Podoplanin is a substrate of presenilin-1/γ-secretase

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Abstract

Podoplanin (PDPN) is a mucin-like transmembrane glycoprotein that plays an important role in development and cancer. Here, we provide evidence that the intracellular domain (ICD) of podoplanin is released into the cytosol following a sequential proteolytic processing by a metalloprotease and γ-secretase. Western blotting and cell fractionation studies revealed that HEK293T and MDCK cells transfected with an eGFP-tagged podoplanin construct (PDPNeGFP, 50-63 kDa) constitutively express two C-terminal fragments (CTFs): a ~33 kDa membrane-bound PCTF33, and a ~29 kDa cytosolic podoplanin ICD (PICD). While pharmacological inhibition of metalloproteases reduced the expression of PCTF33, treatment of cells with γ-secretase inhibitors resulted in enhanced PCTF33 levels. PCTF33 processing by γ-secretase depends on presenilin-1 (PS1) function: cells expressing a dominant negative form of PS1 (PS1 D385N), and mouse embryonic fibroblasts (MEFs) genetically deficient in PS1, but not in PS2, show higher levels of PCTF33 expression with respect to wild-type MEFs. Furthermore, transfection of PS1 deficient MEFs with wild-type PS1 (PS1 wt) decreased PCTF33 levels. N-terminal amino acid sequencing of the affinity purified PICD revealed that the γ-secretase cleavage site was located between valines 150 and 151, but these residues are not critical for proteolysis. We found that podoplanin CTFs are also generated in cells expressing podoplanin mutants harboring heterologous transmembrane regions. Taken together, these results indicate that podoplanin is a novel substrate for PS1/γ-secretase.

key words: podoplanin, γ-secretase, metalloprotease, proteolytic processing

Abbreviations: PDPN, podoplanin; fl-PDPN, full-length PDPN; EC, extracellular; TM, transmembrane; CT, cytoplasmic; ERM, ezrin, radixin, moesin; CTF, C-terminal fragment; ICD, intracellular domain; PS, presenilin; MEF, mouse embryonic fibroblast; RIP, Regulated intramembrane proteolysis.

1. Introduction

Podoplanin (PA2.26 antigen, Aggrus, T1α) is a small mucin-like transmembrane glycoprotein that functions both as a cell adhesion molecule (Cueni and Detmar, 2009; Kato et al., 2003; Tsuneki et al., 2013) and a promoter of cell migration/invasion (Martín-Villar et al., 2006; Scholl et al., 1999; Wicki et al., 2006). It is expressed in several normal cell types and tissues, such as the alveolar epithelium, mesothelia and lymphatic vessels (Schacht et al., 2005; Wicki and Christofori, 2007). Studies with podoplanin-deficient mice suggest that this glycoprotein is involved in lung morphogenesis, lymphatic vasculature formation, and cardiac development (Mahtab et al., 2008, 2009; Ramirez et al., 2003; Schacht et al., 2003). Podoplanin expression is upregulated in a variety of cancers, including squamous cell carcinomas and glioblastomas, where it is associated with malignant progression and metastasis (Astarita et al., 2012; Wicki and Christofori, 2007).

Podoplanin is composed of three structural domains: a highly O-glycosylated extracellular (EC) domain; a hydrophobic transmembrane (TM) domain; and a 9 amino acid-long cytoplasmic (CT) tail that lacks any evident enzymatic motif (Kaneko et al., 2004; Martín-Villar et al., 2005; Scholl et al., 1999). A fraction of podoplanin is localized in detergent-resistant membrane domains or raft platforms (Barth et al., 2010; Fernández-Muñoz et al., 2011). Our laboratory has also demonstrated that recruitment of podoplanin to these structures requires both the TM and CT domains and, more importantly, that the function of podoplanin is affected by its exclusion from them (Fernández-Muñoz et al., 2011).

Podoplanin binding to ezrin and moesin of the ERM (ezrin, radixin, moesin) protein family links the CT domain of podoplanin to the actin cytoskeleton, which leads to the activation of small Rho GTPases and to the induction of cell migration/invasion and epithelial-mesenchymal transition (Martín-Villar et al., 2006; Navarro et al., 2008; Scholl et al., 1999; Wicki et al., 2006). In addition, podoplanin can induce platelet aggregation by interacting with the C-type lectin-like receptor 2 (CLEC-2). This process is important for physiological and pathological events, like the differentiation of the lymphatic vasculature from the blood vascular system (Uhrin et al., 2010), the maintenance of high endothelial venules integrity in lymph nodes during immune responses (Herzog et al., 2013), or podoplanin-mediated pulmonary metastasis of tumor cells (Kunita et al., 2007; Nakazawa et al., 2008). Finally, the interaction of podoplanin with the hyaluronan receptor CD44 seems crucial for podoplanin-dependent stimulation of directional migration in carcinoma cells (Martín-Villar et al., 2010) as well as for tethering tumor cells to hyaluronan-rich extracellular matrices (Tsuneki et al., 2013).

Proteolytic processing of membrane glycoproteins regulates their stability and also plays a crucial role in the regulation of their function and signaling properties (Cullen, 2011; Haapasalo and Kovacs, 2011). In particular, cleavage within the hydrophobic TM domain, or regulated intramembrane proteolysis (RIP), is a process that has emerged as an important mechanism regulating key cellular events, including adhesion and migration (Beel and Sanders, 2008; Haapasalo and Kovacs, 2011). In order to be able to detect the existence of both N- and C-terminal fragments, we used a human podoplanin construct fused to the enhanced green fluorescent protein (PDPNeGFP), given that no available podoplanin antibody reacts with the CT tail of the protein. Previous results from our laboratory showed that the use of a C-terminal eGFP tag does not affect podoplanin subcellular localization and function (Martín-Villar et al., 2005, 2006). We report here that a metalloprotease-dependent cleavage of podoplanin generates a membrane-bound C-terminal stub that is further processed by PS1/γ-secretase, thus releasing the intracellular domain of podoplanin (PICD) into the cytosol.

