N-terminal TMD is in type II orientation. SPPL2b cleaves this TMD only following a membrane-proximal pre-cleavage (i.e. a type II protein fragment), while SPPL3 can cleave FVenv full length (i.e. the type III holoprotein).

Protease	Substrate type	Substrate	Reference
SPP	Signal peptide	Prolactin (bovine)	[10,122]
		HIV gp160 (viral)	[10,122]
		MHC class I (HLA-A*0301)	[34]
		Calreticulin (rat)	[69]
		VSV G (viral)	[69]
		Crumbs (*Drosophila*)	[30]
		Pro-calcitonin	[33]
		Eosinophil cationic protein	[31]
	Multipass proteins	IgSF1	[42]
		HCV polyprotein (viral)	[43]
	Retrotranslocation	MHC class I	[52]
SPPL2a	Type II protein	TNFα	[23,24]
		Bri2	[25]
		FasL	[60]
		CD74 (murine)	[61-63]
		FVenv	[70]

SPPL2b	Type II protein	TNFα	[23,24]
		Bri2	[25]
		Transferrin receptor 1	[123]
	Type III protein*	FVenv (viral)	[70]
SPPL3	Type III protein*	FVenv (viral)	[70]

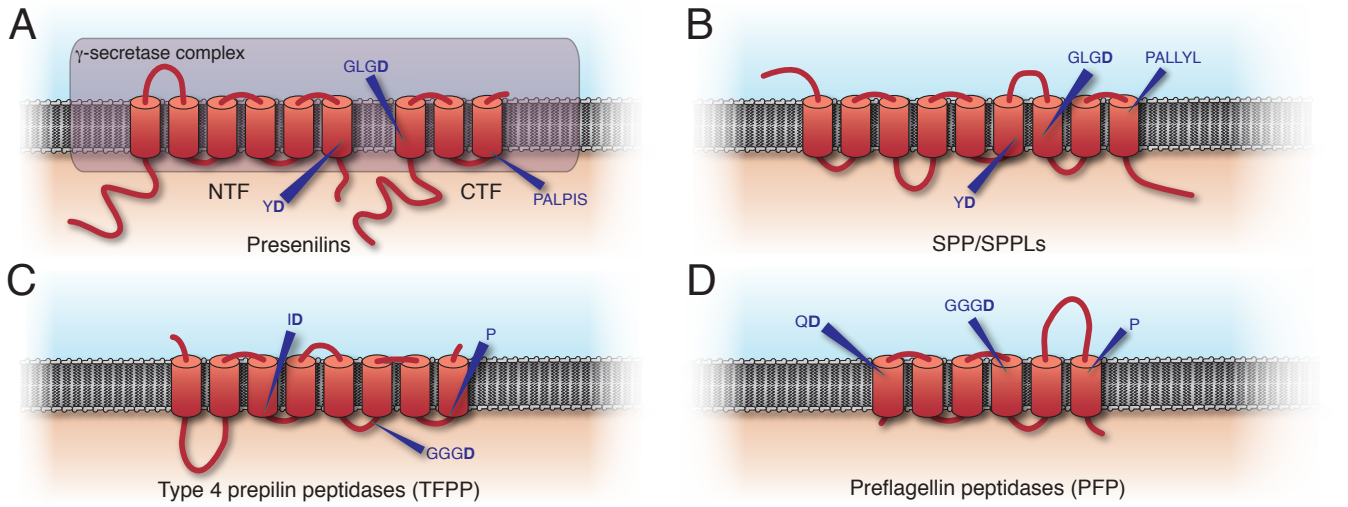

Fig. 1 - Voss et al.

