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Proteolytic ectodomain shedding of membrane proteins in mammals—hardware, concepts, and recent developments

Stefan F Lichtenthaler[1,2,3,*] (iD), Marius K Lemberg[4,**] (iD) & Regina Fluhrer[1,5,***] (iD)

Abstract

Proteolytic removal of membrane protein ectodomains (ectodomain shedding) is a post-translational modification that controls levels and function of hundreds of membrane proteins. The contributing proteases, referred to as sheddases, act as important molecular switches in processes ranging from signaling to cell adhesion. When deregulated, ectodomain shedding is linked to pathologies such as inflammation and Alzheimer's disease. While proteases of the "a disintegrin and metalloprotease" (ADAM) and "beta-site APP cleaving enzyme" (BACE) families are widely considered as sheddases, in recent years a much broader range of proteases, including intramembrane and soluble proteases, were shown to catalyze similar cleavage reactions. This review demonstrates that shedding is a fundamental process in cell biology and discusses the current understanding of sheddases and their substrates, molecular mechanisms and cellular localizations, as well as physiological functions of protein ectodomain shedding. Moreover, we provide an operational definition of shedding and highlight recent conceptual advances in the field. While new developments in proteomics facilitate substrate discovery, we expect that shedding is not a rare exception, but rather the rule for many membrane proteins, and that many more interesting shedding functions await discovery.

Introduction

Membrane proteins are essential for health and disease and have a large variety of fundamental physiological functions. Levels of individual membrane proteins and their functions are tightly controlled through different mechanisms, including post-translational modifications such as proteolytic ectodomain shedding (or briefly shedding). Shedding is a form of limited proteolysis and thus an irreversible post-translational modification (Fig 1). During the shedding process, a protease (referred to as sheddase) cleaves a membrane protein substrate close to or within its transmembrane (TM) domain, resulting in release of the soluble extracellular domain (ectodomain) from the membrane and a fragment that remains bound to the membrane (Fig 1) (Kapeller *et al*, 1973; Black, 1980a; Ehlers & Riordan, 1991). Some sheddases are also referred to as secretases (Selkoe, 1990), as the cleaved substrate ectodomain may be secreted.

Shedding is best understood in mammals, where it has emerged as a key cellular mechanism to control not only abundance, but also activation and inactivation of membrane proteins, for example, through release of membrane-bound growth factors and cytokines or through degradation of surface receptors and cell adhesion proteins (e.g., Black *et al*, 1997; Moss *et al*, 1997; Peschon *et al*, 1998; Colombo *et al*, 2018). Given the large number of substrates, shedding influences many processes in development, physiology, and disease, such as connectivity in the nervous system (e.g., Hattori *et al*, 2000), cholesterol homeostasis (Sakai *et al*, 1996, 1998), Alzheimer's disease (e.g., Vassar *et al*, 1999), and inflammatory disorders (e.g., Black *et al*, 1997; Moss *et al*, 1997). Yet, for other membrane proteins, shedding may simply be a mechanism of protein turnover and may not be coupled to (patho)physiological consequences.

In the literature, the term shedding sometimes also refers to the non-proteolytic release of membrane proteins and the release of

1   German Center for Neurodegenerative Diseases (DZNE), Munich, Germany
2   Neuroproteomics, Klinikum rechts der Isar, School of Medicine, and Institute for Advanced Study, Technical University Munich, Munich, Germany
3   Munich Center for Systems Neurology (SyNergy), Munich, Germany
4   Center for Molecular Biology of Heidelberg University (ZMBH), DKFZ-ZMBH Alliance, Heidelberg, Germany
5   Biomedizinisches Centrum (BMC), Ludwig-Maximilians University of Munich, Munich, Germany
    *Corresponding author. Tel: +49 89 440046426; E-mail: stefan.lichtenthaler@dzne.de
    **Corresponding author. Tel: +49 6221 545889; E-mail: m.lemberg@zmbh.uni-heidelberg.de
    ***Corresponding author. Tel: +49 89 440046505; E-mail: regina.fluhrer@mail03.med.uni-muenchen.de
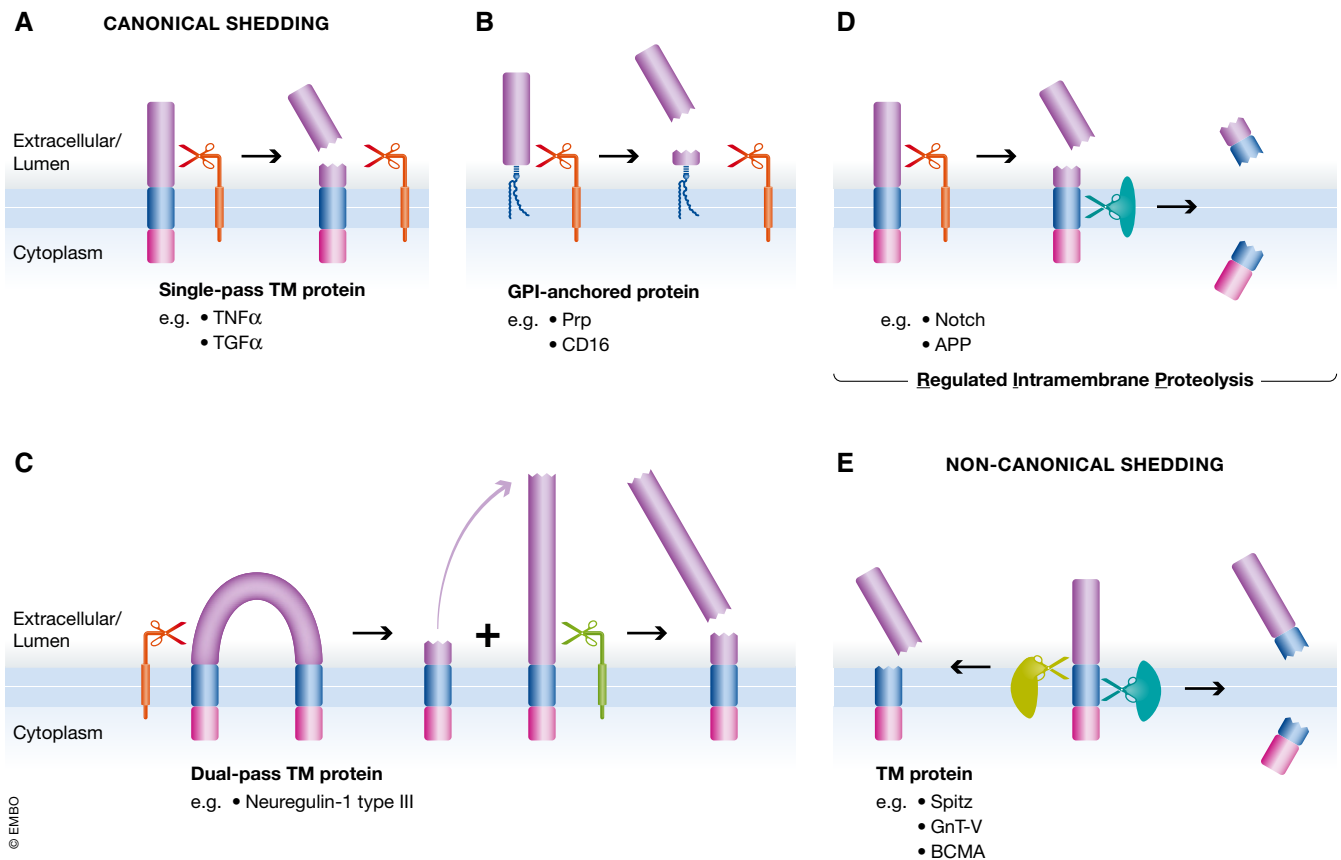
Figure 1. Sheddases trigger the release of a wide range of proteins from the membrane.

(A) Canonical sheddases cleave single-pass TM membrane proteins in their luminal juxtamembrane region, thereby releasing ectodomains from their membrane-integral domains. Ectodomain refers to that part of the protein that is found on the extracellular side of the membrane—in case that the protein localizes to the plasma membrane—or within the lumen of organelles of the secretory and endocytic pathway, which is topologically equivalent to the extracellular space. (B) GPI-anchored proteins are separated from their lipid modification by cleavage within the C-terminus of the protein. (C) Dual-pass and polytopic membrane proteins (not shown) can be cleaved in loops and ectodomains (not shown). Neuregulin-1 type III is cleaved at two sites in its loop domain, thereby releasing a bioactive peptide from its membrane anchors. (D) As a variation of canonical shedding, in regulated intramembrane proteolysis (RIP), the sheddase-generated membrane-integral fragment is further processed in the plane of the lipid bilayer, releasing an intracellular domain and a short extracellular peptide fragment. In this case, shedding is the first step of two subsequent proteolytic cleavages. (E) Non-canonical sheddases cleave their substrate in or close to the TM domain without requiring any preceding cleavage. Depending on the site of cleavage, the intracellular fragment is released from the lipid bilayer or stays anchored by a slightly shortened TM domain.

vesicles from the plasma membrane (Black, 1980a,b), which are different molecular processes and are not covered here.

This review gives an overview of ectodomain shedding, starting with an operational definition of shedding, then highlighting the involved proteases and substrates and their regulation, and finally describing the functional consequences and medical implications of shedding. The aim of this review article is to use selected examples a) to demonstrate that shedding is a fundamental cell biological process, b) to illustrate general principles of shedding that emerge from the comparison of different sheddase families, and c) to highlight new trends and conceptual advances in the field.

Definition of ectodomain shedding

Shedding occurs for single-span TM proteins (Fig 1A), GPI-anchored proteins (Fig 1B), and proteins with two or more TM domains (Fig 1C). For several substrates, shedding is the first proteolytic

cleavage and may be followed by additional proteolytic cleavage(s) within the TM segment. Both cleavages together are conceptually referred to as "regulated intramembrane proteolysis" (Fig 1D) (Brown et al, 2000; Lichtenthaler et al, 2011). In all cases, shedding refers to the release of a protein's ectodomain from the membrane.