2. Materials and methods

2.1. Antibodies and reagents

The human podoplanin monoclonal antibody (mAb) NZ1 was from Acris Antibodies. We used a GFP mAb from Roche for Western blotting, and a rabbit polyclonal antibody from Molecular Probes for GFP-affinity purification of PICD. The $P_{37\text{-}51}$ peptide has been previously described (Martín-Villar et al., 2005). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG was from Amersham and HRP-conjugated goat anti-rat IgG from Pierce. The metalloprotease inhibitors GM6001 and Marimastat were from Calbiochem; all other inhibitors were from Sigma-Aldrich.

2.2. Constructs

The PDPNeGFP plasmid has been described elsewhere (Martín-Villar et al., 2005). The PDPN-RQ* construct was obtained from the PDPNeGFP plasmid that was previously modified to introduce a silent AccI site at position S^{127} (Fernández-Muñoz et al., 2011). AccI digestion of this plasmid was followed by blunting with the Klenow fragment of DNA polymerase I and re-ligation. The internal addition of two nucleotides resulted in a frameshift after S^{127} that introduced a two-amino acid mutation followed by a premature stop codon. Wild-type PS1 (PS1 wt) expression vector was generously

provided by Prof. Michael S. Wolfe (Center for Neurological Diseases, Harvard University, Boston, USA).

2.3. Cell culture conditions and cDNA transfection

MDCK cell transfectants stably expressing either PDPNeGFP or the chimeric PDPN-ECD, PDPN-CD45, or PDPN-TLR9 proteins have been previously described (Fernández-Muñoz et al., 2011; Martín-Villar et al., 2006). Human embryonic kidney (HEK293) cells stably expressing PS1 wt or the dominant negative PS1 D385N were a generous gift from Prof. Christian Haass (Ludwig Maximilian University, Munich, Germany). MEFs derived from wild type, $PS1^{-/-}$, $PS2^{-/-}$ or double $PS1^{-/-}/PS2^{-/-}$ mice were kindly provided by Prof. Bart de Strooper (VIB Center for the Biology of Disease, KU Leuven, Belgium). HEK293 and MDCK cell transfectants were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich). MEFs were cultured in DMEM/F-12 medium supplemented with 100 μg/ml ampicillin and 32 μg/ml gentamicin. Culture media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 μg/ml amphotericin B, and the appropriate antibiotic for selection (200 μg/ml G418 for PDPN transfectants, or 200 μg/ml zeocin for PS1 transfectants).

Cells were seeded onto P60 plates and transfected with 1 μg of plasmid DNA with Lipofectamine (Invitrogen) according to the manufacturer's instructions. For time-course experiments, cells were harvested at the indicated time points. For protease inhibitor treatment, immediately prior to transfection, cells were washed twice and the medium replaced with serum-free medium containing vehicle (DMSO) or the indicated inhibitor at the following working concentrations: 200 μg/ml aprotinin; 10 μM bestatin; 100 μM E-64; 50 μM pepstatin; 50 μM GM6001; 10 μM Marimastat; 10 μM DAPT; and 5 μM L-685,458. Transfection media were replaced with fresh culture medium supplemented with 1% FBS and the corresponding protease inhibitor at 5 and 24 h after transfection. Total cell extracts were obtained 48 h after transfection in RIPA buffer (50 mM Tris-HCl pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl) plus protease and phosphatase inhibitors (2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 mM sodium orthovanadate, 2 mM β-glycerophosphate, and 1 mM NaF). In order to obtain PDPNeGFP-HEK293 stable transfectants for affinity purification of PICD, cells were transfected as indicated above and cultured in complete medium supplemented with 500 μg/ml G418 for three weeks.

2.4. Cell fractionation and preparation of conditioned medium

Cytosolic and membrane fractions were obtained according to the online protocol by Abcam. Briefly, cells grown in P100 dishes were lysed in ice-cold subcellular fractionation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) with protease and phosphatase inhibitors. Cells were scraped, passed ten times through a 25 G needle, and incubated 20 min on ice. The lysate was centrifuged at 720 *g* for 5 min and the supernatant centrifuged for 10 min at 10 000 *g*. The cytosolic (supernatant) and membrane (pellet) fractions were obtained by centrifugation of the resulting supernatant at 100 000 *g* for 1 h. The membrane fraction was washed once with fractionation buffer and centrifuged for 45 min at 100 000 *g*. Conditioned medium of PDPNeGFP-MDCK cell transfectants was obtained by culturing confluent P150 plates in serum-free medium for 24 h, cleared by centrifugation at 1 500 rpm for 5 min, and concentrated by precipitation with 4 volumes of cold acetone at -20ºC for 1 h. The precipitated proteins were pelleted by centrifugation at 14 000 *g* for 20 min. The membrane fraction and precipitated conditioned medium pellets were resuspended in RIPA buffer and centrifuged to remove insoluble material at 13 000 *g*. All centrifugations were carried out at 4ºC.

2.5. Gel electrophoresis and Western blotting

Electrophoresis in 10% or 12% acrylamide gels and Western blot analysis were performed as previously described (Martín-Villar et al., 2005). For determining band specificity, NZ1 was pre-incubated with the P_{37-51} peptide 30 min prior to blotting the membrane. Peroxidase activity was detected with the enhanced chemiluminescence kit (Amersham).

2.6. Affinity purification of the PICD fragment and amino acid sequencing

Affinity purification of PICD from the cytosolic fraction of cells stably expressing PDPNeGFP was performed with the Crosslink Immunoprecipitation Kit (Pierce) according to the manufacturer's instructions. The eluted fragment was precipitated with 4 volumes of acetone, resuspended in RIPA buffer, and loaded onto a 12% SDS-PAGE gel. Proteins were transferred to a 0.2 µm Immobilon-P PVDF membrane (Millipore) and stained with 0.1% Coomassie blue R-250 (Sigma-Aldrich) in 1% methanol. Following destaining with a 50:1:49 (v/v/v) mixture of ethanol, acetic acid and water, the membrane was dried at room temperature. The PICD fragment was excised and sent to the Protein Chemistry Service of the Centro de Investigaciones Biológicas (CSIC, Madrid, Spain) for N-terminal sequencing by Edman degradation.

