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STATE-OF-THE-ART REVIEW

# Structure and function of SPP/SPPL proteases: insights from biochemical evidence and predictive modeling

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More than 20 years ago, signal peptide peptidase (SPP) and its homologues, the signal peptide peptidase-like (SPPL) proteases have been identified based on their sequence similarity to presenilins, a related family of intramembrane aspartyl proteases. Other than those for the presenilins, no high-resolution structures for the SPP/SPPL proteases are available. Despite this limitation, over the years bioinformatical and biochemical data have accumulated, which altogether have provided a picture of the overall structure and topology of these proteases, their localization in the cell, the process of substrate recognition, their cleavage mechanism, and their function. Recently, the artificial intelligence-based structure prediction tool AlphaFold has added high-confidence models of the expected fold of SPP/SPPL proteases. In this review, we summarize known structural aspects of the SPP/SPPL family as well as their substrates. Of particular interest are the emerging substrate recognition and catalytic mechanisms that might lead to the prediction and identification of more potential substrates and deeper insight into physiological and pathophysiological roles of proteolysis.

## Introduction

Intramembrane proteases hydrolyze their transmembrane protein substrate within the hydrophobic phase of cellular membranes. They mediate part of the membrane protein turnover and contribute to signaling pathways [1,2], and by changing their target protein's localization, stability, and function, they modulate protein–protein interactions in an evolutionarily conserved fashion [3-6]. Based on homology—thus overall fold—and their catalytic mechanism human intramembrane-cleaving proteases are divided into four families. Despite the importance of the cellular functions each family, at least in humans, only comprises a

**Abbreviations**
(Z-LL)2-ketone, 1,3-di-(Ncarboxybenzoyl-L-leucyl-L-leucyl) amino acetone; ADAM, A Disintegrin And Metalloproteinase; AI, artificial intelligence; APH-1, anterior pharynx-defective 1; APP, amyloid-beta precursor protein; ATF6, cyclic AMP-dependent transcription factor ATF-6; Cryo-EM, cryo-electron microscopy; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester; DNER, Delta and Notch-like epidermal growth factor-related receptor; GNT-V, mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase; GSI, γ-secretase inhibitor; GSM, γ-secretase modulators; ICD, intracellular domain; LDLR, low-density lipoprotein receptor; PA, protease-associated PEN-2, presenilin enhancer 2; PS, presenilin; RIP, regulated intramembrane proteolysis; S2P, site-2-protease; SNARE, soluble N-ethylmaleimide-sensitive-factor attachment receptor; SPP, signal peptide peptidase; SPPL, signal peptide peptidase-like; SREBP-2, sterol regulatory element-binding protein 2; TA, tail-anchored; TM, transmembrane; TNFα, tumor necrosis factor α; TSA, transition-state analoga; VAMP, vesicle-associated membrane protein.

few members. Rhomboid proteases represented by five human members are serine intramembrane proteases with a core structure comprising six transmembrane (TM) domains. They are involved in cancer development and progression as well as in neurodegenerative, metabolic, and infectious diseases [7]. Site-2-protease (S2P) is a zinc metallo-intramembrane protease cleaving membrane-bound transcription factors of the basic leucine zipper family, like ATF6 and SREBP-2 [8,9]. Due to its unique structure organized in multiple TM domains and alternating hydrophilic regions, human S2P represents its own protease family [10]. Rce1, a seven TM domain intramembrane protease, is the only known human glutamyl protease and processes prenylated proteins by cleaving their C-terminal amino acids [11,12]. The intramembrane aspartyl proteases are represented by two protease families, the presenilin (PS) family and the signal-peptide-peptidase (SPP)/signal-peptide-peptidase-like (SPPL) family, and comprise a core structure of nine TM domains [13,14]. All of them contain a membrane-embedded aqueous active site characterized by a conserved YD motif in TM domain 6 and a GxGD motif in TM domain 7. Therefore, they are often referred to as GxGD-type proteases [15,16]. An equally conserved PAL motif in TM domain 9 completes the common hallmarks of intramembrane aspartyl proteases. Presenilins contribute to the release of the amyloidogenic Aβ-peptide from the amyloid precursor protein (APP) and are involved in the development of Alzheimer's disease [17]. The related signal peptide peptidase (SPP) and its homologues, the signal peptide peptidase-like (SPPL) proteases were first identified based on sequence similarity to presenilins [18]. In the human genome, two members of the PS family, PS1 and PS2, and five members of the SPP/SPPL family, SPP, SPPL2a, SPPL2b, SPPL2c, and SPPL3, are encoded [2,18,19,20]. SPP/SPPL proteases are highly conserved and can be found in eukaryotes, including fungi, protozoa, plants, and animals, highlighting their physiological importance [21]. Among the five members in mammals, SPPL3 is the most conserved, with human and murine proteins even being identical [22]. SPP/SPPL proteases are involved in glycosylation of secretory and membrane proteins [22], vesicular transport [23], and various pathophysiological mechanisms such as carcinogenesis, atherosclerosis [2], immune cell development and function and also play a role in plasmodia causing malaria [24-27]. The importance of SPP/SPPL proteases in the physiological context is discussed in an accompanying review in this issue [28].

Signal peptide peptidase and SPPL2c are localized to the ER membrane [23,29]. SPPL3 is found in the Golgi, SPPL2a in lysosomes/endosomes and SPPL2b at the plasma membrane [30-33]. SPP and SPPL2c exhibit a potential ER retention motif (KKXX) in their cytoplasmic C terminus [18,34]. For SPPL3, a localization to medial/early-trans-Golgi was shown by immunofluorescence [31]. However, whether specific sorting signals are responsible for this subcellular localization remains enigmatic as GOLPH3/GOLPH3L-mediated retrieval did not contribute to the localization of endogenous SPPL3 [31]. SPPL2a is found predominantly in the lysosomes/late endosomes targeted there by a tyrosine motif in its cytoplasmic C-terminal tail [35]. It was shown that the canonical tyrosine-based sorting motif of the YXXø type [36] at position 498 is sufficient for its localization to the lysosomal/late endosomal compartments [35]. This motif is recognized by adapter protein complexes, which can recruit clathrin [36,37].

