

The role of SPP/SPPL intramembrane proteases in membrane protein homeostasis

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Signal peptide peptidase (SPP) and the four SPP-like proteases SPPL2a, SPPL2b, SPPL2c and SPPL3 constitute a family of aspartyl intramembrane proteases with homology to presenilins. The different members reside in distinct cellular localisations within the secretory pathway and the endo-lysosomal system. Despite individual cleavage characteristics, they all cleave single-span transmembrane proteins with a type II orientation exhibiting a cytosolic N-terminus. Though the identification of substrates is not complete, SPP/SPPL-mediated proteolysis appears to be rather selective. Therefore, according to our current understanding cleavage by SPP/SPPL proteases rather seems to serve a regulatory function than being a bulk proteolytic pathway. In the present review, we will summarise our state of knowledge on SPP/SPPL proteases and in particular highlight recently identified substrates and the functional and/or (patho)-physiological implications of these cleavage events. Based on this, we aim to provide an overview of the current open questions in the field. These are connected to the regulation of these proteases at the cellular level but also in context of disease and pathophysiological processes. Furthermore, the interplay with other proteostatic systems capable of degrading membrane proteins is beginning to emerge.

Introduction

Intramembrane proteolysis was initially conceptualised based on analysis of processing of the amyloid precursor protein (APP) [1] and the sterol element-binding protein (SREBP) [2,3]. Since then, it has emerged as an important cellular regulatory pathway. Metallo-, serine, glutamyl and aspartyl intramembrane-cleaving proteases can be distinguished based on their catalytic mechanism [4]. Together with the presenilins, the catalytically active subunits of the γ -secretase complex, signal peptide peptidase (SPP) and the related four SPP-like (SPPL) proteases SPPL2a, SPPL2b, SPPL2c and

SPPL3 constitute the mammalian aspartyl intramembrane proteases [5,6]. Subcellular localisations of the different SPP/SPPL proteases range from the secretory pathway (ER: SPP, SPPL2c; Golgi: SPPL3) to the plasma membrane (SPPL2b) and the endo-lysosomal system (SPPL2a; Table 1) [7–9]. In contrast to the presenilins, no accessory subunits have been identified to date, which are essential for proteolytic activity of SPP/SPPL proteases. However, SPP can form homotetramers [10] and both SPP and SPPL2c are part of distinct high-molecular weight protein complexes of

Abbreviations

BMDC, bone marrow-derived dendritic cells; BMDM, bone marrow-derived macrophages; DC, dendritic cell; HO-1, heme oxygenase 1; RIP, regulated intramembrane proteolysis; SNARE, soluble N-ethylmaleimide-sensitive-factor attachment receptor; SPP, signal peptide peptidase; SPPL, signal peptide peptidase-like; VAMP, vesicle-associated membrane protein.

Table 1. Properties and functions of the different SPP/SPPL proteases. Nd, not observed yet.

	SPP	SPPL2a	SPPL2b	SPPL2c	SPPL3
Subcellular localization	ER	Endosomes, lysosomes	Plasma membrane	ER	Mid-Golgi
Tissue expression in mice	Ubiquitous	Ubiquitous	Strong in brain, presumably ubiquitous at lower level	Testis	Ubiquitous
RIP	Yes	Yes	Yes	Nd	(Yes)
Direct cleavage of substrates with long ectodomains	(Yes)	Yes	Nd	Nd	Yes
TA protein cleavage	Yes	Yes	Yes	Yes	Nd
Cellular and (patho-)physiological pathways	Signal peptide processing Viral infections Carcinogenesis Antigen presentation ER morphogenesis	B cell maturation DC function Atherosclerosis Antimycobacterial immunity Tooth enamel formation Vesicle trafficking?	Atherosclerosis Antifungal immunity Carcinogenesis Vesicle trafficking?	Male germ cell development	Regulation of protein glycosylation Regulation of glycosphingolipid repertoire NK cell maturation NFAT activation

currently unknown composition [11]. Altogether, very little is known about the interactome of SPP/SPPL proteases and how this may influence the function of these enzymes.

All SPP/SPPL proteases cleave single-span transmembrane proteins with a type II topology of their transmembrane segment, so that the N-terminus of the protein faces the cytosol [5,6]. Different types of substrates can be distinguished based on the size of their luminal/ecto-domain. The capability of the individual SPP/SPPL family members to cleave different substrate types is summarised in Table 1. Lists of the identified substrates and also graphical visualisations of the different cleavage modes were provided in recent review articles [6,12] and an accompanying article (Höppner *et al.*). Prototypical substrates of SPP, SPPL2a, SPPL2b and SPPL2c exhibit a short luminal/ectodomain [5,13]. Therefore, in many cases the action of these proteases is part of a proteolytic sequence referred to as regulated intramembrane proteolysis (RIP) [14], in which the size of the luminal/ectodomain of a type II membrane protein is first reduced. The resulting membrane-bound N-terminal fragment (NTF) represents the actual substrate of SPP/SPPL proteases. Luminal/ecto-domains of tail-anchored (TA) proteins, which are post-translationally inserted into the membrane in a type II orientation [15], usually comprise just a few residues. This allows direct cleavage by SPP/SPPL intramembrane proteases without the need of the preceding action of an ectodomain

removing protease. Cleavage of TA proteins was initially reported for SPP [16,17], but later also for SPPL2c [11,18] and recently also for SPPL2a and SPPL2b [19]. SPPL3 cleaves a wide spectrum of type II membrane proteins with larger ectodomains directly, which leads to the shedding and secretion of the substrates' ectodomains [20,21]. This property was initially considered to be unique within the SPPL family. However, also SPP [22] and SPPL2a [23] accept specific substrates with larger ectodomains without any pre-processing. It remains to be determined, if this also applies to further substrates and/or the other family members SPPL2b and SPPL2c, for which this cleavage mode has not been observed yet.

In comparison to the closely related γ -secretase, for which more than 100 different substrates were reported [24], numbers of proteins cleaved by SPP/SPPL proteases are still rather limited. This may indicate that the substrate spectrum of SPP/SPPL proteases is not fully unravelled yet and/or that cleavage occurs with a higher selectivity. The sequence- and/or structural features that determine whether a specific type II or TA protein is cleaved by SPP/SPPL proteases are still poorly understood. Current views on this as well as the structural layout of SPP/SPPL proteases are discussed in an accompanying review in this issue (Höppner *et al.*). In addition to their proteostatic function, SPP/SPPL proteases impact on a wide range of cellular pathways including membrane trafficking, protein glycosylation and signal transduction as compiled

in Table 1. These aspects will be elaborated here with a specific focus on those SPP/SPPL substrates and functions, for which validation *in vivo* has been obtained. In light of a number of previous review articles on this protease family [5,6,12,25,26], which readers are encouraged to consult, we will put a certain focus on the more recent findings being described in more detail in comparison to earlier studies. Beyond summarising our current state of knowledge about SPP/SPPL proteases, we aim to highlight and discuss open questions in the field.

Proteolytic and non-proteolytic functions of SPP/SPPL proteases

SPP – signal peptide degradation, ERAD, cancer and viral protein processing

Signal peptide peptidase was the first member of the SPP/SPPL family to be identified based on its ability to process certain signal peptides [27]. It is still not clear, if this is limited to select signal peptides or represents a universal mechanism for signal peptide clearance. Later it was found that different viral proteins undergo and require processing by host cell SPP in order to allow virus production [25] with the core protein of the hepatitis C virus being the best-characterised example [28]. With regard to cell-intrinsic substrates, SPP-mediated cleavage of different TA proteins has come into focus [16,17,29]. However, the functional relevance of these cleavage events is only partially understood.

Signal peptide peptidase interacts with important components of the ER-associated degradation (ERAD) machinery like Derlin1 and the E3 ubiquitin ligase TRC8 [22], suggesting SPP to be part of the ERAD machinery. Along this line, TRC8 and another E3 ligase, MARCH6, facilitate the degradation of misfolded TA proteins as well as of SPP cleavage products [30]. Furthermore, SPP can cleave mitochondrial TA proteins, which become mis-localised to the ER in the absence of ATP13A1 [31]. However, further studies will be required to fully understand the role of SPP in the context of the unfolded protein response.

Signal peptide peptidase is overexpressed in several different types of cancer [32,33], where high SPP expression was associated with a poor prognosis [33]. Expression of SPP correlated strongly with pan-cancer immune checkpoint gene expression and immune cell infiltration. Knockdown of SPP in different tumour cell lines inhibited proliferation and migration *in vitro* as a correlate for their invasive potential [32,33]. Along this line, SPP depletion also reduced tumour growth

in vivo in a xenograft model with MDA-MB-231 cells [32]. Cleavage of the TA protein heme oxygenase 1 (HO-1) [17] and the type II membrane protein FKBP8 [32] have been proposed as biochemical mechanisms, by which SPP supports growth and invasiveness of malignant cells. Presumably, additional substrates and pathways will be involved.

Beyond the emerging role in tumour biology, the pathophysiological function and therefore also the potential of SPP as a therapeutic target is just beginning to be discovered. A major limitation in this context is that the function of SPP *in vivo* and the physiological relevance of the cleavage events, which have been characterised *in cellulo*, are mostly unknown. Constitutive SPP knockout mice were reported to exhibit perinatal lethality [34]. In this regard, it was surprising, that a systemic knockout induction in mice carrying a conditional floxed SPP allele starting at the age of 2 weeks seemed to be well tolerated over several weeks [35]. This constellation could indicate a specific requirement of SPP around birth, which is bypassed by the postnatal knockout induction. Alternatively, residual amounts of SPP due to incomplete depletion in the inducible system may be sufficient to prevent certain lethal phenotypes. To date, the described conditional system has only been employed to investigate the impact of SPP on corneal herpes simplex virus (HSV) replication. This is based on the finding that binding of the HSV glycoprotein K (gK) to SPP is important for HSV replication in cultured cells [36]. Several studies utilising the described floxed SPP allele to deplete SPP in the eye (Pax6-Cre) or peripheral sensory neurons (Advillin-Cre) now confirmed an important impact of SPP on HSV infection *in vivo* [35,37,38]. In line with the HSV life cycle, blocking SPP expression in the eye reduced primary viral replication but not latency reactivation [38], which was the opposite when SPP was depleted in sensory neurons [37]. A general phenotypic characterisation of the conditional SPP knockout mice and especially the analysis of substrate accumulation will be needed to define the physiological function of SPP and its different substrates *in vivo*, which would also be essential to assess the risks of a pharmacological inhibition potentially being useful in the context of an HSV infection or cancer.

SPPL2a and SPPL2b - two proteases with unique as well as overlapping functions

Based on our current knowledge, SPPL2a and SPPL2b can be regarded as the most closely related members of the SPP/SPPL family. When assessing substrate cleavage in cellular protease-substrate

co-overexpression assays, their substrate spectra mostly overlap [6,12]. Nevertheless, they differ in their primary subcellular localisations as described above as well as in their tissue expression profiles [9]. This constellation may help to explain why the characterisation of SPPL2a/b single and double-deficient mice has revealed both non-redundant and synergistic functions of these two proteases.

The first applies to the cleavage of CD74, the invariant chain of the MHCII complex, for which under endogenous conditions *in vivo* only SPPL2a is responsible [9]. In SPPL2a KO mice, CD74 NTFs accumulate and were linked to a maturation defect of B lymphocytes and loss of certain dendritic cell (DC) subsets by us and others [39–42]. This phenotype was not present in SPPL2b-deficient mice and was not aggravated by additional loss of SPPL2b in the SPPL2a KO [9]. The molecular mechanisms of how the CD74 NTF disturbs the respective immune cells are still only partially understood [43]. A common feature of SPPL2a KO B cells and DCs seems to be disturbed subcellular membrane trafficking manifesting as an accumulation of endosome-derived vacuoles in SPPL2a-deficient B cells and a reduced surface delivery of critical receptor proteins in both cell types [39,42,43]. Human patients carrying mutations within the *SPPL2A* gene exhibit impaired CD74 processing [44,45]. The respective patients fail to mount effective immune responses against mycobacteria reflecting primarily the disturbed differentiation and function of DCs [45]. This confirms a conserved role of SPPL2a in the immune system of mice and humans and was the basis for proposing SPPL2a inhibition as a novel immuno-modulatory approach [46].