3. Results and discussion

3.1. Podoplanin undergoes constitutive proteolytic processing

We studied the expression of PDPNeGFP in transiently transfected MDCK cells by Western blotting. The use of NZ1, a mAb that recognizes amino acids 39-48 in the EC domain of podoplanin (Kato et al., 2006; Ogasawara et al., 2008) revealed the presence of several bands: two broad bands of 60-63 and 37-45 kDa, and an intermediate band of ~48 kDa (Fig. 1A, left panel). Similar results were obtained with a polyclonal antibody previously generated in our laboratory (Martín-Villar et al., 2005) against the peptide P_{37-51} in the EC domain of podoplanin (data not shown). All bands are specific, since they were not detected when NZ1 was pre-incubated with an excess of P_{37-51} (Fig. 1A, middle panel). When the expression of PDPNeGFP was analyzed with an anti-eGFP Ab, a different band pattern emerged: the 60-63 kDa and ~48 kDa bands were visible, but the broad 37-45 kDa band disappeared (Fig. 1A, right panel). Instead, several smaller discrete bands were consistently detected: a doublet of 27-29 kDa, and a ~33 kDa band that was mainly visible at 48 h post-transfection (Fig. 1A, right panel, arrowheads). Given that the 60-63 and ~48 kDa bands are recognized by both antibodies, they must correspond to full-length podoplanin (fl-PDPN). The detection of several fl-PDPN bands is due to the existence of different degrees of EC domain glycosylation (Martín-Villar et al., 2005). On the other hand, the other specific bands correspond to N- or C-terminal podoplanin fragments, since they are detected with only one of the two antibodies. Thus, the 37-45 kDa fragment recognized with NZ1 (Fig. 1A left panel, asterisk) contains at least a portion of the EC domain and lacks all or part of the CT domain of podoplanin. On the other hand, the 27-29 kDa doublet and the ~33 kDa polypeptide, which are not detected with NZ1 lack the N-terminal region of podoplanin.

In order to further analyze the nature of these fragments, we studied their subcellular distribution. To that effect, we obtained membrane and cytosolic fractions of MDCK-PDPNeGFP and MDCK-eGFP stable transfectants, and analyzed the expression of the different polypeptides by Western blotting (Fig. 1B). As expected, the 60-63 and ~48 kDa bands corresponding to fl-PDPN were found exclusively in the membrane fraction. In addition, PCTF27 (~27 kDa), PCTF33 (~33 kDa), and the 37-45 kDa N-terminal polypeptide were also found in this fraction (Fig. 1B middle and right panels), suggesting that they either retain the TM domain or remain associated to a membrane component. On the other hand, the ~29 kDa CTF was detected exclusively in the cytosolic fraction (Fig. 1B, middle panel). This fragment contains only a small

portion of the C-terminal region of podoplanin since the eGFP tag has an apparent molecular mass of 27 kDa (Fig. 1B, left panel). Given that the ICD of podoplanin is 9 amino acid-long, the soluble 29 kDa fragment most likely corresponds to the entire ICD of podoplanin fused to the eGFP tag (PICD).

3.2. Podoplanin EC domain cleavage by a metalloprotease yields PCTF33

Two alternative mechanisms may account for the formation of the newly identified PICD: the existence of an alternative translation initiation site in podoplanin's mRNA, as has been described for the tyrosine kinase receptor HER2 (Anido et al., 2006); or through RIP, as is the case for the Alzheimer's disease-associated amyloid precursor protein and Notch1 (De Strooper et al., 1999; Xia and Wolfe, 2003). Besides methionine at position 1, human podoplanin contains three additional internal methionines: at positions 43, 153 and 156 in the EC, TM and CT domains, respectively (Martín-Villar et al., 2005). In order to test the first hypothesis, we used a truncated podoplanin construct, PDPN-RQ*, which contains a frameshift mutation that introduces a premature stop codon two amino acids downstream of S^{127} in the EC domain of podoplanin (Fig.2A, right). Expression of PDPN-RQ* is expected to produce a podoplanin N-terminal fragment detectable with NZ1 but not with anti-GFP Ab. Smaller GFP-tagged polypeptides would not be detected unless internal methionines could act as alternative translation initiation sites. Transfection of PDPN-RQ* in HEK293T cells yielded a ~40 kDa polypeptide that was detected with NZ1 (Fig. 2A, left blot). Importantly, no polypeptide was detected with the anti-GFP Ab (Fig.2A, middle blot). Thus, podoplanin CTFs are not produced by initiation of translation at alternative sites, but are derived from fl-PDPN (Fig. 2A, right blot).

In order to confirm that the observed CTFs are indeed the products of fl-PDPN proteolytic processing, we treated HEK293T cells transiently expressing PDPNeGFP with a panel of broad spectrum protease inhibitors. This panel included inhibitors that covered all five major protease families: aminopeptidases (bestatin), serine- (aprotinin), cystein- (E-64), aspartyl- (pepstatin), and metallo- (GM6001 and Marimastat) proteases. Whereas none of the inhibitors significantly affected the amount of fl-PDPN, we observed a clear reduction in the levels of PCTF33 in cells treated with the two metalloprotease inhibitors (Fig. 2B). The 37-45 kDa N-terminal fragment, on the other hand, was not downregulated by metalloprotease inhibitor treatment (data not shown). We also observed a significant accumulation of PCTF33 in cells treated with pepstatin, suggesting that this fragment is further processed by an aspartyl-protease. The proteolytic processing we describe here is reminiscent of RIP, which is usually

preceded by a shedding event close to the TM domain of the substrate protein that releases the EC domain into the extracellular medium (Beel and Sanders, 2008; Sannerud and Annaert, 2009). We then analyzed the conditioned medium of MDCK cells expressing PDPNeGFP for the presence of a soluble podoplanin N-terminal fragment. Interestingly, we not only were unable to detect a soluble podoplanin EC fragment, but we observed the same band pattern in the cell lysate and the conditioned medium (Fig. 2C). Preliminary results from our laboratory indicate that this is due to the fact that podoplanin is being released into the conditioned medium associated to exosomes (Carrasco-Ramirez et al., unpublished results). Therefore, PCTF33 is generated by a metalloprotease-dependent cleavage within the EC domain of fl-PDPN. However, we have not been able to detect the resulting soluble EC domain fragment in the exosome-free conditioned medium, suggesting that it is probably being rapidly degraded.

