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Regulation of the trafficking and the function of the metalloprotease ADAM10 by tetraspanins

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Running title: ADAM10 and tetraspanins

Abstract

By interacting directly with partner proteins and with one another, tetraspanins organize a network of interactions referred to as the tetraspanin web. ADAM10, an essential membrane-anchored metalloprotease that cleaves off the ectodomain of a large variety of cell surface proteins including cytokines, adhesion molecules, the precursor of the β -amyloid peptide APP or Notch, has emerged as a major component of the tetraspanin web. Recent studies have shown that ADAM10 associates directly with all members (Tspan5, Tspan10, Tspan14, Tspan15, Tspan17 and Tspan33) of a subgroup of tetraspanins having 8 cysteines in the large extracellular domain and referred to as TspanC8. All TspanC8 regulate ADAM10 exit from the endoplasmic reticulum, but differentially regulate its subsequent trafficking and its function, and have notably a different impact on Notch signaling. TspanC8 orthologs in invertebrates also regulate ADAM10 trafficking and Notch signaling. It may be possible to target TspanC8 tetraspanins to modulate in a tissue or substrate-restricted manner ADAM10 function in pathologies such as cardiovascular diseases, cancer or Alzheimer's disease.

Tetraspanins form a superfamily of proteins characterized by four transmembrane domains and a specific fold in the largest of the two extracellular regions. Tetraspanins have been involved in many physiological and pathological processes. Notably, genetic approaches in human and mouse have demonstrated a key role of some of these molecules in sperm-egg fusion, vision, kidney function, immunity or muscular regeneration [1-3]. Discrete tetraspanins also regulate cancer progression or play an important role in the entry of viruses or parasites into cells. The search for molecules associating with tetraspanins to get insight into their molecular function has shown that at the cell membrane, tetraspanins organize a dynamic network of molecular interactions referred to as the tetraspanin web or tetraspanin-enriched microdomains. Linked to this tetraspanins web are integrins, Ig domain proteins and ectoenzymes [1-3].

ADAM10, a molecular scissor associated with the tetraspanin web

In search for new tetraspanin-associated molecules, we screened the supernatants of hybridomas generated after immunization of mice with Jurkat cells for their ability to co-immunoprecipitate CD81 [4]. One of them was found to specifically recognize ADAM10. Similarly to other proteins associating with the tetraspanin web, ADAM10 was found to associate with all tetraspanins studied, including CD9 [4], confirming and extending an earlier report showing that CD9 associates with ectopically expressed ADAM10 [5] and our own mass-spectrometry analysis of CD9-associated molecules [6].

ADAM10, also known as Kuzbanian in *Drosophila melanogaster*, is a ubiquitous member of the ADAM (A Disintegrin And Metalloprotease) family of membrane-anchored zinc-dependent proteases, and is involved in the proteolytic cleavage of dozens transmembrane substrates within their extracellular region. A large variety of surface proteins undergo this ADAM10-dependent ectodomain shedding including the membrane-anchored forms of cytokine, chemokine and growth factor precursors (CXCL16 and EGF for example), adhesion molecules (N-cadherin, E-cadherin, VE-cadherin, CD44 for example) and Notch receptors [7,

8]. ADAM10 is a type-I transmembrane protein composed of a prodomain removed in the Golgi, a metalloprotease domain corresponding to its catalytic site, a disintegrin domain, a cysteine-rich domain, a stalk domain, a transmembrane domain and a short C-terminal cytoplasmic tail (Figure 1) [7-9]. ADAM10-mediated cleavage allows the cleavage of the transmembrane carboxyterminal fragment (CFT) by the gamma-secretase complex that generates a soluble cytosolic intracellular domain (ICD) which is either fated for degradation or has a signaling activity, notably in the case of Notch (Figure 1). The important role of ADAM10 in Notch signaling is highlighted by the fact that ADAM10 knock-out mice die at early stages of embryonic development with abnormalities reminiscent of Notch signaling defects [10]. Conditional ADAM10 knock-out mice have subsequently highlighted the essential role of ADAM10 in the development and/or homeostasis of several organs, often in relation with Notch defects, including the brain, the skin, the liver, the intestine, the striated muscle, the immune system and the vasculature [11-17]. ADAM10 plays a role in several diseases such as cancer or cardiovascular diseases [7, 18]. Its role in Alzheimer's disease may be especially significant because its cleavage of the amyloid precursor peptide (APP) prevents the production of amyloid-beta (A β) peptides produced through the sequential cleavage by BACE1 and the γ -secretase complex [19].