Unlike presenilin in the γ-secretase complex, SPP/SPPL proteases do not seem to need additional complex partners for activity. Signal peptide peptidase is catalytically active upon purification [38,39], and by overexpression of SPPL proteases, the processing of the respective substrates increases [21]. That does, however, not answer the question whether they need to form higher molecular weight assemblies, for example, by dimerization or tetramerization. In addition, potential additional cellular co-factors needed for proteolytic activity of SPP/SPPLs could simply be highly abundant and, thus, not limiting for catalytic activity in overexpression systems [21]. Moreover, interacting partners important for regulatory functions might not be essential for catalytic activity. In line with this, distinct high-molecular-weight complexes have been reported for SPP. It forms stable homodimers and can be isolated as homo-tetramers of 200 kDa weight. An apparent functional diversification in 500 kDa complexes with proteins including Derlin 1 and the Ring finger protein RNF139 (TRC8) was reported [38,40,41]. Blue native gel purifications also suggest SPPL2c to be part of higher molecular weight assemblies. SPP and SPPL2c both reside in the ER but seem to occur in the distinct heterocomplexes [23]. For the remaining family members SPPL2a, SPPL2b, and SPPL3 so far, no conclusive data on complex formation are available.

## Substrates of SPP/SPPL proteases

It is intriguing that with five members in the SPP/SPPL family the number of so far identified substrates with approx. 30 is relatively small. This might on the one hand argue for a very specific most likely

regulatory cleavage rather than a general removal of membrane proteins [42] as it has been suggested earlier for the presenilin family [43], for which to date more than 100 substrates have been identified [44,45]. On the other hand, lack of appropriate techniques for substrate identification might have hampered the discovery of additional substrates. While members of the presenilin family typically favor membrane protein substrates with type I ($N_{out}$) orientation, known substrates of the SPP/SPPL family (Table 1) either have a type II membrane orientation with the N terminus localizing to the cytoplasm ($N_{in}$) and the C terminus to the

**Table 1.** Currently known SPP/SPPL substrates and their membrane orientation.

| Substrate | Protease | Type | Reference |
|---|---|---|---|
| ASPH | SPPL3 | Type II | [33] |
| ATP1B1 | SPPL3 | Type II | [33,118] |
| CANT1 | SPPL3 | Type II | [33,118] |
| CD74 | SPPL2a, SPPL2b | NTF from type II | [24,25,26,94,141], [32] |
| CHST11 | SPPL3 | Type II | [118] |
| CHST14 | SPPL3 | Type II | [33,118] |
| CHST3 | SPPL3 | Type II | [118] |
| CKAP4 | SPPL3 | Type II | [33,118] |
| CLN5, full-length | SPPL3 | Type II | [142] |
| CLN5, NTF | SPPL2b, SPPL3 | NTF from type II | [142] |
| CYB5A | SPP | Tail-anchored | [46] |
| Dectin-1a/CLEC7a | SPPL2a, SPPL2b | NTF from type II | [96] |
| Epithin/PRSS14 | SPPL2b | NTF from type II | [143] |
| FAM20B | SPPL3 | Type II | [118] |
| FAM234A | SPPL3 | Type II | [118] |
| FasL | SPPL2a | NTF from type II | [75] |
| FKBP8 | SPP | Type II | [144] |
| FVenv | SPPL2a, SPPL2b | NTF from type III | [53] |
| FVenv, full-length | SPPL3 | Type III | [53] |
| GGT7 | SPPL3 | Type II | [33,118] |
| Glycosyltransferases GnT-V (MGAT5), β3GnT1, β4GalT1, EXTL3 and many others | SPPL3 | Type II | [22,33,118,145] |
| HCV polyprotein (viral) | SPP | Type II | [146] |
| HO-1 (Heme oxygenase 1) | SPPL2c, SPP | Tail-anchored | [23], [46,47] |
| HS3ST3A1 | SPPL3 | Type II | [118] |
| HS6ST1 | SPPL3 | Type II | [33] |
| HS6ST2 | SPPL3 | Type II | [33] |
| IgSF1 | SPP | Multipass | [147] |
| ITM2B (Bri2) | SPPL2a, SPPL2b | NTF from type II | [89] |
| LOX-1 | SPPL2a, SPPL2b | NTF from type II | [97] |
| Membrin | SPPL2c | Tail-anchored | [48] |
| NDST1 | SPPL3 | Type II | [33,118] |
| NRG1 type III | SPPL2a, SPPL2b | NTF from type III | [54] |
| OGFOD3 | SPPL3 | Type II | [33,118] |
| Phospholamban | SPPL2c | Tail-anchored | [23] |
| POMK | SPPL3 | Type II | [33,118] |
| RAMP4 | SPP | Tail-anchored | [46] |
| RAMP4-2 | SPP, SPPL2c | Tail-anchored | [23,46] |
| Signal peptides from various targets like prolactin (bovine), HIV gp160 (viral), calreticulin (rat), MHC class I (HLA-A*0301), pro-calcitonin, and many others | SPP | Signal peptide | [148-152] |
| SLC3A2 | SPPL3 | Type II | [33,118] |
| SrbA (*A. nidulans*) | SPP | Type II | [153] |
| Syntaxin-5 | SPPL2c | Tail-anchored | [48] |
| Syntaxin-8 | SPPL2c | Tail-anchored | [23,48] |
| Syntaxin-18 | SPP, SPPL2c | Tail-anchored | [48,154] |
| Teneurin/ODZ1 | SPPL2a | NTF from type III | [155] |

**Table 1.** (Continued).

| Substrate | Protease | Type | Reference |
|---|---|---|---|
| TMEM106b | SPPL2a, SPPL2b | NTF from type II | [156] |
| TNFalpha, full-length | SPPL2a | Type II | [101] |
| TNFalpha, NTF | SPPL2a, SPPL2b | NTF from type II | [30,74] |
| TOR1AIP1 | SPPL3 | Type II | [33] |
| Transferrin receptor 1 | SPPL2b | Type II | [95] |
| TRH4 | SPP | Multipass | [157] |
| VABP | SPPL2c | Tail-anchored | [48] |
| VAMP-1 | SPPL2a, SPPL2b | Tail-anchored | [51,52] |
| VAMP-2 | SPPL2a, SPPl2b | Tail-anchored | [51,52] |
| VAMP-3 | SPPL2a, SPPL2b | Tail-anchored | [51,52] |
| VAMP-4 | SPPL2a, SPPL2b | Tail-anchored | [51,52] |
| VAMP-8 | SPPL2c | Tail-anchored | [48] |
| VAPA | SPPL2c | Tail-anchored | [48] |
| Xbp1u, full-length | SPP | Type II | [40,158] |
| Zrt1 (*S. cerevisiae*) | SPP | Multipass | [159] |