3.3 PCTF33 is a substrate of γ-secretase

The proteolytic release of type I transmembrane protein ICDs into the cytosol is carried out by the members of one of two families of intramembrane cleaving proteases: the serine-protease rhomboid or the aspartyl-protease γ-secretase (Boulton et al., 2008; Xia and Wolfe, 2003). While the former cleaves intact substrates, the latter uses as substrates C-terminal transmembrane stubs produced by cleavage of the EC domain. Several observations suggested that podoplanin is probably a γ-secretase substrate: it is a type I transmembrane protein that is cleaved on its EC domain by a metalloprotease; the resulting membrane-bound PCTF33 fragment is further processed by an aspartyl-protease; and, finally, the PICD fragment is being released into the cytosol. To test this hypothesis, we treated HEK293T cells transiently transfected with PDPNeGFP with two different and highly specific γ-secretase inhibitors. The specific pharmacological inhibition of γ-secretase resulted in the accumulation of this fragment with no significant changes in fl-PDPN levels, indicating that PCTF33 is a substrate of γ-secretase (Fig. 3A).

γ-secretase is a multisubunit enzyme complex that requires at least four proteins to be functional: presenilin (PS), nicastrin, Aph-1, and Pen-2 (Edbauer et al., 2003; Kimberly et al., 2003). The catalytic subunit of the protease is one of the two known isoforms of PS: PS1 or PS2. Therefore, we studied whether PCTF33 cleavage by γ-secretase is dependent on PS activity. As a first approach, we transfected PDPNeGFP into HEK293 cells that stably express exogenous PS1 wt or the dominant negative PS1 D385N (Capell et al., 2000). At similar levels of fl-PDPN expression (Fig.

3B, upper panel), co-expression of podoplanin with PS1 D385N led to a pronounced accumulation of PCTF33 (Fig. 3B, lower panel). Similarly, when PDPNeGFP was transfected into PS1, PS2, or double PS1/PS2 knockout MEFs (Herreman et al., 1999, 2003), we observed an accumulation of PCTF33, even though the levels of exogenous fl-PDPN expression were similar in all cell lines. This accumulation was more pronounced in $PS1^{-/-}$ and $PS1/2^{-/-}$ than in $PS2^{-/-}$ MEFs (Fig. 3C, second panel), indicating that PS1 is the major PS isoform involved in PCTF33 cleavage. Furthermore, PCTF33 levels were reduced in wild-type and PS-deficient MEFs co-transfected with PDPNeGFP and PS1 wt (Wolfe et al., 1999) relative to MEFs transfected only with PDPNeGFP (Fig. 3C, compare second and bottom panels). These results confirm that PCTF33 is a PS1-dependent γ-secretase substrate.

3.4 PDPN is cleaved by γ-secretase between V^{150} and V^{151}

The vast majority of γ-secretase substrates are cleaved at or very close to the boundary of the TM and CT domains, but a consensus sequence has not been identified (Haapasalo and Kovacs, 2011). However, the γ-secretase cleavage site usually flanks a sequence rich in lysine and/or arginines. The TM and CT domains of podoplanin are well conserved across species and contain three highly conserved lysine and arginine residues in the juxtamembrane region (Fig. 4A, underlined). Another common feature of many γ-secretase substrates is the presence of a valine close to the membrane-cytosol interface that lies immediately to the right of the cleavage site (Fig. 4B). Furthermore, mutation of this residue can abrogate proteolysis by γ-secretase in some substrates, including Notch (Huppert et al., 2000) and ErbB4 (Vidal et al., 2005). The TM domain of podoplanin possesses several conserved valine residues in that region (Fig. 4A and B, shaded). N-terminal sequencing of the affinity purified PICD fragment by Edman degradation yielded the sequence VVMXKM, indicating that the γ-secretase cleavage site is located between V^{150} and V^{151} within the TM domain of podoplanin (Fig. 4B). However, mutation of these residues to phenylalanine did not prevent the formation of CTFs (data not shown). In addition, we analyzed the expression of PCTF33 in MDCK cells expressing one of three previously described podoplanin constructs: PDPN-ECD, PDPN-CD45 and PDPN-TLR9 (Fernández-Muñoz et al., 2011). In these constructs, the TM domain of podoplanin was replaced by that of E-cadherin, the leukocyte common antigen CD45, or the Toll-like receptor TLR9, respectively (Fig. 4C). As shown in Fig. 4C, both PDPN-ECD and PDPN-CD45 constitutively produced a 33 kDa CTF. The levels of this CTF diminished upon metalloprotease inhibitor treatment and accumulated following DAPT treatment,

indicating that it is produced by the action of a metalloprotease and that it is a substrate of γ-secretase. Both CD45 and E-cadherin have been previously identified as γ-secretase substrates (Kirchberger et al., 2008; Marambaud et al., 2002). In contrast, we did not observe the formation of CTFs in cells expressing PDPN-TLR9. In contrast to the other two TM chimera, most of PDPN-TLR9 does not reach the cell surface but is retained in cytoplasmic intracellular vesicles (Fernández-Muñoz et al., 2011). The function of γ-secretase is tightly regulated by intracellular transport of its components and substrates (Sannerud and Annaert, 2009). Thus, the localization of the PDPN-TLR9 chimera would prevent it from being processed first by the metalloprotease and then by γ-secretase. Finally, localization in lipid rafts can promote γ-secretase processing of some substrates, including the amyloid precursor protein (Cheng et al., 2007). Podoplanin has been described to associate with lipid rafts (Barth et al., 2010; Fernández-Muñoz et al., 2011). However, podoplanin localization in these microdomains is not necessary for PS1/γ-secretase processing, since the TM chimeras PDPN-ECD and PDPN-CD45, which are cleaved by γ-secretase, have been shown to be excluded from lipid rafts (Fernández-Muñoz et al., 2011).