Direct association of ADAM10 with TspanC8 tetraspanins

The tetraspanin web is hierarchically organized in several levels of interactions: Primary complexes composed of a tetraspanin directly associated with a so-called specific partner protein form the primary level of interaction. These primary complexes associate with one another through tetraspanin to tetraspanin interactions to generate the entire spectrum of interactions inside the tetraspanin web [1-3]. In line with this partner/pair model, the next step after demonstrating that ADAM10 associates with tetraspanins was to identify those that directly associates with ADAM10. To our surprise, we identified six different tetraspanins that directly interact with ADAM10 [20]. These tetraspanins constitute a subgroup of tetraspanins that have 8 cysteines in their large extracellular domain and therefore are referred to as TspanC8: Tspan5, Tspan10, Tspan14, Tspan15, Tspan17 and Tspan33. Similar findings were reported by Haining et al. and in addition a screen for

proteins that associate with ADAM10 using the split-ubiquitin assay uncovered the interaction of Tspan15 with ADAM10 [21, 22].

TspanC8 tetraspanins differentially regulate ADAM10 trafficking and function

The direct association of ADAM10 with 6 tetraspanins is unusual. The only known examples of proteins directly associating with more than one tetraspanin are the Ig domain proteins CD9P-1/EWI-F and IgSF8/EWI-2 that directly associate with CD9 and CD81 [23-26]. So, why does ADAM10 associate with so many tetraspanins? On the one hand they differentially regulate its subcellular trafficking. All 6 TspanC8 tetraspanins promote the exit of the ER and the maturation of ADAM10 [20-22], possibly because the interaction with ADAM10 conceals an ER-retention signal in the cytoplasmic domain of ADAM10 [27]. Two of the TspanC8 tetraspanins target ADAM10 to late endosomes (Tspan10 and Tspan17) and the other 4 stimulate an increased expression of ADAM10 at the plasma membrane (Tspan5, Tspan14, Tspan15 and Tspan33) [20] (Figure 2). On the other hand, TspanC8 tetraspanins differentially regulate ADAM10 substrate selectivity. Three studies indicate that contrary to other TspanC8, Tspan15 is a positive regulator of ADAM10-mediated N-cadherin cleavage [22, 28, 29]. Side by side comparison of the effect of TspanC8 also suggests a differential effect of these tetraspanins on the cleavage of other substrates. For example, only the transfection of Tspan14 was found to inhibit the cleavage of GPVI in HEK cells [28], whereas silencing of Tspan5 but not Tspan15 in PC3 cells reduced CD44 cleavage [29]. There are however some discrepancies among studies. In our hands, transfection of Tspan15 in U2OS cells inhibited APP cleavage, and its silencing in the prostate cancer cell line PC3 slightly stimulated this cleavage [29]. However, transfection of Tspan15 in other cell lines promoted APP shedding [22, 30]. The reason for these discrepancies are unknown. A potential explanation may be that the effect of Tspan15 on the cleavage of APP depends on the expression pattern of the various TspanC8.

TspanC8 differentially regulate Notch signaling

Because the Notch receptors are major ADAM10 substrates, we particularly investigated the effect of TspanC8 on Notch signaling and found that it was also differentially regulated by TspanC8. Silencing the two major TspanC8 expressed by U2OS cells (transduced with Notch1, yielding the U2OS-N1 cell line), Tspan5 and Tspan14, reduced Notch signaling,

whereas their transfection in HeLa cells stimulated this signaling [20]. In these studies, the analysis of the effects of TspanC8 on constitutively active mutants of Notch pointed to an action upstream the γ -secretase cleavage, consistent with an action on the ADAM10 step. The positive effect of Tspan5 on Notch activation is confirmed using HeLa cells transfected by various Notch constructs [5] and during *in vitro* osteoclastogenesis [31].

