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Cellular Functions of γ-Secretase-Related Proteins

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Key Words
Nodal modulator · Presenilin · Signal peptide peptidase · Nodal signaling pathway · Nicastrin-like protein

Abstract
Amyloid-β peptide (Aβ) is generated by γ-secretase, a membrane protein complex with an unusual aspartyl protease activity consisting of the four components presenilin, nicastrin, APH-1 and PEN-2. Presenilin is considered the catalytic subunit of this complex since it represents the prototype of the new family of intramembrane-cleaving GxGD-type aspartyl proteases. Recently, five novel members of this family and a nicastrin-like protein were identified. Whereas one of the GxGD-type proteins was shown to be identical with signal peptide peptidase (SPP), the function of the others, now called SPP-like proteins (SPPLs), is not known. We therefore analyzed SPPL2b and SPPL3 and demonstrated that they localize to different subcellular compartments suggesting nonredundant functions. This was supported by different phenotypes obtained in knockdown studies in zebrafish embryos. In addition, these phenotypes could be phenocopied by ectopic expression of putative active site mutants, providing strong evidence for a proteolytic function of SPPL2b and SPPL3. We also identified and characterized the nicastrin-like protein nicalin which, together with the 130-kDa protein NOMO (Nodal modulator), forms a membrane protein complex different from γ-secretase. We found that during zebrafish embryogenesis this complex is involved in the patterning of the axial mesendoderm, a process controlled by the Nodal signaling pathway.

Introduction

Intramembrane proteolysis is an only recently identified mechanism underlying various important cellular processes such as cholesterol homeostasis, ER stress, cell fate decisions, signal peptide cleavage, and removal of transmembrane domain (TMD) stubs [1]. An extensively studied example of intramembrane proteolysis is the γ-secretase-mediated cleavage of the β-amyloid precursor protein (APP), a critical step in the development of Alzheimer's disease (AD) [2]. Upon initial ectodomain shedding by α- or β-secretase, the remaining APP C-terminal fragment is cleaved within its TMD by γ-secretase, a membrane protein complex of high molecular weight with an unusual aspartyl protease activity. The consecutive cleavage of APP by β- and γ-secretase results in the generation of small peptides, including the 40- to 42-amino acid amyloid-β peptide (Aβ), which are released into the extracellular space [2]. The neurotoxic, highly amyloidogenic 42-amino acid Aβ variant is believed to

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be the pathological agent that initiates AD. The physiological role of the APP processing is not known, but γ-secretase-mediated cleavage of other substrates, most importantly the Notch receptor, is required during development and maintenance of multicellular organisms [3].

Four γ-secretase complex components have been identified: presenilin, nicastrin, APH-1 and PEN-2 [4]. Their simultaneous expression in yeast, an organism that lacks any endogenous γ-secretase activity, results in the reconstitution of γ-secretase complex formation and activity, demonstrating that these four membrane proteins are the core components of the complex [5]. γ-Secretase activity depends on the presence of two conserved aspartate residues in either presenilin 1 or presenilin 2, two polytopic membrane proteins with partially redundant function [4]. Presenilins constitutively undergo endoproteolysis, leading to the generation of N- and C-terminal fragments which remain bound to each other and apparently constitute the catalytic site of the γ-secretase. The active site aspartate residues of the presenilins reside within their TMDs 6 and 7, in agreement with an intramembrane cleavage mechanism. The TMD 7 aspartate is located within the highly conserved sequence motif GxGD [6], which is also found in other intramembrane cleaving proteases, the bacterial type 4 prepilin peptidases and signal peptide peptidase (SPP) (see below). These and other data strongly suggest that the presenilins are the proteolytically active components of the γ-secretase complex. In contrast, the role of the other three subunits is less well understood. The type I glycoprotein nicastrin (see below) and the polytopic membrane protein APH-1 might form a precomplex to which first presenilin holoprotein is added [7]. The subsequent addition of PEN-2 facilitates presenilin endoproteolysis leading to the generation of the active complex. For a detailed description of γ-secretase assembly and function see the paper by Kaether et al. [this issue, pp. 275–283].