3.5 Biological implications of PDPN cleavage

In summary, our results demonstrate that podoplanin undergoes a sequential proteolytic processing that leads to the liberation of its intracellular domain (PICD) into the cytosol. In a first step, a metalloprotease cleaves podoplanin on its EC domain (Fig. 5, step 1). The metalloprotease-dependent cleavage of podoplanin generates a membrane-anchored C-terminal fragment that has an apparent molecular weight of approximately 33 kDa when fused to the eGFP tag (PCTF33). Podoplanin EC domain cleavage most probably occurs at the plasma membrane, since no CTF is formed when cells express a podoplanin TM chimera that does not reach the plasma membrane, PDPN-TLR9. We have not been able to detect the EC domain fragment resulting from metalloprotease cleavage of podoplanin, raising the possibility that this fragment is being rapidly degraded. In addition to PCTF33, we have detected two other membrane-associated podoplanin fragments: a C-terminal fragment of approximately 27 kDa (PCTF27) and a 37-45 kDa N-terminal fragment. These additional fragments probably arise from independent alternative proteolytic processing events that occur simultaneously to the sequential processing that we describe here. We have previously reported that podoplanin is a substrate of calpain-1 (Martín-Villar et al., 2009). However, calpain-1 leads to the complete degradation of podoplanin, and the

fragments described here co-exist with fl-PDPN, suggesting that this protease is not involved in their generation.

Finally, we demonstrate that the membrane-anchored PCTF33 fragment is further processed by γ-secretase, and that this process, which is dependent on PS1 activity, results in the release of a short PICD into the cytosol (Fig. 5, step 2). The ICDs of some of γ-secretase substrates have been shown to function as signaling molecules. This is the case of the amyloid precursor protein, Notch and CD44 (Beckett et al., 2012; Nagano and Saya, 2004), whose intracellular domains, when released by γ-secretase cleavage, are targeted to the nucleus where they regulate gene expression. In contrast, the ICDs of other γ-secretase substrates, like MUC1, are rapidly degraded (Julian et al., 2009). Indeed, it has been suggested that γ-secretase may function as the membrane proteasome (Kopan and Ilagan, 2004). The intracellular domain of podoplanin is extremely short and, therefore, would be expected to readily enter the nucleus. However, the release of a transmembrane protein ICD that functions as a signaling molecule would be expected to be a highly regulated event (Kopan and Ilagan, 2004). Our results indicate that the proteolytic processing of podoplanin does not occur in response to an external signal, but is constitutive. This suggests that γ-secretase-dependent proteolysis is probably involved in regulating the stability of fl-PDPN rather than in generating a bioactive ICD. The cytoplasmic domain of podoplanin contains the binding site for ezrin and moesin, which anchor podoplanin to the actin cytoskeleton. Furthermore, podoplanin-ERM interaction is necessary for the induction of migratory properties or a full epithelial to mesenchymal transition by podoplanin (Martín-Villar et al., 2006). The γ-secretase-dependent liberation of PICD would end the association of podoplanin to the actin cytoskeleton, thus playing an important role in regulating or abrogating podoplanin signaling. Furthermore, binding of PICD to ezrin or moesin in the cytoplasm could modulate the binding of activated ERM proteins to membrane receptors, including fl-PDPN. Therefore, the mechanism we describe here is likely to play an important role in regulating podoplanin function.

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Legends to figures

Figure1. Constitutive expression of podoplanin N- and C-terminal fragments. (A) Time-course analysis of PDPNeGFP expression in transiently transfected MDCK cells by Western blotting with NZ1 in the absence (left panel) or presence of an excess of the P_{37-51} blocking peptide (middle panel), and with an anti-GFP Ab (right panel). Note the expression of a 37-45 kDa N-terminal polypeptide (asterisk) and of several CTFs (arrowheads). (B) Subcellular distribution of podoplanin fragments in MDCK cells stably expressing PDPNeGFP or the empty vector (eGFP). In all panels, the migrating positions of molecular weight protein markers are indicated. T, total cell lysate; C, cytosolic fraction; M, membrane fraction.

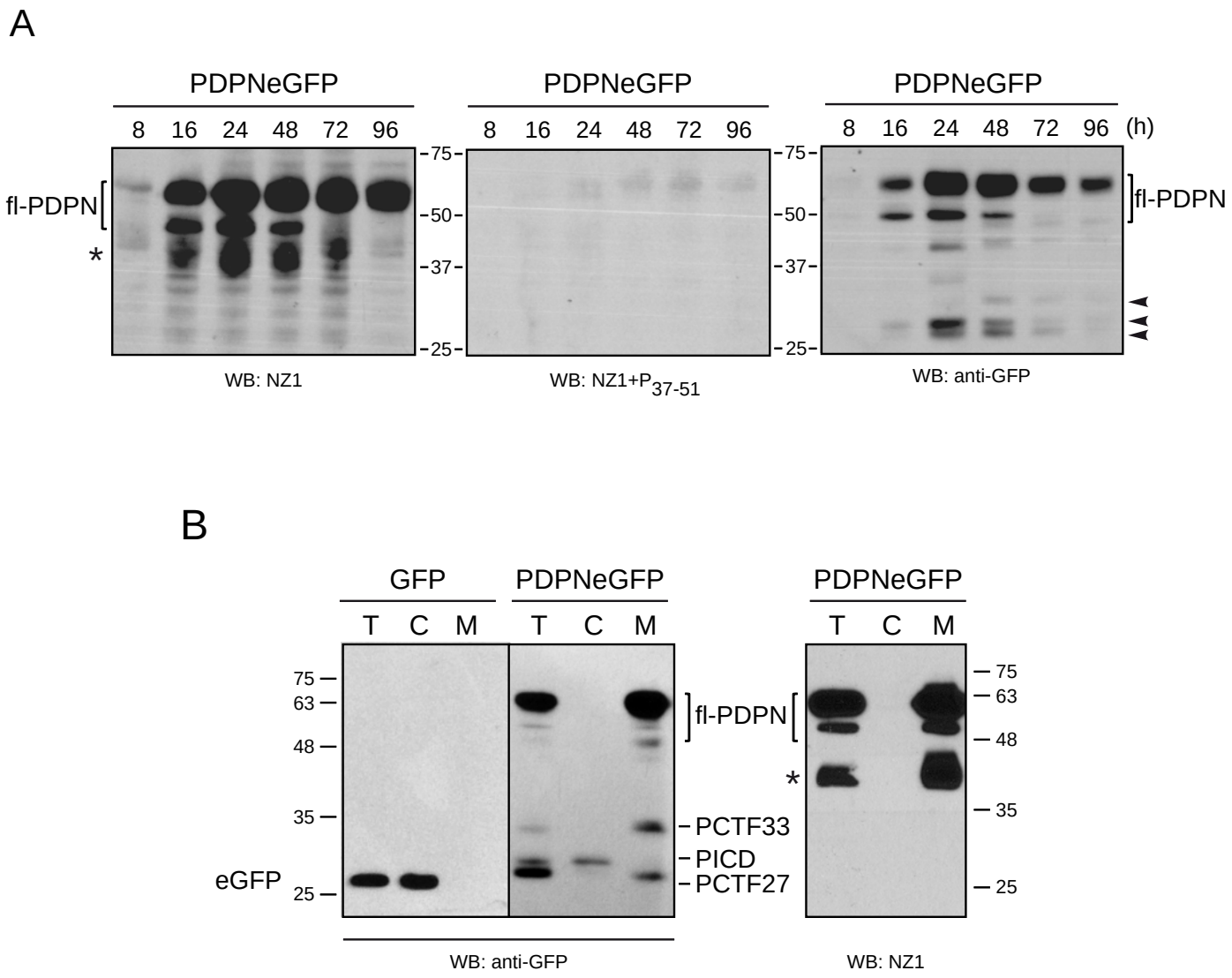
Figure 2. PDPN CTFs arise from proteolytic processing of fl-PDPN. (A) Podoplanin CTFs are observed following transfection with PDPNeGFP but not with the truncated PDPN-RQ* construct. (B) Effect of protease inhibitors on the generation of podoplanin CTFs. HEK293T cells transiently transfected with PDPNeGFP were treated with vehicle (DMSO) or the indicated protease inhibitors for 48 h, and podoplanin expression analyzed by Western blotting. (C) fl-PDPN and the 37-45 kDa N-terminal fragment (asterisk) are detected in the conditioned medium (CM) of MDCK cells stably expressing PDPNeGFP or control vector.