In a second series of experiments, we observed that expression of Tspan15 and Tspan33 in U2OS-N1 cells inhibited Notch signaling. The negative role of Tspan15 was confirmed in PC3 cells in which silencing endogenous Tspan15 stimulates this signaling. Again, Tspan15 acted upstream the γ -secretase cleavage [29]. There is no consensus on the role of Tspan33. Silencing Tspan33 reduced the activity of different Notch constructs transfected in HeLa cells [5], despite the fact that these cells express low levels of this tetraspanin [20, 32], In macrophages, Tspan33 expression is induced by Toll-like receptor activation and by forced Notch expression, and again was a positive regulator of Notch signaling [33]. Of note, in these two studies, it has been suggested that Tspan33 may also regulate Notch signaling by acting on the γ -secretase step.

Potential mechanisms whereby TspanC8 have differential effects on ADAM10 activity

How can TspanC8 differentially regulate the cleavage of ADAM10 substrates? Using ADAM10 deletion mutants and ADAM10/ADAM17 chimeras, Noy et al. have shown that all TspanC8 interact with a region comprising the stalk and cysteine-rich domain of ADAM10 [28]. However, Tspan15 better interacts with an ADAM10 mutant lacking all the extracellular domain but the stalk region than the other TspanC8. This indicates that different TspanC8 engage ADAM10 in subtly different ways, which led the authors suggest that they might direct substrate specificity by constraining ADAM10 into defined conformations [28]. Another explanation is that the different TspanC8 act on ADAM10 function by changing its membrane compartmentalization. In this regard, we have shown using sucrose gradient fractionation and single molecule tracking that Tspan5 and Tspan15 have a different impact on ADAM10 membrane compartmentalization. Notably, in the presence of Tspan15, ADAM10 diffuses faster and shows less frequent confined trajectories, indicating less interactions with other membrane constituents [29]. Label-free quantitative mass spectrometry analysis indicated that Tspan5 interacts better with the tetraspanin web (i.e.

with other tetraspanins and tetraspanin-associated molecules) than Tspan15, and that Tspan15 reduces the ability of ADAM10 to interact with the tetraspanin web [29]. It is therefore possible that the interaction of ADAM10 with the tetraspanin web favors the cleavage of discrete substrates and prevents the cleavage of others. This is consistent with a role of tetraspanins in membrane compartmentalization.

The interaction of ADAM10 with non-TspanC8 tetraspanins.

Several reports have documented the effect of other tetraspanins on ADAM10 function. Of note, none of these other tetraspanins has been shown to regulate ADAM10 exit from the ER. In our initial demonstration that ADAM10 associates with tetraspanins, we showed that the engagement of CD9, CD81 or CD82 by antibodies promotes ADAM10-mediated TNF- α and/or EGF secretion [4]. Because TNF α is usually cleaved by the closely related metalloprotease ADAM17, we hypothesized that these tetraspanins contributed to a cellular inhibition of ADAM10 ability to process TNF α , and that the antibodies overcome this inhibition. Tspan12 was also shown to associate with ADAM10. Overexpression of this tetraspanin was associated with an increased formation of ADAM10 mature form and shedding of APP. The interaction of Tspan12 with ADAM10 depends on the palmitoylation of Tspan12, indicating an indirect interaction [34]. The expression of a non-palmitoylatable Tspan12 mutant had the opposite effect on ADAM10 maturation and APP shedding, and this might be a consequence of a mislocalization of WT Tspan12. The exact mechanisms whereby Tspan12 modulates APP processing are unknown, and may be indirect. Indeed Tspan12 enhances β -catenin signaling in response to norrin by promoting the multimerization of its receptor complex, comprising Frizzled-4 and low-density lipoprotein-related protein 5 co-receptor [35]. Of note, deficiency in Wnt signalling in neurons leads to increase APP processing to A β peptide [36].

Like Tspan15, Tspan3 was identified as an ADAM10-interacting molecule using the split-ubiquitin yeast screening [30]. Tspan3 was shown to co-immunoprecipitate ADAM10, but also APP and presenilin1, a subunit of the γ -secretase complex. Its over-expression led to increased APP CTF production, but the production of APP CTF in the brain of Tspan3 KO mice was not modified, possibly due to functional redundancy by other tetraspanins [30].