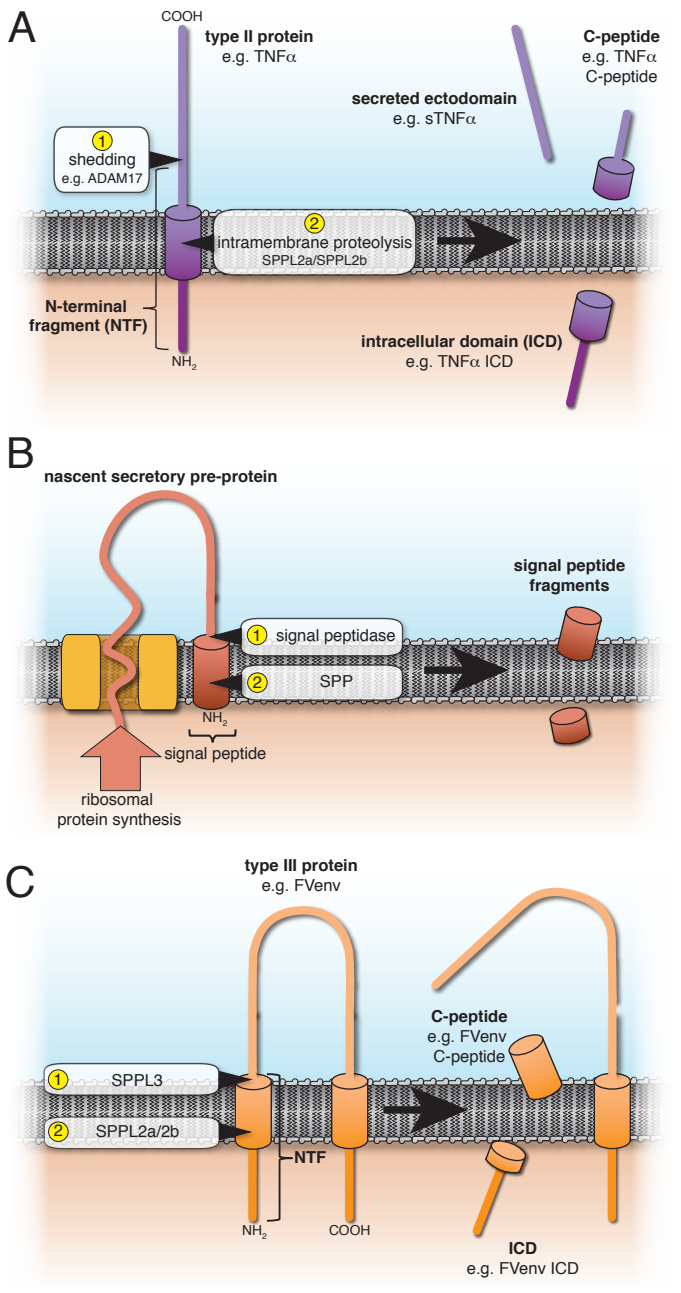

Fig. 2 - Voss et al.