Figure 3. PCTF33 is a substrate of γ-secretase. (A) PCTF33 levels accumulate in cells transiently transfected with PDPNeGFP and treated for 48 h with γ-secretase inhibitors but not with vehicle (DMSO) or metalloprotease inhibitors. (B) Following transient transfection with PDPNeGFP, PCTF33 accumulates in HEK293 cells expressing a dominant-negative form of PS1 (PS1 DN385N) but not wild-type PS1. (C) PCTF33 accumulation in PS1 knockout MEFs transiently transfected with PDPNeGFP is reversed by co-transfection with PS1 wt. In all experiments, expression of fl-PDPN was used as transfection efficiency control.

Figure 4. Identification of the γ-secretase cleavage site within the TM region of podoplanin. (A) Alignment of the TM and CT domain sequences of podoplanin from different species. A stretch of valine residues in the TM region proximal to the CT domain (shaded) is highly conserved. Basic residues involved in ERM binding are underlined. (B) Sequence comparison of the TM regions of podoplanin and representative γ-secretase substrates around the cleavage site (arrowhead). The N-terminal sequence of the affinity purified PICD, as determined by Edman degradation, is indicated at the bottom. (C) Effect of metalloprotease and γ-secretase inhibitors on

the generation of PCTF33 in cells expressing PDPNeGFP or eGFP-tagged podoplanin TM chimeras. MDCK cells transiently expressing PDPNeGFP or the indicated mutant proteins were treated for 48 h with either vehicle or the indicated inhibitors and the levels of PCTF33 analyzed by Western blotting with an anti-GFP Ab. The sequence of the TM domain of podoplanin, CD45, ECD and TLR9 are indicated on the right. Valine residues close to the CT domain are shaded.

Figure 5. Model of podoplanin proteolytic processing. Schematic representation of the proteolytic events involved in podoplanin processing described in this article. First, podoplanin is cleaved by a metalloprotease at some point within its EC domain, generating a membrane-bound CTF (PCTF33) and an EC fragment that is probably being rapidly degraded. In a second step, the intracellular domain of podoplanin (PICD) is released into the cytosol following the cleavage of PCTF33 between V^{150} and V^{151} by PS1/γ-secretase. The NZ1 epitope is indicated (shaded amino acids). The glycosylation sites have been experimentally identified in dog podoplanin (Zimmer et al., 1997). PCTF33 and PICD could only be detected with an anti-GFP Ab, since currently, no available podoplanin Ab recognizes the CT domain of the protein.