TspanC8 tetraspanin functions in invertebrates

Phylogenetic analysis has suggested that each phylum has developed its own repertoire of tetraspanins [37]. Coherent with this idea, classical tetraspanins such as CD9, CD81, CD63 or CD151, have no clear orthologs in invertebrates. In contrast, TspanC8 tetraspanins do have unambiguous orthologs in insects and worms. Notably, Tspan5, the most conserved tetraspanin, shares 44% and 38% identity with the closest orthologs in *Drosophila melanogaster* and *Caenorhabditis elegans* respectively [20]. Three studies have shown that these invertebrate TspanC8 regulate Notch or ADAM10-dependent developmental processes. Although *Tsp-12*, the only TspanC8 tetraspanin in *C. elegans*, does not have 8 cysteines in the LEL, it can be considered as a TspanC8 because it has other TspanC8-specific residues in this domain [20]. *Tsp-12* was identified in a RNAi screen as the only tetraspanin contributing to the sterility produced by a mutation causing constitutive or elevated activity of *GLP-1*, one of two Notch-like receptors in *C. elegans* [38]. More generally, in several cellular contexts, a null mutation of *Tsp-12* abolished the effect of Notch (*GLP-1* or *Lin-12*) elevated activity, and synergized with partial loss-of-function Notch mutants. In addition, phenotypic enhancement was observed when a null mutation of *tsp-12* was combined with a partial loss-of-function allele of *sup-17*, the ADAM10 ortholog [38]. More recently, *Tsp-12* and *Tsp-14* (a non-TspanC8 tetraspanin) mutants were shown to have anomalies in developmental processes dependent on the highly conserved bone morphogenetic protein (BMP) pathway [39]. *Tsp-12* was shown to play its role in the signal-receiving cells and to control the trafficking of ADAM10/*sup-17* to the plasma membrane (which *Tsp-14* does not do). *Sup-17* similarly regulates BMP signaling, through a mechanism that probably does not involve Notch signaling but may rather involve in part the cleavage of the neogenin homolog *unc-40*. Of note neogenin is an ADAM10 substrate in the neurons of mammals [40].

In *Drosophila*, two of the three TspanC8 (*Tsp3A* and *Tsp86D*) were identified in a genome-wide RNAi screen as regulators of the formation of sensory bristles, a well-characterized Notch-dependent developmental process. Decreasing the expression level of only one of these tetraspanins, or that of *Tsp26A*, the third *drosophila* TspanC8 not tested in the initial screen, produced only a mild phenotype. In contrast, down-regulation of all three TspanC8 caused a dramatic diminution of sensory bristles, indicating a potential redundancy between these three tetraspanins. Here again, diminution of TspanC8 expression impacted several

Notch-dependent developmental processes. Importantly, the diminution of TspanC8 levels was associated with a reduced Notch activity *in vivo* and a mislocalization of Kuzbanian, the fly ADAM10 ortholog [20]. Of note, in another screen focusing on intracellular trafficking regulators, several other tetraspanins were shown to regulate the formation of sensory bristles. The underlying mechanisms are unknown [41].

TspanC8 tetraspanins in physiological processes and disease in mammals

There is not yet evidence that TspanC8 regulate Notch activity *in vivo* in mammals. The only mouse genetic model for TspanC8 is a Tspan33 knock-out mouse. These mice developed anemia and splenomegaly, and had abnormal circulating reticulocytes. *In vitro* experiments revealed a differentiation defect of Tspan33^{-/-} erythroid progenitors. Importantly, Tspan33-null erythrocytes show substantially reduced ADAM10 expression [42]. The importance of Notch2 signaling for erythropoiesis has recently been highlighted using elegant genetic approaches [43].

