Harnessing Notch signaling to decode mechanisms of proteolytic regulation in diverse cell-surface receptors

Amanda N. Hayward, Eric J. Aird, and Wendy R. Gordon*

Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN, USA

* Please address correspondence to: wrgordon@umn.edu

Summary

Proteolysis of transmembrane receptors is a critical cellular communication mechanism often dysregulated in disease, yet proteolytic regulation mechanisms of ~400 receptors estimated to be cleaved from the surface remain elusive due to difficulties in controlling stimulus or unknown fates of cleavage products. In Notch receptors, intercellular forces drive exposure of a cryptic protease site within a juxtamembrane “proteolytic switch” domain to activate transcriptional programs inside the cell. The facile control and readout of proteolysis in Notch has been harnessed for therapeutics and synthetic biology. Thus, we created a Synthetic Notch Assay for Proteolytic Switches (SNAPS) to screen for putative proteolytic switches that might functionally substitute for the analogous Notch domain and initiate Notch signaling. We find conformational control of cryptic protease site exposure in cell-surface receptors is not unique to Notch but is potentially common to diverse classes of receptors. We also demonstrate the assay can be used to measure proteolytic modulation by potential therapeutics.

Keywords: signaling, proteolysis, synthetic biology, cell surface receptors, proteolytic switch, drug screening, mechanotransduction, ectodomain shedding, cryptic protease sites

Introduction

Proteolysis of cell surface transmembrane proteins is a tightly regulated cellular mechanism that controls intercellular communication and the interaction of cells with their extracellular environment. Diverse adhesion receptors involved in cell-cell contact and cell-extracellular matrix (ECM) interactions, as well as receptors that respond to soluble factors, have been shown to be cleaved at sites close to the extracellular side of the membrane by metalloproteinases such as 'A Disintegrin And Metalloproteinases' (ADAMs) and matrix metalloproteinases (MMPs) (Miller, Sullivan, and Lauffenburger 2017; Kessenbrock, Plaks, and Werb 2010; McCawley and Matrisian 2001; Seals and Courtneidge 2003; White 2003), resulting in ectodomain shedding. In many of these receptors, ectodomain shedding is a prerequisite for further cleavage inside the membrane by the γ-secretase/presenilin protease complex in a process known as Regulated Intramembrane Proteolysis (RIP) (Brown et al. 2000; Selkoe and Wolfe 2007). These proteolytic events not only provide a mechanism to break cell-cell and cell-ECM contacts to modulate processes such as cell migration, but it may also result in biologically-active fragments outside and inside of the cell, such as the intracellular fragment of Notch, which acts as a transcriptional co-activator (Bray 2006), and the

soluble extracellular fragment of IL6R, which binds to IL-6 and acts as an agonist to control cellular processes (Garbers et al. 2011).

Dysregulated proteolysis contributes to disease pathogenesis by several mechanisms, including: 1) accumulation of pathogenic fragments, such as the amyloid β-peptide in Alzheimer's disease (Goate et al. 1991; Scheuner et al. 1996) and the ubiquitous cancer biomarker soluble E-cadherin (S. M. Brouxhon et al. 2014; Katayama et al. 1994), 2) abnormal signaling, as in Notch mutations that drive unregulated proteolysis and transcription of target genes in T-Cell Acute Lymphocytic Leukemia (Weng et al. 2004; Malecki et al. 2006), 3) premature disruption of cellular adhesions to favor cell migration (Maretzky et al. 2005), and 4) removal of epitopes required for normal cell communication, as cancer cells evade the immune response by shedding MICA receptors (Groh et al. 2002; Kaiser et al. 2007; Boutet et al. 2009; Waldhauer et al. 2008), normally deployed to the cell surface in response to cellular damage. Receptor tyrosine kinase (RTK) are well established proteolysis substrates (Merilahti and Elenius 2018) and provide an interesting example of how both proteolytic enhancement and deficiency are detrimental to cells. For example, increased HER2 proteolysis leads to accumulation of a constitutively active kinase at the membrane in breast cancer (Arribas et al. 2011) while decreases in RTK shedding caused by MEK kinase inhibition lead to drug resistance (Miller et al. 2016).

The common dysregulation of receptor proteolysis in disease has prompted the development of therapeutics that modulate proteolysis, either by inhibiting proteases or by preventing access to protease sites in a specific receptor. Many protease inhibitors

have been developed but have failed clinically due to significant off-target effects caused by substrate promiscuity and lack of protease specificity (Dufour and Overall 2013; Vandenbroucke and Libert 2014; Turk 2006). Conversely, relatively few examples of modulating access to protease sites in specific receptors have been reported, despite the clinical success of the monoclonal antibody trastuzumab (Herceptin) that was found to act, in part, by blocking proteolysis of the receptor tyrosine kinase HER2 (Molina et al. 2001). Similarly, successful development of modulatory antibodies targeting proteolysis of Notch (Li et al. 2008; Aste-Amézaga et al. 2010; Wu et al. 2010; Tiyanont et al. 2013; Qiu et al. 2013; Agnusdei et al. 2014; Falk et al. 2012) and MICA (Ferrari de Andrade et al. 2018) receptors have recently been reported. However, even though 8% of the annotated human transmembrane proteins are predicted to be shed from the surface (Tien, Chen, and Wu 2017), mechanisms of proteolytic regulation that inform development of specific modulators have remained elusive.