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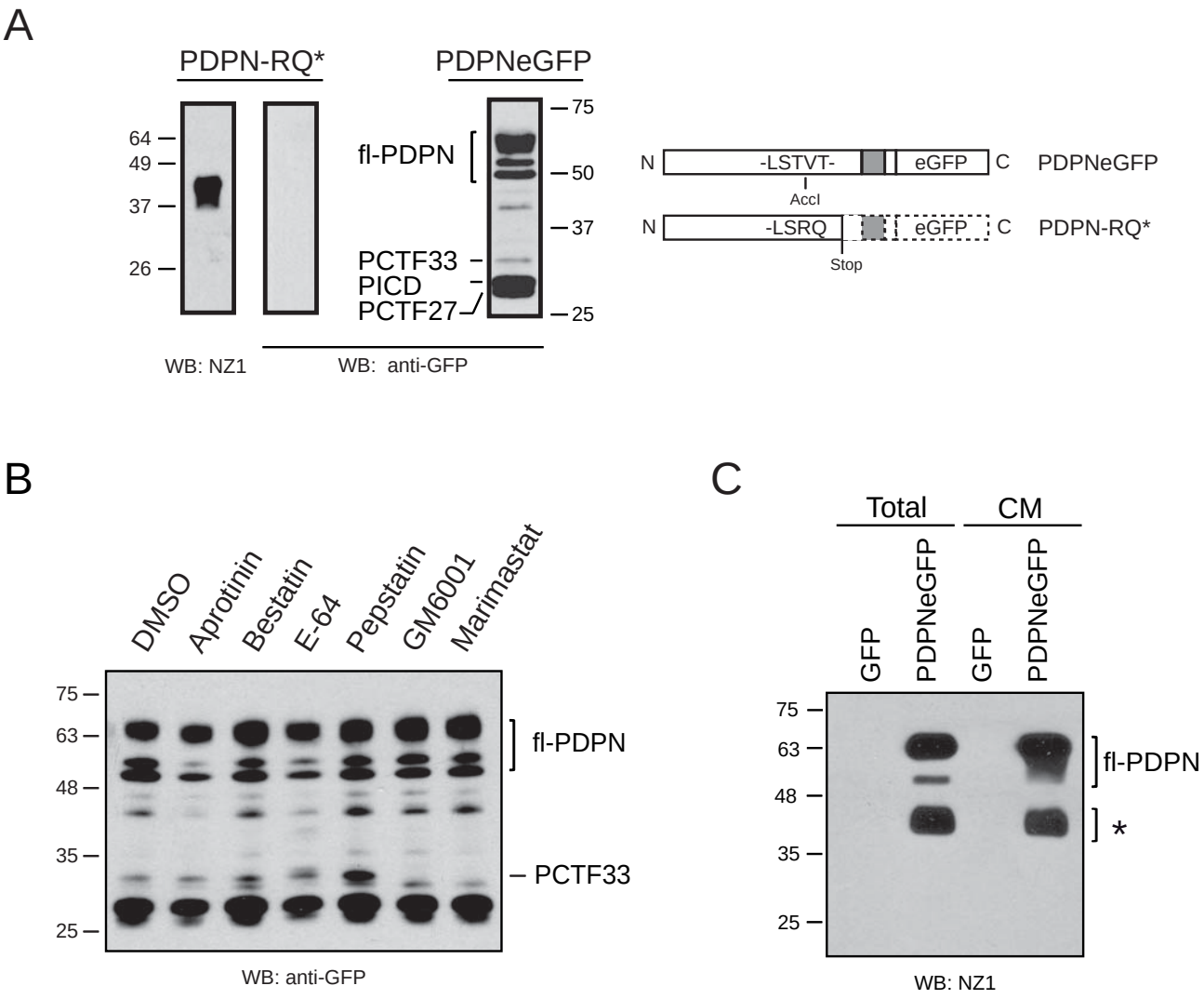
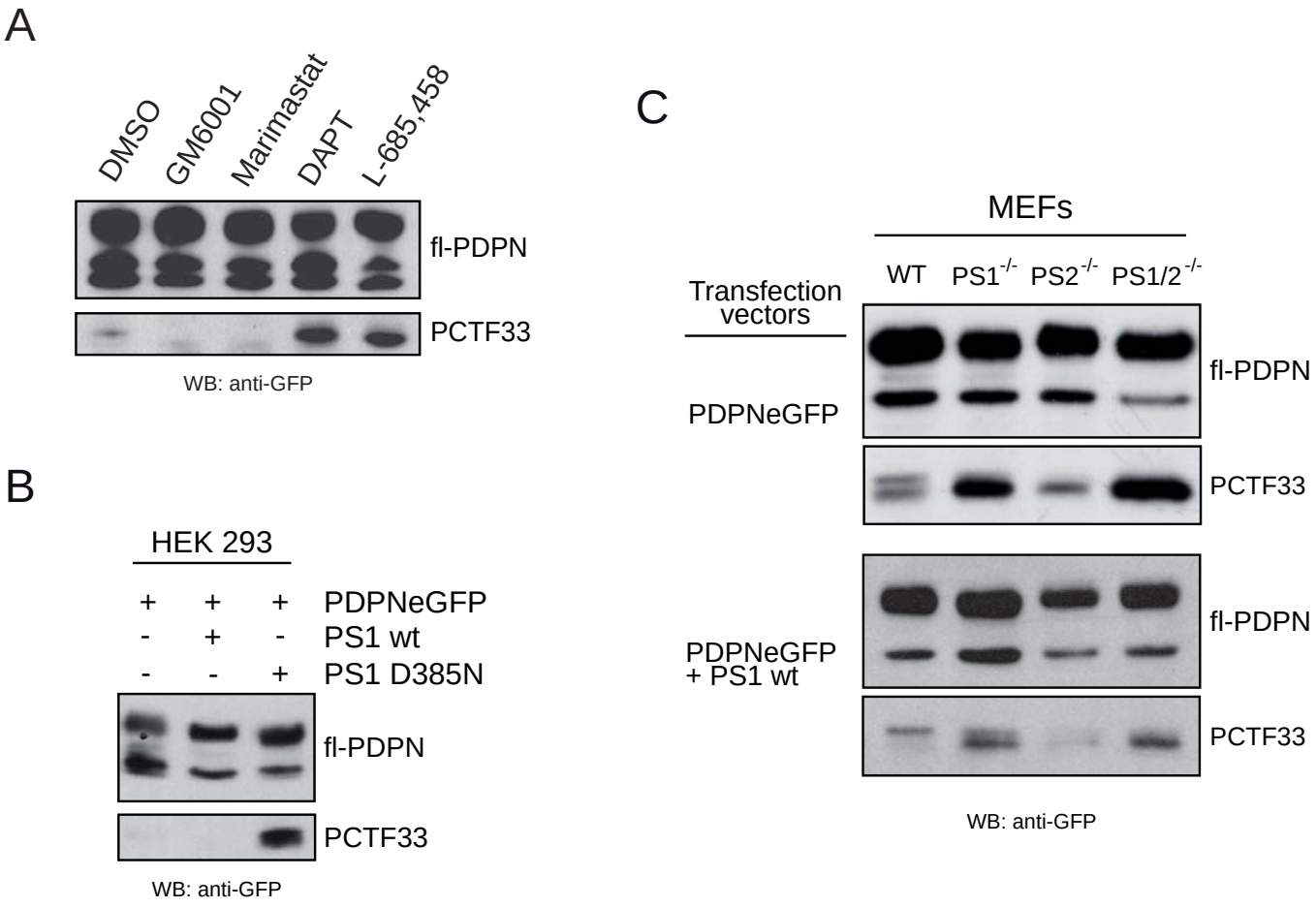


Yurrita et al., Figure 2.