extracellular space/lumen or are type IV tail-anchored proteins [23,46,47,48]. Tail-anchored (TA) proteins are a diverse class of membrane proteins that are post-translationally inserted with their C terminus into membranes by a specialized machinery [49]. Initially, only SPP and SPPL2c had been shown to cleave type IV proteins [50], but very recently the type IV SNARE proteins VAMP1-4 have been added to the list of SPPL2a and SPPL2b substrates [51]. All four VAMPs are so-called R-SNAREs with a smaller cytosolic domain than Q-SNAREs. Except for the SPPL3 substrates, most SPP/SPPL substrates undergo ectodomain shedding to remove the bulky C-terminal domains (Table 1) [1]. In line with this, type IV TA proteins exhibit only short C-terminal amino acid stretches facing the lumen or extracellular space and, thus, resemble type II proteins that have undergone shedding. Their larger N-terminal domain co-localizes with the cytosolic C-terminal domains of the SPP/SPPL proteases. *In vitro* experiments point to a size limitation of the cytosolic domain of cleavable type IV proteins [51,52]. In addition, a few polytopic type III membrane proteins have been found to be substrates to cleavage by SPP/SPPL proteases (Table 1). However, also in this case SPP/SPPL proteases hydrolyze the TM helix, which spans from an N-terminal cytoplasmic site to the C-terminal luminal site. Hence, the substrate exhibits the same orientation as the single-pass TM domains [53]. Mostly these substrate's TM domains have been released from the full-length protein by an independent proteolytic cleavage in a neighboring loop domain ([53,54].

SPPL3 releases the catalytic domain of a variety of type II glycosyltransferases to the Golgi lumen from where they are secreted, thus deactivating their function in the Golgi [22,31,55]. Whether this is a part of a signaling process or a degradation/downregulation mechanism has been extensively discussed and remains to be unraveled [42].

## Structure of SPP/SPPL proteases

### Known structure of presenilins and AI predictions as a model for the SPP/SPPL family.

The 3D structure of a PS1- and PS2-containing γ-secretase complex was determined experimentally by cryo-EM [56,57]. Utilizing sequence alignments, secondary structure predictions, and inhibitor studies, it was postulated that the 9 TMD catalytic core of the SPP/SPPL family should exhibit a very similar overall fold as the homologous presenilins [18,34,58,59,60,61]. Early after the homology-based discovery of SPP/SPPLs, it was postulated that their orientation in the membrane is inverted compared with presenilins. Thus, the N-terminal domain of SPP/SPPLs was suggested to be located on the luminal or extracellular site while the C-terminal domain is cytoplasmic [18-20]. This was confirmed through utilization of artificial glycosylation sites throughout the different proteases. Murine SPP, SPPL2a, SPPL2b, and SPPL2c are N-glycosylated on their N-terminal domains, and SPPL2b contains an additional consensus sequence for N-glycosylation in the hydrophilic loop between TM6 and TM7, while SPPL3 is nonglycosylated [23,34]. To our knowledge, the exact sites of glycosylation have not been confirmed for human SPP/SPPL so far. It is hypothesized that the membrane topology of the SPP/SPPL family causes their selectivity for type II and type IV TM protein substrates [62] [42]. PS1 and PS2 exhibit an inverted membrane orientation and consequently

prefer type I ($N_{out}$) substrates like Notch, APP, LDLR, CD44, and DNER [43,63,64].

To date, only a very low-resolution structural dataset for SPP [41] and no experimental data at atomic resolution for any member of the SPP/SPPL family are available. Since 2020, structure prediction has been revolutionized by the machine learning-based artificial intelligence (AI) AlphaFold [65]. Its prediction for large parts of the catalytic core of the five human SPP/SPPL family members displays a very high confidence level of > 90% (Fig. 1). Despite a common fold in the catalytic core, SPP/SPPLs differ substantially from presenilins as well as among each other in their N-and C-terminal domains and in the TM domain-connecting loop regions.

## N-terminal domains

Members of the SPPL2 subfamily comprise long glycosylated N-terminal domains that show a predicted globular fold that SPPL3 lacks completely (Fig. 1). SPPL2 proteases are therefore considered a subfamily within the SPP/SPPL family [42]. By sequence homology, the SPPL2 N-terminal domains were annotated in the UniProt database as a protease-associated (PA) domain [66,67]. Despite experimental structural data being available for such evolutionary highly conserved PA domains [67], a blast against deposited structures in the pdb database with the N-terminal sequences of SPPL2s does not pick up the deposited PA domain structures. However, we analyzed the AlphaFold predicted structures of the N-terminal SPPL2 domains in more detail (confidence level of the core up to > 90%) and performed a manual 3D alignment with part of the PA domains from *Bacillus subtilis* zinc aminopeptidase (pdb code: 6HC6) and human ferritin receptor. The resulting overlay strongly supports the prediction of the N-terminal SPPL2 domains exhibiting the canonical PA domain fold (Fig. 2). The N-terminal domain of SPP is special as it is much shorter and not homologous to that of the SPPL2 subfamily, and no defined fold is predicted for it.

It had been speculated whether the globular N-terminal domains of the SPPL2 subfamily fulfill a similar role as Nicastrin in the γ-secretase complex [50]. Nicastrin forms a lid on top of the catalytic site [56] and is discussed to act like a gatekeeper involved in substrate recognition [68]. However, Nicastrin with a total of 78 kDa and four domains, namely a short cytoplasmic domain, a TM domain, the Nicastrin small lobe and the Nicastrin domain [56,66], is much larger than the N-terminal domains of the SPPL2 subfamily with an approximate molecular weight of 16 kDa. Nonetheless, PA domains are known to be