SPP-Like Proteins

SPP and SPP-like proteins (SPPLs) were originally identified as presenilin homologues (PSHs) in a database search [8]. Five PSH genes were described and shown to encode polytopic membrane proteins with a predicted topology similar, but inverted to that of presenilins (fig. 1a). Moreover, their sequences contained two conserved aspartate residues within putative TMDs and a highly conserved PALLYL motif reminiscent of the presenilin PALP sequence, suggesting the existence of a family of unusual aspartyl proteases with the sequence GxGD as a signature motif [9]. This hypothesis was confirmed by the finding that one of the PSHs is identical to SPP [10], a protease cleaving the hydrophobic signal peptides after their removal from newly synthesized secreted or membrane proteins by signal peptidase. Mutation of the aspartate within the GxGD motif of SPP abolished this activity, supporting the idea that SPP is indeed an aspartyl protease. Subsequently, γ-secretase inhibitors were shown to also block SPP activity, suggesting a common cleavage mechanism of γ-secretase and SPP [11]. In addition, the liberation of signal peptides by signal peptidase, a step analogous to the ectodomain shedding of γ-secretase substrates, was shown to be required for intramembrane processing, indicating that SPP and γ-secretase use a similar substrate recognition mechanism [12]. Thus, the discrimination of SPP and γ-secretase substrates appears to be primarily based on their membrane orientation: γ-secretase cleaves only type I substrates, SPP accepts only type II substrates, in agreement with the inverted membrane orientation of their active site-forming TMDs [13]. However, SPP differs from presenilin in that it does not undergo endoproteolysis and that it does not need a high-molecular-weight complex for activity. Although SPP dimerization has been shown to occur [13–15], its biological relevance is not clear.

One of the physiological functions of signal peptide processing in mammalian cells is the generation of fragments from MHC class I molecules which serve as human lymphocyte antigen E epitopes to report the immunocompetence of antigen-presenting cells [16]. In *Caenorhabditis elegans*, SPP has been shown to be required for embryonic development and suggested to be involved in the lipoprotein receptor pathway [17]. In contrast, nothing is known about the function of the other four PSHs, now known as SPPLs. Based on their sequence homology to SPP [8] and their membrane topology [13], they might represent intramembrane cleaving aspartyl proteases as well, but no substrates have been identified so far. Two of the four human SPPLs, SPPL2b and SPPL3, are conserved in all vertebrates, including the zebrafish, and were chosen for a biochemical and functional study [15]. Analysis of their subcellular localization in cultured human embryonic kidney HEK293 cells by immunofluorescence microscopy revealed that SPPL3, like SPP, localizes to the endoplasmic reticulum, whereas SPPL2b is predominantly found in endocytic/lysosomal compartments (fig. 1b). This suggested that at least one of the SPPLs might have a function different from SPP. To examine this further, we studied the role of SPP, SPPL2b and SPPL3