Initially, the term ectodomain shedding was used in a narrow manner with regard to cellular localization (plasma membrane) (Kapeller et al, 1973; Black, 1980a; Arribas et al, 1996), the position of the cleavage sites within the substrates (lumenal juxtamembrane domain) and the number of proteases and substrates involved (Ehlers & Riordan, 1991; Massague & Pandiella, 1993). However, several key studies over the past years, which will be discussed in more detail below, demonstrated that shedding occurs in all cellular organelles of the secretory and endocytic pathway, happens both outside and even within the substrates' TM domain (Fig 1E), and is mediated by many more proteases than previously thought, including membrane-bound, intramembrane, and even soluble proteases. Moreover, it now has become clear that shedding impacts on many,

if not all single-span membrane proteins and numerous polytopic TM proteins at some stage during their lifetime. In order to reflect these new findings, we propose a broader definition of shedding. Ectodomain shedding is the proteolytic release of the bulk or even the entire ectodomain of a mature membrane protein into the luminal or extracellular space and often alters the substrate's function. Depending on the cellular compartment where shedding occurs, the ectodomain is released into the extracellular space (at the plasma membrane) or into the lumen of the organelles (e.g., Golgi or endosome), which is topologically equivalent to the extracellular space (Schatz & Dobberstein, 1996), and from where it may subsequently be secreted into the extracellular space. The proteolytic cut occurs within the extracellular or luminal juxtamembrane (membrane-proximal) region or within the TM anchor of a membrane protein substrate. Cleavage sites within the juxtamembrane region are typically at a short distance of often 10 - 35 amino acids from the TM segment, but more distant cleavage sites are possible and, in fact, the exact cleavage sites have only been determined for few shedding substrates (e.g., summarized for ADAMs and BACE1 in Caescu *et al*, 2009; Yan, 2017).

Several other proteolytic events in cells, such as removal of a signal peptide by signal peptidase (Blobel & Dobberstein, 1975) and proteolytic cleavages by mitochondrial AAA proteases (Levytskyy *et al*, 2017), formally share similarities to ectodomain shedding, but will not be discussed in this review, as they occur either during protein biosynthesis but not on the mature protein (signal peptidase), or do not occur in the secretory or endocytic pathway (mitochondria).

Hardware: canonical sheddases

The human genome contains nearly 600 protease-encoding genes (Lopez-Otin & Bond, 2008), and an increasing number of them are recognized to act as sheddases, with some having many shedding substrates and others so far having only a single substrate reported to undergo shedding, as will be discussed below. Proteases are commonly considered as sheddases if they cleave their substrates in the luminal juxtamembrane domain with a short distance to the membrane-anchoring domain (Ehlers & Riordan, 1991). We refer to these proteases as *canonical sheddases* (Table 1) to distinguish them from the more recently described *non-canonical sheddases* (described below) that cleave within a substrates' TM domain or at the membrane boundary (Table 2). Canonical sheddases are typically themselves membrane-bound, but more and more soluble proteases, such as matrix metalloproteases (MMPs), are also reported to mediate shedding, as will be discussed below. In Tables 1 and 2, we additionally distinguish between sheddases whose primary function is ectodomain release and proteases that mostly have non-shedding functions, but can additionally act as secondary or "part-time" sheddases. Some of the best-characterized sheddases, such as "a disintegrin and metalloprotease 10" (ADAM10), ADAM17 (also known as TACE for TNFα-converting enzyme), and "β-site APP cleaving enzyme" (BACE1), have many shedding substrates and act as "full-time" sheddases. In contrast, other proteases, such as matrix metalloproteases (MMPs) or pro-protein convertases, have mostly non-shedding functions, because they cleave soluble proteins (MMPs) or remove pro-peptides

(pro-protein convertases) without shedding the whole ectodomain. As will be discussed below, such proteases are increasingly found to additionally act as sheddases on a few selected substrates. This qualifies them as "part-time" sheddases.

The following paragraphs first describe the canonical sheddases, starting with membrane-bound sheddases, followed by soluble ones. As some sheddases have more than 100 substrates, only selected substrates are listed, in particular those that have been validated under protease-deficient conditions or through *in vivo* studies.

ADAM10 and ADAM17

The best-characterized canonical sheddases, ADAM10 and ADAM17, are most likely active in the trans-Golgi network (TGN), in later secretory pathway compartments, and at the plasma membrane (Fig 2). More than 100 substrates for ADAM10 and similar numbers for ADAM17 have been identified in different tissues and cells using candidate testing and advanced proteomics, although not all of them have been validated under physiological conditions and with *in vitro* assays (for detailed lists, see e.g., Pruessmeyer & Ludwig, 2009; Weber & Saftig, 2012; Kawahara *et al*, 2014; Saftig & Lichtenthaler, 2015; Kuhn *et al*, 2016; Zunke & Rose-John, 2017). Selected substrates are highlighted in Table 3. Substrate cleavage by ADAM10 often happens constitutively under non-stimulated conditions, whereas substrate shedding by ADAM17 is mostly observed, when cells are stimulated, either with physiological activators or phorbol esters such as PMA (phorbol-12-myristat-13-acetat, also known as TPA, 12-*O*-tetradecanoylphorbol-13-acetat).

ADAM10 is essential for ligand-dependent shedding of the Notch1 receptor and its subsequent signaling (Pan & Rubin, 1997; Bozkulak & Weinmaster, 2009; van Tetering *et al*, 2009), which is required for embryonic development but also in several adult tissues (Sato *et al*, 2012; reviewed in Alabi *et al*, 2018). Additionally, it acts as α-secretase for the amyloid precursor protein (APP) thereby preventing the generation of the neurotoxic amyloid-β peptide (Lammich *et al*, 1999; Postina *et al*, 2004; Jorissen *et al*, 2010; Kuhn *et al*, 2010; Suh *et al*, 2013), and is, thus, considered a drug target for Alzheimer's disease. Numerous phenotypes have been identified in ADAM10-deficient mice, for example, in the nervous system (Prox *et al*, 2013), but the many substrates still need to be assigned to the individual phenotypes and functions. It is also possible that some phenotypes are not just caused by the loss of cleavage of a single, but of multiple substrates simultaneously.

ADAM17 has a key function in tissue homeostasis through cleavage of several members of the epidermal growth factor (EGF) receptor (EGFR) ligand family, including TGFα (Peschon *et al*, 1998), and may be a drug target for EGFR-dependent tumors (e.g., Schmidt *et al*, 2018). Additionally, ADAM17 has a fundamental role in inflammation by being the major sheddase for the cytokine tumor necrosis factor α (TNFα). Thus, ADAM17 is considered a major drug target for inflammatory diseases such as sepsis, rheumatoid arthritis, and inflammatory bowel disease (reviewed in Rose-John, 2013).

Besides ADAM10 and ADAM17, the ADAM family has ten more members with proven or assumed proteolytic activity (Weber & Saftig, 2012), but only few (or in some cases no) physiological substrates for them have been identified to date (Table 1).

Table 1.   List of canonical, mammalian sheddase families.

Sheddase type	Protease family	Protease type	Cellular localization	References
Full-time sheddases	**ADAM proteases** (metalloproteases) **ADAM8, ADAM9, ADAM10, ADAM12**, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28, ADAM30, ADAM33	Membrane-anchored, type I	Late secretory pathway and plasma membrane	Pruessmeyer and Ludwig (2009), Saftig and Lichtenthaler (2015), Weber and Saftig (2012), Zunke and Rose-John (2017)
	BACE proteases (aspartyl proteases) **BACE1, BACE2**	Membrane-anchored, type I	Trans-Golgi network and endosomes	Barao *et al* (2016), Dislich and Lichtenthaler (2012), Vassar *et al* (2014), Yan (2017)
	Site-1 protease (serine protease), also known as SKI-1 or S1P	Membrane-anchored, type I	Golgi	Seidah *et al* (2017), Seidah and Prat (2012)
Part-time sheddases	**Meprin β** (metalloprotease)	Membrane-anchored, type I		Broder and Becker-Pauly (2013)
	MT-MMPs (metalloproteases) **MT1-MMP**, MT2-MMP, **MT3-MMP**, MT4-MMP, **MT5-MMP**, MT6-MMP, also named MMP14-MMP17, MMP24, MMP25	Membrane-anchored, type I or GPI-anchored	Late secretory pathway and plasma membrane	Hayashida *et al* (2010), Itoh (2015)
	Pro-protein convertases (serine proteases) PCSK1/3, PCSK2, **furin**, PCSK4, PCSK5/6, PACE4, **PCSK7** and PCSK9	Membrane-anchored, type I or soluble	Late secretory pathway and plasma membrane	Seidah *et al* (2017), Seidah and Prat (2012)
	Transmembrane serine proteases Matriptase, **Matriptase-2**, Matriptase-3, Polyserase-1, Corin, Hepsin, TMPRSS2, TMPRSS3, TMPRSS4, MSPL, Spinesin, Enteropeptidase, HAT, DESC1, TMPRSS11A, HAT-like 4, HAT-like 5	Membrane-anchored, type II		Szabo and Bugge (2011), Tanabe and List (2017)
	Matrix metalloproteases (MMPs) MMP1, MMP2, MMP3, MMP7, MMP8, **MMP9**, MMP10, MMP11, MMP12, MMP13, MMP19, MMP20, MMP21, MMP23, MMP26, MMP27, MMP28	Soluble	Extracellular space	Freitas-Rodriguez *et al* (2017), Klein and Bischoff (2011), Peixoto *et al* (2012)
	Legumain (δ-secretase) cysteine protease	Soluble		Zhang *et al* (2016)
	Cathepsin S and L (cysteine protease)	Soluble	Extracellular space	Sobotic *et al* (2015)

Family members with known shedding function are indicated in bold and italics. Selected review articles that typically describe the whole protease family are given. Some articles also contain lists of identified substrates. For proteases with few shedding substrates, the original study is cited. Site-1 protease belongs to the family of pro-protein convertases, but is listed separately to highlight that it acts as a full-time sheddase in contrast to the other members of the same family.

BACE1 and BACE2

Another class of sheddases in the endomembrane system are BACE1 and BACE2 (Fig 2), which were initially identified as APP sheddases (Hussain *et al*, 1999; Sinha *et al*, 1999; Vassar *et al*, 1999; Yan *et al*, 1999, 2001; Lin *et al*, 2000; Fluhrer *et al*, 2002). Candidate approaches and, more recently, proteomic studies identified more than 40 substrates and substrate candidates each for BACE1 and BACE2 (see Table 3 for selected substrates) (Hemming *et al*, 2009; Esterhazy *et al*, 2011; Kuhn *et al*, 2012; Zhou *et al*, 2012; Stutzer *et al*, 2013; Dislich *et al*, 2015), but many of them have not yet been validated under physiological conditions. Since BACE1 acts as the major β-secretase for APP and catalyzes formation of the pathogenic amyloid-β peptide, several inhibitors targeting BACE1 are currently in advanced clinical trials for Alzheimer's disease. However, BACE1 has additional functions in neurobiology, including in myelination, muscle spindle formation and maintenance, synapse formation, and

axon targeting (Hu *et al*, 2006; Willem *et al*, 2006; Rajapaksha *et al*, 2011; Cao *et al*, 2012; Hitt *et al*, 2012; Cheret *et al*, 2013; Barao *et al*, 2015; Zhu *et al*, 2018).

For most substrates, the functional consequences of their cleavage by BACE1 have not yet been investigated, largely for lack of tools such as antibodies targeted to the substrates' ectodomains or intracellular domains, or because little is known about the substrates.