Whereas CD74 degradation is specifically conducted by SPPL2a *in vivo*, we observed that SPPL2a and SPPL2b synergistically cleave NTFs of the Lectin-like oxidised lipoprotein receptor 1 (LOX-1) *in vivo* [47,48]. LOX-1 is a receptor protein, which can mediate the uptake of oxidised LDL (oxLDL) into endothelial cells and macrophages [49]. At the same time, ligand binding triggers a signalling response promoting endothelial dysfunction and atherosclerotic plaque development [48,50]. We found that the signalling function of LOX-1 is modulated by NTFs derived from this receptor [47]. LOX-1 NTFs can be generated both by ectodomain shedding as well as lysosomal proteolysis. Irrespective of their generation, these fragments require SPPL2a/b for their degradation, which is why they accumulate in the vasculature of SPPL2a/b-deficient (SPPL2a/b dKO) mice [47]. LOX-1 NTFs can interact with the full-length receptor and by this means enhance its ligand-induced signalling.

Furthermore, the NTFs can self-associate and thereby initiate signal transduction independent of ligand stimulation. Altogether, this leads to enhanced pro-atherogenic and pro-fibrotic signalling in the absence of SPPL2a/b. In agreement, we found that the development of atherosclerotic plaques is significantly enhanced in SPPL2a/b dKO mice [47].

In LOX-1 over-expressing HeLa cells steady state LOX-1 NTF levels were not modulated by the presence of oxLDL, suggesting that proteolytic processing of LOX-1 involving generation and removal of NTFs occurs independent of ligand-binding and takes place constitutively. Thus, although the NTF levels at least in the analysed experimental systems were not dependent on the induction of signalling, their presence was able to enhance signalling. Due to these properties of the LOX-1 NTF, the removal of this fragment by intramembrane proteolysis provides a critical negative regulation of pro-atherogenic signalling. LOX-1 belongs to the family of C-type lectin receptors (CLRs), which includes several other proteins with a type II membrane topology. Based on this, we recently identified another member of this family, Dectin-1 (CLEC7A) as SPPL2a/b substrate [51].

SPPL2a/b as regulator of an anti-fungal pattern recognition receptor

Dectin-1 is a type-II-oriented pattern recognition receptor (PRR) that participates in the activation of cellular pathways initiating the secretion of pro-inflammatory cytokines and reactive oxygen species (ROS) upon recognition of fungal pathogens (Fig. 1) [52,53]. Upon ligand-binding, tyrosine phosphorylation of the cytoplasmic hemITAM motif of Dectin-1 initiates various signalling cascades via recruitment and activation of the tyrosine kinase Syk as well as the internalisation of the receptor/ligand complex into the phagosome, where it is degraded [54].

Dectin-1 exists in two major isoforms termed Dectin-1a and -1b. These are solely distinguished by the presence of the juxtamembrane stalk domain present in Dectin-1a, but not Dectin-1b [55,56]. Interestingly, both isoforms follow different degradative pathways upon their internalisation [51,57,58]. As reported by three independent groups [58–60], the smaller Dectin-1b isoform is degraded in an ubiquitin-dependent manner most likely within intraluminal vesicles of multivesicular bodies, which is also required for termination of receptor-mediated signal transduction. By contrast, we demonstrated that a stable NTF is generated from the longer Dectin-1a variant within the phagosomal membrane by endo-/lysosomal proteases

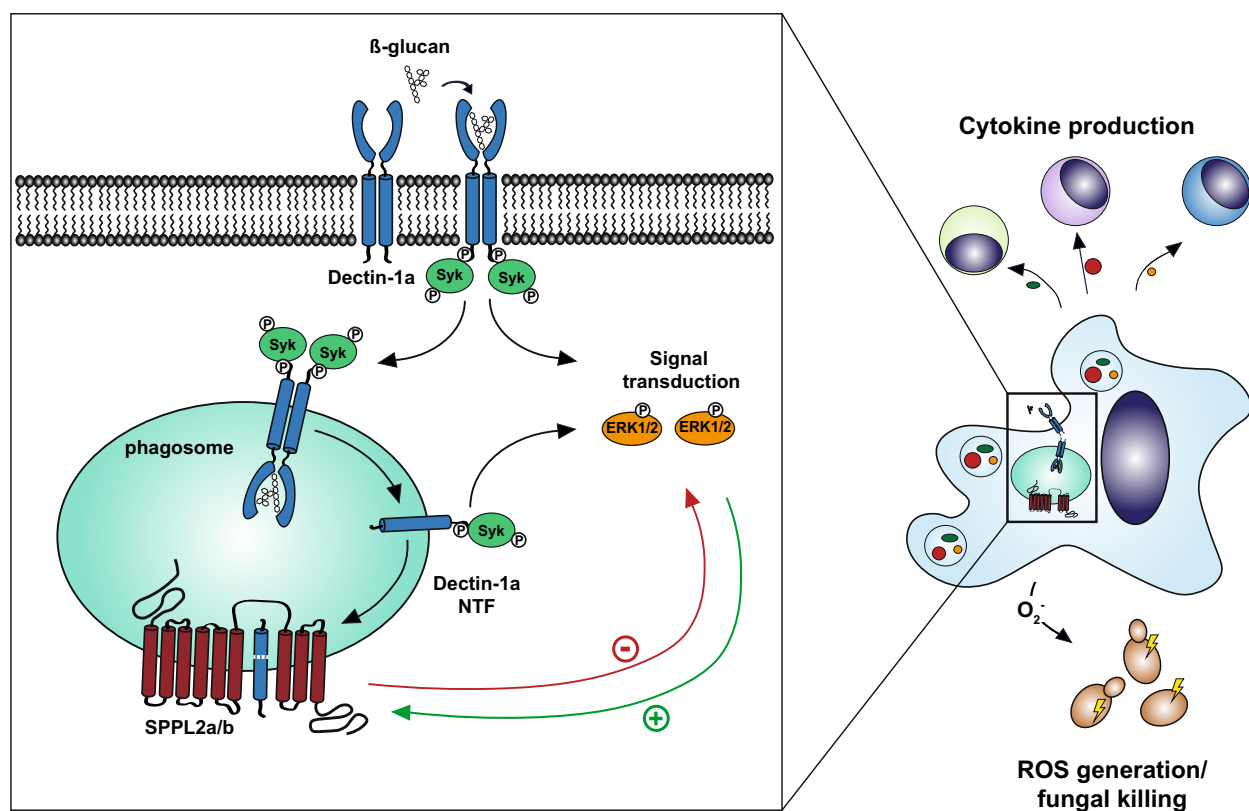


Fig. 1. SPPL2a/b terminate Dectin-1-dependent anti-fungal immune responses. Upon binding of fungal β -glucans, Dectin-1a initiates signalling cascades via Syk and the MAP kinase pathway that lead to cytokine secretion and ROS generation as anti-fungal response. Ligand binding simultaneously induces the internalisation of the receptor to phagosomes, where a stable Dectin-1a NTF is generated that still participates in signal transduction. Dectin-1a-dependent signalling is finally terminated by SPPL2a/b-mediated intramembrane proteolysis of the NTF. The expression of SPPL2a/b is controlled in a feedback loop by Dectin-1-induced signalling cascades thereby allowing efficient fine-tuning of Dectin-1-dependent anti-fungal immune responses.

(Fig. 1). The mechanisms stabilising the Dectin-1a NTF in the limiting phagosomal membrane remain unclear. Importantly, the NTF was found to significantly contribute to Dectin-1 signalling because of its ability to recruit essential factors of the signalling machinery downstream of the receptor including the spleen tyrosine kinase Syk [51]. This isoform-specific Dectin-1 signalling mode combines rapid delivery of the receptor and the bound pathogens to degradative phagosomes, while at the same time allowing a persistent signalling response based on the retention of the Dectin-1a NTF.

The termination of this Dectin-1a NTF-mediated signal transduction requires its proteolytic processing by SPPL2a/b (Fig. 1). As intramembrane proteases are considered to be intrinsically slow enzymes [61], these may be particularly well suited for this purpose. While SPPL2a naturally resides within endosomal/lysosomal compartments [8], SPPL2b is co-internalised with Dectin-1a to the phagosome where cleavage of the Dectin-1a NTF takes place. In contrast to

the constitutive processing of LOX-1, generation and the subsequent intramembrane proteolysis of the Dectin-1a NTF are ligand-induced. Clearance of the Dectin-1a NTF was delayed in mouse embryonic fibroblasts lacking both SPPL2a and SPPL2b and in HEK cells with pharmacological inhibition or knockdown of SPPL2a/b [51]. Analysis of SPPL2a/b single-deficient cells, also in combination with additional pharmacological inhibition, suggests a synergistic contribution of SPPL2a and SPPL2b to degradation of the Dectin-1a NTF.

To demonstrate the functional relevance of this proteolytic pathway, we have analysed Dectin-1a induced responses in bone marrow-derived dendritic cells (BMDC) and macrophages (BMDM). Although SPPL2b single deficiency may only provide partial inhibition of Dectin-1a NTF intramembrane processing, this system was selected since SPPL2a deficiency leads to CD74 NTF accumulation which has widespread impact on DC function as described before

[42]. In mice, Dectin-1 isoform expression differs in a strain-dependent way [56]. As C57BL/6 mice express very little Dectin-1a, this pathway had to be investigated on a Balb/c background. Due to the delayed turnover of endogenous Dectin-1a NTF, BMDCs and BMDMs derived from SPPL2b-deficient mice demonstrated enhanced Dectin-1a signalling, which translated into enhanced ROS production upon stimulation with fungal ligands or living *Candida albicans* [51]. In the latter case, also secretion of pro-inflammatory cytokines was significantly increased in the protease-deficient cells. This confirms a relevant role of intramembrane proteolysis in order to limit and terminate anti-fungal immune responses.

Intriguingly, activation of the immune cells by the described stimulants upregulated expression of SPPL2a and SPPL2b [51] thereby creating a negative feedback loop preventing exacerbated signalling responses upon contact with fungal pathogens (Fig. 1). As the described proteolytic pathway only applies to Dectin-1a, it is important to mention that in humans the SPPL2a/b-regulated Dectin-1a isoform constitutes about 50% of the total Dectin-1 pool. Therefore, this pathway can be anticipated to be of functional and pathophysiological relevance also in humans. Further members of the CLR family act as anti-fungal pattern

recognition receptors [62]. It was found that also NTFs derived from the CLRs Mincle and DC-SIGN were stabilised by SPPL2 inhibition [51]. However, these NTFs seemed to be generated in a constitutive way independent of ligand binding. How processing of these or potentially further CLRs by SPPL2a/b, contributes to the immunomodulatory function of these proteases remains to be analysed in detail.

SNARE proteins as substrates of SPPL2a/b

Soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins comprise a family of 36 proteins in humans, which are responsible for catalysing membrane fusion events [63,64]. This is achieved by the zipper-like complex formation between SNARE proteins of different subfamilies on the opposing membranes (Fig. 2). In addition to this canonical function, SNARE proteins are also discussed to contribute to earlier steps in vesicular trafficking, for example, by interacting with tethering factors and thereby helping in targeting vesicles to their correct destinations [64]. Though initially identified and investigated based on their role in synaptic vesicle exocytosis [65], SNARE proteins function broadly in cellular membrane trafficking.