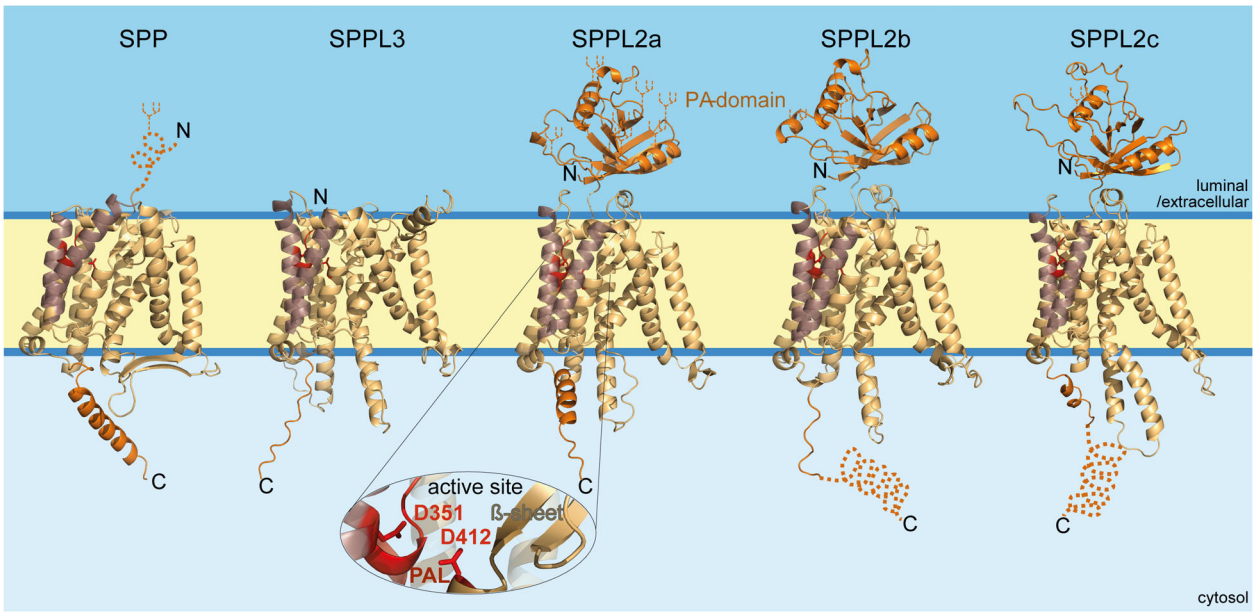


**Fig. 1.** AlphaFold predicted overall structures of all human SPP/SPPL family members. The active site aspartates and PAL motive are marked in red. The N-terminal PA domains and C-terminal domains are labeled in orange, and dashed lines indicate domains with no distinctive fold prediction. TM domains are depicted in olive and the predicted substrate entry site between TM domains 2 and 6 in dark salmon. Predicted N-glycosylation sites are marked with orange dashed tree-like structures. Structure representations were drawn using PyMol [160].

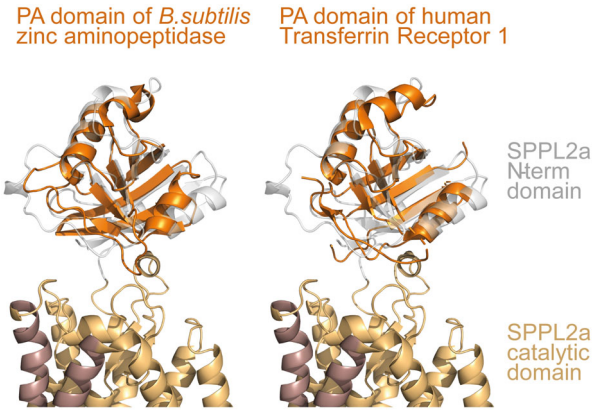


**Fig. 2.** Predicted 3D structures of SPPL2 N-terminal domains exhibit a PA domain fold. 3D alignment with core parts of experimentally solved structures from PA domains (pdb codes 6HC6, 6OKD) with AlphaFold model for SPPL2a N-terminal domain. Structure representations were drawn using PyMol [160].

involved in substrate recognition and dimerization processes [67], yet their function in SPPL2 proteases still remains enigmatic.

## C-terminal domains

The C-terminal domains differ even much more between the individual SPP/SPPL family members and are less well conserved across species. While SPPL3 and SPPL2a comprise only very short C-terminal sequence stretches of approximately 25 and 35 residues, respectively, these domains are much longer with predicted approximately 90 residues in SPPL2b and 165 residues in human SPPL2c. For neither of them, a distinctive fold is predicted and no similarities to known domains in other proteins are annotated in the database. Such intrinsically disordered regions (IDRs) point to a diverse folding upon binding of interaction partners as well as potential post-translational modifications *in vivo*. Those regions in many cases serve as 'molecular hubs' as part of regulatory and signaling processes as they are available for interaction with a wide array of macromolecular targets [69]. We therefore speculate that these domains fold *in vivo* in the presence of interaction partners in the cytoplasm yet to be identified. For SPPL2b, potential phosphorylation sites are predicted in this region [70,71]. For murine SPPL2c, two isoforms differing in their C terminus were reported [23,42] and SPP displays a short C-terminal domain with two potential phosphorylation sites on a predicted α-helix [65,70,71]. For comparison, the C termini of human presenilin 1 and 2 are short helices at the membrane/extracellular interface buried partly into APH-1 as shown by experimentally solved structures [56,57].

## Loop structures

A striking difference between the family members is their loop structures connecting the transmembrane domains. The largest difference is observed in the loop connecting the two active site TM domains 6 and 7, which faces the luminal/extracellular side in the SPP/SPPL family and the cytosolic side in the PS family. In each member, it contains many cross-species conserved residues, but the length and predicted fold of the loops differ between family members. In PS1, part of the respective sequence was shown to form a small hybrid β-sheet with the substrate apparently upon its binding [72,73]. Interestingly, the residues forming this β-sheet with the substrate in γ-secretase are not conserved between the PS family and the SPP/SPPL family members, but are conserved within the SPP/SPPL family (Fig. 3). No substrate complex structure of PS2 is available, but human PS1 and PS2 exhibit an identical sequence in this stretch. The short β-sheet is also predicted for SPP/SPPLs in this very position by AlphaFold (Fig. 3). The AI predicts this β-sheet to be present in the apo-form of the enzyme. This might in our opinion however be a prediction bias as the program has learned from all published structures of the γ-secretase complex—supposedly including the two substrate-bound complexes. The fact that the AlphaFold model of apo-presenilin 1 also exhibits the β-sheet hints to such bias and highlights the necessity for atomic resolution experimental structural data.

## Catalytic cleft architecture

The catalytic cleft architecture of the SPP/SPPL family is expected to be very similar to that of PS1. The catalytic function of the two conserved aspartyl residues has been verified by various mutational studies. The aspartyl to alanine mutation of either residue or both leads to an inactivation of the enzymes [18,30,53,74,75,76]. The general mechanism for peptide bond hydrolysis suggested for the intramembrane aspartyl proteases is similar to that known from soluble aspartyl proteases. In an acid–base system, one of the aspartates acts as a base and the other as an acid. Through the deprotonated aspartyl residue, the water molecule is polarized and initiates enzymatic catalysis by a nucleophilic attack on the carbonyl group of the scissile bond [77,78], while the protonated aspartyl residue polarizes the peptide carbonyl. A gem-diol intermediate forms before the aspartic residues return to their initial protonation state and the proteolytic products are released [77].