While BACE1 is highly expressed in the nervous system, its homolog BACE2 is strongly expressed in pancreas (Vassar *et al*, 1999). *In vivo* experiments using BACE2-deficient mice revealed that BACE2 regulates pancreatic β-cell function and mass through cleavage of "transmembrane protein 27" (TMEM27) (Esterhazy *et al*, 2011), making BACE2 a potential drug target for diabetes, which needs to be further evaluated. Another BACE2 substrate is PMEL17, the cleavage of which is required for pigment production in

Table 2. List of non-canonical, mammalian sheddase families.

Sheddase type	Protease family and members	Protease type	Cellular localization	References
Full-time sheddases	**Rhomboid proteases** (serine proteases) RHBDL1, ***RHBDL2***, RHBDL3, RHBDL4	Integral multi-pass TM protein	Golgi (RHBDL1), plasma membrane (RHBDL2), endosomes (RHBDL3), ER (RHBDL4)	Freeman (2014), Lemberg (2013)
Part-time sheddases	**SPP/SPPL family** (aspartyl proteases) ***SPPL3*** **SPP**, SPPL2a, SPPL2b, SPPL2c, (SPPL3 is a major sheddase; SPP acts as a sheddase only in exceptional cases)	Integral multi-pass TM protein	ER (SPP), lysosomes (SPPL2a), cell surface (SPPL2b), ER (SPPL2c), Golgi (SPPL3)	Kuhn *et al* (2015), Voss *et al* (2014) Boname *et al* (2014), Chen *et al* (2014)
	Presenilin/γ-secretase (aspartyl protease) ***Presenilin-1***, Presenilin-2 (γ-secretase acts as a sheddase only in exceptional cases)	Integral multi-pass TM protein,	Plasma membrane, endosomes	Laurent *et al* (2015), Schauenburg *et al* (2018)

Family members with known shedding function are indicated in bold and italics. Selected review articles are given that typically describe the whole protease family. Some articles also contain lists of identified substrates. For proteases with few shedding substrates, the original study is cited.

melanocytes and thus, for pigmentation of hair, skin, and mucosa, at least in rodents (Rochin *et al*, 2013).

Meprin β

Meprin β is a homodimeric TM metalloprotease. Soluble and TM substrates as well as substrate candidates were identified proteomically from cell lines overexpressing or exposed to soluble meprin β (Bien *et al*, 2012; Jefferson *et al*, 2013). Several TM proteins were cleaved within their ectodomain at a large distance from the membrane, which is not seen as a shedding event, as a large part of the ectodomain remains. However, meprin β also acts as a sheddase and cleaves close to the TM domain in CD99 to promote transendothelial cell migration, and in APP, for which it acts as an alternative β-secretase (Jefferson *et al*, 2011; Arolas *et al*, 2012; Bedau *et al*, 2017). To what extent meprin β may contribute to Alzheimer's disease still needs to be explored in more detail.

Membrane-type matrix metalloproteases (MT-MMPs)

The six MT-MMPs are a subgroup of the larger MMP family. They are assumed to be active at the plasma membrane and are mostly known for their cleavage of soluble substrates (see Table 1), in particular extracellular matrix proteins, such as collagens and fibronectin (Itoh, 2015). Increasingly, they are reported to also act as canonical sheddases for TM proteins (see Table 3 and reviewed in Hayashida *et al*, 2010; Itoh, 2015), and more shedding substrates are likely to be identified in the future. For example, MT1-MMP sheds RANKL (Receptor activator of NF-kappaB ligand) and negatively regulates osteoclastogenesis (Hikita *et al*, 2006). MT3-MMP was recently shown to shed the GPI-anchored Nogo receptor 1, which promotes excitatory synapse formation *in vitro* and *in vivo* (Sanz *et al*, 2018). MT5-MMP sheds N-cadherin and controls peripheral thermal nociception, presumably through modulation of cell adhesion between mast cells and sensory fibers (Folgueras *et al*, 2009). MT5-MMP shedding of N-cadherin also controls adhesion of neuronal stem cells to ependymocytes and thereby stem cell quiescence versus proliferation (Folgueras *et al*, 2009; Porlan *et al*, 2014). MT5-MMP was recently furthermore identified as the APP η-secretase, and its inactivation reduced inflammation and amyloid

pathology in an Alzheimer's disease mouse model (Willem *et al*, 2015; Baranger *et al*, 2016). However, the APP η-secretase cleavage is more distant (~120 amino acids) from the membrane than most other shedding events. It is not yet clear for all examples mentioned above how exactly the MT-MMPs contribute to the indicated (patho)physiological processes, and it is likely that more shedding functions of MT-MMPs will be discovered.

Pro-protein convertases, including site-1-protease (S1P)

Pro-protein convertases are a family of nine soluble and membrane-bound serine proteases that are commonly found in the TGN and later compartments of the secretory pathway (Fig 2). Several of them, such as furin, remove pro-peptides from soluble or membrane-bound inactive protein precursors (reviewed in Seidah & Prat, 2012), including ADAM and BACE proteases. These pro-peptide cleavages are not considered as shedding event, since they often occur several hundred amino acids distant from the substrates' TM domains and, therefore, do not remove the majority of the substrates' ectodomains. Yet, pro-protein convertases are increasingly reported to additionally act as sheddases for selected substrates. For example, PCSK7 sheds the transferrin receptor (Guillemot *et al*, 2013), whereas furin or another pro-protein convertase sheds MT5-MMP (Wang & Pei, 2001), with both cleavages occurring < 25 amino acids away from the substrates' TM domains. Thus, while the functional consequences of these shedding events are not yet fully understood, pro-protein convertases can act as "part-time" sheddases.

One family member, site-1 protease (S1P), also known as subtilins/kexin-isozyme 1, stands out from the other family members in that it functions primarily as a sheddase (reviewed in Seidah *et al*, 2017). Known substrates of this Golgi-resident protease include viral proteins as well as the latent transcription factors SREBP, involved in cholesterol homeostasis, and ATF6, which is proteolytically activated during the endoplasmic reticulum (ER) unfolded protein response, as well as the inactive α/β-subunit precursor of GlcNAc-1-phosphotransferase, where proteolysis is required for lysosomal homeostasis (Sakai *et al*, 1998; Ye *et al*, 2000; Marschner *et al*, 2011). Both SREBP and ATF6 are shed at a distance of < 30 amino
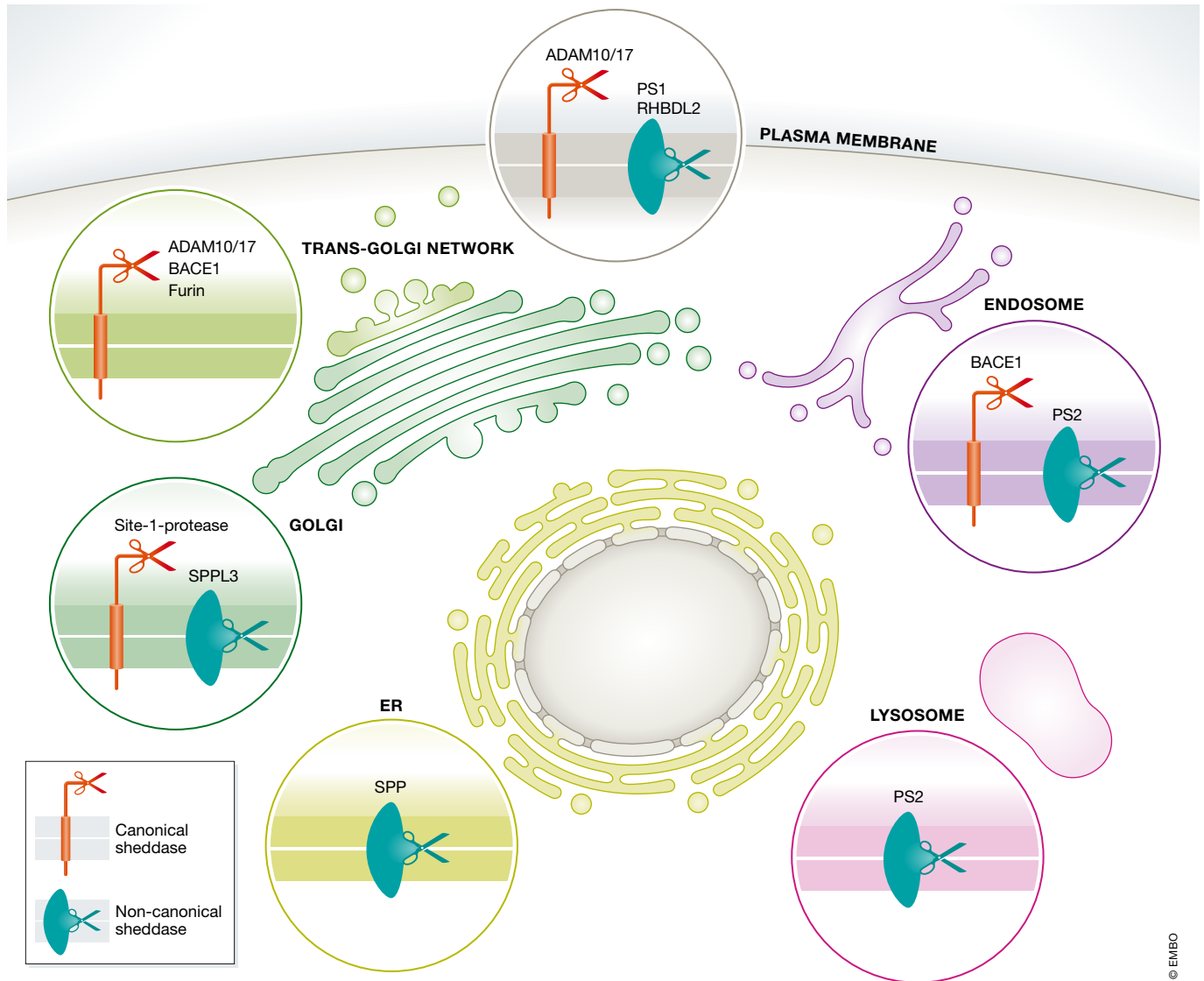
Figure 2. Cellular localization of sheddases.
Catalytically active canonical and non-canonical sheddases not only localize to the cell surface but also to different subcellular compartments. The localization of selected canonical (red) and non-canonical (green) sheddases is indicated.

acids from the membrane and are further processed by site-2 protease in the paradigm of regulated intramembrane proteolysis (Brown *et al*, 2000).