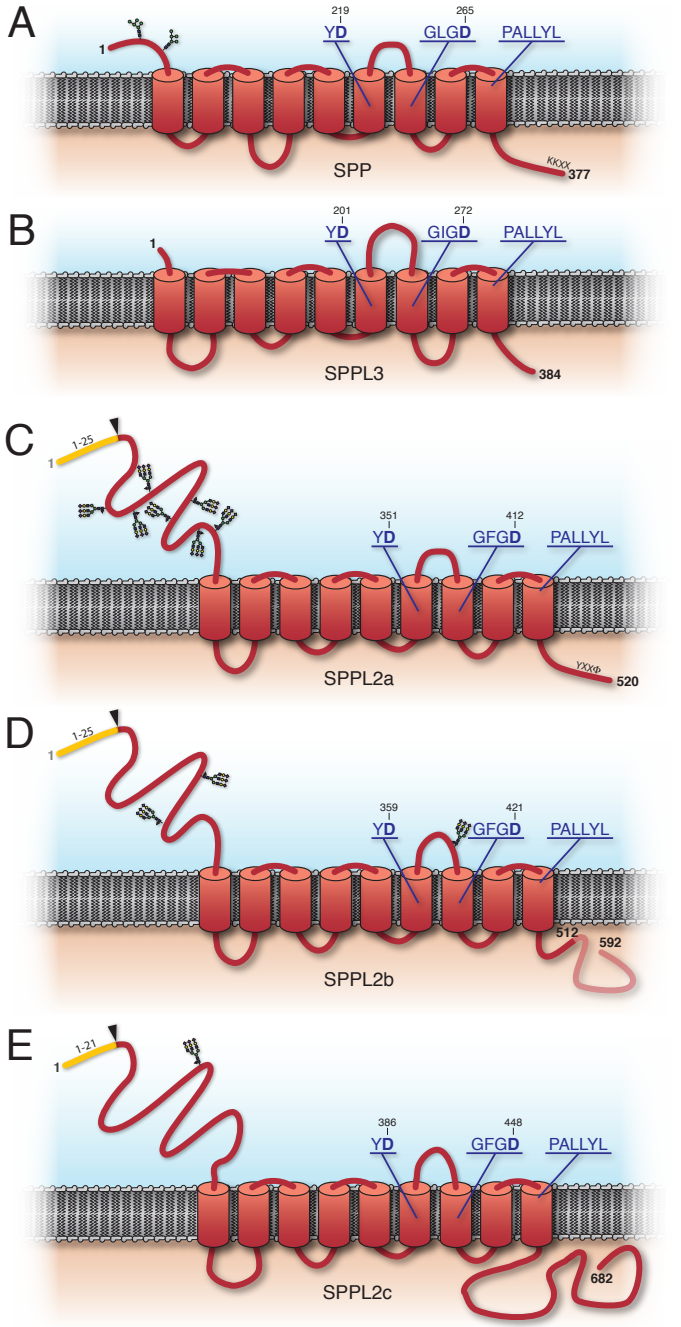

Fig. 3 - Voss et al.

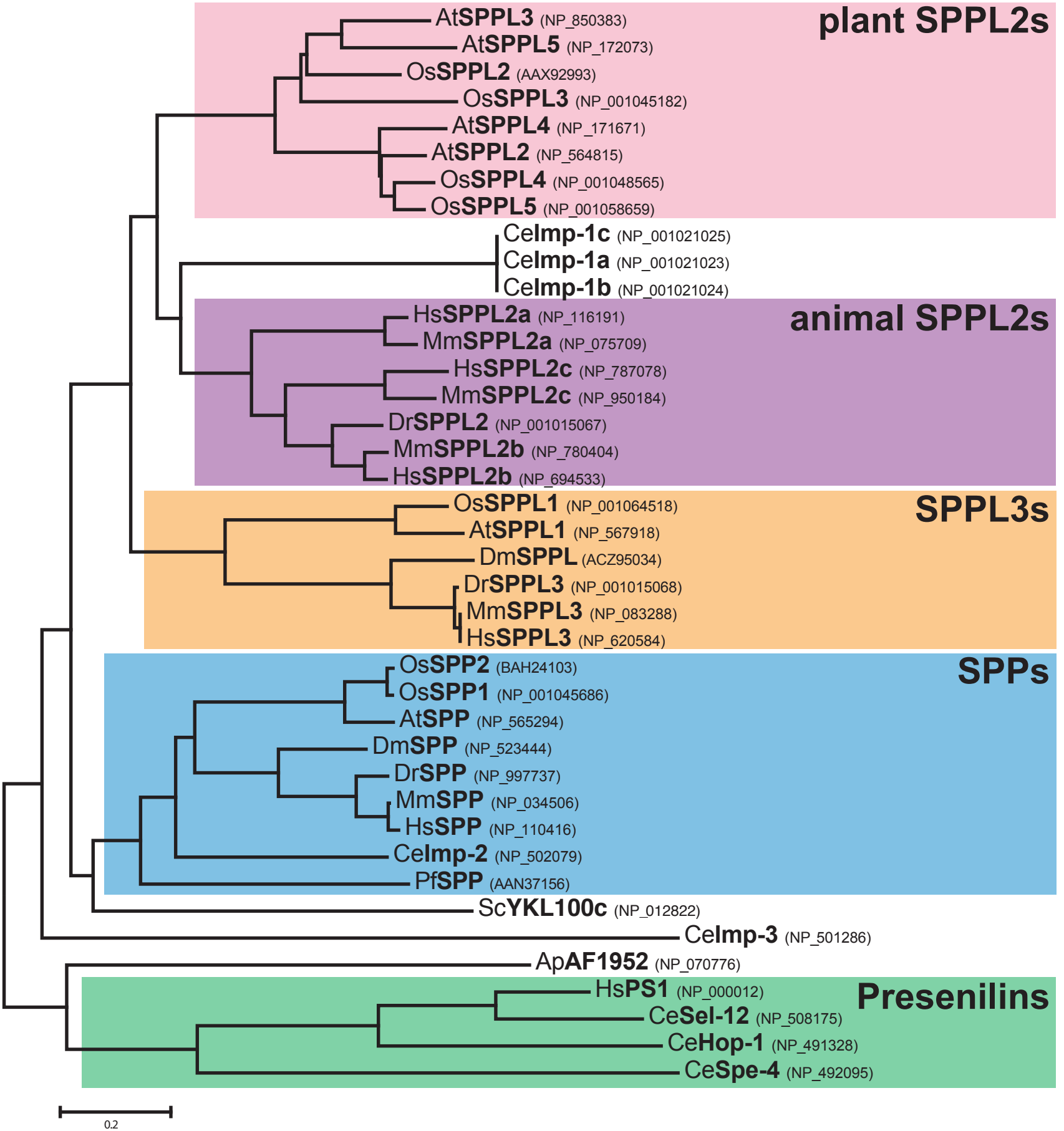

Fig. 4 - Voss et al.