A relatively unique proteolytic regulation mechanism has recently come to light in which a stimulus alters protein conformation to induce exposure of a cryptic protease site. For example, the secreted von Willebrand factor is cleaved in its A2 domain in response to shear stress in the bloodstream, which regulates blood clotting (Dong et al. 2002). Transmembrane Notch receptors also control exposure of a cryptic protease recognition site via the conformation of a juxtamembrane domain called the Negative Regulatory Region (NRR) (Gordon et al. 2015). The NRR normally exists in a proteolytic cleavage-resistant state in which the protease site is buried by interdomain interactions but can be switched to a protease-sensitive state when it undergoes a conformational

change upon binding a ligand on a neighboring cell (Gordon et al. 2015; Sanchez-Irizarry et al. 2004) or if it harbors disease-related mutations that destabilize the domain (Malecki et al. 2006; Gordon et al. 2009; Weng et al. 2004). Notch's proteolytic switch has been exploited to develop conformation-specific modulatory antibodies (Li et al. 2008; Aste-Amézaga et al. 2010; Wu et al. 2010; Tiyanont et al. 2013; Qiu et al. 2013; Agnusdei et al. 2014; Falk et al. 2012) and harnessed for synthetic biology applications (Morsut et al. 2016) to turn on transcription in response to any desired cell-cell contact.

Crystal structures (Gordon et al. 2007, 2009; Xu et al. 2015) reveal that the NRR is comprised of an N-terminal cysteine-rich, calcium binding domain, called the Lin-12 Notch Repeat (LNR) domain, which intimately interacts with a juxtamembrane heterodimerization domain that can be structurally characterized as a Sea urchin Enterokinase Agrin-like (SEA-like) domain. SEA-like domains have high structural homology to canonical SEA domains of mucins (Macao et al. 2006; Maeda et al. 2004) but lack the characteristic autoproteolytic site. Moreover, a recent bioinformatic study identified additional proteins predicted to contain SEA-like domains adjacent to the transmembrane domain (Pei and Grishin 2017). In many cases, SEA-like domains are found in tandem with a defined neighboring N-terminal domain, such as Notch, while some SEA-like domains are adjacent to heavily glycosylated regions with little predicted secondary structure as in mucins.

Despite the knowledge that several cell-surface receptors harbor extracellular juxtamembrane domains with structural homology to Notch's proteolytic switch and that

more than 100 receptors undergo a Notch-like proteolytic cascade (Brown et al. 2000; Selkoe and Wolfe 2007), other membrane resident proteolytic switches have not been identified, in large part due to difficulties in controlling stimulus (e.g. homotypic interactions) or unknown fates of cleavage products. Thus we aimed to harness the well-defined inputs and outputs of Notch signaling to interrogate proteolytic mechanisms of diverse cell-surface receptors using a Synthetic Notch Assay for Proteolytic Switches (SNAPS). Here, we find that proteolysis regions of several receptors with structural homology to Notch can substitute for the Notch "proteolytic switch" and facilitate signaling in response to cell contact. Moreover, several RTKs, CD44, and PCDH12, also exhibit increases in proteolysis when "induced" by a Notch ligand, suggesting potential control of protease site exposure by the conformation of the receptor. Finally, we demonstrate that the assay can be used to screen modulators of proteolysis.

Results

Design of SNAPS

To identify receptors in which proteolysis is regulated by exposure of the protease site, which are attractive targets for development of specific proteolysis modulators, we exploited the well-established modularity and easily controlled inputs and outputs of Notch signaling (Malecki et al. 2006; Gordon et al. 2015; Roybal et al. 2016) to create a Synthetic Notch Assay for Proteolytic Switches (SNAPS). SNAPS