Transmembrane serine proteases (TTSPs)
Matriptase-2 is a member of the type II transmembrane serine proteases (TTSPs), an understudied group of 17 membrane-bound serine proteases (Szabo & Bugge, 2011), and has been reported to shed APP within the amyloid β domain, at least in transfected cells or *in vitro* (Beckmann *et al*, 2016). Other TTSPs appear to cleave predominantly soluble proteins or activate membrane-bound proteins, but without shedding them in their juxtamembrane domains (Jackle *et al*, 2015; Murray *et al*, 2016). Thus, at present TTSPs belong to the group of "part-time" sheddases. However, it is

well possible that the future will reveal more TTSP shedding substrates.

Soluble sheddases
Canonical sheddases are typically single-span TM or GPI-anchored proteins (Table 1). Yet, several soluble proteases, which typically cleave non-membrane-bound substrates, are increasingly reported to also act as "part-time" sheddases by cleaving within the substrate's juxtamembrane domain. For example, MMP9, a soluble MMP, sheds neuroligin 1 in the nervous system (Peixoto *et al*, 2012). Another example, the cysteine protease legumain (also known as asparagine endopeptidase), was recently shown to act as APP δ-secretase (Zhang *et al*, 2015, 2017). This cleavage occurs in vicinity to the BACE1 cleavage site in APP and enhances amyloid-β

Table 3. Examples of substrates of selected canonical and non-canonical, mammalian sheddases.[a]

Sheddase	Selected substrates	References
ADAM10	Notch, APP, PrP, EGF, ephrin-A5, N-cadherin, DR6, CD23	Altmeppen *et al* (2011), Colombo *et al* (2018), Hartmann *et al* (2002), Janes *et al* (2005), Jorissen *et al* (2010), Kuhn *et al* (2016, 2010), Pan and Rubin (1997), Postina *et al* (2004), Reiss *et al* (2005), Sahin *et al* (2004), Suh *et al* (2013), Vincent *et al* (2001), Weskamp *et al* (2006)
ADAM17	TGFα, TNFα, IL6R, amphiregulin, epiregulin, heparin-binding EGF-like growth factor, L-selectin, TNFR2	Althoff *et al* (2000), Black *et al* (1997), Ludwig *et al* (2005), Moss *et al* (1997), Peschon *et al* (1998), Sahin *et al* (2004)
BACE1	APP, NRG1, SEZ6, CHL1	Dislich *et al* (2015), Esterhazy *et al* (2011), Hemming *et al* (2009), Kuhn *et al* (2012), Stutzer *et al* (2013), Zhou *et al* (2012)
BACE2	TMEM27, PMEL17	Esterhazy *et al* (2011), Rochin *et al* (2013)
Meprin β	CD99, APP	Arolas *et al* (2012), Bedau *et al* (2017), Jefferson *et al* (2011)
MT1-MMP	CD44, syndecan, RANKL	Endo *et al* (2003), Hikita *et al* (2006), Kajita *et al* (2001), Tam *et al* (2004)
MT3-MMP	NgR1	Ferraro *et al* (2011), Sanz *et al* (2018)
MT5-MMP	N-cadherin, APP	Baranger *et al* (2016), Folgueras *et al* (2009), Porlan *et al* (2014), Willem *et al* (2015)
MMP9, MMP12	N-cadherin, NLG1	Dwivedi *et al* (2009), Peixoto *et al* (2012)
PC7	Transferrin receptor	Guillemot *et al* (2013), Wang and Pei (2001)
Site-1 protease (S1P, SKI-1)	SREBP, ATF6, GlcNAc-1-phosphotransferase	Marschner *et al* (2011), Sakai *et al* (1998), Seidah *et al* (2017), Ye *et al* (2000)
RHBDL2	Thrombomodulin, EGF, BCAM, Spint-1, CLCP1	Adrain *et al* (2011), Cheng *et al* (2011), Johnson *et al* (2017), Lohi *et al* (2004)
RHBDL4	ERAD substrates, APP	Fleig *et al* (2012), Johnson *et al* (2017), Paschkowsky *et al* (2016)
SPP	XBP1u	Chen *et al* (2014)
SPPL3	GnT-V and other glycan-modifying enzymes	Kuhn *et al* (2015), Voss *et al* (2014)
γ-secretase	BCMA	Laurent *et al* (2015)

Sheddases with many substrates and examples of recent studies are listed.
[a]Only such substrates are listed that have been validated, preferentially under sheddase-inactivating conditions or through *in vivo* experiments.

generation, which makes legumain a potential drug target in Alzheimer's disease. Likewise, the soluble protease cathepsin S sheds the cell adhesion proteins ALCAM and CD44 *in vivo* and, at least *in vitro,* numerous additional cell surface proteins (Sobotic *et al*, 2015). Taken together, these examples demonstrate that membrane attachment is not required for a protease to act as a sheddase. However, a membrane-anchor offers the advantage to position the active site close to the membrane surface, facilitating rapid accessibility to the substrate's cleavage site.

Hardware: non-canonical sheddases

Based on the position of their cleavage sites outside the substrates' TM region, canonical sheddases were long thought to be clearly different from intramembrane proteases, which cleave their substrates within the TM domain. The best-investigated intramembrane protease, γ-secretase, cleaves its first-identified substrates, which typically have a long ectodomain, only when their ectodomain is truncated by a canonical sheddase. Consequently, γ-secretase and other intramembrane proteases initially were assumed to not directly shed membrane proteins, but only act secondary to a primary shedding event. However, recent studies demonstrated that some intramembrane proteases, in particular rhomboids and SPPL3, act as *bona fide* sheddases and that other intramembrane proteases,

such as SPP and γ-secretase, can—at least occasionally—also shed membrane protein ectodomains directly and thus act as "part-time" sheddases. Thus, proteases cleaving in juxtamembrane and TM regions share more functional properties than previously expected.

Rhomboids

Rhomboids are intramembrane serine proteases first discovered in *Drosophila*, where they act as key activators of EGFR signaling (Lee *et al*, 2001; Urban *et al*, 2001). *Drosophila* Rhomboid-1 is a Golgi-resident protease that triggers secretion of the EGFR-receptor ligand Spitz by cleaving within its TM domain, paralleling the physiological function of ADAM proteases in mammals (Fig 3A). Rhomboids are universally conserved, and different functions ranging from protein degradation to cleavage of cell adhesion molecules during parasite invasion have been described (for recent reviews see Lemberg, 2013; Freeman, 2014; Urban, 2016). The crystal structures of the *E. coli* rhomboid protease GlpG revealed a conserved six-TM helix-bundle forming a rhomboid active site cavity that opens to the periplasmic (luminal) side of the membrane (Wang *et al*, 2006). Consistent with this, rhomboid proteases have been shown to also cleave substrates within their ectodomains and within loops of multi-TM domain proteins (Erez & Bibi, 2009; Fleig *et al*, 2012). Interestingly, in contrast to most other intramembrane proteases, rhomboids can directly act as sheddases on full-length proteins with long ectodomains and do not require the substrate's ectodomain to
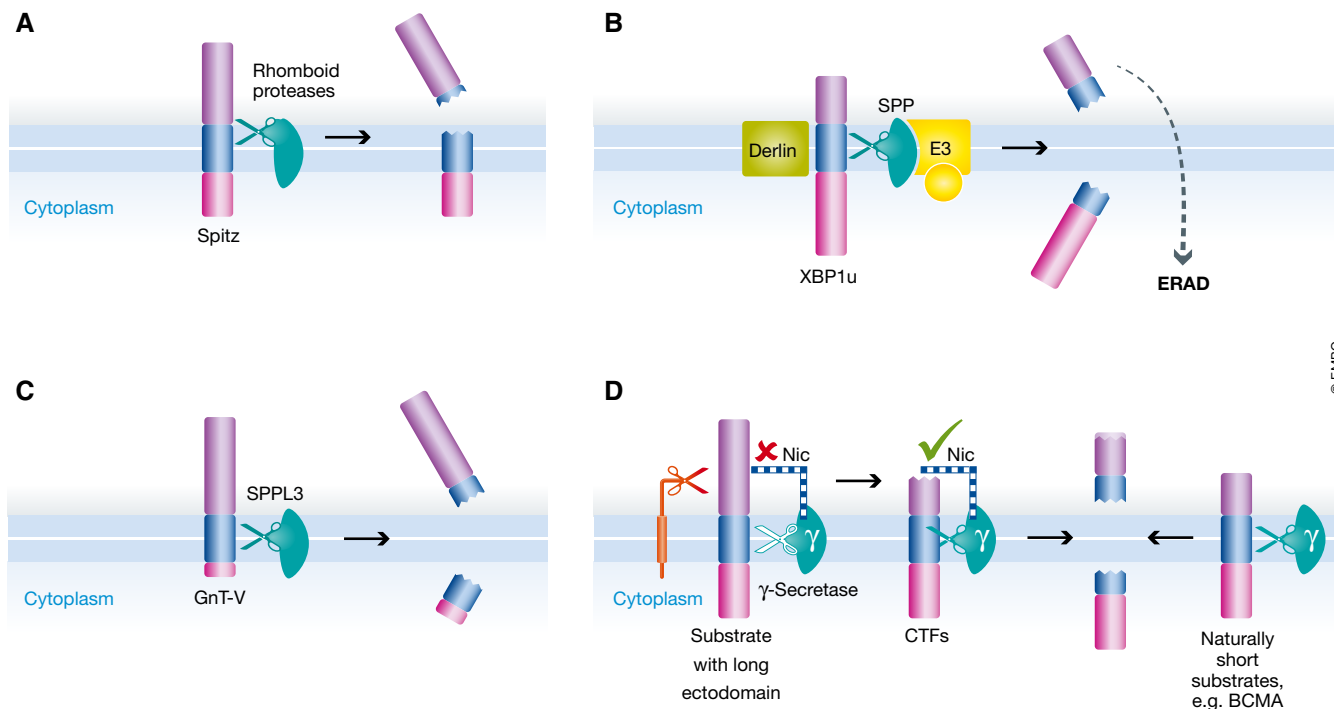
Figure 3. Non-canonical sheddases. One representative substrate per non-canonical sheddase is given.