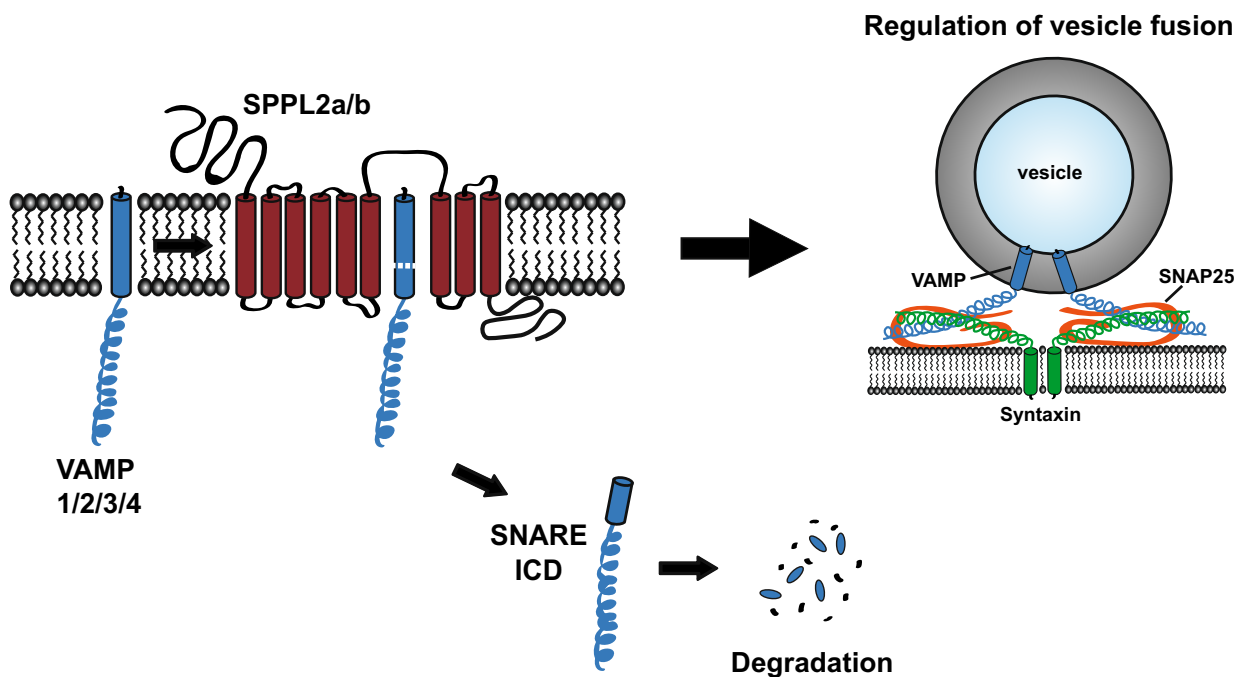


Fig. 2. Regulation of SNARE homeostasis by SPPL2a/b. VAMP1/2/3/4 can be directly cleaved by SPPL2a/b without any preceding cleavage event, which might impact on vesicle fusion. The major cleavage fragment, the SNARE intracellular domain (ICD) is further degraded by an unknown mechanism. VAMP1-4 accumulate upon inhibition of SPPL2a/b or in tissues of SPPL2a/b dKO mice. It is currently unknown if this SNARE protein accumulation affects membrane trafficking pathways.

The vast majority of SNARE proteins are TA proteins [66] qualifying them as potential SPP/SPPL protease substrates. As two ER-resident syntaxins had been found to be substrates of SPP or SPPL2c [11,18,29], we aimed to assess if any SNARE proteins in the late secretory and endo-lysosomal pathway are substrates of SPPL2a and/or SPPL2b. Therefore, we screened 18 SNARE proteins in cellular protease-substrate co-expression assays [19]. Of these, only the vesicle-associated membrane proteins (VAMP) VAMP1-4 were cleaved by SPPL2a/b (Fig. 2). Surprisingly, none of the evaluated syntaxins was processed despite members of this family being SPP and SPPL2c substrates. Most of the SNARE proteins, for which no cleavage was observed, showed overlapping subcellular distributions with the proteases. This points to additional, yet unknown substrate features which are required for cleavage (Höppner *et al.*, accompanying review). Fusion of GFP to the N- and C-termini of VAMP2 and VAMP4 abolished cleavage [19]. Thus, the size and/or confirmation of the extramembraneous domains—in addition to other factors—may play a role in this context.

Importantly, we could confirm cleavage of endogenous VAMP1-4 by SPPL2a/b based on the accumulation of these proteins upon SPPL2a/b inhibition in cell lines and in tissues of SPPL2a/b dKO mice [19]. Altogether, cleavage of the four VAMP proteins occurs in a tissue- and cell-type-specific manner as different patterns of substrate accumulation were observed. Surprisingly, in total brain lysates from SPPL2a/b dKO mice, VAMP1 and VAMP3 levels were only very mildly increased, while cerebellum showed a more robust increase in VAMP1, VAMP3 and VAMP4. VAMP2 levels in the CNS were not affected by SPPL2a/b deficiency. Altogether, the impact of SPPL2a/b-induced VAMP2-4 accumulation was much more pronounced in non-neuronal tissues. Heart, pancreas, liver, spleen, thymus and also immune cells like DCs and macrophages, showed clear accumulations of VAMP2 and VAMP4, while VAMP3 varied in a tissue-dependent way [19]. This unambiguously confirms a role of SPPL2a/b in the turnover of VAMP1-4 (Fig. 2). At the endogenous level, the individual contribution of SPPL2a and SPPL2b to this process has not been analysed yet. In the overexpression assay, both were comparably capable of proteolysing VAMP1-4.

It is currently unknown if the SPPL2a/b-induced degradation of VAMP1-4 occurs constitutively or in a regulated way, for example, following certain trafficking events involving the delivery of the VAMP proteins to compartments where SPPL2a/b reside. It seems conceivable, but it has not been demonstrated

yet that the modulation of cellular VAMP1-4 levels, possibly even restricted to certain compartments, has a relevant impact on SNARE-mediated membrane trafficking and fusion. VAMP1-4 have been broadly detected in the late secretory and endo-lysosomal pathway [66]. In addition to exocytosis, they have also been implicated in endosomal recycling [67–69] as well as in antero- and retrograde endosome-Golgi trafficking [66,70]. It remains to be determined if the degree of VAMP1-4 accumulation seen in SPPL2a/b dKO mice influences any of these routes and can be linked with any yet unknown phenotypes of these mice. As the activity of SNARE proteins is also regulated by other mechanisms [64], it is not clear yet to which extent the increased amount of SNARE proteins contributes to the active pool of these proteins.

SPPL2c and sperm-oocyte fusion

With regard to tissue expression, SPPL2c is much more restricted than the other family members as it has only been detected in developing male germ cells so far [11]. There, SPPL2c has been implicated in the formation of the sperm acrosome and regulation of sperm motility. As we have demonstrated, this may be based on the intramembrane proteolysis of either selected SNARE molecules including Syntaxin-8 or the Ca^{2+} -regulating protein Phospholamban [11,18]. While the above-mentioned phenotypes of SPPL2c-deficient male mice, that reproduce rather normally when bred with wild-type females, rely on the proteolytic activity of the enzyme, the protease also impacts male fertility in a non-catalytic mode by stabilising the membrane protein Frey1 (also called C11orf94 or 1700029I15Rik; Fig. 3) [71].

Frey1 is a small type II-oriented protein that resides within the ER. In mice, it is exclusively expressed in round spermatids from stages V to VIII but absent from mature spermatozoa [72]. As demonstrated by two independent groups, Frey1 physically interacts with SPPL2c both in cell-based overexpression systems but also *in vivo* in the murine testis, while not being proteolytically processed [71,73]. This interaction is essential to stabilise the intrinsically unstable Frey1 molecule (Fig. 3). In the absence of SPPL2c, Frey1 is depleted by a yet unknown, post-transcriptional mechanism, most likely representing degradation at the protein level [11,71]. Remarkably, the interaction of SPPL2c and Frey1 depends on the catalytically relevant aspartates within the active site of the enzyme, suggesting that Frey1 is able to directly bind to these residues. In line with these findings, Frey1 efficiently inhibits SPPL2c-mediated intramembrane proteolysis in cell-based overexpression assays without affecting

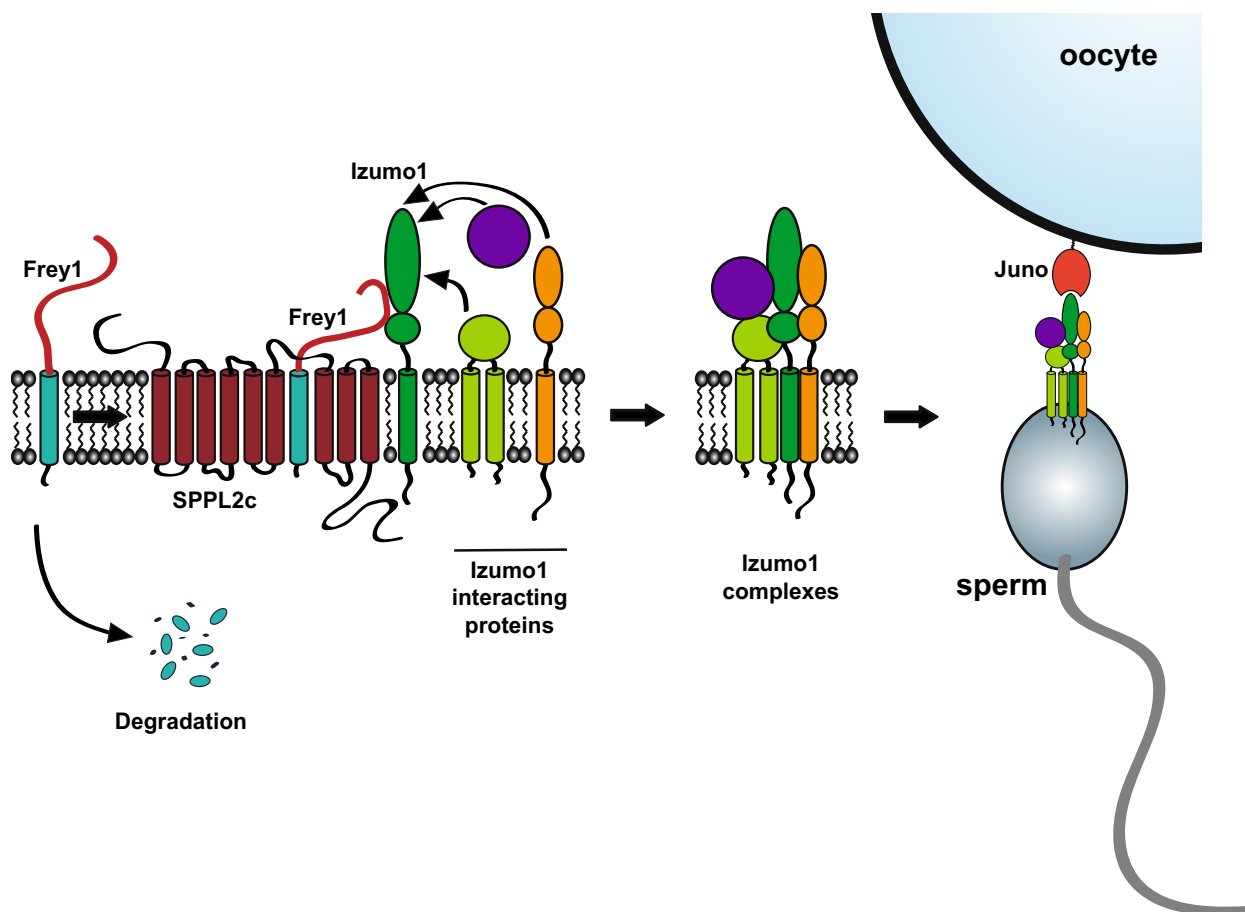


Fig. 3. Role of SPPL2c and Frey1 in sperm-oocyte fusion. SPPL2c stabilises the small ER-resident Frey1 molecule within spermatids. By doing so, SPPL2c acts as a platform for assembly of Izumo1-containing protein complexes, which are essential for fusion of sperm cells with oocytes. In the absence of SPPL2c, Frey1 is unstable and rapidly degraded.

SPP-dependent cleavage of the same substrates [71]. Even though this establishes Frey1 as the first direct and highly specific protein inhibitor of an aspartyl intramembrane protease in general, so far, there is no direct evidence for Frey1-mediated inhibition of SPPL2c *in vivo*. This might point to the existence of different pools of SPPL2c in developing male germ cells that take over non-redundant functions.