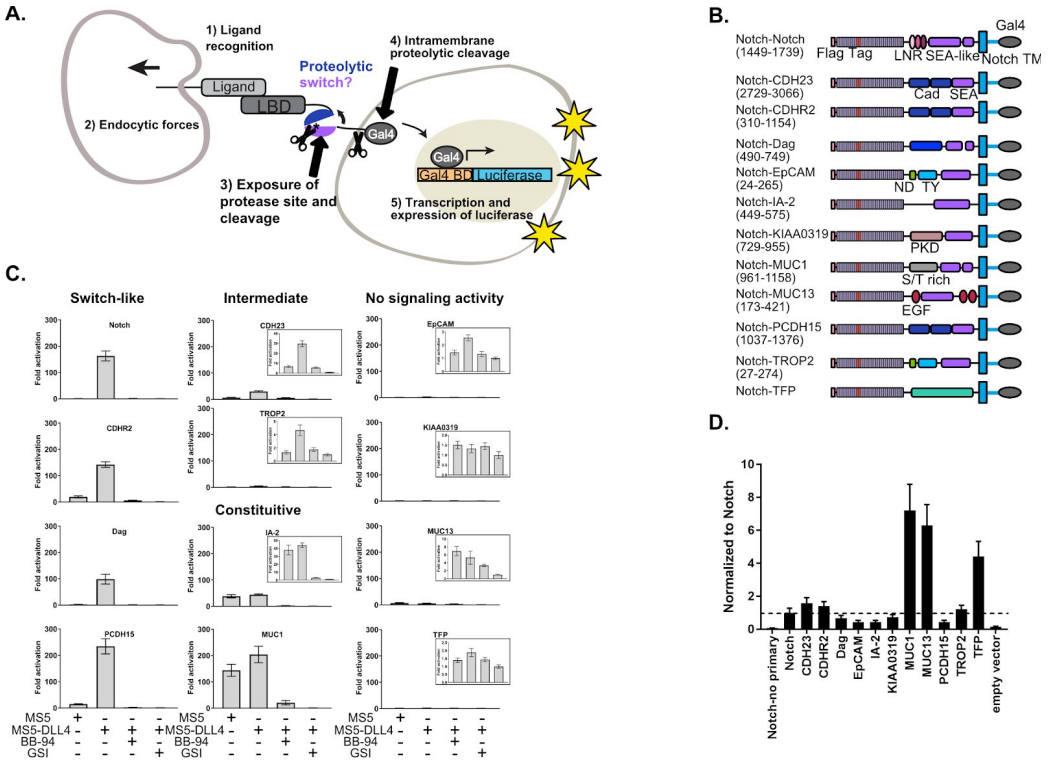

Fig. 1: SEA-like domains cooperate with adjacent domains to behave as proteolytic switches (**A**) Schematic of Synthetic Notch Assay for Proteolytic Switches (SNAPS). Cells co-expressing Flag-Notch-X-Gal4 chimeras, where X is a putative proteolysis region of another receptor, and luciferase reporter constructs are co-cultured with DLL4 ligand-expressing cells. Luciferase signal corresponds to Notch activation. (**B**) Schematic of chimeric constructs utilized in the signaling assay. Protein domains are color coded and labeled below. Amino acid ranges used for each construct are in parentheses under the names. Note that Notch's SEA-like domain is also referred to as the Heterodimerization Domain (HD) in the literature. Abbreviations used: ADAM10: A Disintegrin and metalloproteinase domain-containing protein 10. Cad: cadherin. CDH23: Cadherin-23. CDHR2: Cadherin-related family member 2 Dag: Dystroglycan. DLL4: Delta-like ligand 4. EGF: Epidermal growth factor. EpCAM: Epithelial cell adhesion molecule. LBD: Ligand binding domain. LNR: Lin-12 Notch-like repeats. MUC1: Mucin-1. MUC13: Mucin-13. ND: N-terminal domain. PCDH15: Protocadherin-15. PKD: polycystic kidney disease domain. S/T rich: serine-threonine rich. SEA: sea urchin enterokinase agrin. TFP: Teal fluorescent protein. TM: transmembrane domain. TROP2: Tumor-associated calcium signal transducer 2. TY: thyroglobulin type-1A domain. (**C**) Luciferase reporter gene activity profile of Notch in comparison to the Notch chimera constructs co-cultured with MS5 cells or MS5 cells stably transfected with DLL4. BB-94=Batimastat (pan-metalloproteinase inhibitor) GSI= Compound E (γ-secretase inhibitor). Error bars represent the SEM of triplicate measurements (**D**) Cell surface ELISA of Notch and Notch chimera constructs. Anti-Flag primary and goat anti-mouse HRP secondary antibodies were used to detect cell surface expression levels of each chimera. HRP activity was read out using a luminescence plate reader, and the signal was normalized to Notch. The horizontal dotted line corresponds to Notch expression levels. Error bars represent the SEM of triplicate measurements.

uses the native Notch ligand-binding interaction with DLL4 as the input and the Gal4 transcriptional response as the output (Fig. 1A). “Proteolysis region” chimeras were created by replacing the NRR proteolytic switch with protease site-containing juxtamembrane domains of diverse cell-surface receptors. Then, chimeric constructs together with Gal4-responsive and control luciferase reporter constructs were transfected into U2OS cells, co-cultured with cells stably expressing Notch ligands, and luciferase activity measured in a high-throughput format.

SEA-like domains cooperate with adjacent domains to behave as proteolytic switches

To determine if receptors bearing juxtamembrane domains predicted to be structurally homologous to Notch could also function as proteolytic switches, we created chimeric receptors in which we replaced the Notch NRR proteolytic switch domain with SEA-like domains from other cell surface receptors and included any previously characterized tandem N-terminal domains (Fig. 1B). We also made a negative control chimera where the NRR was replaced by the fluorescent protein mTFP. We hypothesized that other putative proteolytic switches could functionally substitute for the Notch NRR and initiate a transcriptional response in response to contact with a cell expressing DLL4.

Surprisingly, we found the ECM receptor dystroglycan and two protocadherins involved in intercellular adhesion, Protocadherin-15 (PCDH15) and Cadherin-related protein 2 (CDHR2), could functionally substitute for Notch's NRR (Fig. 1C). These chimeric receptors signaled robustly only in the presence of cells expressing DLL4, and

the signal was abrogated by both a global metalloproteinase inhibitor (BB-94) and an inhibitor of the subsequent intramembrane γ-secretase cleavage event (γ-secretase inhibitor; GSI). The putative cell adhesion molecules Trop2 and Cadherin-23 (CADH23) displayed a more moderate signaling activity in response to DLL4. Interestingly, these receptors all contain SEA-like domains in tandem with an N-terminal domain. On the other hand, SEA-like domains without a structured neighboring domain, such as Mucin-1 (MUC1) and receptor tyrosine phosphatase-related islet antigen 2 (IA-2), exhibited a high level of proteolysis even in the absence of DLL4, suggesting they contain a constitutively exposed protease site in the context of this assay.

A few chimeras showed very little signal in the assay, suggesting a lack of proteolysis or lack of cell-surface expression. To further probe the receptors exhibiting low levels of activation in the signaling assay, we performed a cell-surface ELISA assay. Briefly, Flag-tagged Notch chimera constructs were transfected into U2OS cells, fixed, stained, and cell-surface levels quantified by measuring HRP activity. Our negative control mTFP chimera expressed substantially better than Notch (Fig. 1D), suggesting its lack of response in the signaling assay is due to an absence of proteolysis in the assay, as expected. Most of the chimeras lacking signaling activity also expressed at lower levels than Notch, suggesting defects in expression or trafficking due to incorrect choice of domain termini. Most of the receptors with robust signaling activity, either constitutive or switch-like, expressed at similar levels to Notch, with some notable exceptions. MUC1 expressed at higher levels than Notch but also had high signaling activity. In contrast, Mucin-13 (MUC13) expressed at substantially higher levels than

Notch but had low signaling activity, suggesting that MUC13 might lack a proteolysis site. Indeed, unlike the other receptors containing SEA-like domains, MUC13 has two EGF-like domains between its SEA-like and transmembrane domains that likely do not contain an accessible protease site. Similarly, KIAA0319 expresses at similar levels to Notch but has no observable signaling activity. Lack of proteolysis for these chimeras could be due to the absence of protease sites, incorrect protease expression or localization, or abnormal protein folding due to artificial joints to Notch's ligand binding and transmembrane domain.