(A) Rhomboid proteases cleave the TM domain of their substrates in the luminal membrane leaflet, thereby triggering release of the ectodomain. (B) SPP assembles with the rhomboid pseudoprotease Derlin1, and ERAD E3 ubiquitin ligases TRC8 and MARCH6 to form a proteolytic ERAD complex that recognizes membrane proteins without preceding cleavage. In a concerted action, fragments are released to both sides of the membrane and degraded by further components of the ERAD pathway. (C) SPPL3 cleaves glycan-modifying enzymes at the luminal border of their TM domains, releasing the active site containing ectodomain. (D) Membrane proteins with large ectodomains need shedding to truncate their ectodomain before their C-terminal fragment (CTF) can be further processed by γ-secretase. In contrast, substrates with a naturally short ectodomain are directly shed by γ-secretase leading to secretion of their entire ectodomains. Nicastrin (Nic) serves as a molecular ruler accepting only membrane proteins with a short ectodomain.

be trimmed by a preceding canonical shedding event (Fig 3A). In mammals, four secretory pathway rhomboid proteases are known, which are referred to as RHBDL1 to 4 (Fig 2 and Table 2). The first known physiological substrate of a mammalian rhomboid protease was thrombomodulin, which is cleaved by RHBDL2 at the plasma membrane (Lohi *et al*, 2004; Cheng *et al*, 2011). RHBDL2 also cleaves EGF, but likely only to modulate its signaling, a process that may nevertheless be deregulated in certain cancer cells (Adrain *et al*, 2011). More recently, substrate proteomics identified several additional proteins to be cleaved by RHBDL2 (Table 3) (Johnson *et al*, 2017). RHBDL4 has been linked to the ER-associated degradation (ERAD) pathway (Fleig *et al*, 2012) and has been suggested to act as a non-canonical sheddase of APP (Paschkowsky *et al*, 2016).

Signal peptide peptidase (SPP)

Signal peptide peptidase (SPP) is a member of the heterogenous group of GxGD intramembrane aspartyl proteases (Ponting *et al*, 2002; Weihofen *et al*, 2002). A recent crystal structure of the archaeal GxGD protease MCMJR1 revealed the catalytic aspartate residues forming an aqueous active site 8 Å below the membrane surface (Li *et al*, 2013). SPP was first characterized as the activity that clears signal peptides from the ER following their removal from nascent secretory proteins by signal peptidase (Weihofen *et al*, 2000; Lemberg & Martoglio, 2002). More recently, SPP has also been recognized as an ERAD factor that may under certain circumstances

act as a non-canonical sheddase, where—different to its common role in regulated intramembrane proteolysis of signal peptides—it does not require initial activatory cleavage by signal peptidase (Boname *et al*, 2014; Chen *et al*, 2014; Hsu *et al*, 2015). Consistent with this dual role, SPP assembles as a homo-tetramer that processes signal peptides (Schrul *et al*, 2010) and—for its sheddase function—as higher molecular weight assembly with the ERAD factor Derlin1 and ERAD E3 ubiquitin ligases (Fig 3B) (Stagg *et al*, 2009; Chen *et al*, 2014; Stefanovic-Barrett *et al*, 2018). While SPP had initially been hypothesized to contribute to non-proteolytic dislocation of certain ERAD substrates (Loureiro *et al*, 2006; Lee *et al*, 2010), heme oxigenase-1 and XBP1u were recently shown to be shed by SPP, leading to their rapid degradation by the proteasome (Boname *et al*, 2014; Chen *et al*, 2014). Although the C-terminal, luminal portion of SPP-dependent ERAD substrates is not secreted (Fig 3B), cleavage without preceding substrate processing formally ranks SPP as a "part-time" non-canonical sheddase.

SPP-like proteases (SPPL)

Besides SPP four SPP homologs, the SPP-like (SPPL) proteases, SPPL2a, SPPL2b, SPPL2c, and SPPL3, have been identified in mammals (Grigorenko *et al*, 2002; Ponting *et al*, 2002; Weihofen *et al*, 2002). While all known SPPL2b substrates require processing by a canonical sheddase before SPPL2b processing can occur within the TM segment (Fluhrer *et al*, 2006; Martin *et al*, 2008, 2009; Zahn

et al, 2013), SPPL3 was recently found to act as a non-canonical sheddase independently of the substrates' ectodomain length (Fig 3C; Voss *et al*, 2012). Proteomic approaches have identified many substrate candidates, in particular with functions in regulation of cellular N-glycosylation (Voss *et al*, 2014; Kuhn *et al*, 2015). By shedding of various glycosyltransferases and glycosidases, SPPL3 removes the catalytic domain of these enzymes in the Golgi. Subsequent secretion of these domains results in inactivation of the glycan-modifying enzymes. Consequently, increased expression of SPPL3 leads to protein hypoglycosylation, while reduced SPPL3 expression induces hyperglycosylation of cellular proteins. Thus, shedding mediated by SPPL3 may serve as a potent cellular switch that allows adaption of a cell's glycan pattern to environmental changes (Voss *et al*, 2014). SPPL2a is involved in processing of CD74, the invariant chain of the Major Histocompatibility Complex II (MHCII) (Beisner *et al*, 2013; Bergmann *et al*, 2013; Schneppenheim *et al*, 2013). Under physiological conditions, the type II-oriented CD74 molecule is sequentially processed by several serine and cysteine proteases generating a stable membrane-bound CD74 fragment that is subject to SPPL2a cleavage. Thus, CD74 processing reflects a classical cascade of regulated intramembrane proteolysis. Whether SPPL2a and its presently still orphan sister protease SPPL2c can also act as a non-canonical sheddases remains to be elucidated.

γ-secretase

The last member of the GxGD-type aspartyl proteases is γ-secretase, which has presenilin-1 or -2 as the catalytic subunit and which acts as a sheddase on full-length proteins only in exceptional cases. Initially identified because of its link to APP processing in Alzheimer's disease (Sherrington *et al*, 1995; De Strooper *et al*, 1998; Wolfe *et al*, 1999), γ-secretase currently has more than 100 known substrates (for an overview see Haapasalo & Kovacs, 2011). Interestingly, presenilins are distant homologs of the SPP/SPPL family members, but have opposite membrane topology and therefore only cleave type I membrane protein substrates, whereas SPP/SPPL proteases selectively cleave type II-oriented TM segments (Weihofen *et al*, 2002). Besides presenilin, γ-secretase consists of three additional proteins (Aph-1, nicastrin, and PEN2) (Fig 3D), which are essential for γ-secretase maturation and activity (Edbauer *et al*, 2003; Kimberly *et al*, 2003; Takasugi *et al*, 2003). Cryo-electron microscopy shows that the nicastrin subunit has a compactly folded ectodomain that forms a lid on top of the active site of γ-secretase (Bai *et al*, 2015), making nicastrin a molecular ruler that prevents membrane proteins with long ectodomains from getting cleaved by γ-secretase (Bolduc *et al*, 2016). As a consequence, γ-secretase substrates with long ectodomains require prior shedding by canonical sheddases, reducing ectodomain length to < ~50 amino acids and allowing subsequent intramembrane proteolysis by γ-secretase (Struhl & Adachi, 2000). Thus, γ-secretase generally does not act as a sheddase on intact membrane proteins, with the striking exception of the recently described γ-secretase shedding of the B cell maturation antigen (BCMA) that alters its function as a B cell surface receptor required for NFκB signaling and maintenance of long-lived plasma cells (Laurent *et al*, 2015). The cleavage takes place within the BCMA TM domain, but—in contrast to other γ-secretase substrates—does not require prior ectodomain shedding, as the ectodomain is naturally short enough (54 amino acids) for direct

non-canonical shedding by γ-secretase. The mammalian proteome contains additional type I membrane proteins with naturally short ectodomains, and BCMA may therefore be the founding member of a new class of naturally short γ-secretase shedding substrates. Interestingly, a recent study reported that also the APP-homolog APLP1, which has a large ectodomain of several hundred amino acids, may be directly shed by γ-secretase, at least to a small extent, in addition to its usual shedding by ADAM10 and BACE1 (Schauenburg *et al*, 2018). However, it is in this case not yet clear how the long APLP1 ectodomain could mechanistically bypass the strict, short ectodomain length requirement imposed by nicastrin.

Taken together, intramembrane proteases can act as non-canonical sheddases and thus directly influence the physiological functions of their substrates. While some intramembrane proteases, like rhomboids and SPPL3, primarily act as sheddases and have multiple substrates, others including SPP and γ-secretase appear to have exceptional "part-time" sheddase functions only on selected targets under specific conditions, and otherwise mostly act as intramembrane proteases requiring a prior shedding event.

Hardware: higher order assembly and non-proteolytic subunits

Most sheddases are assumed to act as monomers or, as observed for BACE1, homodimers (Schmechel *et al*, 2004; Westmeyer *et al*, 2004), but there is increasing evidence that certain sheddases may assemble into higher order complexes, as has been described for ADAM10 that interacts with γ-secretase (Chen *et al*, 2015). While this may allow efficient coupling of shedding and subsequent intramembrane proteolysis, it is unknown whether all ADAM10 substrates are further processed by γ-secretase, and it remains to be determined which fraction of both proteases is found in the complex. In addition, ADAM10 was also reported to associate with certain members of another class of multi-pass TM proteins, the tetraspanins (reviewed in Matthews *et al*, 2017). These non-proteolytic partners have been attributed functions in ADAM10 maturation (Arduise *et al*, 2008; Dornier *et al*, 2012; Haining *et al*, 2012; Prox *et al*, 2012), but their exact impact on activity, regulation, and substrate specificity of ADAM10 remains to be determined. Similarly, larger complexes have been observed for ADAM17, which associates with the catalytically inactive rhomboid-family proteins iRhom1 or -2 (Adrain *et al*, 2012; McIlwain *et al*, 2012; Christova *et al*, 2013; Maretzky *et al*, 2013; Cavadas *et al*, 2017; Grieve *et al*, 2017), and for SPP, which assembles with Derlin1 (Chen *et al*, 2014). iRhoms and derlins can be seen as part of the sheddase hardware and serve as substrate adaptors or trafficking regulators for their active protease partners ADAM17 or SPP (Maretzky *et al*, 2013). This is reminiscent of γ-secretase with its three non-proteolytic subunits required for maturation, trafficking, and activity of the whole protease complex (Edbauer *et al*, 2003; Kimberly *et al*, 2003; Takasugi *et al*, 2003).

Hardware: substrates

To date, the number of shedding substrates is unknown, but given the large numbers of canonical sheddase substrates mentioned

above (see substrates and review articles cited in Table 3), it is clear that shedding affects at least a few hundred different mammalian membrane proteins. Moreover, a systematic screening of published reports led to a database (SheddomeDB) listing over 400, mostly human, shed proteins (Tien *et al*, 2017). Furthermore, several recent proteomic studies detected hundreds of membrane proteins in conditioned medium of various cell lines and in body fluids (e.g., Faca *et al*, 2008; Kuhn *et al*, 2012; Meissner *et al*, 2013; Kim *et al*, 2014; Wilhelm *et al*, 2014; Dislich *et al*, 2015). These proteins may well constitute cleavage products of shedding substrates, although not all of them have been validated by independent methods. Interestingly, sets of shed proteins differed significantly between different cell types or even between distinct cancer cell lines (Faca *et al*, 2008; Kuhn *et al*, 2012). Given the large variety of cell types in mammals, it thus seems reasonable to estimate that the total number of shed proteins in a given organism may exceed 1,000. Potentially, all of the more than 2,000 human single-span membrane proteins listed in UniProt may undergo shedding, at least at some point during their life cycle. In fact, it has proven difficult to find proteins that are not shed at all, in particular when proteins are overexpressed in cell lines. Thus, it appears possible that shedding substrates fall into two distinct categories. One of them includes substrates for which shedding is coupled to physiological consequences, as discussed in the next paragraph. The other category comprises membrane proteins where shedding does not lead to major functional changes but may be a mechanism of protein turnover. Yet, more functional studies with a larger number of shedding substrates are required to firmly distinguish between both categories.