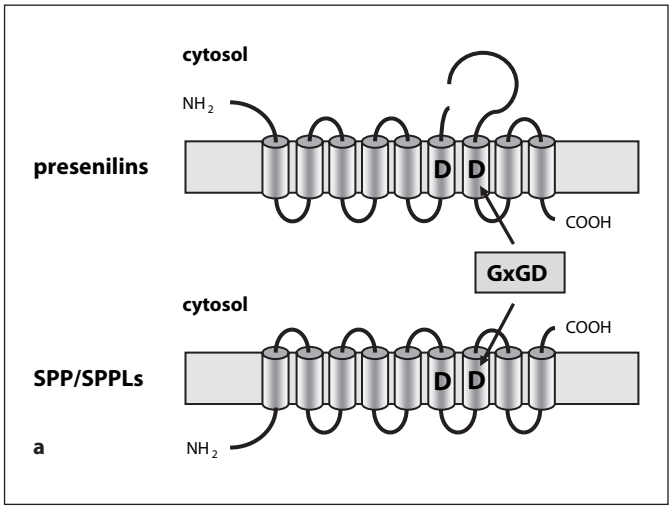
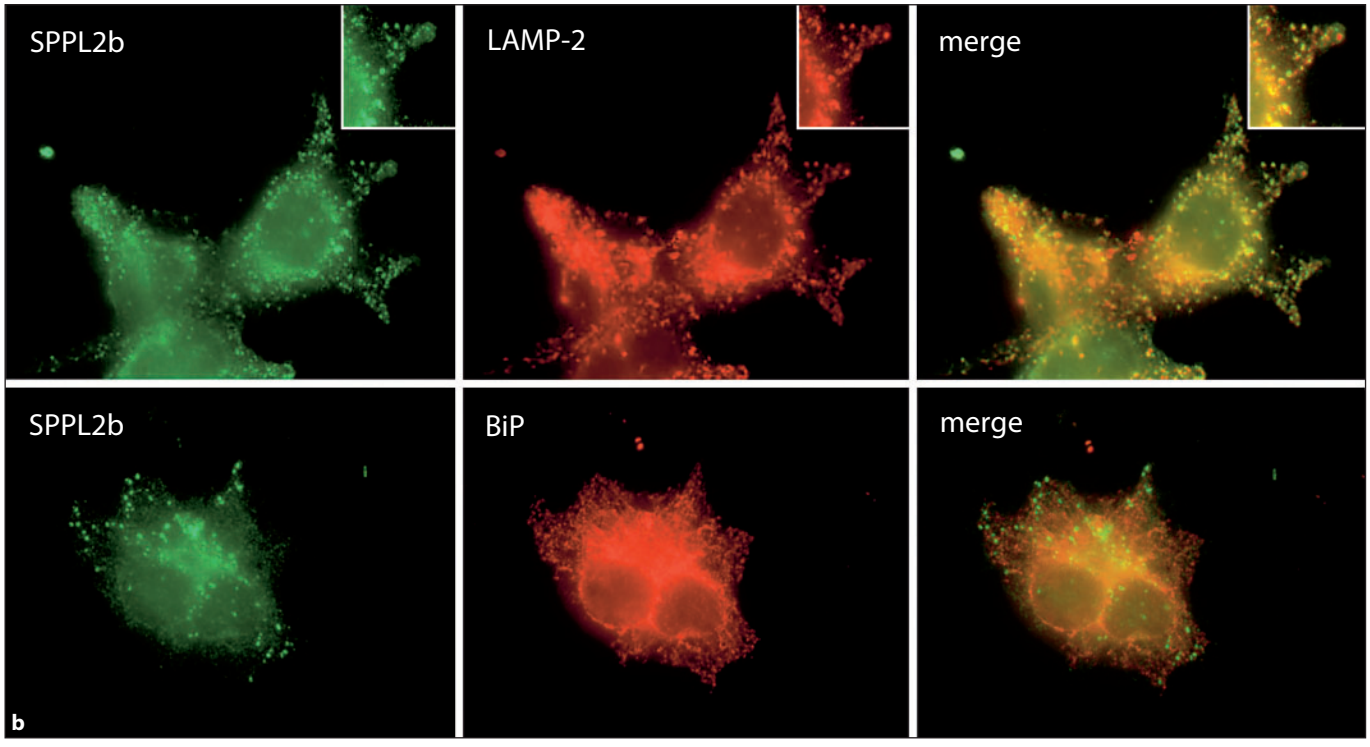


Fig. 1. a Presenilins and SPP/SPPLs have opposite membrane topology. Both are believed to contain nine TMDs with the presenilins' N-termini in the cytosol and the SPP/SPPLs' N-termini in the lumen (SPPL2a, b, c also contain a signal peptide which is not shown here). In contrast to SPP/SPPLs, presenilins are endoproteolyzed within the large cytoplasmic loop during γ-secretase complex formation. **b** SPPL2b localizes to endosomal/lysosomal compartments. Immunofluorescent microscopy of HEK293 cells shows a high degree of colocalization of SPPL2b with LAMP-2, a marker for lysosomes, and only little colocalization with BiP, an endoplasmic reticulum-resident protein.