In contrast, the ELISA showed that IA-2 expressed at much lower levels than Notch yet exhibited robust constitutive signaling activity. We reasoned that high rates of shedding could result in apparently low cell-surface levels in the ELISA assay, so we repeated the ELISA assay with the addition of the metalloproteinase inhibitor BB-94. Indeed, IA-2 surface expression was substantially increased in the presence of BB-94 (Fig. S1), while surface levels of other receptors that exhibited constitutive signaling activity were not drastically affected by inhibitor treatment. This suggests that IA-2 undergoes much higher rates of proteolysis than the other proteins studied.

Since we observed variable cell-surface levels of the receptors in the ELISA assay, we also performed titrations of the chimeric receptors in the SNAPS signaling assay to ensure that high surface level expression was not masking proteolytic switch like behavior (Fig. S2). Most receptors showed decreasing signaling activity with

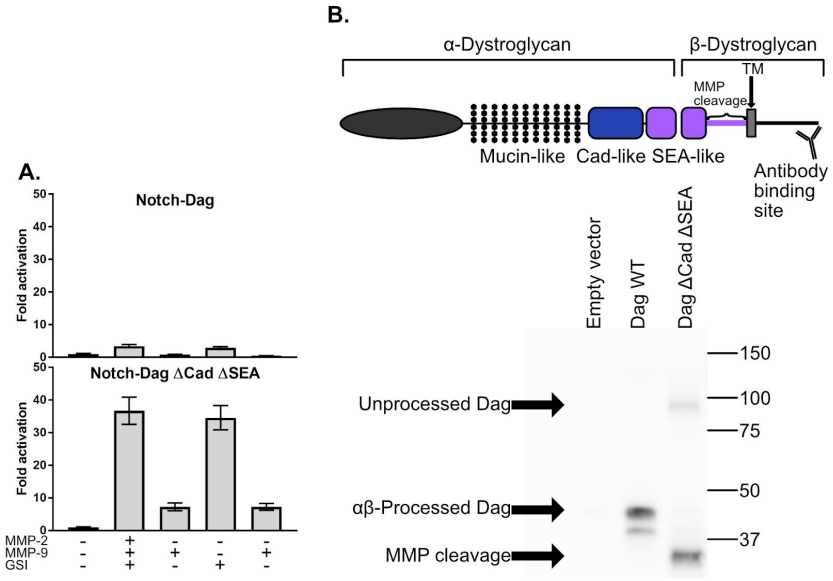

Fig. 2: The dystroglycan proteolytic switch is partially protected from treatment with exogenous MMPs (**A**) Luciferase reporter gene activity comparing dystroglycan and dystroglycan containing constitutive MMP sites (dag ΔCadΔSEA) Error bars represent the SEM of triplicate measurements (**B**) β-dystroglycan western blot of Cos7 cell lysates after transfection with empty vector, wild-type dystroglycan or dag ΔCadΔSEA. Bands for unprocessed, αβ-processed Dag, and MMP cleavage have been denoted.

decreasing concentration of receptor, as expected. Interestingly, IA-2 signal increased as receptor concentration decreased, perhaps related to its high expression levels and turnover rates.

The dystroglycan proteolytic switch is partially protected from treatment with exogenous MMPs

We next wanted to probe the observed proteolytic-switch behavior of dystroglycan more deeply and validate the assay. Dystroglycan is an adhesion receptor that provides a critical mechanical link between the ECM and the actin cytoskeleton to help muscle cells withstand contraction and neural cells maintain the blood brain barrier

(Barresi and Campbell 2006). It is post-translationally processed into two subunits, termed α- and β-dystroglycan in it's SEA-like domain, akin to mucins. Cleavage of β-dystroglycan by matrix metalloproteinases (MMPs) is greatly enhanced in pathogenic states, such as muscular dystrophy and cancer (Agrawal et al. 2006; Matsumura et al. 2005; Singh et al. 2004). SNAPS reveals that dystroglycan contains a putative Notch-like proteolytic switch, in which the conformation of a juxtamembrane domain controls access to protease sites. While the ADAM10 likely present in these experiments is known to cleave dystroglycan in the brain (Kuhn et al. 2016), MMPs have been shown to cleave dystroglycan in muscles and are upregulated in biopsies of patients with muscular dystrophy (Matsumura et al. 2005). Thus, we wanted to determine whether the protease site(s) were protected in the presence of the dystroglycan-relevant proteases MMP-2 and MMP-9. Since U2OS cells are not known to secrete high levels of MMPs (Geiger et al. 2012; Beck et al. 2011), we added exogenous MMP-2/MMP-9 and compared MMP proteolysis in the dystroglycan chimera containing the entire “proteolytic switch” domain or a chimera that only contained the 39 amino acids containing putative protease site(s) (ΔCadΔSEA). In this construct, the protease sites are expected to be constitutively exposed. Indeed, we saw that adding MMPs to the chimera containing the intact proteolytic switch resulted in a ~2-3 fold increase in signaling, while the chimera containing only protease site(s) exhibited up to a 30-fold higher protease sensitivity (Fig. 2A). The difference in sensitivity to MMP treatment of the two constructs supports our model that exposure of dystroglycan MMP sites is also controlled by the proteolytic switch domain. We also observed a similar

effect by adding MMP buffer alone, which contains MMP activator 4-aminophenylmercuric acetate (APMA) (Fig. S3).