A future establishment of a more comprehensive catalog of sheddase substrates appears possible and is facilitated by the recent development of new proteomic methods for *in vitro* and *in vivo* substrate identification (Gevaert *et al*, 2003; Kleifeld *et al*, 2010; Eichelbaum *et al*, 2012; Kuhn *et al*, 2012; Dislich *et al*, 2015;

reviewed in Muller *et al*, 2016). However, before concluding that all proteins identified in a given proteomic study or from overexpression studies are new sheddase substrates, careful validation by independent methods must be executed. This includes *in vitro* assays to test the direct cleavage and the demonstration of physiological relevance, in particular when substrates have been identified upon protease overexpression or exogenous addition of recombinant protease in *in vitro* assays.

How does shedding alter membrane protein function?

As for all other enzymes, the function of sheddases is determined by their substrates. Given the large number and diversity of their substrates, it is clear that shedding affects numerous physiological processes. In the following, we will illustrate three fundamental ways in which the shedding process can alter a substrate protein's function.

First, where the full-length membrane-bound form of the substrate displays its physiological function, shedding provides a mechanism to *terminate the function of a full-length membrane protein* (Fig 4A). Examples are cell adhesion proteins (e.g., selectins), glycosyltransferases (e.g., GnT-V), and cell surface receptors (e.g., TNFRs). This is not just a mechanism leading to membrane protein degradation, but the shed ectodomain can even further block the physiological function of the remaining full-length proteins. For example, the shed ectodomain of the B cell maturation antigen (BCMA) acts as a decoy receptor that sequesters the cognate ligand and thereby further attenuates cell signaling in addition to shedding of the full-length BCMA receptor (Laurent *et al*, 2015).

Second, if cleavage releases the biologically active ectodomain of the membrane protein, shedding can *activate a membrane protein* (Fig 4B). Examples are growth factors (e.g., TGFα) and cytokines
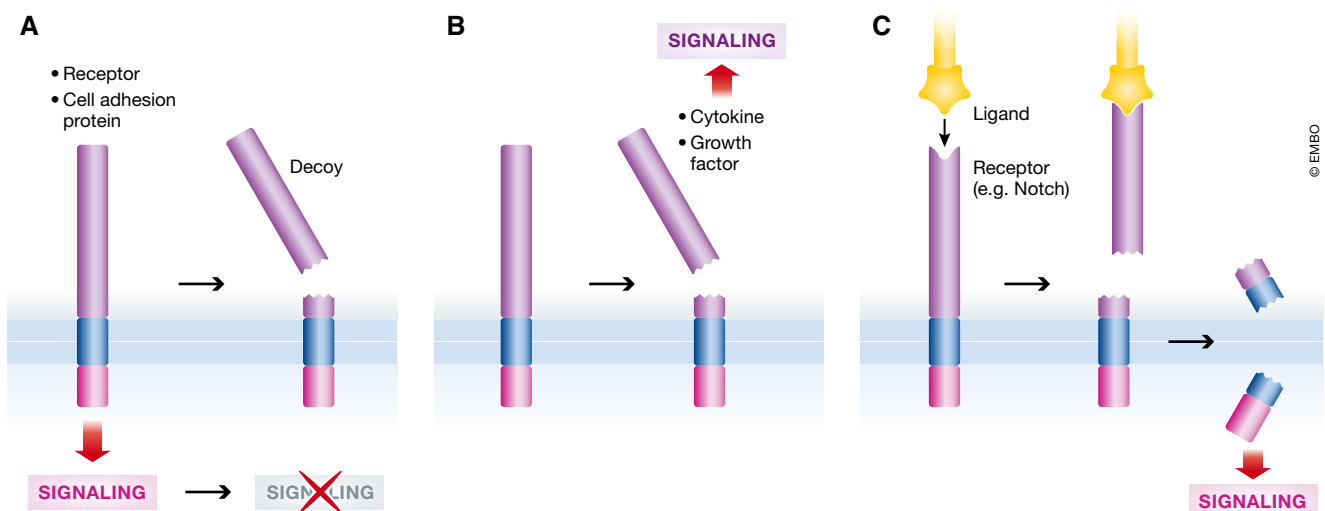

Figure 4. Functional consequences of shedding.
(A) By proteolytic processing of TM proteins that display a physiological function, like cell adhesion or receptor-mediated signaling, sheddases terminate these functions. (B) Shedding generates biologically active signaling molecules from membrane-anchored precursors, e.g., cytokines or growth factors that act on neighboring or far distant cells. (C) As part of regulated intramembrane proteolysis, sheddases induce a proteolytic cascade ultimately activating signaling molecules, like the Notch intracellular domain, that act within the same cell. Shedding may be ligand-induced and the ligand may even be a membrane-anchored protein itself, as in case of Notch.

(e.g., TNFα). In these cases, shedding is a mechanism to timely and spatially control release and activity of the biomolecule. For example, shed TNFα acts in a paracrine manner to activate proinflammatory TNFR1 signaling, whereas membrane-bound TNFα activates anti-inflammatory TNFR2 (Grell *et al*, 1995), but can only do this upon direct cell–cell contact. Interestingly, also the membrane-bound fragment remaining after shedding can be biologically active. This is observed for the BACE1-generated C-terminal fragment of CHL1, which functions in growth cone collapse during axon guidance in the nervous system (Barao *et al*, 2015).

Third, shedding can *induce subsequent processing by an intramembrane protease*. A key example is activation of the Notch receptor, which is induced by a membrane-anchored ligand on another cell. Ligand-binding triggers endocytosis of both the ligand and of Notch into the opposing cell. This exposes the membrane-proximal ADAM10 cleavage site. ADAM10 cleavage is followed by γ-secretase-mediated processing to release the intracellular domain, which then acts as a transcription activator (Pan & Rubin, 1997; De Strooper *et al*, 1999; Tiyanont *et al*, 2011; Fig 4C).

Taken together, shedding is a versatile mechanism to control the activity of membrane proteins. Yet, for most shedding substrates, it has not yet been explored how shedding controls or alters their function. As a result, more functional consequences in addition to the three categories described above may be discovered in the future. Likewise, it appears possible that—for some substrates—the shedding process is simply contributing to protein turnover and may not be coupled to a major functional consequence, for example, in cell signaling.

Cellular localization of ectodomain shedding

Initially, the term shedding referred to cleavages occurring at or very close to the plasma membrane (Arribas *et al*, 1996), where the cleaved, soluble ectodomain was released from cells into conditioned medium or into body fluids. It is now clear that shedding additionally takes place in multiple cellular compartments, including all organelles of the secretory and endocytic pathway (Fig 2). Related proteases may be active in different cellular compartments, as seen for SPP cleaving in the ER (Weihofen *et al*, 2002) and SPPL3 being active in the Golgi (Voss *et al*, 2014), or presenilin-1-containing γ-secretase being more active at the plasma membrane while γ-secretase complexes containing the presenilin-2 paralog are predominantly active in endo- and lysosomes, but potentially also in the trans-Golgi network (Meckler & Checler, 2016; Sannerud *et al*, 2016). In which cellular compartment a given sheddase cleaves its substrate is largely determined by where the substrate meets the active enzyme. Many sheddases, including ADAMs, BACEs, and MT-MMPs, require removal of their pro-peptide by furin or related pro-protein convertases for gaining their full proteolytic activity (e.g., Lopez-Perez *et al*, 1999, 2001; Bennett *et al*, 2000; Capell *et al*, 2000; Huse *et al*, 2000; Schlondorff *et al*, 2000; Creemers *et al*, 2001). Pro-peptide removal often occurs in the trans-Golgi compartment and, thus, efficiently prevents premature substrate cleavage in the ER and the Golgi compartment. Activity of other sheddases is pH-dependent. For instance, BACE1 has an acidic pH optimum (Vassar *et al*, 1999; Shimizu *et al*, 2005), thus only cleaving in acidic cellular compartments. Therefore, depending on

the substrates' localization, some BACE1 substrates are predominantly cleaved in endosomes, whereas others are mostly cleaved in the TGN. These different cellular localizations of BACE1 activity are even exploited for the development of substrate-specific BACE1 inhibitors, which selectively target BACE1 in endosomes (Rajendran *et al*, 2008; Mitterreiter *et al*, 2010; Ben Halima *et al*, 2016). On the other hand, partly due to lack of suitable reagents, the exact cleavage compartment—assumed to be late in the secretory pathway or at the plasma membrane—has been identified for only few substrates of ADAM10. For instance, that ADAM10 cleavage of the Eph-receptor ligand ephrin-A5 takes place at the plasma membrane has been inferred mainly because this cleavage happens *in trans* with ephrin-A5 being expressed on the surface of one cell and ADAM10 on the surface of another cell (Janes *et al*, 2005). Otherwise, shedding events known to date happen *in cis*, with substrate and sheddase expressed within the same cell.

Substrate recognition of sheddases

The increasing number of substrates that are assigned to sheddases allows to highlight two major requirements governing substrate recognition by sheddases: (i) substrate sequence and structure and (ii) vicinity of the cleavage site close to or within the membrane.

Sheddases recognize amino acid motifs and/or secondary structures in their substrates

Sheddases often have preferences for certain amino acid motifs, which is seen in *in vitro* assays (e.g., Gruninger-Leitch *et al*, 2002; Caescu *et al*, 2009), by mutational analyses (e.g., Sisodia, 1992) and by sheddase structure determinations (e.g., Hong *et al*, 2000; Seegar *et al*, 2017). However, this requirement is less pronounced compared to many soluble proteases, such as trypsin and caspases. As a consequence, point mutations in the substrates close to the cleavage site rarely fully abolish cleavage, as for example shown for APP (Sisodia, 1992; Citron *et al*, 1995). Additionally, it is possible that mutations simply shift the cleavage site by a few amino acids to an alternative, cryptic cleavage site, or that other proteases cleave at a site close by. This may be overlooked in typical cellular shedding experiments, where levels of one of the cleavage products are measured, but where the exact cleavage sites have mostly not been determined. Not only the amino acid sequence, but also secondary structure elements around the substrate's cleavage site may contribute to specificity of the shedding event. For example, cleavage sites for rhomboids are within or at the border of substrates' TM domains and are part of helical structures, which need to be unfolded before cleavage (Urban & Freeman, 2003; Strisovsky *et al*, 2009). Consequently, point mutations increasing or decreasing the propensity to unfold the helical structure increased or decreased the extent of substrate cleavage, respectively (Strisovsky *et al*, 2009; Moin & Urban, 2012; Strisovsky, 2016). Overall, substrate specificity of sheddases is not only determined by a binary interaction between sequence surrounding the scissile peptide bond and protease active site, but additional protein interactions to so-called exosites located either on the sheddase or adaptor proteins. For example, it has been suggested that iRhoms and tetraspanins present substrates to ADAM17 and ADAM10 (Maretzky *et al*, 2013; Matthews *et al*,

2017), respectively, proposing an interaction of the substrate with both the adapter and the protease. Similarly, SPP is primed toward the full-length type II membrane protein XBP1u by the rhomboid pseudoprotease Derlin1 (Chen *et al*, 2014). Likewise, substrates are thought to interact with different subunits of the four-member γ-secretase complex before they reach the active site (Fukumori & Steiner, 2016).