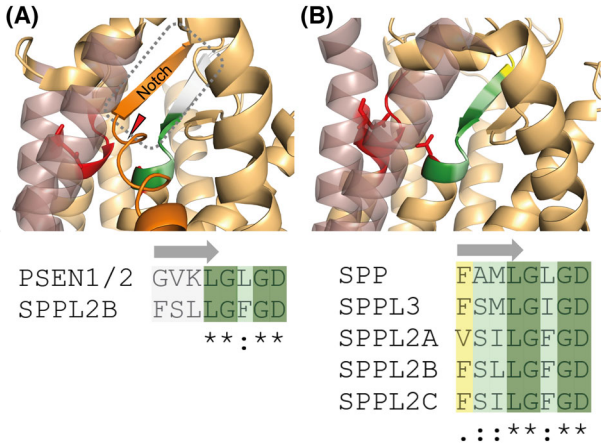


**Fig. 3.** Homology of substrate binding β-strand in aspartyl intramembrane proteases. The site of initial substrate cleavage is marked with a red arrow, and the hybrid β-sheet (PS) and the predicted hybrid β-sheet (SPP/SPPL) are highlighted in dashed gray boxes. (A) *Cryo-EM structure of the PS1/Notch complex*. Amino acids potentially contributing to β-strand formation in the enzyme are not conserved between PS and the SPP/SPPL family as indicated by comparison of PS1/2 and SPPL2b. (B) *AlphaFold predicted apo-structure SPPL2a*. Amino acids potentially contributing to β-strand formation in the human SPP/SPPL family exhibit a high degree of conservation. Structure representations were drawn using PyMol [160].

Consistent with this, the cryo-EM structure of presenilin in complex with the transition-state analogon L-685,458 reveals positioning of the inhibitor in the middle between the catalytic aspartates [79]. In the activated conformation, the two catalytic aspartates were assumed to be aligned in close proximity [79]. The glycine residues in the GxGD motif have been suggested to allow for a fluctuation of the distance between the two catalytic aspartates [6]. Very recently, it was shown that substrate binding to presenilin changes the protonation state of the catalytic residues and the acidity of the aspartate in the GxGD motif is shifted. It acts as the general acid during the cleavage mechanism, while the aspartate in the YD motif acts as general base. The PAL motif stabilizes the deprotonated state of the latter upon substrate binding [77]. In the SPP/SPPL family, the PAL motif is found in a highly conserved QPALLY sequence stretch in TM domain 9 [2,34]. The close conservation of the active site architecture within the presenilin and the SPP/SPPL family implies a similar mechanism of peptide bond hydrolysis.

## Inhibitors of SPP/SPPL proteases

Inhibitor studies have improved knowledge of the catalytic cleft architecture, potential allosteric sites, and substrate recognition mechanisms. For γ-secretase, inhibitors (GSIs) and modulators (GSMs) have been developed [80]. The GSIs are either transition-state analoga (TSA)-like Semagacestat (LY-450139) and Avagacestat (BMS-708163) or noncompetitive non-TSAs like L-685,458, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester (DAPT) and others [80,81]. In an attempt to alter the cleavage product spectrum, GSMs like E2012 have been developed [82]. E2012 binds at an allosteric site 25 Å from the active site and consequently is able to synergize with active site binding GSIs to improve inhibition of substrate cleavage [79].

For the SPP/SPPL family, not many specific inhibitors are available. However, as expected by homology, they are inhibited to some extent by γ-secretase inhibitors, and thus, knowledge about the binding modes derived from experimental structural data of such inhibitors in complex with γ-secretase might be transferred to some extent to the SPP/SPPL family. So far, all solved ligand–enzyme complex structures with GSIs show them occupying the pocket where the hybrid β-sheet is established. This holds true for TSA (L685,458) and non-TSA (Semagacestat, Avagacestat, MRK-560 [83]), for which structures in complex with γ-ecretase have been solved [57,79]. It was speculated that they inhibit the enzyme by impeding the hybrid β-sheet formation [79]. Despite the occupation of the same pocket, they are differentially recognized [79]. L-685,458 inhibits the activity of SPP, SPPL2a, and SPPL2b to some extent [61,84,85,86]. It is thus tempting to speculate it would occupy the equivalent position in SPP. However, inhibitors might even act in a distinct way on SPP/SPPL and γ-secretase, since the selective SPP inhibitor 1,3-di-(Ncarboxybenzoyl-L-leucyl-L-leucyl) amino acetone ((Z-LL)$_2$-ketone) does not show an inhibiting, but product spectrum modulating activity on γ-secretase [86-88]. SPPL2a and SPPL2b are also inhibited by (Z-LL)$_2$-ketone [21,89]. In contrast, it does not inhibit SPPL3, the closest homolog of SPP [53]. SPL-707 is a selective inhibitor for SPPL2a, which to a much lesser extent inhibits SPPL2b, and even SPP, and γ-secretase [90]. Similar to SPPL3, SPPL2c was not inhibited by (Z-LL)$_2$-ketone, but by DAPT in an assay with the model substrate RAMP4-2 [23]. Based on this inhibitor profile, it was suggested that SPPL2c exhibits crucial differences in its active site architecture compared with the other family members [23], which are not inhibited by DAPT [22,53]. It had previously been discussed whether DAPT could act as an allosteric inhibitor [91]; however, the cryo-EM structure proposed by Yang *et al.* [79] suggests its binding in the active site rather at the same position as the other peptidomimetic inhibitors. For DAPT however, the authors draw the conclusion

that it must occupy the same pocket as the other GSIs after re-evaluation of a previously solved structure of γ-secretase in complex with DAPT. Yet, conclusive high-resolution electron density for the inhibitor is still lacking, and thus, the evaluation of structural differences in the active sites of SPPL2c versus SPPL2a and SPPL2b remains enigmatic. In addition, all studies on inhibitory profiles and kinetics are greatly hampered by the fact that for the SPP/SPPL family no *in vitro* assay is available as of yet.