To further validate that SNAPS is revealing aspects of dystroglycan biology, we transfected full-length dystroglycan and a mutant receptor with constitutively exposed protease sites (ΔCadΔSEA) in MMP expressing Cos7 cells, in which dystroglycan proteolysis has been previously studied (Herzog et al. 2004). We performed a Western blot on cell lysates using a β-dystroglycan antibody to determine if it is cleaved by proteases. As expected from the chimera signaling assays, wild-type dystroglycan shows no apparent 31-kDa cleavage product. However, a substantial increase in the protease cleaved 31-kDa fragment is observed for the mutant with constitutively exposed cleavage sites (DagΔCadΔSEA) compared to full-length dystroglycan with an intact proteolytic switch (Fig. 2B). These results suggest the proteolytic switch-like behavior may be relevant to regulation of dystroglycan's cleavage by MMPs in its native context, and that this assay can be used to further probe receptor biology.

Proteolysis of receptors lacking juxtamembrane SEA-like domains may also be controlled by exposure of the protease-site

We next wanted to determine whether SNAPS could be used to assess proteolytic regulatory mechanisms in receptors not containing SEA-like domains. Proteolysis plays a major role in the function of cell surface receptors such as E-cadherin, CD44, and in RTKs (Merilahti et al. 2017; Okamoto et al. 1999; Katayama et al. 1994), and dysregulation of proteolysis in these receptors is linked to many

diseases. We chose a diverse set of receptors to test in our chimeric Notch signaling assay (Fig. 3A). In this cohort, several receptors also showed a significant fold change when co-cultured with DLL4 expressing cells, suggesting some degree of protection of the protease sites. Most of the chimeras also exhibited higher basal levels of signaling

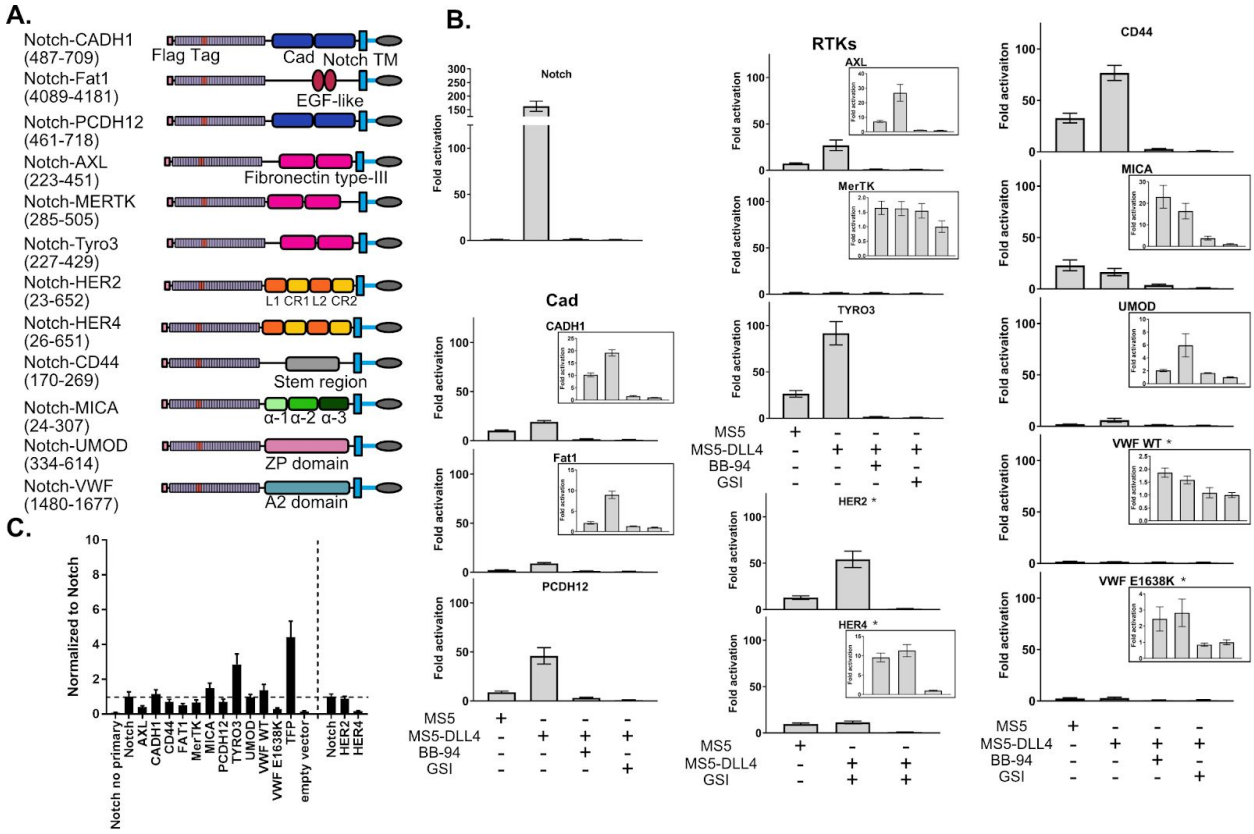

Fig. 3: Proteolysis of receptors lacking juxtamembrane SEA-like domains may also be controlled by exposure of the protease-site. (**A**) Chimera constructs for proteins without SEA domains. Protein domains are color coded and labeled. Abbreviations: AXL: Tyrosine-protein kinase receptor UFO. Cad: Cadherin. CADH1: E-cadherin. CR: Cysteine rich. EGF: Epidermal growth factor. Fat1: Protocadherin Fat 1. HER2: human epidermal growth factor receptor 2. HER4: Human epidermal growth factor receptor 4. L: Leucine-rich. MerTK: Tyrosine-protein kinase Mer. MICA: MHC class I polypeptide related sequence A. PCDH12: Protocadherin-12. Tyro3: Tyrosine-protein kinase receptor TYRO3. UMOD: Uromodulin. VWF: Von Willebrand Factor. ZP: zona pellucida (**B**) Luciferase reporter gene activity profile of Notch in comparison to the Notch chimera constructs co-cultured with MS5 cells or MS5 cells stably transfected with DLL4. Asterisked graphs denote experiments performed on different days and with 2ng DNA/well instead of 1ng/well of DNA. Both wild-type and mutated VWF luciferase

assays were performed with 100 ng DNA for each well. BB-94: Batimastat (pan-metalloproteinase inhibitor). GSI: Compound E (gamma-secretase inhibitor). RTK: receptor tyrosine kinase Error bars represent the SEM of triplicate measurements (**C**) Cell surface ELISA data of chimera constructs in comparison to Notch. 100 ng of each construct was transfected into U2OS cells in triplicate. Horizontal line depicts the level of Notch expression. A vertical line separates ELISAs that were performed on different days. Error bars represent the SEM of triplicate measurements.