Intriguingly, a full ablation of Frey1 by genetic knockout of the corresponding gene has much stronger consequences on male germ cell function than that of SPPL2c, although Frey1 protein levels are already substantially reduced in testes from SPPL2c-deficient mice [71]. Frey1-deficient male mice suffer from normozoospermic infertility, meaning that they generate normal or only slightly reduced amounts of morphologically intact sperm cells that, however, fail to fertilise oocytes even in *in vitro* fertilisation set-ups [73]. Additional ablation of SPPL2c on a Frey1 knockout background

does not restore fertility of the double knockout males [71]. Therefore, the phenotype of Frey1-deficient mice is independent of a potential increase in SPPL2c activity in the absence of the inhibitory function of Frey1. Instead, it reflects a role of Frey1 in the organisation of protein complexes required for sperm-oocyte fusion and the control of stability as well as post-translational modifications of acrosomal proteins (Fig. 3) [71,73]. Although the precise role of SPPL2c in this context remains to be investigated, in cell-based assays, the protease supports binding of Frey1 to the male fusion factor Izumo1, suggesting that it might aid the formation of Izumo1-containing protein complexes by providing a molecular platform for their assembly. This scaffold-like function is reminiscent of that of iRhom1/2, that regulate the proper trafficking of ADAM17 [74–77], suggesting that intramembrane proteases might be a useful cellular scaffold for the arrangement of protein complexes in general.

SPPL3 - regulation of protein glycosylation

SPPL3 is unique within the SPP/SPPL family with its broad preference for direct cleavage of type II transmembrane proteins with large ectodomains without any pre-processing [20,21,78]. Most of the known substrates are Golgi-resident glycosyltransferases, which become secreted by SPPL3 cleavage so that their catalytic activity is depleted from the cell. Systematic proteomic approaches for identification of SPPL3 substrates have uncovered a variety of candidate substrates by analysing changes in the cellular secretome associated with modulation of SPPL3 [21,78]. More than 20 of these have been experimentally verified as substrates [20,21,78]. It should be kept in mind that SPPL3-mediated cleavage events, which do not result in substrate secretion would not have been uncovered by these approaches. Modulation of SPPL3 activity has significant impact on cellular protein glycosylation [20,78]. However, the primary physiological purpose and the regulation of this pathway are still not fully resolved. Constitutive SPPL3 knockout mice were smaller than their wild-type littermates and died shortly after birth [79], for which the reasons have not been further analysed yet. While supporting a critical function of SPPL3, conditional depletion of SPPL3 in the haematopoietic system demonstrated a rather specific, cell-intrinsic impact on the development and function of natural killer (NK) cells, while B- and T-cell numbers were not affected [79]. It remains unclear if the protein hyper-glycosylation observed in SPPL3-deficient NK cells is causally connected to the described phenotype [79].

A cell-based screen has identified SPPL3 as a modulator of HLA-class I-driven immune responses. These were linked to SPPL3-induced changes in the cell surface glyco-sphingolipid repertoire, which influence protein interactions of HLA-class I [80]. Upregulation of SPPL3 and activation of the described pathway may help tumour cells to evade immune responses. In the opposite direction, loss of SPPL3 expression in malignant B cells results in hyperglycosylation of CD19 and by this means accounts for a resistance against chimeric antigen receptor (CAR) T cells and impairment of anti-tumour cytotoxicity [81]. These examples indicate that despite the general modulation of glycan synthesis, the pathways affected by SPPL3 in a critical way are quite cell-type specific. So far, no further tissue-specific SPPL3 knockout mouse models have been reported in the literature so that the cell-type-specific functions of SPPL3 *in vivo* in a physiological context remain an open question.

Open questions in the field

Over the recent years, our knowledge on SPP/SPPL intramembrane proteases has significantly increased by defining general cleavage modes and in particular by identifying substrates. At the same time, characterisation of protease-deficient mouse models has indicated in which cell types SPP/SPPL are active and have critical functions under physiological conditions. Nevertheless, many very general aspects are still poorly understood (Fig. 4).

In general, the proteolytic cleavage of a target protein can pave the way to its degradation or represent a selective post-translational modification, which alters its functional state and/or generates cleavage fragments with new biological functions. This functional dualism applies similarly to intramembrane proteases, for example, when thinking of γ -secretase as a 'membrane proteasome' [82] or vice versa the transcription factor function of the Notch or SREBP cytosolic domains released by such enzymes [3,83]. With regard to SPP/SPPL proteases, many studies support that these enzymes have a degradative and proteostatic function. When these proteases are inhibited or knocked out, the uncleaved substrates start to accumulate, irrespective of whether these are TA proteins, NTFs within a RIP cascade or full-length type II membrane proteins. In several examples, the accumulation or delayed clearance of substrates such as the CD74 [39], LOX-1 [47] or Dectin-1 [51] NTFs could be linked to major functional consequences on membrane trafficking and or signalling pathways. In other cases, for example, for the accumulation of VAMP1-4 in SPPL2a/b dKO mice [19] similar effects have not been demonstrated yet. Nevertheless, these findings suggest a picture that SPP/SPPL proteases are meant to titrate cellular levels of certain membrane proteins. As cells possess other systems to degrade membrane proteins, this leads to the question discussed below how SPP/SPPL proteases cooperate with these other pathways and which factors determine whether a given protein takes the one or the other route for degradation (Fig. 4). Like other intramembrane proteases, also SPP/SPPL proteases generate and release cleavage products into the cytosol and/or into the extracellular/luminal space. The fate of these SPP/SPPL-derived cleavage products and whether they have biological functions or are merely degradation intermediates is only barely understood. In any case, whether clearance of substrate proteins from cellular membranes or generation of functional soluble cleavage products may be regarded as the primary purpose of SPP/SPPL proteases, the question arises to what extent cleavage by

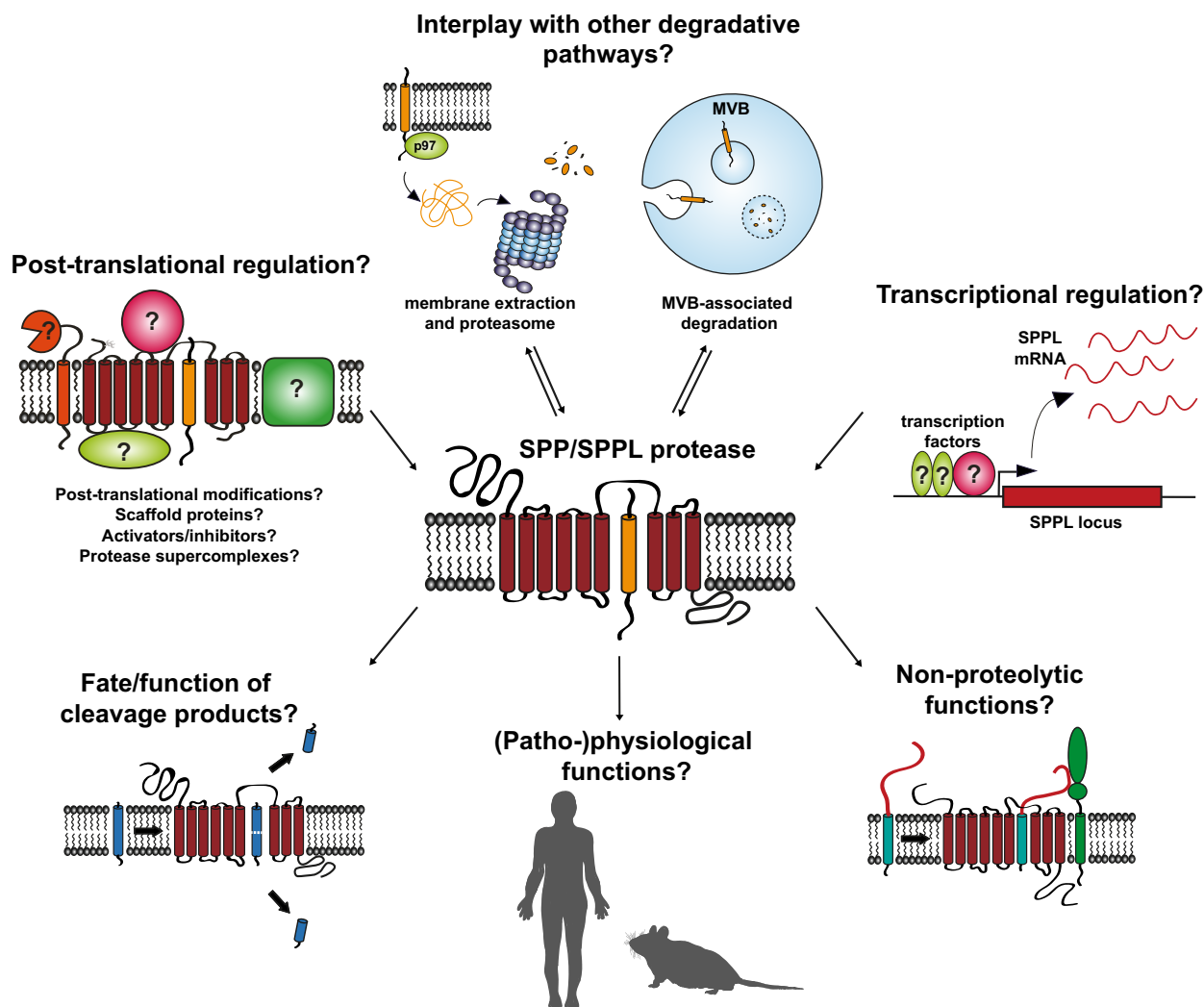


Fig. 4. Open questions in SPP/SPPL protease research. Despite recent advances in our understanding of these enzymes, several substantial questions regarding the function of these enzymes as well as their embedding in cellular pathways remain to be answered. This, among others, include the post-translational as well as transcriptional and translational regulation of these enzymes, the fate of their cleavage products and their interplay with other degradative pathways. Analysis of these aspects will aid our understanding of the patho-physiological relevance of these druggable enzymes.

these enzymes occurs in a constitutive homeostatic or a regulated way. Therefore, trying to understand if processing by SPP/SPPL proteases is regulated and, if so, which mechanisms are involved here is one central question in the field. This applies to the cellular level but is not limited to this. When analysing SPP/SPPL function *in vivo* using knockout mice, the focus so far has been to characterise their basal phenotype, which is the obvious starting point. A potential dysregulation in pathophysiological and disease contexts has not been systematically analysed yet but is required considering that these proteases are druggable enzymes.

What is the interplay of SPP/SPPL proteases with other membrane degradation pathways?

Being embedded in a phospholipid bilayer degradation of integral membrane proteins requires mechanisms to liberate these from the membrane. In addition to intramembrane proteases, other mechanisms have evolved to solve this problem (Fig. 4). In ER-associated degradation, the p97 ATPase (in yeast *cdc48*) uses the energy from ATP hydrolysis to extract the target protein from the membrane in order to deliver it to the proteasome [84,85]. In the endo-lysosomal system, cargo membrane proteins can be segregated into

intraluminal vesicles, for example, by the ESCRT (endosomal sorting complexes required for transport) system [86], and are then degraded in the lysosomal lumen.

For several SPP/SPPL substrates, also the utilisation of degradation pathways other than the intramembrane cleavage has been described. For instance, in vascular smooth muscle cells, the ER-resident SPP substrate HO-1 was found to be degraded by ERAD and the proteasome, which was dependent on p97 [87]. In contrast, Bri2, a SPPL2a/b substrate, was found to undergo intra-lysosomal degradation, as inhibition of lysosomal acidification with chloroquine stabilised both the Bri2 full-length protein as well as the Bri2 NTF [88]. Also, for the SNARE proteins VAMP2 and VAMP4 delivery to lysosomes for degradation has been reported [89,90]. In case of VAMP2, dependence on ESCRT was demonstrated [89], whereas the precise mechanism used for turnover of VAMP4 in this context remains unknown [90]. These examples demonstrate that the role of SPP/SPPL proteases for the turnover of these proteins is not exclusive. Nevertheless, the alternative degradative pathways in most cases cannot completely compensate for the abolished intramembrane proteolysis, since SPP/SPPL inhibition or knockout goes along with an accumulation of the respective substrates. This might indicate that separate protein pools destined to use a certain pathway for turnover exist. It remains to be analysed for each substrate, to what extent the different degradative pathways are utilised (Fig. 4), which is likely to vary in a cell type- and context-dependent manner.