In humans, several studies have linked genetic variants of TspanC8 with diseases. The *Tspan15* locus has been recently identified by a meta-analysis of 12 genome wide association studies (GWAS) as a susceptibility locus for venous thromboembolism. The single nucleotide polymorphism identified is common, intronic and is associated with an increased risk of 1.31-fold [44]. In another study, a single nucleotide polymorphism (SNP) in the *Tspan10* locus was associated with the retinal microvascular diameter, which is itself associated with cardiovascular and cerebrovascular conditions [45]. Another GWAS for plasma serotonin concentrations in relation with major depressive disorders revealed a significant SNP cluster on chromosome four 5' of *Tspan5* [46]. These SNP are associated with higher baseline plasma serotonin concentrations and greater decreases in plasma serotonin concentration during selective serotonin reuptake inhibitor therapy, and were shown to influence the expression level of Tspan5 RNA. *In vitro* experiments indicated that Tspan5 is a positive regulator of the expression of several serotonin pathway enzymes [46]. Finally, in another genome-wide study, aiming at better understanding the pathogenesis of type 2 diabetes, Tspan33 expression in pancreatic islets was shown to be associated with the concentration of Glycated hemoglobin (HbA1c), a measure of long-term hyperglycemia. In addition, a SNP in Tspan33 was shown to correlate with a lower Tspan33 gene expression,

and silencing Tspan33 expression in INS-1 cells was associated with significantly reduced glucose-stimulated insulin secretion [47].

Several tetraspanins have been shown to regulate the cell entry or the pathogenicity of infectious agents [1, 48]. In this regard, two genome-wide loss-of-function screens [49, 50] identified Tspan33 and Tspan14 as proteins contributing to the toxicity of the pore-forming toxin alpha hemolysin (α HL) of *Staphylococcus aureus*, a bacteria that causes a wide variety of infections. These findings are consistent with ADAM10 being the receptor for the toxin. In this regard, its surface expression was strongly decreased in cells with a disruption of the Tspan14 gene [49], confirming the role of this tetraspanin in the regulation of ADAM10 surface expression levels. One explanation for the identification of two different TspanC8 in these two studies, may be that these TspanC8 tetraspanins are the most abundant in the cell types used in the screening, and therefore have, compared to other TspanC8, a major role in the regulation of ADAM10 surface expression levels.

Finally, a number of studies suggest that ADAM10 may regulate the progression or aggressiveness of several types of cancers [8, 18]. Considering the key role of TspanC8 in the regulation of ADAM10, it is likely that future studies will demonstrate a role for TspanC8 as well. So far only one recent study has addressed the role of these tetraspanins in cancer. It was shown in this study that the expression of Tspan5 in human gastric adenocarcinomas is correlated with a low overall survival in patients. Transfection of Tspan5 *in vitro* reduced cell proliferation by inhibiting the cycle transition from G1/S phase, and inhibited the growth of xenografts in mice [51].

Conclusions and perspectives

Over the years, dozens of ADAM10 substrates have been identified. Considering the diversity of functions of these substrates, that include among others, adhesion molecules, receptors, cytokines or guidance molecules, it is not surprising that ADAM10 has been shown to be involved in a number of diseases, and has been suggested as a therapeutic target. This is notably the case for neurodegenerative diseases, and particularly in Alzheimer's disease, cardiovascular diseases or cancer [8]. However, considering the large distribution of ADAM10 and its numerous targets, a medium to severe toxicity of chemical inhibitors is

expected. The different cellular distribution of TspanC8 and their different impact on ADAM10 substrate selectivity, suggest that it may be possible, via these tetraspanins, to target selectively ADAM10 proteolytic activities and restricts the modulation of its activities to certain tissues or substrates. Targeting ADAM10 through TspanC8 could be especially useful to avoid toxicity due to wide range alterations of Notch signaling

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Figure legends

Figure 1:

ADAM10 structure, maturation and function. ADAM10 is expressed as a zymogen the prodomain of which is removed in the Golgi apparatus through the action of proprotein convertases. At the plasma membrane, ADAM10 cleaves off the ectodomain of its transmembrane substrates leading to the release of a soluble extracellular fragment and a remnant transmembrane carboxyterminal fragment (CTF). The latter is then processed by the gamma-secretase complex, yielding an intracellular domain (ICD) which is fated to either degradation or has a signaling activity, as well described for Notch.

Figure 2:

TspanC8 differentially regulate ADAM10 subcellular localization, membrane compartmentalization and substrate selectivity. The interaction of TspanC8 with ADAM10 allows its exit from the endoplasmic reticulum and its accumulation in either late endosomes (Tspan10 and Tspan17) or at the plasma membrane (Tspan5, Tspan14, Tspan15 and Tspan33). At the plasma membrane, Tspan5, Tspan14 and Tspan15 differentially associate with the tetraspanin web, and have a different impact on the cleavage of CD44, N-cadherin and GP VI, as well as a different impact on Notch signaling.