than the SEA-like-containing proteins. Interestingly, most of the RTKs fell into the proteolytic switch class, as well as the extracellular matrix receptor CD44 and Protocadherin-12. E-cadherin exhibited a modest but very consistent 2-fold increase in ligand-induced signaling. The immune receptor MICA and RTK HER4 exhibited constitutive signaling in the assay. Interestingly, HER4 showed low surface expression in the ELISA that was significantly increased in the presence of BB-94 (Fig. S1), reminiscent of the SEA-like receptor IA-2. Several receptors exhibited low activity in the signaling assay (Fig. 3B) and either low (MERTK, Fat1) or normal surface expression (Umod) in the ELISA assay (Fig. 3C), suggesting defects in expression or lack of proteolysis, respectively. Similarly, neither wild-type or mutated von Willebrand factor (VWF) A2 domain chimeras, which have been previously tested as functional replacements of the Notch NRR in *Drosophila* (Langridge and Struhl 2017), signaled in the context in this assay. We again performed receptor titrations in the chimera signaling assay (Fig. S 2B and 2D) to ensure that proteolytic switch behavior was not being masked by high expression levels.

SNAPS can be used to screen for proteolysis modulators of HER2 and E-cadherin

We next reasoned that SNAPS could provide a powerful means to screen for receptor-specific modulators of proteolysis. For example, the Notch signaling assay was used to screen activating and inhibitory therapeutic antibodies targeting the Notch receptor (Li et al. 2008; Aste-Amézaga et al. 2010; Wu et al. 2010). The monoclonal antibody trastuzumab (Herceptin), used to treat HER2+ breast cancer (Pegram et al. 1998; Baselga et al. 1996)

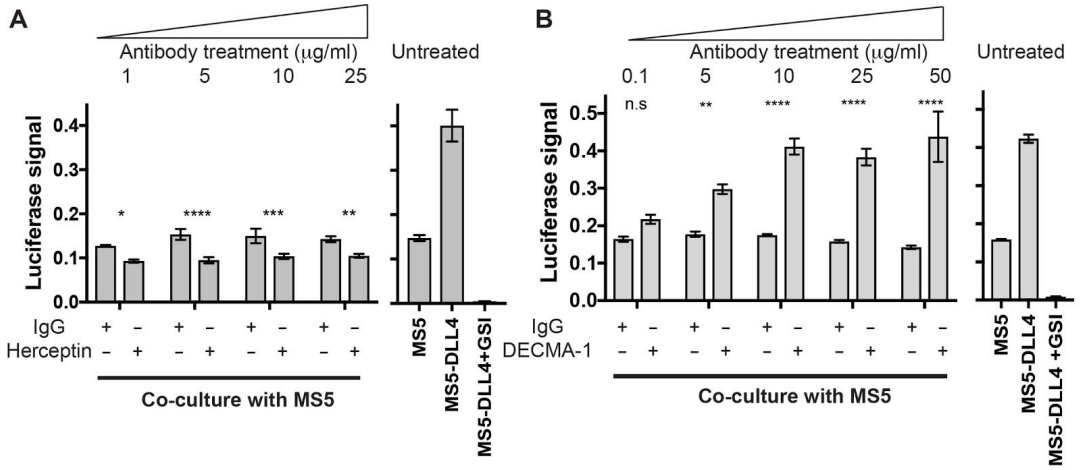

Fig. 4: SNAPS can be used to screen for proteolysis modulators of HER2 and E-cadherin. (**A**) Left panel shows effects of Herceptin on basal signaling of HER2-Notch chimeras (i.e. co-culture with MS5 cells). HER2-Notch chimera expressing cells co-cultured with MS5 cells were treated with 1-25 ug/ml Herceptin or IgG control. Right panel shows untreated cells in co-culture with MS5 or MS5-DLL4 cells +/- GSI for reference. Error bars represent the SEM of triplicate measurements (**B**) Left panel shows effects of DECMA-1 on basal signaling of Cad4-5-Notch chimeras. Cad4-5-Notch chimeras co-cultured with MS5 cells were treated with 0.1-50 ug/ml of DECMA-1 or IgG control. Right panel shows untreated chimera basal and ligand-induced proteolysis shown for reference. Error bars represent the SEM of triplicate measurements. Statistical significance was determined with a two-way ANOVA followed by a post hoc Bonferroni test. ****: $p<0.0001$, ***: $p<0.001$, **:$p<0.01$ *: $p<0.02$

(Pegram et al. 1998; Baselga et al. 1996), has been shown to block proteolysis of the HER2 receptor tyrosine kinase as part of its mechanism of action (Molina et al. 2001). Therefore, we tested whether Herceptin could modulate the basal proteolysis of HER2 observed in the Notch-HER2 chimeras, in which the Notch NRR is replaced with the ectodomain of HER2. We treated HER2-chimera expressing cells with increasing concentrations of Herceptin or an IgG control. We observed reproducible and statistically significant decreases in proteolysis in cells treated with Herceptin as compared to the IgG control (Fig. 4A). Proteolysis was reduced up to 40%. This robust effect demonstrates the potential utility of the chimeric assay in drug screening.