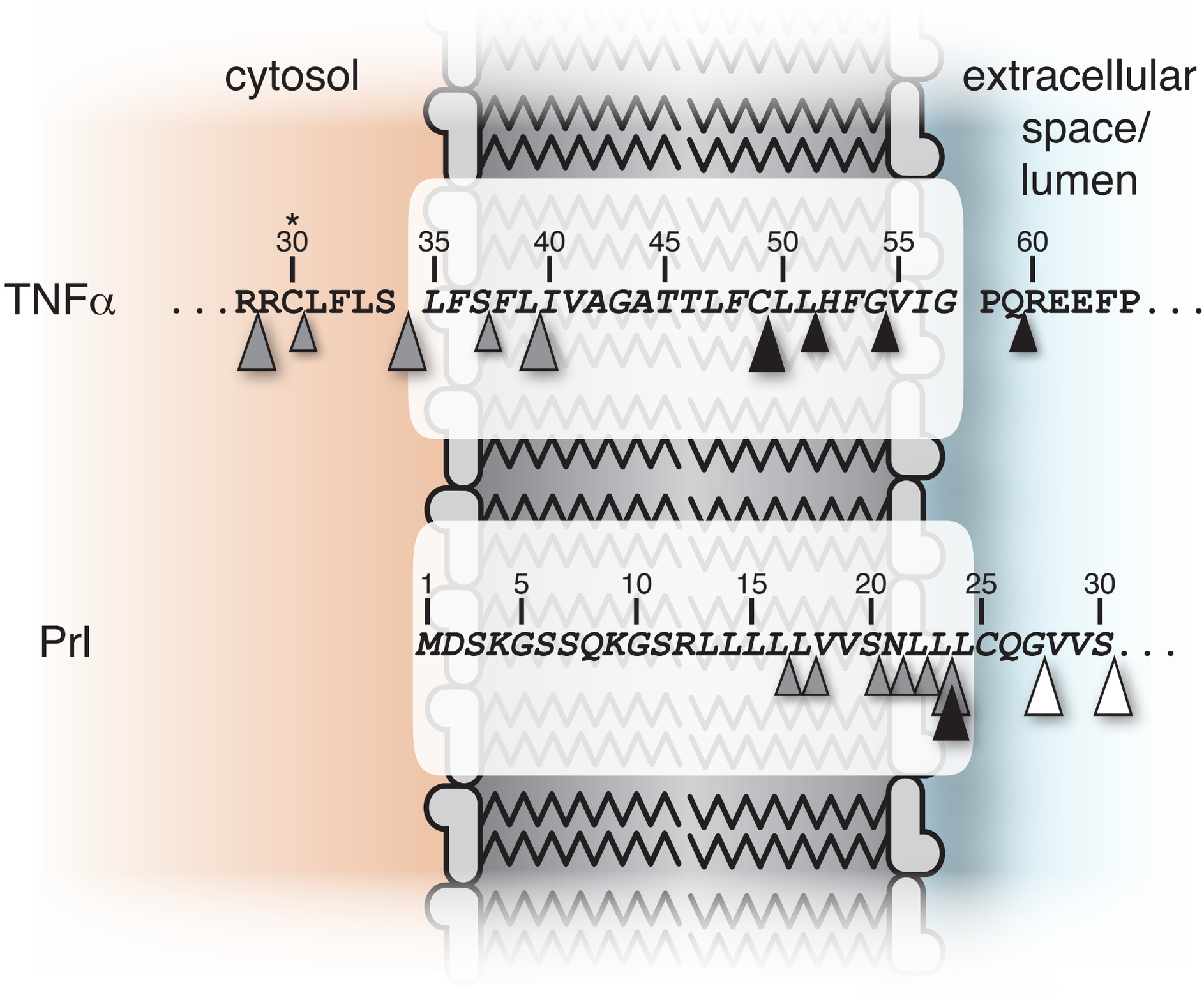

Fig. 5 - Voss et al.