The close inter-connection of SPP/SPPL proteases with other membrane protein degradation pathways is most obvious for Dectin-1, where the presence of the stalk domain in Dectin-1a determines its degradation by SPPL proteases [51]. It is currently unknown how Dectin-1a at least partially evades processes that efficiently remove the shorter Dectin-1b. These were suggested to involve ubiquitination and ESCRT-dependent sorting [58–60]. Attachment and subsequent recognition of ubiquitin by the sorting machinery takes place at the cytosolic domain, which is identical in Dectin-1a and Dectin-1b. Therefore, it is not obvious how the stalk domain, which is in the extracellular/luminal part of the receptor, helps to avoid this and retains the Dectin-1a NTF in the endosomal membrane, where it then can be cleaved by SPPL2a/b. It is tempting to speculate that the stalk mediates an interaction with a phagosomal membrane protein, which then prevents the packaging into intraluminal vesicles.

Both for ERAD and ESCRT-mediated sorting in the lysosomal system, specific types of ubiquitination

of the target protein represent a canonical trigger. It remains to be systematically investigated if ubiquitination of substrates influences cleavage by SPP/SPPL proteases. A VAMP2 mutant where all lysine residues have been mutated to arginines was cleaved by ectopically co-expressed SPPL2a/b similar to wild-type VAMP2. This argues against canonical lysine-directed ubiquitination as a cleavage pre-requisite at least for this substrate [19]. A thorough analysis of further post-translational modifications of substrates including phosphorylation and lipidation may be helpful in identifying switches between different degradative routes. This is of particular relevance as protein removal by a SPP/SPPL intramembrane protease in contrast to the other pathways has the potential to release a functional cleavage product into the cytoplasm.

What is the fate and/or function of cleavage products released by SPP/SPPL proteases?

In general, intramembrane proteases release cleavage products to either side of the membrane (Fig. 4). Depending on the substrate type, the size of the released fragments differs significantly. The fragments liberated into the cytosol (intracellular domain, ICD) of TA proteins comprise nearly the entire protein, whereas ICDs of type II membrane proteins released at the end of a RIP sequence typically comprise < 100 amino acids. As a common feature, these cytosolic cleavage fragments were found to be difficult to detect in many cases suggesting that they are rather short-lived. If so, the question arises how their degradation is achieved. An involvement of the proteasome may seem obvious and was supported for some substrates, for example, HO-1 [16], Stx18 [29] and CD74 [91]. However, the requirement and involved pathways of ubiquitin conjugation have not been analysed in most cases. In case of HO-1, a role of the E3 ligases MARCH6 and TRC8 has been demonstrated, as degradation of the released ICD is delayed in MARCH6/TRC8 double-deficient cells [30]. It would also need to be resolved what the precise sequence of events is: Does intramembrane proteolysis trigger ubiquitination of the cleavage fragment? Or does ubiquitination of the membrane-bound substrate initiate the intramembrane cleavage? Even if canonical ubiquitination, as seen in case of VAMP2, is not an essential pre-requisite for cleavage [19], it could still be an activating factor.

Although a role of the proteasome has been demonstrated for degrading cleavage products of certain substrates, there are hints to assume a relevant contribution of other cytosolic proteases. For many substrates, proteasome inhibition seemed to stabilise

the released ICDs only partially. Furthermore, in case of VAMP1-4 no stabilisation of the cleavage products was observed upon proteasome inhibition with epoxomicin [19]. These were very efficiently turned over and could be only detected at very low levels not corresponding to the amount of cleaved full-length proteins. This may be necessary as in other contexts soluble variants of SNARE proteins were found capable of acting in a dominant-negative way by forming non-functional complexes with membrane-bound SNARE proteins [92]. Altogether, analysing the further degradative fate of cytosolic domains released by SPP/SPPL proteins is strongly advocated, which should include the evaluation of proteasome-independent pathways.

Despite their inherent instability, biological functions of different cytosolic cleavage fragments have been reported. These include HO-1 [17,93], TNF α [7], CD74 [91,94] and the transmembrane serine protease epithin/PRSS14 [95]. These different fragments are assumed to influence transcriptional regulation in the nucleus, where they can translocate after being released from the membrane. The molecular details and the transcription factors involved have not been very well characterised. In particular, the TNF α , CD74 and PRSS14 ICDs with sizes < 60 residues would most likely require partners for this task, which remain to be identified. The pathophysiological role of the HO-1 and PRSS14 ICDs was demonstrated with regard to cancer. Overexpression of these protein fragments in tumour cells enhanced their malignant potential in xenograft models in mice [17,95]. It is currently unclear how relevant these proteolytically released fragments in non-transformed cells are. So far, no basal loss-of function phenotypes of SPP/SPPL-deficient mice could be linked with this. This also applies to the TNF α and CD74 ICDs. However, in case of the CD74 ICD, which was suggested to work as a transcriptional regulator in B cells [94], these would probably be masked by the major changes induced in these cells by the accumulating CD74 NTF [39,43]. Obviously, when analysing SPPL2a-deficient mice depletion of the CD74 cleavage fragment and accumulation of the uncleaved substrate cannot be uncoupled. Altogether, based on our current knowledge it may be concluded that in a physiological context, the functional consequences of losing the proteostatic function of SPP/SPPL proteases seem to dominate over effects associated with the depletion of certain cleavage fragments.

In parallel to the cytosolic cleavage fragments, processing by SPP/SPPL proteases also releases corresponding fragments into the lumen of organelles or the extracellular space depending on the localisation of the individual protease. The extracellular/luminal

domains of tail-anchored proteins typically just comprise a few residues and may therefore be disregarded. However, the cleavage of full-length type II membrane domains, for example, glycosyltransferases by SPPL3 releases potentially functional proteins. Even if cleavage does not occur at the cell surface like in case of SPPL3, such fragments can follow the secretory pathway, reach the extracellular space [20] and are even detected in human blood [78,96]. It was proposed that these secreted glycosyltransferases are active outside of a cell, although it is an ongoing question how the required UDP-activated sugars are provided there. This process has been implicated in producing sialylated IgG immunoglobulins in the circulation as well as modulating cell surface glycans on bone marrow progenitor cells, which may suppress granulopoiesis in the context of multiple myeloma [97].

In addition to processing TNF α NTFs, SPPL2a was recently shown to also be able to cleave full-length TNF α , which leads to a secretion of the entire ectodomain, thus a slightly longer fragment than that released by its canonical sheddase ADAM17 [98]. So far, these experiments were performed under overexpression conditions. It remains unknown, if this alternative soluble TNF α exists under physiological conditions and if it has the same affinity to TNF receptors and pro-inflammatory activity as canonically released soluble TNF α .

Finally, it should be mentioned that also the cleavage of NTFs in a RIP sequence like of LOX-1 at the plasma membrane, in the secretory or endocytic pathway produces C-terminal cleavage fragments, often referred to as C-peptides, which have access to the extracellular space and the circulation *in vivo*. The fate of these cleavage fragments has not been investigated so far. With their rather small size of 30–50 amino acids, they may be anticipated to be rather short-lived after release from a cell. Nevertheless, it may not be justified to fully ignore these fragments, even if currently no function of any of these C-peptides produced by SPP/SPPL proteases is known.

How is cleavage by SPP/SPPL proteases regulated?

One possible idea, why intramembrane proteolysis has evolved in addition to other degradative pathways, is that the level of the protease in principle allows an additional layer of regulation. However, our knowledge about the control of aspartyl intramembrane proteases in general and of SPP/SPPL proteases in particular is still quite limited. In fact, for a long period, these enzymes were believed to function as a

kind of unregulated proteasome of the membrane removing membrane-embedded stubs of single span membrane proteins [82]. This concept has been challenged by the notion that γ -secretase but also SPP/SPPL proteases are capable of cleaving proteins with naturally short ectodomains without any regulatory preceding cleavage event [11,16,18,19,99]. This implies that the activity of these enzymes needs to be fine-tuned to avoid depletion or accumulation of such substrates under homeostatic conditions. In principle, regulation of intramembrane protease activity could take place translationally, post-translationally but also on the transcriptional level based on altered gene expression (Fig. 4). There is at least some experimental evidence for the latter mechanism for SPPL2a/b. Both SPPL2a and SPPL2b can be upregulated by microbial pro-inflammatory factors including bacterial LPS as well as fungal ligands of pattern recognition receptors including Zymosan (a cell wall preparation of *S. cerevisiae*) and heat-killed *C. albicans* yeasts [51]. Since the receptors targeted by these ligands typically transmit signals by MAPK and NF- κ B pathways [100], this might suggest that transcription factors responsive to these signal transduction pathways affect SPPL2 gene expression in an inflammatory context. Furthermore, SPPL2b protein levels are significantly higher in SPPL2a-deficient BMDCs [9], suggesting a compensatory upregulation of SPPL2b under this condition. However, as this was only assessed by western blotting, it is currently unknown, whether the observed changes were caused at the transcriptional or translational level or by post-translational stabilisation of the intramembrane protease. In-depth analysis of transcriptome data combined with a bioinformatic analysis of the SPPL2a/b promoters might shed further light into the transcriptional regulation of these enzymes. In light of the therapeutic potential of SPPL and γ -secretase inhibitors that in many cases also target related proteases to various degrees [101], it is also of translational relevance to understand any compensatory regulatory mechanisms within these protease families.

Beyond the level of transcription and translation that allows the adjustment of available enzymes in a rather delayed time frame, enzymatic activity of SPP/SPPL proteases may also be controlled more dynamically in a post-translational manner. In fact, post-translational modifications impact on the activity of the closely related γ -secretase. Although phosphorylation of presenilin 1 only has minor effects on the activity of the enzyme complex [102], attachment of phosphate groups to the accessory nicastrin molecule is connected to destabilisation of this subunit thereby

also affecting the activity of the whole enzyme complex [103,104]. In addition to covalent modifications of γ -secretase, its catalytic activity can also be modulated by interaction with several accessory proteins that affect γ -secretase in different ways. These include the hypoxia-inducible factor-1 α (HIF-1 α) [105], the small ER-resident stress-associated ER protein 1 (SERP1), which itself is cleaved by SPP [11,16,106], the γ -secretase activating protein (GSAP) [107,108], the interferon-induced transmembrane protein 3 (IFITM3) [109] and the ER-Golgi-resident Transmembrane protein 21 (TMP21) [110,111]. While in case of γ -secretase, the unbiased analysis of the interactome of this enzyme has yielded significant progress in our understanding of regulatory processes for intramembrane proteolysis, our knowledge about the post-translational control of SPP/SPPL protease activity is still very limited. In fact, Frey1 represents the first *in vivo* validated regulator of a member of this intramembrane protease family [71]. However, based on the broad expression of SPP/SPPL proteases and their position as crucial switches in several disease-relevant pathways [47], it is tempting to speculate that Frey1 will not remain the only modulatory interaction partner of a SPP/SPPL protease. As exemplified by the above-mentioned examples of γ -secretase, analysis of the interactome of these enzymes by mass-spectrometric approaches might provide novel insights into their regulation. Validation of potential regulators would also substantially benefit from the availability of an *in vitro* assay as it is available for γ -secretase that would allow the analysis of the proteolytic activity of SPP/SPPL proteases in a non-cellular and defined context. However, so far despite several attempts such an assay has not been established for these enzymes representing a major limitation for the field.

Physiological role: are challenges needed to discover function?

Unravelling the (patho-)physiological relevance of SPP/SPPL proteases may be regarded as one of the most relevant open questions in the context of these enzymes (Fig. 4). In case of SPP and SPPL3, loss of these enzymes is linked to perinatal lethality suggesting an important role in embryonic development [34,79]. This is supported by a rather high expression of SPP in the developing embryo, which decreases around birth [112]. Furthermore, global deletion of SPP employing a tamoxifen-inducible system—when initiated 2 weeks after birth - does not lead to similarly drastic phenotypes [35]. However, the molecular mechanisms underlying the perinatal death of the SPP and

also SPPL3 knockout mice remain elusive until to date, highlighting the need of a more in-depth analysis of death-causing events in these animals. In addition to this approach, the analysis of conditional knockout mice might significantly aid our understanding of these enzymes as already successfully demonstrated for SPPL3 [79]. With regard to different substrates, depletion of SPP in different cell types and tissue will be of interest. Based on the documented function of SPP in the degradation of HO-1 [16], which fulfils crucial functions in the turnover of heme, macrophages would represent an interesting starting point to study the effects of an SPP knockout. Analysing the function of SPP in HO-1 turnover under conditions in which high amounts of heme need to be processed, for example, hemolytic anaemias, might provide insights into the relevance of proteolytic cleavage of HO-1 by SPP under pathophysiological conditions.