Location of the cleavage site with respect to the membrane

A second major determinant of substrate recognition by sheddases is the location of the substrate's cleavage site. The cleavage site typically localizes in the luminal juxtamembrane domains (canonical sheddases) and TM domains (non-canonical sheddases) of the substrate. Thus, a "perfect" sequence motif for a membrane-bound sheddase may still not be sufficient for cleavage if the sequence motif in the substrate is too far away from the membrane. Likewise, once the membrane is removed, e.g., in *in vitro* sheddase assays, the substrate specificity may be different compared to cellular and *in vivo* experiments, as the active site may gain access to potential cleavage sites that would not be reachable and therefore never get cleaved under physiological conditions (e.g., Schlondorff *et al*, 2000; Brummer *et al*, 2018). Conversely, protease cleavage specificities determined *in vitro* using peptide libraries may not necessarily be relevant in full-length substrates in cellular membranes. Thus, it is difficult to predict sheddase substrates simply based on sequence analysis, and experimental substrate identification is required.

Taken together, substrate recognition of sheddases requires a permissive sequence and structure around the cleavage site of the substrate and the correct positioning of the sheddase's active site toward the substrate's scissile peptide bond.

Substrate repertoire of sheddases

For most known shedding substrates, the protease has not yet been identified. And conversely, only for few sheddases, a comprehensive list of substrates is known. In the following, we will summarize key lessons about shedding substrates learned from the comparative study of different sheddases with multiple substrates.

Developmental stage matters

Numerous substrates are known for ADAM10 and ADAM17. Yet, only one or at most few of them were assumed to be of major physiological relevance. This conclusion was largely based on the most obvious phenotype(s) of the corresponding sheddase-deficient mice. For example, ADAM10-deficient mice die at embryonic day 9.5, because the ADAM10 substrate Notch is no longer cleaved, thus preventing Notch signaling during embryonic development (Hartmann *et al*, 2002). However, conditional postnatal ADAM10 deletion circumventing embryonic lethality demonstrated additional phenotypes, particularly in the brain, that are not related to Notch but to other substrates; for example, defects in synaptic connectivity were caused by defective processing of NrCAM (Jorissen *et al*, 2010; Prox *et al*, 2013; Kuhn *et al*, 2016). Thus, the most pertinent physiological function of a sheddase may be mediated by different substrates at distinct developmental or adult stages.

Tissue-dependent shedding

A substrate may be cleaved by different sheddases in a tissue-dependent manner, depending on the expression pattern of substrate and sheddases. For example, BACE1 is highly expressed in the brain, but at low levels in most other tissues (Vassar *et al*, 1999). As a result, APP and the cell adhesion protein L1 are mostly shed by BACE1 in the brain, but predominantly by ADAM10 in peripheral cells and tissues (Gutwein *et al*, 2003; Kuhn *et al*, 2012, 2016; Colombo *et al*, 2013). Likewise, the surface protein SEZ6L is mostly cleaved by BACE1 in the brain, but by BACE2 in the pancreas (Stutzer *et al*, 2013; Pigoni *et al*, 2016).

Subcellular localization

The substrate spectrum may also depend on the subcellular localization of protease and substrate, in particular in polarized cells such as neurons. For example, neuronal ADAM10 predominantly localizes to the somatodendritic compartment (Marcello *et al*, 2007), whereas BACE1 is found more in axons (Kandalepas *et al*, 2013). Similar differences are seen in polarized epithelial cells (Capell *et al*, 2002; Wild-Bode *et al*, 2006). SPPL3 mainly localizes to the Golgi and preferentially sheds glycan-modifying enzymes in this compartment, while glycosyltransferases localizing to the ER are not affected by SPPL3 (Voss *et al*, 2014; Kuhn *et al*, 2015). Thus, it is likely that these proteases mostly cleave substrates that localize to the same subcellular compartment.

One substrate may be cleaved by multiple sheddases within one cell type

A major insight from recent quantitative proteomic studies for substrate identification is that some targets are predominantly shed by a single protease within one cell type, whereas other substrates within the same cell type may be cleaved by more than one protease. For instance, in neurons, SEZ6 is only cleaved by BACE1 and MMP17 is only shed by ADAM10, whereas the cell adhesion protein CHL1 is cleaved to more than 40% by each BACE1 and ADAM10 (Kuhn *et al*, 2012, 2016) and to some extent by ADAM8 (Naus *et al*, 2004). For most substrates with multiple sheddases, it remains unclear which additional sheddase(s) contribute and whether the cleavage sites of the different proteases are identical or different. For example, numerous glycan-modifying enzymes are shed by SPPL3, but even upon loss of the protease some substrates still undergo significant shedding (Kuhn *et al*, 2015). Additionally, compensatory effects need to be considered, when one sheddase is inactivated. For example, blocking BACE1-mediated shedding of the Alzheimer's disease-linked APP protein in neurons leads to a compensatory increase in APP shedding by ADAM10, as observed in neurons and even in humans in an Alzheimer clinical trial (May *et al*, 2011; Colombo *et al*, 2013). A key challenge for the future will be to understand whether the different proteases that cleave a single protein have redundant functions or whether the corresponding cleavages lead to different functional consequences, as in the case of APP and neuregulin (Hu *et al*, 2006; Willem *et al*, 2006; Ring *et al*, 2007; Li *et al*, 2010; La Marca *et al*, 2011).

Substrates can have major and minor sheddases

Another future challenge will be to detect (patho)physiologically relevant, but minor proteolytic cleavage events in a given substrate. For example, APP in neurons is mostly shed by the α-secretase

ADAM10 and the β-secretase BACE1. Yet, to an apparently smaller extent, APP is also shed by additional proteases, including meprin β and BACE2 (Farzan *et al*, 2000; Yan *et al*, 2001; Fluhrer *et al*, 2002; Jefferson *et al*, 2011) and the recently identified δ- and η-secretases legumain and MT5-MMP, respectively, which may be relevant for Alzheimer's disease (Willem *et al*, 2015; Zhang *et al*, 2015). APP in non-neuronal cells may further be cleaved by RHBDL4 (Paschkowsky *et al*, 2016), but the pathophysiological relevance of this cleavage still needs to be demonstrated. Other examples are the Nogo-66 receptor (NgR1), which is predominantly shed by MT3-MMP in neurons (Sanz *et al*, 2018), and the transferrin receptor that is mostly shed by PC7 in hepatocytes (Guillemot *et al*, 2013), with presumed minor sheddases in these cases yet to be identified. The glycosylation enzyme ST6GalI is a shedding substrate for both SPPL3 (Kuhn *et al*, 2015) and BACE1 (Kitazume *et al*, 2001).

Establishment of minor cleavage events is difficult, because the major sheddase would still cut a substrate protein even when the minor sheddase is inactivated. However, proteomic methods specifically determining the neo-N-/C-termini of cleavage products can determine even such minor cleavage sites (reviewed in Muller *et al*, 2016). Another difficulty in detecting minor or even major cleavage events is that the shedding by a specific protease may only take place under activated cellular conditions, such as inflammation, infection, or cell stress. Thus, as long as the stimulus is unknown, the cleavage event will not be detected.

Taken together, sheddases can have different substrates and substrates can have different sheddases. As a last expansion of the complexity, a protease that cleaves a given substrate may differ between various organisms. This still appears as an exception, but during evolution different proteases have evolved in distinct organisms to cleave similar types of substrates. One prominent example is EGFR ligands, which are cleaved by ADAM10 and ADAM17 in mammals but by rhomboid proteases in *Drosophila*, despite both classes of sheddases being conserved between the two species (Lee *et al*, 2001). Interestingly, mammalian rhomboid proteases still retain the ability to cleave EGFR ligands, but they appear to be less prominent, as ADAM17 has taken the lead during mammalian evolution. Hence, EGFR ligand cleavage by rhomboids only becomes visible upon ADAM17 inhibition or deficiency (Adrain *et al*, 2011). Over all, we can expect that the substrate spectrum in common model organisms will be more comprehensively defined so that we shall learn more about the fascinating evolution of canonical and non-canonical sheddases.

Regulation of shedding

Shedding is frequently regulated by mechanisms ranging from trafficking control to natural protein inhibitors and activators. Given the number of sheddases, substrates, and mechanisms, there is a wealth of studies on this topic (reviewed e.g., in Hayashida *et al*, 2010; Lichtenthaler, 2012; Adrain & Freeman, 2014; Clark, 2014). In the following, we will illustrate key principles with selected examples and highlight new developments in shedding regulation.

Trafficking introduces a major layer of control

Protein trafficking is arguably one of the most important regulatory mechanisms for ectodomain shedding. While a soluble protease may meet its substrate through diffusion, membrane-bound sheddases and their TM substrates need to be transported to the same organelle for cleavage to occur. This was first shown for the pathway regulating shedding and activation of SREBP. When cellular cholesterol levels drop, ER-localized SREBP translocates to the Golgi, where it gets shed by S1P and subsequently cleaved by site-2-protease within its TM domain (Rawson *et al*, 1997; Sakai *et al*, 1998). This dual cleavage results in release and activation of the cytoplasmic SREBP domain, which induces transcription of genes involved in cholesterol biosynthesis (Sakai *et al*, 1996). Regulated trafficking also happens for other shedding substrates such as APP, where endocytic trafficking controls APP cleavage by either the α-secretase ADAM10 or the β-secretase BACE1 (e.g., Haass *et al*, 1993; Koo & Squazzo, 1994; Chyung & Selkoe, 2003; Carey *et al*, 2005; Schobel *et al*, 2008) (and reviewed in Lichtenthaler, 2012). Trafficking also controls the activity of sheddases. For example, upon activation of NMDA receptors, the cytoplasmic adaptor protein Sap97 binds the cytoplasmic tail of ADAM10, thereby promoting its trafficking and activation (Marcello *et al*, 2007). ADAM10 trafficking is also controlled by tetraspanins (Dornier *et al*, 2012). Likewise, iRhoms have been implicated in trafficking and regulation of ADAM17 (Adrain *et al*, 2012; McIlwain *et al*, 2012).