during zebrafish embryonic development [15]. The corresponding zebrafish genes, *spp*, *sppl2* and *sppl3* are highly homologous to their human counterparts and expressed throughout early embryogenesis. Knockdown of each of these genes using antisense oligonucleotides resulted in embryonic lethal phenotypes demonstrating an essential role in zebrafish development. Whereas injection of *spp* and *sppl3*-specific oligonucleotides led to a neuronal degeneration phenotype due to enhanced cell death, knockdown of *sppl2b* resulted in the development of an abnormal caudal vein [15]. Importantly, we obtained identical phenotypes by injecting mRNAs encoding SPP or SPPL proteins harboring a mutation of the aspartate residue within the GxGD motif. This mutation has already been shown to act as dominant-negative mutation in human SPP [10]. We, therefore, concluded that SPPLs most likely represent aspartyl proteases with non-redundant functions.

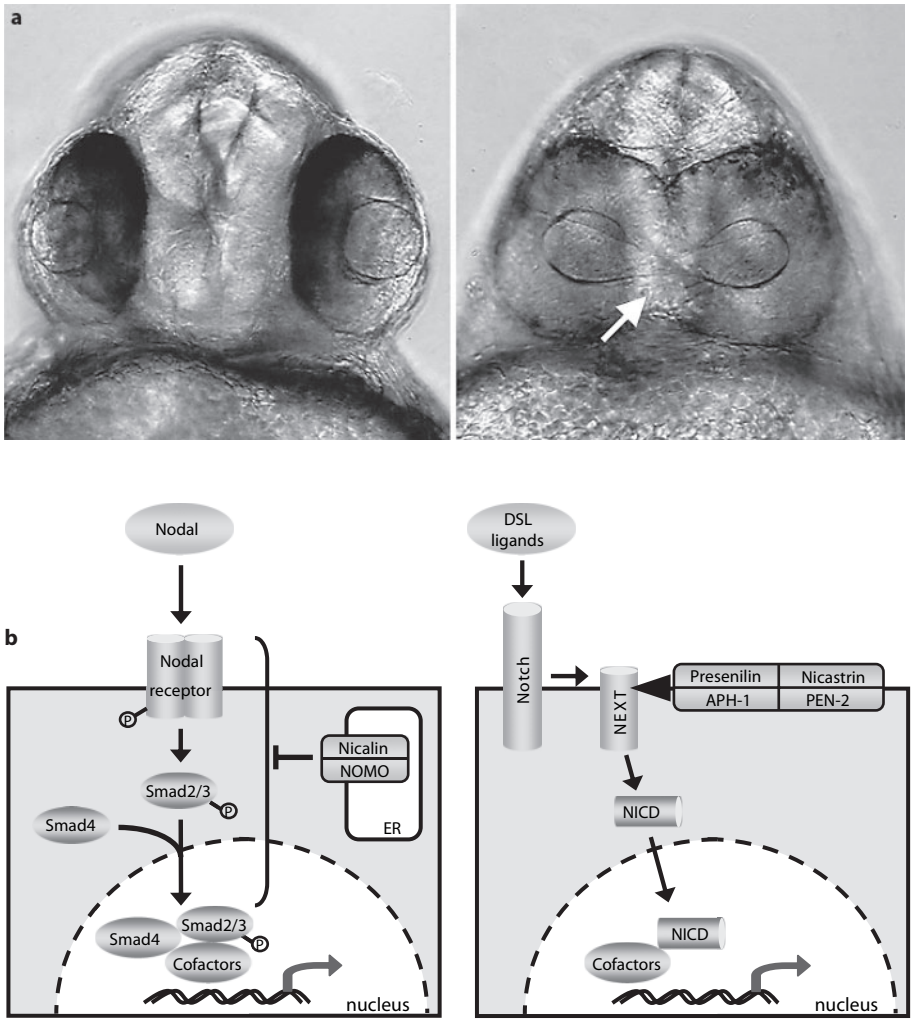


Fig. 2. a Co-expression of *ncl1* and *nomo* in zebrafish embryos results in cyclopia. Morphology of a wild-type embryo (left panel) and an embryo co-injected with *ncl1* and *nomo* capped RNAs (right panel) 36 h after fertilization. Note the prominent cyclopia of the injected embryo (arrow). **b** Roles of nicalin and nicastrin in the Nodal (left panel) and Notch (right panel) signaling pathways, respectively. The Nodal signal is transmitted by kinase receptors which phosphorylate intracellular signal transducers of the Smad family leading to the formation and nuclear translocation of heteromeric Smad complexes. In the nucleus, these complexes associate with cofactors to activate the transcription of target genes. The nicalin/NOMO complex localizes to the endoplasmic reticulum (ER) membrane and antagonizes the Nodal pathway at an undefined step. The Notch receptor is activated by ligands of the Delta/Serrate/LAG-2 (DSL) family and undergoes two proteolytic cleavage events. The second cleavage is mediated by γ-secretase, a membrane protein complex consisting of at least four components including nicastrin. The generated Notch intracellular domain (NICD) translocates to the nucleus and, together with cofactors, regulates the transcription of target genes. NEXT = Notch extracellular truncation; APH-1 = anterior pharynx defective-1; PEN-2 = presenilin enhancer-2.

Nicalin (Nicastrin-Like Protein)