With the exception of SPP and SPPL3, of which constitutive ablation is more problematic [34,79], loss of SPPL2 proteases is in general well tolerated by mice that are typically housed in a rather protective environment. Many of the validated substrates of SPPL2a/b identified so far fulfil specified functions in the immune system (CD74, TNF α , FasL, Dectin-1) or are functionally linked to certain diseases (LOX-1, Epithin) [7,39,47,51,95,113]. Along this line, large parts of our knowledge about the physiological functions of SPPL2 proteases originate from experiments, in which SPPL2a/b-deficient immune cells or mice were challenged by various means including nutritional changes [47] or stimulation with pathogens like mycobacteria or *C. albicans* [42,51] as well as the analysis of human patients lacking SPPL2a suffering from Mendelian susceptibility for mycobacterial disease [45]. Together with the above-mentioned increased expression of SPPL2a/b under inflammatory conditions, this might point to the fact that beyond their general proteostatic functions, the role of these enzymes might be more pronounced in specific stress or disease conditions. This might hamper both the validation of known substrates identified in cell-based overexpression systems and the identification of novel proteins that depend on SPPL2a/b proteases for their degradation in non-challenged mice or other model organisms. In light of these thoughts, it may be advocated to study the function of these enzymes in a broad setup of inflammatory diseases or models thereof, for example, rheumatoid arthritis. Given the fact that SPP/SPPL proteases are druggable enzymes for which potent, bioavailable and at least partially selective compounds have been developed [114,115], this might also open the door towards novel treatment strategies of these diseases. Importantly, this approach is not limited

to models of inflammation or immune diseases, but could also be extended to other pathophysiological relevant processes based on the molecular pathways known substrates of SPPL2a/b are implicated in. Based on the processing of the NTF of Transferrin receptor 1 by SPPL2b [116], the physiological relevance of the cleavage event which was identified in overexpression setups and has not been validated in a more physiological context, yet, might be analysed in mouse models of iron depletion or overload. Another example is provided by VAMP1-4, which accumulate in the absence of SPPL2a/b in several organs of respective knockout mice [19]. So far, no physiological function has been attributed to the SPPL2a/b-mediated VAMP degradation. Considering this, one could envision to monitor processes with increased vesicular trafficking rates like excitation of neurons, pathogen phagocytosis or stimulated exocytosis to validate the physiological relevance of SPPL2a/b-dependent turnover of selected SNARE proteins.

In a more unbiased way, genomic approaches might point to disease conditions in which SPPL2a/b proteases could be involved. For example, several genome-wide association studies (GWAS) suggest an association of SPPL2a with Alzheimer's disease [117–121]. Albeit being expressed at rather low levels in the mouse brain as compared to other tissues [9], expression of SPPL2a might be of high relevance in specific cell types such as microglia, which express several already characterised SPPL2a/b substrates including CD74 [122,123] and Dectin-1 [124]. Based on the high expression of SPPL2b in brain, specifically within the hippocampus as well as the piriform cortex and the cerebellum [9], it could be speculated about an involvement of this enzyme into neurodegenerative disorders.

As demonstrated by these examples, our knowledge about the pathophysiological functions of SPP/SPPL proteases still remains limited. This also applies to many other aspects of the biology of these enzymes including their interplay with other degradative pathways as well as their embedding into cell biological pathways. Since SPP/SPPL proteases in principle represent ideal drug targets, addressing these questions in addition to broadening our understanding of intramembrane proteolysis in general might also open the perspective of novel therapeutic strategies based on selective targeting of SPP/SPPL proteases.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

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References

- De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K & Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387–390.
- Sakai J, Duncan EA, Rawson RB, Hua X, Brown MS & Goldstein JL (1996) Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. *Cell* **85**, 1037–1046.
- Brown MS & Goldstein JL (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331–340.
- Kuhnle N, Dederer V & Lemberg MK (2019) Intramembrane proteolysis at a glance: from signalling to protein degradation. *J Cell Sci* **132**, jcs217745.
- Mentrup T, Cabrera-Cabrera F, Fluhrer R & Schroder B (2020) Physiological functions of SPP/SPPL intramembrane proteases. *Cell Mol Life Sci* **77**, 2959–2979.
- Mentrup T & Schroder B (2022) Signal peptide peptidase-like 2 proteases: regulatory switches or proteasome of the membrane? *Biochim Biophys Acta Mol Cell Res* **1869**, 119163.
- Friedmann E, Hauben E, Maylandt K, Schleege S, Vreugde S, Lichtenthaler SF, Kuhn PH, Stauffer D, Rovelli G & Martoglio B (2006) SPPL2a and SPPL2b promote intramembrane proteolysis of TNF α in activated dendritic cells to trigger IL-12 production. *Nat Cell Biol* **8**, 843–848.
- Behnke J, Schneppenheim J, Koch-Nolte F, Haag F, Saftig P & Schroder B (2011) Signal-peptide-peptidase-like 2a (SPPL2a) is targeted to lysosomes/late endosomes by a tyrosine motif in its C-terminal tail. *FEBS Lett* **585**, 2951–2957.
- Schneppenheim J, Hüttl S, Mentrup T, Lüllmann-Rauch R, Rothaug M, Engelke M, Dittmann K, Dressel R, Araki M, Araki K *et al.* (2014) The intramembrane proteases signal peptide peptidase-like 2a and 2b have distinct functions in vivo. *Mol Cell Biol* **34**, 1398–1411.
- Miyashita H, Maruyama Y, Isshiki H, Osawa S, Ogura T, Mio K, Sato C, Tomita T & Iwatsubo T (2011) Three-dimensional structure of the signal peptide peptidase. *J Biol Chem* **286**, 26188–26197.
- Niemeyer J, Mentrup T, Heidasch R, Muller SA, Biswas U, Meyer R, Papadopoulou AA, Dederer V, Haug-Kroper M, Adamski V *et al.* (2019) The intramembrane protease SPPL2c promotes male germ cell development by cleaving phospholamban. *EMBO Rep* **20**, e46449.
- Mentrup T, Fluhrer R & Schroder B (2017) Latest emerging functions of SPP/SPPL intramembrane proteases. *Eur J Cell Biol* **96**, 372–382.
- Martin L, Fluhrer R & Haass C (2009) Substrate requirements for SPPL2b-dependent regulated intramembrane proteolysis. *J Biol Chem* **284**, 5662–5670.
- Lichtenthaler SF, Lemberg MK & Fluhrer R (2018) Proteolytic ectodomain shedding of membrane proteins in mammals—hardware, concepts, and recent developments. *EMBO J* **37**, e99456.
- Stefanovic S & Hegde RS (2007) Identification of a targeting factor for posttranslational membrane protein insertion into the ER. *Cell* **128**, 1147–1159.
- Boname JM, Bloor S, Wandel MP, Nathan JA, Antrobus R, Dingwell KS, Thurston TL, Smith DL, Smith JC, Randow F *et al.* (2014) Cleavage by signal peptide peptidase is required for the degradation of selected tail-anchored proteins. *J Cell Biol* **205**, 847–862.
- Hsu FF, Yeh CT, Sun YJ, Chiang MT, Lan WM, Li FA, Lee WH & Chau LY (2015) Signal peptide peptidase-mediated nuclear localization of heme oxygenase-1 promotes cancer cell proliferation and invasion independent of its enzymatic activity. *Oncogene* **34**, 2360–2370.
- Papadopoulou AA, Muller SA, Mentrup T, Shmueli MD, Niemeyer J, Haug-Kroper M, von Blume J, Mayerhofer A, Feederle R, Schroder B *et al.* (2019) Signal peptide peptidase-like 2c (SPPL2c) impairs vesicular transport and cleavage of SNARE proteins. *EMBO Rep* **20**, e46451.
- Ballin M, Griep W, Patel M, Karl M, Mentrup T, Rivera-Monroy J, Foo B, Schwappach B & Schroder B (2023) The intramembrane proteases SPPL2a and SPPL2b regulate the homeostasis of selected SNARE proteins. *FEBS J* **290**, 2320–2337.
- Voss M, Kunzel U, Higel F, Kuhn PH, Colombo A, Fukumori A, Haug-Kroper M, Klier B, Grammer G,

- Seidl A *et al.* (2014) Shedding of glycan-modifying enzymes by signal peptide peptidase-like 3 (SPPL3) regulates cellular N-glycosylation. *EMBO J* **33**, 2890–2905.
- 21 Kuhn PH, Voss M, Haug-Kroper M, Schroder B, Schepers U, Brase S, Haass C, Lichtenthaler SF & Fluhrer R (2015) Secretome analysis identifies novel signal peptide peptidase-like 3 (Sppl3) substrates and reveals a role of Sppl3 in multiple Golgi glycosylation pathways. *Mol Cell Proteomics* **14**, 1584–1598.
- 22 Chen CY, Malchus NS, Hehn B, Stelzer W, Avci D, Langosch D & Lemberg MK (2014) Signal peptide peptidase functions in ERAD to cleave the unfolded protein response regulator XBP1u. *EMBO J* **33**, 2492–2506.
- 23 Spitz C, Schlosser C, Guschtschin-Schmidt N, Stelzer W, Menig S, Gotz A, Haug-Kroper M, Scharnagl C, Langosch D, Muhle-Goll C *et al.* (2020) Non-canonical shedding of TNFalpha by SPPL2a is determined by the conformational flexibility of its transmembrane helix. *iScience* **23**, 101775.
- 24 Guner G & Lichtenthaler SF (2020) The substrate repertoire of gamma-secretase/presenilin. *Semin Cell Dev Biol* **105**, 27–42.
- 25 Mentrup T, Loock AC, Fluhrer R & Schroder B (2017) Signal peptide peptidase and SPP-like proteases – possible therapeutic targets? *Biochim Biophys Acta* **1864**, 2169–2182.
- 26 Papadopoulou AA & Fluhrer R (2020) Signaling functions of intramembrane aspartyl-proteases. *Front Cardiovasc Med* **7**, 591787.
- 27 Weihofen A, Lemberg MK, Ploegh HL, Bogoy M & Martoglio B (2000) Release of signal peptide fragments into the cytosol requires cleavage in the transmembrane region by a protease activity that is specifically blocked by a novel cysteine protease inhibitor. *J Biol Chem* **275**, 30951–30956.
- 28 McLauchlan J, Lemberg MK, Hope G & Martoglio B (2002) Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* **21**, 3980–3988.
- 29 Avci D, Malchus NS, Heidasch R, Lorenz H, Richter K, Nessling M & Lemberg MK (2019) The intramembrane protease SPP impacts morphology of the endoplasmic reticulum by triggering degradation of morphogenic proteins. *J Biol Chem* **294**, 2786–2800.
- 30 Stefanovic-Barrett S, Dickson AS, Burr SP, Williamson JC, Lobb IT, van den Boomen DJ, Lehner PJ & Nathan JA (2018) MARCH6 and TRC8 facilitate the quality control of cytosolic and tail-anchored proteins. *EMBO Rep* **19**, e45603.
- 31 McKenna MJ, Adams BM, Chu V, Paulo JA & Shao S (2022) ATP13A1 prevents ERAD of folding-competent mislocalized and misoriented proteins. *Mol Cell* **82**, 4277–4289.e10.
- 32 Hsu FF, Chou YT, Chiang MT, Li FA, Yeh CT, Lee WH & Chau LY (2019) Signal peptide peptidase promotes tumor progression via facilitating FKBP8 degradation. *Oncogene* **38**, 1688–1701.
- 33 Liu J, Li W & Wu L (2022) Pan-cancer analysis suggests histocompatibility minor 13 is an unfavorable prognostic biomarker promoting cell proliferation, migration, and invasion in hepatocellular carcinoma. *Front Pharmacol* **13**, 950156.
- 34 Aizawa S, Okamoto T, Sugiyama Y, Kouwaki T, Ito A, Suzuki T, Ono C, Fukuhara T, Yamamoto M, Okochi M *et al.* (2016) TRC8-dependent degradation of hepatitis C virus immature core protein regulates viral propagation and pathogenesis. *Nat Commun* **7**, 11379.
- 35 Wang S & Ghiasi H (2019) Absence of signal peptide peptidase, an essential herpes simplex virus 1 glycoprotein K binding partner, reduces virus infectivity in vivo. *J Virol* **93**, e01309-19.
- 36 Allen SJ, Mott KR, Matsuura Y, Moriishi K, Kousoulas KG & Ghiasi H (2014) Binding of HSV-1 glycoprotein K (gK) to signal peptide peptidase (SPP) is required for virus infectivity. *PLoS One* **9**, e85360.
- 37 Wang S, Jaggi U, Tormanen K, Hirose S & Ghiasi H (2022) Absence of signal peptide peptidase in peripheral sensory neurons affects latency-reactivation in HSV-1 ocularly infected mice. *PLoS Pathog* **18**, e1010281.
- 38 Wang S, Jaggi U & Ghiasi H (2022) Knockout of signal peptide peptidase in the eye reduces HSV-1 replication and eye disease in ocularly infected mice. *PLoS Pathog* **18**, e1010898.
- 39 Schneppenheim J, Dressel R, Hüttl S, Lüllmann-Rauch R, Engelke M, Dittmann K, Wienands J, Eskelinen EL, Hermans-Borgmeyer I, Fluhrer R *et al.* (2013) The intramembrane protease SPPL2a promotes B cell development and controls endosomal traffic by cleavage of the invariant chain. *J Exp Med* **210**, 41–58.
- 40 Beisner DR, Langerak P, Parker AE, Dahlberg C, Otero FJ, Sutton SE, Poirot L, Barnes W, Young MA, Niessen S *et al.* (2013) The intramembrane protease Sppl2a is required for B cell and DC development and survival via cleavage of the invariant chain. *J Exp Med* **210**, 23–30.
- 41 Bergmann H, Yabas M, Short A, Miosge L, Barthel N, Teh CE, Roots CM, Bull KR, Jeelall Y, Horikawa K *et al.* (2013) B cell survival, surface BCR and BAFFR expression, CD74 metabolism, and CD8-dendritic cells require the intramembrane endopeptidase SPPL2A. *J Exp Med* **210**, 31–40.
- 42 Gradtke AC, Mentrup T, Lehmann CHK, Cabrera-Cabrera F, Desel C, Okakpu D, Assmann M, Dalpke A, Schaible UE, Dudziak D *et al.* (2021) Deficiency of the intramembrane protease SPPL2a alters