Abundance control is key

More sheddase or more substrate typically results in more cleavage, and levels of both enzyme and substrates are typically controlled through transcription, translation, and protein degradation. For example, LPS stimulation of immune cells induces TNFα transcription followed by increased TNFα shedding through ADAM17 (Black *et al*, 1997; Moss *et al*, 1997). Translational repression has been intensively studied for BACE1 (De Pietri Tonelli *et al*, 2004; Lammich *et al*, 2004; Rogers *et al*, 2004; Zhou & Song, 2006; Mihailovich *et al*, 2007; Faghihi *et al*, 2008; Hebert *et al*, 2008; Wang *et al*, 2008), and this repression may be relieved upon cellular stress or during disease (e.g., O'Connor *et al*, 2008). Lysosomal protein degradation is an additional mechanism to control levels of sheddases and substrates. For instance, binding of BACE1 to the adaptor protein GGA promotes degradation (Tesco *et al*, 2007) and this is blocked by a specific sugar modification, bisecting N-acetyl-glucosamine (Kizuka *et al*, 2015). As an alternative to lysosomal degradation, classical sheddases may be shed themselves, e.g., ADAM10, BACE1, and meprin β (Hussain *et al*, 2003; Tousseyn *et al*, 2009), which may be considered a mechanism for inactivating a sheddase (Wichert *et al*, 2017).

Integration of signaling at the level of sheddase and regulatory subunits

Besides stimulation through regulation of trafficking and protein abundance, signaling pathways can also acutely stimulate shedding within minutes, allowing cells to quickly respond to external stimuli without the need for time-consuming protein biosynthesis. A prime example is the fast activation of ADAM17 by the phorbol ester PMA (Peschon *et al*, 1998; Doedens *et al*, 2003; Sahin *et al*, 2004). This occurs through phosphorylation of the ADAM17-associated iRhom protein independently of the cytoplasmic tail of ADAM17 and appears to induce a fast structural change in ADAM17 (Doedens *et al*, 2003; Le Gall *et al*, 2010; Cavadas *et al*, 2017; Grieve *et al*, 2017). This fast ADAM17 activation also occurs for a physiological

process called transactivation, in which agonists of G protein-coupled receptors indirectly activate the EGFR (Prenzel *et al*, 1999; Maretzky *et al*, 2011). Although this fast activation appears to be independent of phosphorylation of ADAM17 itself, other sheddases can be regulated by direct phosphorylation as observed for the δ-secretase legumain (Wang *et al*, 2017). Another mechanism that is increasingly linked to the shedding control is calcium signaling, as observed for human meprin β (Arnold *et al*, 2015) or certain rhomboid proteases (Baker & Urban, 2015).

Inhibitors and activators of protein shedding
In addition to direct activation of the enzymes, various mechanisms tune access of sheddases to their substrates. One prominent example is the soluble tissue inhibitors of metalloproteases that (to various extent) block ADAM10, ADAM17, and MT-MMPs (e.g., Amour *et al*, 1998, 2000). Conversely, other ligands are known to induce shedding as observed for Notch, RGMa, BAFFR, and DDR1 (Pan & Rubin, 1997; Bozkulak & Weinmaster, 2009; van Tetering *et al*, 2009; van Erp *et al*, 2015; Shitomi *et al*, 2015; Smulski *et al*, 2017). An emerging layer of regulation is post-translational modification at the substrate level. For instance, O-glycosylation at amino acids close to the cleavage site controls substrate shedding by ADAM proteases (Goth *et al*, 2015) and in context of the ER, substrate

ubiquitination has been shown to trigger intramembrane proteolysis (Fleig *et al*, 2012).

Modulation by lipids
Since sheddases and their substrates are mostly membrane proteins, they are in direct contact with membrane lipids. It is now becoming clear that lipids are not only bystanders, but can directly control proteolytic activity, as is clearly seen for intramembrane sheddases, in particular γ-secretase and rhomboids (Urban & Wolfe, 2005; Bondar *et al*, 2009; Holmes *et al*, 2012; Winkler *et al*, 2012). Yet, even the activity of canonical sheddases can be affected by lipids, for example, BACE1 by cholesterol (Ehehalt *et al*, 2003; Kalvodova *et al*, 2005) or ADAM17 by phosphatidylserine (Sommer *et al*, 2016). However, the exact mechanisms by which lipids regulate sheddase activity await further clarification.

Disease association and shedding-based drugs

Deregulation of shedding may result in diseases caused by too much or too little of the substrate or the sheddase or the cleavage activity. Sheddases and the relevant substrates are consequently considered as drug targets. A key example is an excessive level of shed TNFα,

Table 4. Shedding-related diseases and drugs.

Sheddase	Substrate	Disease	Therapeutic strategy and stage of development	References (review articles or original study)
ADAM8	Unknown	Breast cancer	Inhibition of ADAM8 (mouse study)	Romagnoli *et al* (2014)
	Unknown	Pancreatic cancer	Inhibition of ADAM8 (mouse study)	Schlomann *et al* (2015)
ADAM9	EGF, FGFR2iiib	Prostate cancer	Inhibition of ADAM9 (mouse study)	Peduto *et al* (2005)
ADAM10	APP	Alzheimer's disease	Activation of ADAM10 (phase II clinical trial terminated),	Endres *et al* (2014), Suh *et al* (2013)
	CD23	Asthma	Inhibition of ADAM10 (mouse study)	Mathews *et al* (2011), Weskamp *et al* (2006)
	Ephrin-B2	Lung fibrosis	Inhibition of ADAM10 (mouse study)	Lagares *et al* (2017)
	PrP	Prion diseases	Activation of ADAM10 (mouse study)	Altmeppen *et al* (2015), Endres *et al* (2009)
ADAM17	TNFα	Inflammatory diseases *Sepsis Rheumatoid arthritis Crohn's disease Psoriasis Lupus nephritis*	Inhibition of TNFα, (approved drugs), blocking ADAM17 through exosite inhibitors, soluble prodomain or iRhoms (mouse study)	Adrain *et al* (2012), Horiuchi *et al* (2007), Issuree *et al* (2013), McIlwain *et al* (2012), Qing *et al* (2018), Udalova *et al* (2016), Wong *et al* (2016)
	IL6 receptor	Inflammatory diseases *Intestinal inflammation, intestinal cancer, Rheumatoid arthritis, Lupus erythematosus, Asthma, Sepsis, Nephrotoxic nephritis, Arterosclerosis, Lung emphysema and others*	Inhibition of IL6 signaling through soluble gp130 (clinical trials ongoing)	Rose-John (2017), Schmidt *et al* (2018)
BACE1	APP	Alzheimer's disease	Inhibition of BACE1 with small-molecule drugs (clinical trials ongoing)	Barao *et al* (2016), Vassar *et al* (2014)
δ-secretase	APP	Alzheimer's disease	Inhibition (mouse study)	Zhang *et al* (2017, 2015)

The table lists selected examples where the pathological role of a sheddase or its substrate to a disease has been established in animal models and through human genetics. The many instances where altered sheddase expression only correlates with disease are not listed.

which is implicated in numerous inflammatory diseases, including sepsis, rheumatoid arthritis, and lupus. Clinical treatment blocks the function of shed TNFα by neutralizing it with antibodies or antibody-like proteins, which are among the best-selling drugs worldwide (Udalova *et al*, 2016). Other approaches were less successful, such as the use of small-molecule inhibitors of the TNFα sheddase ADAM17, because of the broad ADAM17 substrate spectrum and the cross-reactivity of inhibitors with ADAM17-related metalloproteases. Besides small-molecule drugs and antibodies, shed substrate ectodomains may be employed as decoy receptors. For example, recombinant soluble gp130, corresponding to the shed gp130 ectodomain, is used to block excessive IL-6 signaling in inflammatory conditions and is currently being tested in a phase II clinical trial (reviewed in Rose-John, 2017). Another important example of a shedding-related condition is Alzheimer's disease, where pathogenic amyloid-β peptide results from shedding of APP by BACE1 followed by γ-secretase-mediated intramembrane proteolysis. Naturally occurring mutations at the BACE1 cleavage site in APP result in enhanced APP shedding by BACE1 and increased amyloid-β levels, thus causing an inherited form of the disease (Citron *et al*, 1992). For some diseases, increased or reduced levels of sheddases have been reported, but it is not always clear whether this is a cause or a consequence of the disease pathogenesis and whether it might be therapeutically exploited, for example, in cancer (reviewed in Murphy, 2008). Selected examples of shedding-related diseases and potential drugs are listed in Table 4. The shed substrate ectodomains may also serve as potential companion diagnostics to monitor drug responses in patients upon sheddase inhibition or activation. For example, the BACE1-cleaved APP ectodomain serves as a marker to monitor BACE1 activity in clinical trials with BACE1 inhibitors against AD (May *et al*, 2011).

Conclusion and outlook

ADAM10 and ADAM17, the first known proteases with sheddase activity, were identified 21 years ago (Black *et al*, 1997; Moss *et al*, 1997; Pan & Rubin, 1997). Initially considered as a process affecting selected membrane protein substrates only (Ehlers & Riordan, 1991; Massague & Pandiella, 1993; Arribas *et al*, 1996), ectodomain shedding is now a fundamental process in cell biology. It controls the communication between cells and their environment and impacts on many areas in life sciences and medicine. It is becoming increasingly clear that proteolysis of membrane proteins is not the exception, but rather the rule for many membrane proteins. Despite the tremendous progress over the past years, there are many central open questions and challenges that lie ahead. Given the large number of known and still to be identified shedding substrates and the increasing number of sheddases, a major challenge for the future will be to assign individual substrates to proteases and determine how the proteolytic cleavage alters the substrates' function. This is particularly important as sheddases are used as drug targets. Their inhibition or activation may not only interfere in the desired way with the function of the disease-linked protein, but may affect the function of multiple other substrates of the same protease as well. Yet, these hurdles may be overcome by developing substrate-selective inhibitors or by designing drugs targeting protease exosites where only a subset of substrates binds. This will require a better understanding of the molecular mechanisms underlying the sheddases' substrate specificity. It will be equally important to understand the spatial organization of proteolysis, for example, within cells or even whole tissues, as well as the timing, kinetics, and regulation of shedding, including the potential identification of more non-proteolytic subunits of sheddases. Taken together, it is a fascinating time to study ectodomain shedding and we can stay tuned for more major discoveries over the years to come.

Conflict of interest

The authors declare that they have no conflict of interest.

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