We next wanted to test an antibody with no previously reported effects on proteolysis. DECMA-1 is a function-blocking E-cadherin antibody known to break cell-cell contacts thereby decreasing cell adhesion. Treatment of a mouse breast cancer model with DECMA-1 was shown to reduce the number of tumors formed as well as the tumor weight (Sabine M. Brouxhon et al. 2013), highlighting its effectiveness of treating cancer in mice. However, the mechanism of breaking cell-adhesions has remained elusive; the antibody binds to E-cadherin at the interface of the last two cadherin repeats (EC4 and EC5) near the membrane, but the N-terminal repeats EC1 and EC2 are responsible for the homotypic interactions presumed to be disrupted by the antibody. We hypothesized that DECMA-1 might affect E-cadherin shedding since the antibody epitope maps to the “proteolysis region” of E-cadherin. In the absence of antibody or when treated with IgG control antibodies, the Notch-cadherin chimera, in which cadherin repeats EC4 and EC5 have replaced the Notch proteolytic switch,

displays a moderate level of constitutive proteolysis and a 2-fold increase in activity in response to DLL4 expressing cells (Fig. 4B). When the cells are treated with DECMA-1, we observe a dose-dependent increase in the basal level of signaling, in comparison to control antibody, almost to the level of ligand induced signaling. The apparent EC50 of DECMA-1 measured by the assay is ~0.8 ug/mL (Fig. S5). These data suggest that the mechanism of DECMA-1 breaking adhesive contacts could, in part, be due to increased shedding of the receptor from the membrane.

Discussion

The dysregulation of receptor proteolysis near the plasma membrane is common to many diseases and has prompted the development of therapeutics that modulate proteolysis, either by targeting the protease or access to the protease site for a given receptor. Major efforts to develop protease inhibitors have yielded modest results, as the high ratio of receptor substrates for a given protease leads to significant off-target effects. However, a few examples of receptor-specific modulators of proteolysis have proven successful. Antibodies specific to HER2, Notch, and MICA have effectively perturbed proteolysis of these receptors (Li et al. 2008; Aste-Amézaga et al. 2010; Wu et al. 2010; Tiyanont et al. 2013; Qiu et al. 2013; Agnusdei et al. 2014; Falk et al. 2012; Molina et al. 2001; Ferrari de Andrade et al. 2018). Development of additional receptor-specific modulators has been limited due to gaps in understanding of stimuli driving proteolysis and roles of proteolytic fragments for the majority of receptors, leading to a difficulty in both measuring and controlling activity.

We created SNAPS utilizing well-understood stimuli and responses of Notch signaling to probe proteolytic mechanisms of a range of cell-surface receptors. Using this assay, we find that Notch's mechanism of proteolytic regulation via conformational control of a cryptic protease site is not a unique phenomenon and is rather a potentially common mechanism of control for several SEA-like domain-containing receptors that share structural homology to Notch. Moreover, response by other transmembrane proteins such as HER2, AXL, CD44, and PCDH12 suggests that cryptic protease site exposure by the conformation of the receptor is potentially a common mechanism of

regulation. SNAPS can also be used to screen for modulators of proteolysis; we observe that Herceptin treatment causes significant decreases in basal proteolysis of HER2, while DECMA-1 treatment results in substantial increases in basal proteolysis of E-cadherin. These results reveal new mechanistic insights into DECMA-1's function in breaking cellular adhesions.

Cryptic protease site exposure in diverse receptors - structure function analysis

Notch's proteolytic switch has been exploited to develop conformation-specific modulatory antibodies and harnessed for synthetic biology applications to turn on transcription in response to any desired cell to cell contact (Li et al. 2008; Aste-Amézaga et al. 2010; Wu et al. 2010; Tiyanont et al. 2013; Qiu et al. 2013; Agnusdei et al. 2014; Falk et al. 2012). Thus we aimed to determine if other receptors utilize proteolytic switches in control exposure of protease sites. We observed that several receptors containing juxtamembrane SEA-like domains could functionally substitute for Notch, suggesting that these receptors may function as proteolytic switches to convey cellular stimuli.

We were struck by the fact that almost all of the receptors harboring SEA-like domains in tandem with neighboring domains showed a similar switch-like behavior in the intercellular signaling assay, despite having neighboring domains with very different predicted structural characteristics. In Notch, the neighboring LNR domain is disulfide-rich and binds calcium, with little to no secondary structure. Dystroglycan and the Protocadherins have neighboring cadherin-like domains, characterized by high

β-strand content, while EpCAM and Trop2 have a cysteine-rich thyroglobulin domain. The existing crystal structures of several of these domains also reveal differential modes of interaction with the SEA-like domain. For example, in the EpCAM and NRR structures, the neighboring domain contacts the α-helix in close proximity to the β-strand containing putative proteolytic sites. In contrast, the cadherin-like domain interacts with the opposite face of the SEA-like domain in the Protocadherin-15 structure. (Fig. S4). These different modes of interdomain interactions suggest that the proteolytic switches may have different propensities to “switch on” as well as potentially different requirements for direction of applied force. Future studies probing comparative anatomy of putative proteolytic switches may reveal whether the structural differences are a consequence of cellular context; e.g. receptor involved in intercellular versus ECM interactions. On the other hand, the SEA-like domains lacking structured neighboring domains exhibit constitutive signaling, likely due to a more dynamic domain where protease site exposure occurs more frequently.

Interestingly, many receptors with juxtamembrane domains where protease sites are not expected to be buried in a structured domain exhibited switch-like behavior. Secondary structure calculations of the CD44 juxtamembrane region predict an unstructured region, while the putative protease sites in E-cadherin, Protocadherin 12 and HER2 map to the region between the terminal structured repeat and the transmembrane domain. Thus other mechanisms of protease site occlusion besides intramolecular interactions must be considered for these receptors, such as dimerization or other intermolecular interactions.