## Cleavage mechanisms of aspartyl intramembrane proteases

The specific cleavage or degradation of a protein upon intra- and extracellular stimuli is referred to as regulated proteolysis. Within the plane of the membrane, such process is termed regulated intramembrane proteolysis (RIP) [3,92]. Prior to the cleavage of the TM domain by an intramembrane protease, the ectodomain of the membrane protein substrate is removed by so-called canonical sheddases. The remaining membrane-bound fragment typically comprises a rather short ectodomain and can be subject to intramembrane proteolysis [93]. In the context of type II membrane substrates, intramembrane proteases of the SPP/SPPL family release a short C-terminal peptide to the extracellular/luminal space. This cleavage is termed initial cleavage, which is not precise and mostly happens on different cleavage sites in a defined cleavage region. The counterpart of the secreted peptide still comprises the substrate's TM helix and might—at least in some cases—be further processed by the intramembrane protease by consecutive cleavages in the substrate's TM domain. An N-terminal fragment termed intracellular domain (ICD) is then released to the cytosol [54,74,94,95,96,97]. Note that γ-secretase releases the fragments exactly in the opposite way, the C-terminal ICD to the cytosol and the N-terminal peptide to the extracellular/luminal space [45]. The consecutive cleavage mechanism has been demonstrated for some presenilin substrates, like CD44, APP and Notch [98-100] as well as for the SPPL2a and SPPL2b substrate TNFα [74,101] and is referred to as processivity (Fig. 4). For all other known aspartyl intramembrane proteases, cleavage sites are only known for either the secreted peptide or the ICD and it remains to be demonstrated whether SPP, SPPL2c, and SPPL3 also utilize a similar cleavage mechanism and whether processivity is commonly applied to PS, SPPL2a, and SPPL2b substrates.

### Substrate recognition

Substrates of intramembrane proteolysis encounter their protease in the membrane most likely via lateral diffusion. A gating mechanism for the transfer of the TM domain into the active site has previously been identified using structural and biochemical analysis of S2P and rhomboids [102-104]. Based on biochemical analysis and structural data from presenilin in complex with Notch, it was concluded that substrate lateral gating to the active site happens at TM domains 2 and 6 of presenilin [56,72,105,106,107]. The postulated substrate entry site of presenilin is projected onto the structure predictions for SPP/SPPL proteases (Figs 1 and 4). In the experimentally determined γ-secretase structures, the substrates Notch (V1721 to R1761) and APP (L688 to K726) interact with the enzyme via a short helix or loop in their N-terminal juxtamembrane domain, followed by their transmembrane domain and a β-strand in their C-terminal juxtamembrane domain. The N-terminal stretch interacts with a hydrophilic cavity of Nicastrin and the C-terminal β-strand on the opposite side of the membrane and forms the hybrid β-sheet with the β-strand of γ-secretase (see above) next to its active site [72,73]. The β-sheet induced upon substrate binding might be responsible for positioning of the substrate's backbone in the active site aligning the two catalytic aspartic acid residues for activity [72,73]. Note that despite the inverted topology of presenilin, the relative positioning of the substrate's TM helix and, thus, of the initial cleavage site region, to the catalytic aspartates is most likely equivalent in SPP/SPPL proteases as the substrates are also inverted (Fig. 4). The PAL motif in TM domain 9 of PS1 seems to play a crucial role in substrate recognition by triggering the alignment of the two aspartates, since within the membrane plane the localization of the catalytic aspartate residues and the PAL motif is close [56,72,108,109]. Supporting this, mutations within the PAL motif of presenilin significantly affected the catalytic activity of the enzyme [108-111]. While the PAL motif of presenilin faces the cytoplasmic side [56], it localizes to the luminal part of TM domain 9 in the SPP/SPPL family. AlphaFold structural predictions suggest that the PAL motif of SPP/SPPL proteases is also close to the catalytic center (Fig. 1) and it was shown by mutation to be required for SPP activity [109].

For quite some time, it was believed that aspartyl intramembrane proteases in general strictly require a short luminal/extracellular domain not exceeding a length of about 60 amino acids for efficient catalysis [112,113]. This assumption still holds true for substrate processing by γ-secretases, SPPL2b and SPPL2c, while with a more detailed analysis of SPPL3 it became evident that intramembrane aspartyl proteases are also capable of directly accepting substrates with long and