The type I transmembrane protein nicastrin was originally identified as presenilin-binding factor that affects APP processing [18] as well as Notch signaling in *C. elegans* [18, 19]. It was subsequently shown to represent an obligate component of the γ-secretase complex [20] and to be required for the reconstitution of γ-secretase activity [5]. Its extracytosolic domain contains N-linked carbohydrates which undergo maturation during passage through the secretory pathway [20, 21]. This maturation, together with a conformational change leading to trypsin resistance [22], has been used as an indicator of γ-secretase complex assembly and trafficking. Within nicastrin's ectodomain, a ~200-amino acid region was found which is predicted to adopt a fold similar to the aminopeptidase (AP) domain. Although this domain does not confer peptidase activity [23], its integrity is necessary for γ-secretase activity [18, 22]. We have used a generalized sequence profile constructed from nicastrin ectodomains of various species to search databases for nicastrin-related sequences and identified a novel protein which we termed nicalin (nicastrin-like protein) [24]. The sequence similarity is confined to a region of 180 residues, which roughly corresponds to nicastrin's AP domain. Northern and Western blot analysis revealed ubiquitous expression of nicalin in human tissues and cell lines, albeit at varying levels. Examination of native, endogenous nicalin from HEK293 cells by Blue-Native polyacrylamid gel electrophoresis revealed its presence in a high-molecular-weight membrane protein complex of ~200 kDa [Haffner and Haass, unpubl. data], a size clearly differing from the 500–550 kDa determined for γ-secretase [20]. Moreover, γ-secretase components were not detected in nicalin im-

munoprecipitates and vice versa, demonstrating that these complexes are unrelated. Immunoaffinity purification of nicalin resulted in the co-purification of a 130-kDa protein, which was identified by mass spectrometry as pM5 [24], a membrane protein of unknown function. Based on the results of our analysis in zebrafish embryos (see below) this protein was termed NOMO (Nodal modulator). Like nicalin, NOMO is highly conserved in higher eukaryotes and expressed ubiquitously. Native, endogenous NOMO has a molecular weight of ~200 kDa strongly suggesting its presence in the Nicalin complex. Glycosylation and subcellular localization studies revealed that both proteins have type I topology and localize to the endoplasmic reticulum [Haffner and Haass, unpubl. data].