- antimycobacterial cytokine responses of dendritic cells. *J Immunol* **206**, 164–180.
- 43 Hüttl S, Klasener K, Schweizer M, Schneppenheim J, Oberg HH, Kabelitz D, Reth M, Saftig P & Schröder B (2015) Processing of CD74 by the intramembrane protease SPPL2a is critical for B cell receptor signaling in transitional B cells. *J Immunol* **195**, 1548–1563.
- 44 Schneppenheim J, Hüttl S, Kruchen A, Fluhrer R, Müller I, Saftig P, Schneppenheim R, Martin CL & Schröder B (2014) Signal-peptide-peptidase-like 2a is required for CD74 intramembrane proteolysis in human B cells. *Biochem Biophys Res Commun* **451**, 48–53.
- 45 Kong XF, Martinez-Barricarte R, Kennedy J, Mele F, Lazarov T, Deenick EK, Ma CS, Breton G, Lucero KB, Langlais D *et al.* (2018) Disruption of an antimycobacterial circuit between dendritic and helper T cells in human SPPL2a deficiency. *Nat Immunol* **19**, 973–985.
- 46 Sabnis RW (2022) Diazepinone compounds as Sppl2a inhibitors for treating autoimmune diseases and lymphomas. *ACS Med Chem Lett* **13**, 881–882.
- 47 Mentrup T, Theodorou K, Cabrera-Cabrera F, Helbig AO, Happ K, Gijbels M, Gradtke AC, Rabe B, Fukumori A, Steiner H *et al.* (2019) Atherogenic LOX-1 signaling is controlled by SPPL2-mediated intramembrane proteolysis. *J Exp Med* **216**, 807–830.
- 48 Mentrup T, Cabrera-Cabrera F & Schroder B (2020) Proteolytic regulation of the lectin-like oxidized lipoprotein receptor LOX-1. *Front Cardiovasc Med* **7**, 594441.
- 49 Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T *et al.* (1997) An endothelial receptor for oxidized low-density lipoprotein. *Nature* **386**, 73–77.
- 50 Akhmedov A, Sawamura T, Chen CH, Kraler S, Vdovenko D & Luscher TF (2021) Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1): a crucial driver of atherosclerotic cardiovascular disease. *Eur Heart J* **42**, 1797–1807.
- 51 Mentrup T, Stumpff-Niggemann AY, Leinung N, Schlosser C, Schubert K, Wehner R, Tunger A, Schatz V, Neubert P, Gradtke AC *et al.* (2022) Phagosomal signalling of the C-type lectin receptor Dectin-1 is terminated by intramembrane proteolysis. *Nat Commun* **13**, 1880.
- 52 Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, Williams DL, Gordon S, Tybulewicz VL, Brown GD *et al.* (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* **22**, 507–517.
- 53 Underhill DM, Rossnagle E, Lowell CA & Simmons RM (2005) Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* **106**, 2543–2550.
- 54 Herre J, Marshall AS, Caron E, Edwards AD, Williams DL, Schweighoffer E, Tybulewicz V, Reis e Sousa C, Gordon S & Brown GD (2004) Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* **104**, 4038–4045.
- 55 Willment JA, Gordon S & Brown GD (2001) Characterization of the human beta -glucan receptor and its alternatively spliced isoforms. *J Biol Chem* **276**, 43818–43823.
- 56 Heinsbroek SE, Taylor PR, Rosas M, Willment JA, Williams DL, Gordon S & Brown GD (2006) Expression of functionally different dectin-1 isoforms by murine macrophages. *J Immunol* **176**, 5513–5518.
- 57 Hernanz-Falcon P, Joffre O, Williams DL & Reis e Sousa C (2009) Internalization of Dectin-1 terminates induction of inflammatory responses. *Eur J Immunol* **39**, 507–513.
- 58 Wirsberger G, Zwolanek F, Asaoka T, Koziaradzki I, Tortola L, Wimmer RA, Kavirayani A, Fresser F, Baier G, Langdon WY *et al.* (2016) Inhibition of CBLB protects from lethal *Candida albicans* sepsis. *Nat Med* **22**, 915–923.
- 59 Xiao Y, Tang J, Guo H, Zhao Y, Tang R, Ouyang S, Zeng Q, Rappleye CA, Rajaram MV, Schlesinger LS *et al.* (2016) Targeting CBLB as a potential therapeutic approach for disseminated candidiasis. *Nat Med* **22**, 906–914.
- 60 Zhu LL, Luo TM, Xu X, Guo YH, Zhao XQ, Wang TT, Tang B, Jiang YY, Xu JF, Lin X *et al.* (2016) E3 ubiquitin ligase Cbl-b negatively regulates C-type lectin receptor-mediated antifungal innate immunity. *J Exp Med* **213**, 1555–1570.
- 61 Dickey SW, Baker RP, Cho S & Urban S (2013) Proteolysis inside the membrane is a rate-governed reaction not driven by substrate affinity. *Cell* **155**, 1270–1281.
- 62 Brown GD, Willment JA & Whitehead L (2018) C-type lectins in immunity and homeostasis. *Nat Rev Immunol* **18**, 374–389.
- 63 Wang T, Li L & Hong W (2017) SNARE proteins in membrane trafficking. *Traffic* **18**, 767–775.
- 64 Koike S & Jahn R (2022) SNARE proteins: zip codes in vesicle targeting? *Biochem J* **479**, 273–288.
- 65 Jahn R & Fasshauer D (2012) Molecular machines governing exocytosis of synaptic vesicles. *Nature* **490**, 201–207.
- 66 Dingjan I, Linders PTA, Verboogen DRJ, Revelo NH, Ter Beest M & van den Bogaart G (2018) Endosomal and phagosomal SNAREs. *Physiol Rev* **98**, 1465–1492.
- 67 Bakr M, Jullie D, Krapivkina J, Paget-Blanc V, Bouit L, Petersen JD, Retailliau N, Breillat C, Herzog E, Choquet D *et al.* (2021) The vSNAREs VAMP2 and VAMP4 control recycling and intracellular sorting of post-synaptic receptors in neuronal dendrites. *Cell Rep* **36**, 109678.

- 68 Chen H, Weinberg ZY, Kumar GA & Puthenveedu MA (2023) Vesicle-associated membrane protein 2 is a cargo-selective v-SNARE for a subset of GPCRs. *J Cell Biol* **222**, e202207070.
- 69 Galli T, Chilcote T, Mundigl O, Binz T, Niemann H & De Camilli P (1994) Tetanus toxin-mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor-containing vesicles in CHO cells. *J Cell Biol* **125**, 1015–1024.
- 70 Mallard F, Tang BL, Galli T, Tenza D, Saint-Pol A, Yue X, Antony C, Hong W, Goud B & Johannes L (2002) Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. *J Cell Biol* **156**, 653–664.
- 71 Contreras W, Wieschofer C, Schreier D, Leinung N, Peche P, Wennemuth G, Gentzel M, Schroder B & Mentrup T (2022) C11orf94/Frey is a key regulator for male fertility by controlling Izumo1 complex assembly. *Sci Adv* **8**, eabo6049.
- 72 Hao H, Shi B, Zhang J, Dai A, Li W, Chen H, Ji W, Gong C, Zhang C, Li J *et al.* (2022) The vertebrate- and testis- specific transmembrane protein C11ORF94 plays a critical role in sperm-oocyte membrane binding. *Mol Biomed* **3**, 27.
- 73 Lu Y, Shimada K, Tang S, Zhang J, Ogawa Y, Noda T, Shibuya H & Ikawa M (2023) 1700029I15Rik orchestrates the biosynthesis of acrosomal membrane proteins required for sperm-egg interaction. *Proc Natl Acad Sci USA* **120**, e2207263120.
- 74 Adrain C, Zettl M, Christova Y, Taylor N & Freeman M (2012) Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. *Science* **335**, 225–228.
- 75 McIlwain DR, Lang PA, Maretzky T, Hamada K, Ohishi K, Maney SK, Berger T, Murthy A, Duncan G, Xu HC *et al.* (2012) iRhom2 regulation of TACE controls TNF-mediated protection against listeria and responses to LPS. *Science* **335**, 229–232.
- 76 Siggs OM, Xiao N, Wang Y, Shi H, Tomisato W, Li X, Xia Y & Beutler B (2012) iRhom2 is required for the secretion of mouse TNFalpha. *Blood* **119**, 5769–5771.
- 77 Li X, Maretzky T, Weskamp G, Monette S, Qing X, Issuree PD, Crawford HC, McIlwain DR, Mak TW, Salmon JE *et al.* (2015) iRhoms 1 and 2 are essential upstream regulators of ADAM17-dependent EGFR signaling. *Proc Natl Acad Sci USA* **112**, 6080–6085.
- 78 Hobohm L, Koudelka T, Bahr FH, Truberg J, Kapell S, Schacht SS, Meisinger D, Mengel M, Jochimsen A, Hofmann A *et al.* (2022) N-terminome analyses underscore the prevalence of SPPL3-mediated intramembrane proteolysis among Golgi-resident enzymes and its role in Golgi enzyme secretion. *Cell Mol Life Sci* **79**, 185.
- 79 Hamblet CE, Makowski SL, Tritapoe JM & Pomerantz JL (2016) NK cell maturation and cytotoxicity are controlled by the intramembrane aspartyl protease SPPL3. *J Immunol* **196**, 2614–2626.
- 80 Jongsma MLM, de Waard AA, Raaben M, Zhang T, Cabukusta B, Platzer R, Blomen VA, Xagara A, Verkerk T, Bliss S *et al.* (2021) The SPPL3-defined glycosphingolipid repertoire orchestrates HLA class I-mediated immune responses. *Immunity* **54**, 132–150.e9.
- 81 Heard A, Landmann JH, Hansen AR, Papadopoulou A, Hsu YS, Selli ME, Warrington JM, Lattin J, Chang J, Ha H *et al.* (2022) Antigen glycosylation regulates efficacy of CAR T cells targeting CD19. *Nat Commun* **13**, 3367.
- 82 Kopan R & Ilagan MX (2004) Gamma-secretase: proteasome of the membrane? *Nat Rev Mol Cell Biol* **5**, 499–504.
- 83 Schroeter EH, Kisslinger JA & Kopan R (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382–386.
- 84 Wu X & Rapoport TA (2018) Mechanistic insights into ER-associated protein degradation. *Curr Opin Cell Biol* **53**, 22–28.
- 85 Bodnar NO & Rapoport TA (2017) Molecular mechanism of substrate processing by the Cdc48 ATPase complex. *Cell* **169**, 722–735.e9.
- 86 Szymanska E, Budick-Harmelin N & Miaczynska M (2018) Endosomal “sort” of signaling control: the role of ESCRT machinery in regulation of receptor-mediated signaling pathways. *Semin Cell Dev Biol* **74**, 11–20.
- 87 Lin PH, Chiang MT & Chau LY (2008) Ubiquitin-proteasome system mediates heme oxygenase-1 degradation through endoplasmic reticulum-associated degradation pathway. *Biochim Biophys Acta* **1783**, 1826–1834.
- 88 Yin T, Yao W, Lemenze AD & D’Adamio L (2021) Danish and British dementia ITM2b/BRI2 mutations reduce BRI2 protein stability and impair glutamatergic synaptic transmission. *J Biol Chem* **296**, 100054.
- 89 Sheehan P, Zhu M, Beskow A, Vollmer C & Waites CL (2016) Activity-dependent degradation of synaptic vesicle proteins requires Rab35 and the ESCRT pathway. *J Neurosci* **36**, 8668–8686.
- 90 Ivanova D, Dobson KL, Gajbhiye A, Davenport EC, Hacker D, Ultanir SK, Trost M & Cousin MA (2021) Control of synaptic vesicle release probability via VAMP4 targeting to endolysosomes. *Sci Adv* **7**, eabf3873.
- 91 Mentrup T, Hasler R, Fluhrer R, Saftig P & Schröder B (2015) A cell-based assay reveals nuclear translocation of intracellular domains released by SPPL proteases. *Traffic* **16**, 871–892.
- 92 Scales SJ, Chen YA, Yoo BY, Patel SM, Doung YC & Scheller RH (2000) SNAREs contribute to the specificity of membrane fusion. *Neuron* **26**, 457–464.