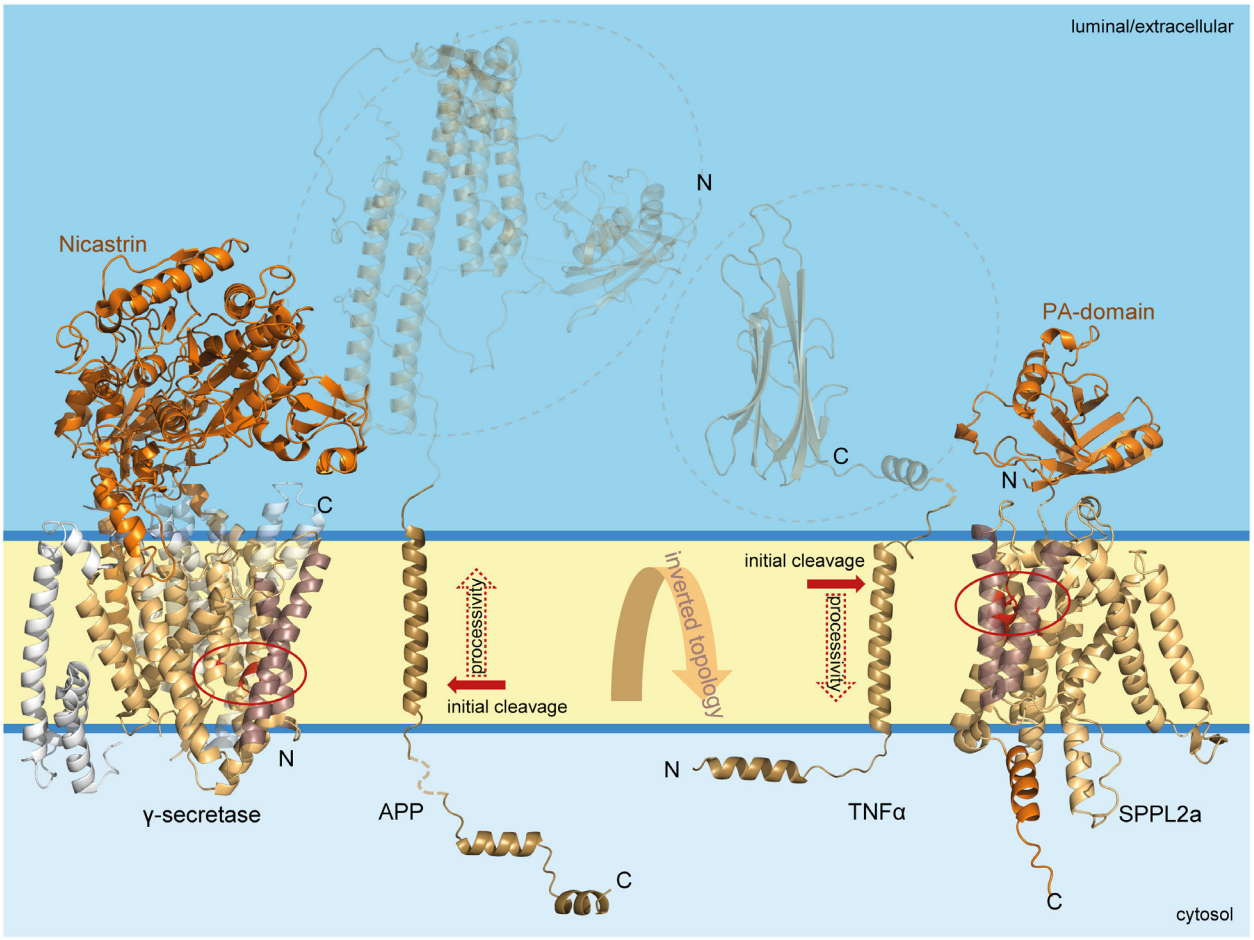


**Fig. 4.** Comparison of topology and substrate cleavage sites of γ-secretase (cryo-EM structure) and SPPL2a (AlphaFold prediction). Substrate representations result from predictions, and shedded ectodomains are faded. Active site aspartates and PAL motive are depicted in red; N-terminal PA domains, C-terminal domains, and Nicastrin in orange; and TM domains of PS1 and SPPL2a in olive. APH-1 and PEN-2 are shown in gray. The substrate entry between TM domains 2 and 6 (predicted for SPPL2a) is highlighted in dark salmon. The dashed red arrow indicates the direction of processivity, and the catalytic site of the enzyme is marked by a dashed red circle. Structure representations were drawn using PyMol [160].

bulky ectodomains [22,33,53]. Initial cleavage of these substrates by an intramembrane protease leads to the secretion of a large, often glycosylated protein fragment and release of a cytosolic ICD. Intramembrane proteases catalyzing such cleavages are termed noncanonical sheddases [93]. In addition to SPPL3, which acts as a bona fide noncanonical sheddase, SPP and SPPL2a have also been demonstrated to act as a noncanonical sheddases on individual substrates or in certain contexts, although they predominantly accept substrates with short ectodomains in the context of RIP [40,101].

The reason for the exclusion of substrates with large and bulky ectodomains by presenilins is attributed to Nicastrin [114]. The N-terminal extracellular residues of APP and Notch C-terminal fragments interact with the same cavity in Nicastrin in the 3D structures [72,73] pointing to a recognition mechanism in this area. Nicastrin is however located on the opposite site of the enzyme when compared to the N-terminal domain of the SPPL2 proteases (Fig. 4). However, due to the inverted topology of enzymes and substrates, recognition of the substrates in the luminal/extracellular space would be possible in both cases, but with the opposite side relative to the active site (Fig. 4). In terms of position relative to the active site of the enzymes, the substrate interaction region of Nicastrin would hence rather correspond to the C-terminal domains of SPP/SPPL proteases.

Since SPPL3 is lacking the N-terminal domain (Fig. 1), a lack of steric hindrance might be responsible

for acceptance of substrates with large ectodomains. However, this raises the question how full-length TNFα can enter the catalytic site of SPPL2a bypassing the steric exclusion of its N-terminal domain. It may be speculated that large movements of the TNFα ectodomain support the entry, but experimental proof for this hypothesis is so far missing.

Co-localization of enzyme and substrate is crucial for successful peptide bond hydrolysis. For quite some time, it was believed that co-localization of a given substrate with an intramembrane protease, when depicting the correct membrane orientation and the correct ectodomain length, is sufficient for cleavage. However, processing by intramembrane proteases seems to be highly specific as most membrane proteins are not turned over [6]. This might be attributed to the requirement of previous shedding by specific proteases like ADAMs (***A Disintegrin And Metalloproteinase***), constituting a specific upstream recognition step combined with a less specific cleavage of the remaining membrane-bound fragments by the intramembrane proteases [6,115,116].

Supporting this view, an elegant study with endogenously tagged SPPL3 ruled out that the intra-Golgi co-localization alone would be sufficient for SPPL3 to cleave a given type II Golgi protein, like GOLM1 whereas well-established substrates were shown to be cleaved under the same setting. [31]. Hence, the authors conclude that sequence-intrinsic substrate properties are required for substrate recognition. This is well in line with observations by others, as for instance SPPL2a and SPPL2b selectively recognize R-SNAREs while none of the analyzed Q-SNAREs were processed despite the presence in the same compartments [51].

## Initial cleavage

For γ-secretase, the site of initial substrate cleavage was trapped in the substrate complex structures for Notch and APP. It takes place three residues upstream of the hydrophilic sequence on the C-terminal (intracellular) side of the TM domain. This region adjacent to the cleavage site is forming the hybrid β-sheet with enzyme, and the intervening sequences between the substrate transmembrane helix and the β-strand harbor the primary cleavage site (Fig. 3) [72,73]. Initial cleavage sites have been mapped for some SPP/SPPL substrates [42,55]. In these, the initial cleavage sites are also located just a few residues upstream of a hydrophilic stretch. Hence, it is tempting to speculate that also here the primary cleavage site would be located between such β-strand and the TM helix. Experimental structural data as proof are however missing. Sequence analysis of cleavage regions so far has not led to the identification of consensus recognition sequences within the substrate's TM domains [2,55,117]. For SPPL3, it was suggested that an M or Y in position P1 might be favorable [33]; however, a recent N-terminomics study on the enzyme did not detect a consensus sequence [118]. Yet, the authors found that when substrate and SPPL3 are located in the same compartment, the exchange of the TM domain can turn a nonsubstrate to a substrate [119]. They conclude that despite the lack of a clear consensus sequence structural properties of the TM domain determine cleavability. For γ-secretase, it has also recently been shown that an artificial nonsubstrate poly-leucine TM domain was turned into a substrate by reintroducing a few selected residues from the APP TM domain [119]. The lack of a consensus sequence is a fundamental difference to most soluble proteases and holds true for many other intramembrane proteases, including the members of the presenilin family [120]. Only for some rhomboids, a specific amino acid sequence which may represent a recognition site for cleavage was reported [121]. Based on this, there is an ongoing debate whether intramembrane proteases rather sense structural properties like flexibility and dynamics of the TM domains or other 3D structural motifs in their substrates [6,101,115]. In this context, it was shown that certain amino acids in the substrate's TM helix, although not being part of a consensus recognition site, do influence cleavage efficiency and can even promote distant shifts in cleavage product spectrum [101,122,123,124]. The reduction of helical content within the substrate seems to be a general requirement for limited proteolysis [117,125] and is also reported for intramembrane proteolysis [72,73]. Soluble proteases are known to cleave in flexible loop regions and β-strands [117,126]. Natural mutants in the APP-gene prone to early development of Alzheimer's disease have been analyzed [45,127], and in several studies, mutations were introduced into the TM domain of substrates for rhomboids, γ-secretase, SPP, SPPL2a, SPPL2b, and SPPL3 to decipher the underlying mechanisms [6,55,101,122,128,129,130,131,132]. The overarching observation is that changes leading to a greater flexibility in the TM helix mostly increase cleavage [6,115,122]. This goes well in line with the general requirement for local unfolding in proteolysis. In this scenario, exchange of residues at the cleavage sites that decrease helix flexibility, like exchanges to P, should increase cleavage, whereas exchanges to L, which are known to stabilize α-helices, should result in decreased cleavage [101,120]. This was confirmed for