To identify the biological function of this novel protein complex, we examined the role of nicalin and NOMO in the zebrafish. One NOMO *(nomo)* and two nicalin orthologs *(ncl1, ncl2)* were found in the zebrafish genome, of which *nomo* and *ncl1* are expressed during early development. Interfering with their expression levels in embryos failed to produce phenotypes related to Notch signaling deficiencies, indicating distinct functions of γ-secretase and the nicalin/NOMO complex in vivo [24]. In contrast, we found that simultaneous ectopic expression of *nomo* and *ncl1*, but not of each factor alone, led to cyclopic, 'squint-eyed' embryos (fig. 2a). Blocking expression of *nomo* with an antisense oligonucleotide resulted in the development of a massively enlarged hatching gland. Both phenotypes can arise through a failure in the proper patterning of embryonic mesendodermal tissue, a process regulated by the TGFβ factor Nodal [25]. In situ hybridization experiments demonstrated that downregulation of *nomo* reduced the amount of posterior axial mesendoderm, indicating enhanced Nodal signaling. These data suggested an antagonistic role of NOMO in the Nodal pathway, and were confirmed by the finding that enhanced inhibition of Nodal signaling by overexpressing the specific Nodal inhibitor *lefty* was counteracted by blocking *nomo* expression [24]. We therefore concluded that the nicalin/NOMO complex acts as inhibitor of the Nodal signaling pathway.

Concluding Remarks

We characterized three novel γ-secretase-related proteins, SPPL2b, SPPL3 and nicalin. SPPL2b and SPPL3 are members of the GxGD family of aspartyl proteases and our data strongly support the hypothesis that they are active proteases [15]. This is confirmed by very recent results from our lab identifying a substrate for SPPL2b [Fluhrer et al., in press]. Moreover, it appears that SPPL2b and SPPL3 fulfill nonredundant functions important for vertebrate development. The characterization of substrates for SPPLs and their mode of action will provide important insights into the mechanisms underlying the process of intramembrane proteolysis.

Our analysis of the nicastrin-like protein nicalin and its binding partner NOMO led to the identification of a novel membrane protein complex involved in the Nodal signaling pathway [24]. Thus, nicalin as well as nicastrin are both part of high-molecular-weight protein complexes which are involved in signaling pathways controlling cell fate decisions during embryonic development (fig. 2b). Very recently, it has been suggested that nicastrin functions as a receptor for γ-secretase substrates and that its AP domain is involved in this process by recognizing the N-termini of processed peptides [26]. Future studies of nicalin and NOMO might help to understand the precise role of nicastrin within the γ-secretase complex.

References

1 Weihofen A, Martoglio B: Intramembrane-cleaving proteases: controlled liberation of functional proteins and peptides from membranes. Trends Cell Biol 2003;13:71–78.
2 Steiner H, Haass C: Intramembrane proteolysis by presenilins. Nat Rev Mol Cell Biol 2000;1:217–224.
3 Fortini ME: Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. Nat Rev Mol Cell Biol 2002;3:673–684.
4 Haass C: Take five – BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. EMBO J 2004;23:483–488.
5 Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C: Reconstitution of gamma-secretase activity. Nat Cell Biol 2003;5:486–488.
6 Steiner H, Kostka M, Romig H, Basset G, Pesold B, Hardy J, Capell A, Meyn L, Grim MG, Baumeister R, Fechteler K, Haass C: Glycine 384 is required for presenilin-1 function and is conserved in polytopic bacterial aspartyl proteases. Nat Cell Biol 2000;2:848–851.
7 Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, Thinakaran G, Iwatsubo T: The role of presenilin cofactors in the gamma-secretase complex. Nature 2003;422:438–441.