Fig 1

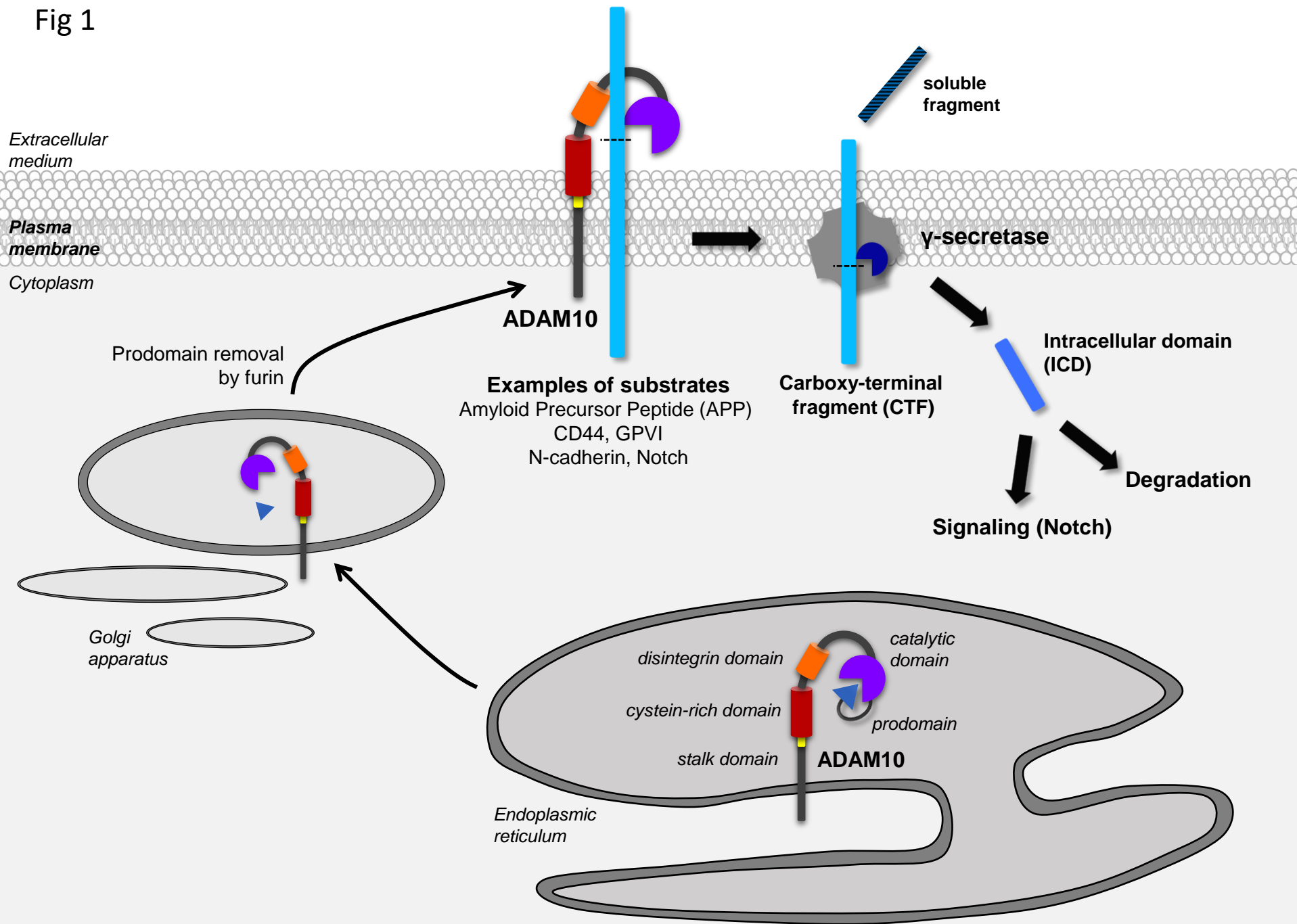


Fig 2