noncanonical shedding of TNFα by SPPL2a, since several proline substitutions resulted in increased initial cleavage, while exchanges to leucine had the opposite effect [101]. Interestingly, the strongest increase in cleavage was observed when the TNFα TM helix was destabilized distant from the initial cleavage site, while stabilization directly at the initial cleavage site resulted in the most prominent reduction of cleavage [101]. A similar effect was observed for a cleavage site in APP (G38) where the mutation to leucine reduced the cleavage [120]. However, a mutation to proline at the same position also resulted in cleavage reduction and it was hence concluded that the overall substrate positioning was altered, since a proline at the cleavage site could present too much of a sterical hindrance for efficient cleavage [120]. In line with that a proline substitution at the SPPL2a, initial cleavage site in TNFα had no quantitative impact on processing but changed the product spectrum [101]. For the SPPL2b substrate Bri2, only one out of four glycines of the TM helix was identified as critical for cleavage efficiency [122]. Mutation of this glycine to alanine resulted in significantly reduced cleavage [122]. Also for SPP, a helix break in the substrate was shown to be required for efficient initial cleavage [130].

### Processivity

The precise mechanism underlying the processive cleavage following the initial cleavage still remains enigmatic. To date, two models exist: In the unwinding model, the substrate's TM helix would unwind successively allowing for the next cleavage sites to reach the catalytic aspartates, while in the piston model the substrates maintain helical conformation and are shifted to the active site [72,133,134]. The later model would require the hydrophilic sequences N-terminal of the TM domain (extracellular in case of γ-secretase; intracellular in case of SPP/SPPL proteases) to enter the membrane [72] and, thus, the unwinding model with successive β-strand formation accessibility of new cleavage sites was favored [72]. In line with this substrate unwinding theory, some mutations were shown to influence the processivity of GxGD proteases. In the logic of processivity, the shifts toward larger fragments might be due to hindered processivity whereas the disappearance of fragments could result from very fast processive cleavage, which prevents detection in the experiment. For γ-secretase, processive substrate cleavage is best studied for Notch and APP [99,100] and SPPL2a and SPPL2b were reported to act on TNFα in a processive manner [62,101]. Some mutations leading to early onset Alzheimer's disease localize to the TM domain of APP, affect the processivity of γ-secretase and, thus, cause a shift in ratio of the different product species, resulting in an increase of longer and more aggregation-prone Aβ species [45].

### Impact of flexible hinges in the substrate on cleavage and processivity

Apart from local unwinding, helical stability could also impact substrate processing by other mechanisms. A flexible hinge region within the substrate's TM domain was suggested as crucial factor for efficient cleavage [6]. Such flexible hinges might, for instance, allow for proper substrate positioning in the catalytic center, facilitate entry of the substrate to the active site or support large-scale bending movements resulting in close substrate–protease interaction [6]. Flexible hinge regions or curve-inducing bendings have been identified in the TM domains of several substrates of intramembrane proteolysis such as APP [120,135,136], TatA [6,137,138], TNFα [101], and GnT-V [55]. Mutations at the proposed hinge in TNFα from AGA to helix stabilizing LLL reduced initial cleavage by SPPL2a [101]. In line with this, a proline mutation in a proposed hinge region in the TM domain of GNT-V, a substrate of SPPL3, resulted in increased initial cleavage of the substrate [55]. Computational analysis of 23 SPPL3 substrates indicates a modest enrichment of glycine residues in the middle of the cleavable TM domain [118]. A recent study including deuterium exchange experiments on the APP substrate's TM domain analyzed the effect of mutations on the N-and C-terminal part of the substrate's TM domain as well as of a hinge region between them. The authors conclude that flexibility in N-terminal part as well as in the hinge region promotes efficient cleavage. Flexibility in the C-terminal part of the substrate was suggested to allow for the formation of a cleavage-competent state near the active site [119]. These observations are in our opinion in line with the unwinding model [72,134] and nicely correlate with the data on TNFα and SPPL2a [101].

## Outlook

Despite significant progress in understanding the structure and function of the SPP/SPPL family, there are still several challenges that need to be addressed to fully comprehend their catalytic mechanisms and physiological roles. A major limitation is the reliance on mainly cellular assays to study substrate recognition and processing, which makes it difficult to obtain accurate kinetic and affinity data. One reason for this

is the localization of these proteases in membranes of different subcellular compartments, which hampers a purely biochemical analysis of their interaction with substrates. To overcome this limitation, the development of *in vitro* proteolysis assays analogous to γ-secretase [139,140] is necessary to unambiguously validate substrates, nonsubstrates, and inhibitors. Such assays with at least partially purified proteases and substrates could be extended to include interacting proteins that may influence substrate recognition and processing.

While the AI predictions exhibit a high confidence level in the core of the enzymes, there is still a lack of information on the parts involved in substrate recruitment and potential higher molecular weight complex formation.

To finally completely decipher substrate recognition and cleavage mechanism of aspartyl intramembrane proteases, more high-resolution 3D structural data would be most valuable.

The elucidation of a structure-based substrate recognition mechanism could facilitate the process of substrate identification and enable a more comprehensive understanding of the molecular mechanism and physiological function of this protease family.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

SH and RF wrote the manuscript, and SH provided all the figs; BS gave essential conceptual advice and input. All authors contributed to the editing of the manuscript.

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