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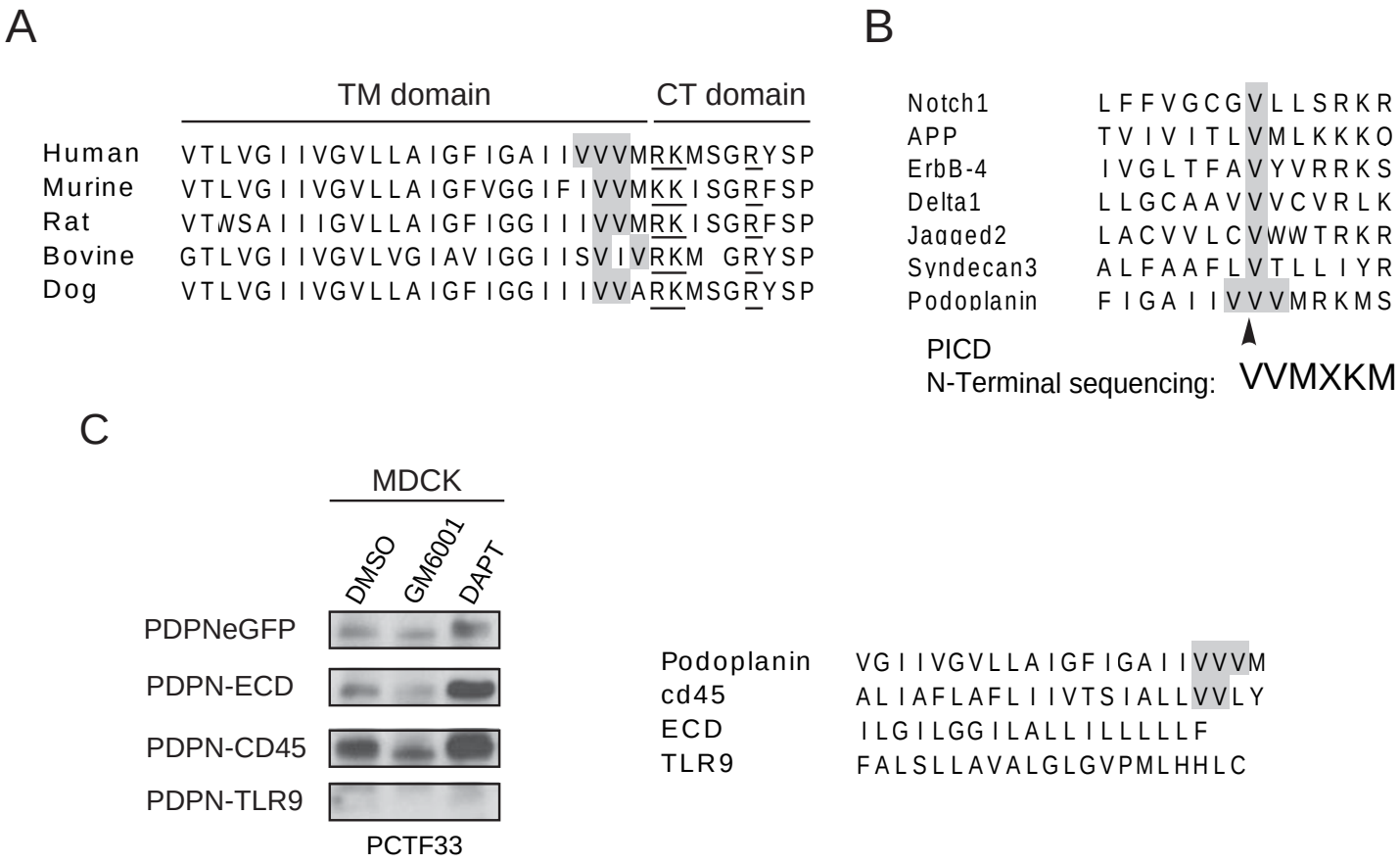

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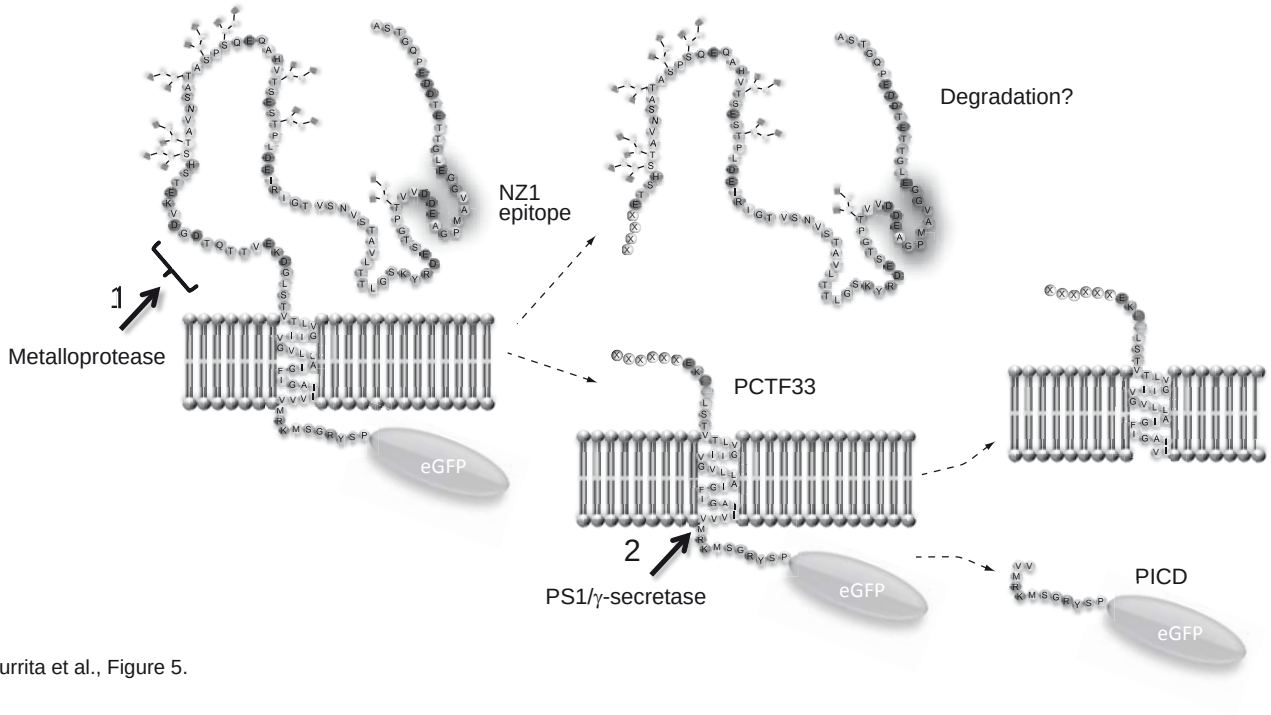

Yurrita et al., Figure 5.