- 93 Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F *et al.* (2007) Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *J Biol Chem* **282**, 20621–20633.
- 94 Gil-Yarom N, Radomir L, Sever L, Kramer MP, Lewinsky H, Bornstein C, Blecher-Gonen R, Barnett-Itzhaki Z, Mirkin V, Friedlander G *et al.* (2017) CD74 is a novel transcription regulator. *Proc Natl Acad Sci USA* **114**, 562–567.
- 95 Cho Y, Kim SB, Kim J, Pham AVQ, Yoon MJ, Park JH, Hwang KT, Park D, Cho Y, Kim MG *et al.* (2020) Intramembrane proteolysis of an extracellular serine protease, epithin/PRSS14, enables its intracellular nuclear function. *BMC Biol* **18**, 60.
- 96 Lee-Sundlov MM, Ashline DJ, Hanneman AJ, Grozovsky R, Reinhold VN, Hoffmeister KM & Lau JT (2017) Circulating blood and platelets supply glycosyltransferases that enable extrinsic extracellular glycosylation. *Glycobiology* **27**, 188–198.
- 97 Irons EE, Lee-Sundlov MM, Zhu Y, Neelamegham S, Hoffmeister KM & Lau JT (2019) B cells suppress medullary granulopoiesis by an extracellular glycosylation-dependent mechanism. *Elife* **8**, e47328.
- 98 Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S *et al.* (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature* **385**, 729–733.
- 99 Laurent SA, Hoffmann FS, Kuhn PH, Cheng Q, Chu Y, Schmidt-Suppran M, Hauck SM, Schuh E, Krumbholz M, Rubsamen H *et al.* (2015) Gamma-secretase directly sheds the survival receptor BCMA from plasma cells. *Nat Commun* **6**, 7333.
- 100 Li D & Wu M (2021) Pattern recognition receptors in health and diseases. *Signal Transduct Target Ther* **6**, 291.
- 101 Ran Y, Ladd GZ, Ceballos-Diaz C, Jung JI, Greenbaum D, Felsenstein KM & Golde TE (2015) Differential inhibition of signal peptide peptidase family members by established gamma-secretase inhibitors. *PLoS One* **10**, e0128619.
- 102 Matz A, Halamoda-Kenzaoui B, Hamelin R, Mosser S, Alattia JR, Dimitrov M, Moniatte M & Fraering PC (2015) Identification of new Presenilin-1 phosphosites: implication for gamma-secretase activity and Abeta production. *J Neurochem* **133**, 409–421.
- 103 Mo JS, Yoon JH, Hong JA, Kim MY, Ann EJ, Ahn JS, Kim SM, Baek HJ, Lang F, Choi EJ *et al.* (2012) Phosphorylation of nicastrin by SGK1 leads to its degradation through lysosomal and proteasomal pathways. *PLoS ONE* **7**, e37111.
- 104 Jo EH, Ahn JS, Mo JS, Yoon JH, Ann EJ, Baek HJ, Lee HJ, Kim SH, Kim MY & Park HS (2015) Akt1 phosphorylates Nicastrin to regulate its protein stability and activity. *J Neurochem* **134**, 799–810.
- 105 Villa JC, Chiu D, Brandes AH, Escorcía FE, Villa CH, Maguire WF, Hu CJ, de Stanchina E, Simon MC, Sisodia SS *et al.* (2014) Nontranscriptional role of Hif-1 α in activation of gamma-secretase and notch signaling in breast cancer. *Cell Rep* **8**, 1077–1092.
- 106 Jung S, Hyun J, Nah J, Han J, Kim SH, Park J, Oh Y, Gwon Y, Moon S, Jo DG *et al.* (2020) SERP1 is an assembly regulator of gamma-secretase in metabolic stress conditions. *Sci Signal* **13**, eaax8949.
- 107 He G, Luo W, Li P, Remmers C, Netzer WJ, Hendrick J, Bettayeb K, Flajolet M, Gorelick F, Wennogle LP *et al.* (2010) Gamma-secretase activating protein is a therapeutic target for Alzheimer's disease. *Nature* **467**, 95–98.
- 108 Wong E, Liao GP, Chang JC, Xu P, Li YM & Greengard P (2019) GSAP modulates gamma-secretase specificity by inducing conformational change in PS1. *Proc Natl Acad Sci USA* **116**, 6385–6390.
- 109 Hur JY, Frost GR, Wu X, Crump C, Pan SJ, Wong E, Barros M, Li T, Nie P, Zhai Y *et al.* (2020) The innate immunity protein IFITM3 modulates gamma-secretase in Alzheimer's disease. *Nature* **586**, 735–740.
- 110 Pardossi-Piquard R, Bohm C, Chen F, Kanemoto S, Checler F, Schmitt-Ulms G, St George-Hyslop P & Fraser PE (2009) TMP21 transmembrane domain regulates gamma-secretase cleavage. *J Biol Chem* **284**, 28634–28641.
- 111 Chen F, Hasegawa H, Schmitt-Ulms G, Kawarai T, Bohm C, Katayama T, Gu Y, Sanjo N, Glista M, Rogava E *et al.* (2006) TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. *Nature* **440**, 1208–1212.
- 112 Huang Z, Han Z, Zhang F, He H, Yu S & Wu Q (2014) Spatiotemporal expression of retrogene-host pair Mcts2/H13 in mouse embryo, and Mcts2 has no influence on H13 transcription pattern in NIH/3T3 cells. *Acta Histochem* **116**, 312–318.
- 113 Kirkin V, Cahuzac N, Guardiola-Serrano F, Huault S, Luckerath K, Friedmann E, Novac N, Wels WS, Martoglio B, Hueber AO *et al.* (2007) The Fas ligand intracellular domain is released by ADAM10 and SPPL2a cleavage in T-cells. *Cell Death Differ* **14**, 1678–1687.
- 114 Velcicky J, Bodendorf U, Rigollier P, Epple R, Beisner DR, Guerini D, Smith P, Liu B, Feifel R, Wipfli P *et al.* (2018) Discovery of the first potent, selective, and orally bioavailable signal peptide peptidase-like 2a (SPPL2a) inhibitor displaying pronounced immunomodulatory effects in vivo. *J Med Chem* **61**, 865–880.
- 115 Velcicky J, Mathison CJN, Nikulin V, Pflieger D, Epple R, Azimioara M, Cow C, Michellys PY, Rigollier P, Beisner DR *et al.* (2019) Discovery of

- orally active hydroxyethylamine based SPPL2a inhibitors. *ACS Med Chem Lett* **10**, 887–892.
- 116 Zahn C, Kaup M, Fluhrer R & Fuchs H (2013) The transferrin receptor-1 membrane stub undergoes intramembrane proteolysis by signal peptide peptidase-like 2b. *FEBS J* **280**, 1653–1663.
- 117 Liu JZ, Erlich Y & Pickrell JK (2017) Case-control association mapping by proxy using family history of disease. *Nat Genet* **49**, 325–331.
- 118 Marioni RE, Harris SE, Zhang Q, McRae AF, Hagenaars SP, Hill WD, Davies G, Ritchie CW, Gale CR, Starr JM *et al.* (2018) GWAS on family history of Alzheimer's disease. *Transl Psychiatry* **8**, 99.
- 119 Kunkle BW, Grenier-Boley B, Sims R, Bis JC, Damotte V, Naj AC, Boland A, Vronskaya M, van der Lee SJ, Amlie-Wolf A *et al.* (2019) Genetic meta-analysis of diagnosed Alzheimer's disease identifies new risk loci and implicates Abeta, tau, immunity and lipid processing. *Nat Genet* **51**, 414–430.
- 120 de Rojas I, Moreno-Grau S, Tesi N, Grenier-Boley B, Andrade V, Jansen IE, Pedersen NL, Stringa N, Zettergren A, Hernandez I *et al.* (2021) Common variants in Alzheimer's disease and risk stratification by polygenic risk scores. *Nat Commun* **12**, 3417.
- 121 Schwartzenruber J, Cooper S, Liu JZ, Barrio-Hernandez I, Bello E, Kumasaka N, Young AMH, Franklin RJM, Johnson T, Estrada K *et al.* (2021) Genome-wide meta-analysis, fine-mapping and integrative prioritization implicate new Alzheimer's disease risk genes. *Nat Genet* **53**, 392–402.
- 122 Sebastian Monasor L, Muller SA, Colombo AV, Tanriover G, Konig J, Roth S, Liesz A, Berghofer A, Piechotta A, Prestel M *et al.* (2020) Fibrillar Abeta triggers microglial proteome alterations and dysfunction in Alzheimer mouse models. *Elife* **9**, e54083.
- 123 Mathys H, Adaiக்கan C, Gao F, Young JZ, Manet E, Hemberg M, De Jager PL, Ransohoff RM, Regev A & Tsai LH (2017) Temporal tracking of microglia activation in neurodegeneration at single-cell resolution. *Cell Rep* **21**, 366–380.
- 124 Shah VB, Huang Y, Keshwara R, Ozment-Skelton T, Williams DL & Keshwara L (2008) Beta-glucan activates microglia without inducing cytokine production in Dectin-1-dependent manner. *J Immunol* **180**, 2777–2785.