8 Ponting CP, Hutton M, Nyborg A, Baker M, Jansen K, Golde TE: Identification of a novel family of presenilin homologues. Hum Mol Genet 2002;11:1037–1044.
9 Haass C, Steiner H: Alzheimer disease gamma-secretase: a complex story of GxGD-type presenilin proteases. Trends Cell Biol 2002; 12:556–562.
10 Weihofen A, Binns K, Lemberg MK, Ashman K, Martoglio B: Identification of signal peptide peptidase, a presenilin-type aspartic protease. Science 2002;296:2215–2218.
11 Weihofen A, Lemberg MK, Friedmann E, Rueeger H, Schmitz A, Paganetti P, Rovelli G, Martoglio B: Targeting presenilin-type aspartic protease signal peptide peptidase with gamma-secretase inhibitors. J Biol Chem 2003;278:16528–16533.
12 Lemberg MK, Martoglio B: Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. Mol Cell 2002;10: 735–744.
13 Friedmann E, Lemberg MK, Weihofen A, Dev KK, Dengler U, Rovelli G, Martoglio B: Consensus analysis of signal peptide peptidase and homologous human aspartic proteases reveals opposite topology of catalytic domains compared with presenilins. J Biol Chem 2004;279:50790–50798.
14 Nyborg AC, Kornilova AY, Jansen K, Ladd TB, Wolfe MS, Golde TE: Signal peptide peptidase forms a homodimer that is labeled by an active site-directed gamma-secretase inhibitor. J Biol Chem 2004;279:15153–15160.
15 Krawitz P, Haffner C, Fluhrer R, Steiner H, Schmid B, Haass C: Differential localization and identification of a critical aspartate suggest non-redundant proteolytic functions of the presenilin homologues SPPL2b and SPPL3. J Biol Chem 2005;280:39515–39523.
16 Lemberg MK, Bland FA, Weihofen A, Braud VM, Martoglio B: Intramembrane proteolysis of signal peptides: an essential step in the generation of HLA-E epitopes. J Immunol 2001;167:6441–6446.
17 Grigorenko AP, Moliaka YK, Soto MC, Mello CC, Rogaev EI: The *Caenorhabditis elegans* IMPAS gene, imp-2, is essential for development and is functionally distinct from related presenilins. Proc Natl Acad Sci USA 2004;101:14955–14960.
18 Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T, Supala A, Levesque L, Yu H, Yang DS, Holmes E, Milman P, Liang Y, Zhang DM, Xu DH, Sato C, Rogaev E, Smith M, Janus C, Zhang Y, Aebersold R, Farrer LS, Sorbi S, Bruni A, Fraser P, St George-Hyslop P: Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and beta-APP processing. Nature 2000;407:48–54.
19 Goutte C, Hepler W, Mickey KM, Priess JR: aph-2 encodes a novel extracellular protein required for GLP-1-mediated signaling. Development 2000;127:2481–2492.
20 Edbauer D, Winkler E, Haass C, Steiner H: Presenilin and nicastrin regulate each other and determine amyloid beta-peptide production via complex formation. Proc Natl Acad Sci USA 2002;99:8666–8671.
21 Herreman A, Van Gassen G, Bentahir M, Nyabi O, Craessaerts K, Mueller U, Annaert W, De Strooper B: Gamma-secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. J Cell Sci 2003;116:1127–1136.
22 Shirotani K, Edbauer D, Capell A, Schmitz J, Steiner H, Haass C: Gamma-secretase activity is associated with a conformational change of nicastrin. J Biol Chem 2003;278: 16474–16477.
23 Fergani A, Yu G, St George-Hyslop P, Checler F: Wild-type and mutated nicastrins do not display aminopeptidase M- and B-like activities. Biochem Biophys Res Commun 2001;289:678–680.
24 Haffner C, Frauli M, Topp S, Irmler M, Hofmann K, Regula JT, Bally-Cuif L, Haass C: Nicalin and its binding partner Nomo are novel Nodal signaling antagonists. EMBO J 2004;23:3041–3050.
25 Schier AF: Nodal signaling in vertebrate development. Annu Rev Cell Dev Biol 2003;19: 589–621.
26 Shah S, Lee SF, Tabuchi K, Hao YH, Yu C, Laplant Q, Ball H, Dann CE, 3rd, Sudhof T, Yu G: Nicastrin functions as a gamma-secretase-substrate receptor. Cell 2005;122:435–447.