Exposure of cryptic protease site may be a common mechanotransduction mechanism

In this study, mechanical force derived from intercellular contact is applied to cell-surface receptors to identify cryptic protease sites. While mechanical force may not play a role in the function of some receptors studied here, several of the receptors probed have been implicated in mechanosensing. Like Notch (Gordon et al. 2015), E-cadherin (Schwartz and DeSimone 2008) and protocadherin-15 are involved in intercellular adhesions and transmission of mechanical stimuli. Protocadherin-15, for example, is involved in sensing sound vibrations across stereocilia tip links in the process of hearing (Kazmierczak et al. 2007). Mechanical forces are also known to be sensed at adhesions of cells with the ECM, as ECM stiffness dictates multiple cellular processes such as cell migration (Lo et al. 2000) and stem cell differentiation (Engler et al. 2006). For example, the ECM receptor CD44 is hypothesized to sense the stiffness of the ECM resulting in increased cell migration (Kim and Kumar 2014; Razinia et al. 2017). Additionally, the ECM receptor dystroglycan is thought to act as a shock absorber to protect the sarcolemma during muscle contraction (Barresi and Campbell 2006). Finally, even receptors that do not reside at canonical force sensing structures of cells have been implicated in mechanosensing. The RTK AXL which binds to a secreted ligand Gas6 has been shown to be a rigidity sensor (Yang et al. 2016) and facilitate a decrease in cellular stiffness in lung cancer (Iida et al. 2017). Thus, our studies showing that many receptors exhibit increased proteolysis in response to mechanical forces

suggest that proteolysis may be a common mechanism used by cells to communicate mechanical stimuli. This assay could be used in the future to measure how varying the mechanical microenvironment affects receptor proteolysis.

Limitations/caveats of assay

In the chimeric signaling assay, putative regions of proteolysis are evaluated in the context of artificial linkages at their N- and C-termini as well as potentially non-native stimuli and non-physiological presentation of proteases. In most cases, a small region of a receptor was excised and inserted into a larger receptor, resulting in non-native links to Notch's ligand binding and transmembrane domains. One might expect the artificiality of the chimeras would result in a majority of chimeric receptors signaling either constitutively or not at all. However, several receptors exhibited "switch-like" behavior, underscoring the validity of SNAPS and the modular nature of cell-surface receptors. The use of the Notch transmembrane domain in the chimeric receptors also introduces some caveats as the domain, together with membrane proteins such as tetraspanins (Zimmerman et al. 2016), likely associates with the Notch membrane-tethered proteases ADAM10 and ADAM17. Though many of the chimeras studied have been reported to be cleaved by ADAM10 and ADAM17, some receptors may not typically reside in close proximity to these proteases and therefore not normally be cleaved. However, these proteases are upregulated in many diseases suggesting that the cleavage observed in this assay may be biologically relevant in certain cellular contexts. Finally, the chimeric Notch signaling assay provides a stimulus for exposing protease

sites involving a 2-5 pN force normal to the cell surface. While many of the receptors studied here are also involved in cell-cell contacts likely involving similar mechanical forces, many interact with the ECM or have soluble ligands and perhaps may not normally be exposed to mechanical allostery. The main goal, however, was to provide a means to determine the presence of cryptic protease sites regardless of mechanical sensitivity. Harnessing this assay to study proteolytic regulation mechanisms is more specific than using, for instance, APMA to non-specifically activate metalloproteinases. (Ogata, Itoh, and Nagase 1995; Stetler-Stevenson et al. 1989)

Conclusions

We have identified several putative proteolytic switches using SNAPS. These findings may drive development of conformation-specific modulatory antibodies as well as find use in synthetic biology applications that use cell to cell contact to drive transcriptional events; perhaps using a conformational switch domain that is less sensitive to cell-cell contact could be preferably be switched on when it encounters a stiffer tumor cell, for example. Our results provide a starting point for determining whether mechanisms of proteolytic regulation observed here are relevant to the biology of a given receptor. The convenient stimulus and response to proteolysis can be used to make additional chimeras to move closer to the native system and discover more about proteolytic regulation in the native receptor. For example, the luciferase response can be measured when systematically replacing chimeric domains with native transmembrane domains, ligand recognition domains, and intracellular tails. We also

demonstrate that this assay provides a convenient platform for evaluating modulators of proteolysis.

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Materials and Methods

Reagents

Recombinant DLL4, MMP-2, and MMP-9 were purchased from R&D Systems. Batimastat (BB-94) was purchased from Sigma Aldrich. Compound E (GSI) was purchased from Fisher Scientific (Catalog # AAJ65131EXD). DECMA-1 antibody was purchased from Sigma-Aldrich (U3254). U2OS cells were purchased from ATCC. MS5 and MS5-DLL4 cells were a kind gift from Dr. Stephen Blacklow. 4-aminophenylmercuric acetate (APMA) was purchased from Sigma-Aldrich. Herceptin was purchased from MedChemExpress (HY-P9907). β-dystroglycan antibody was purchased from Leica Biosystems (B-DG-CE)

Cloning

An Nhe1 site was added in Notch between amino acids 1735 and 1736 near the transmembrane region in a previously described Notch1-Gal4 construct (Andrawes et al. 2013) containing an N-terminal Flag tag, an AvrII site between the last EGF-like repeat and NRR, and a Bsu36i restriction site C-terminal to Notch transmembrane domain. All of the constructs were cloned using In-Fusion (Clontech).

CD44 was cloned using CD44S pWZL-Blast from Addgene (Item ID 19126). APP was cloned using pEGFP-n1-APP from Addgene (Item ID 69924). Dystroglycan was

cloned from cDNA from Origene (Cat#: SC117393). mTFP was cloned from TS module from Addgene (Item ID 26021). AXL, MerTK, and Tyro3 were originally ordered as *E.coli* optimized gBlocks (IDT) for different constructs and then cloned into the Notch chimera using primers with In-Fusion ends. HER2 and HER4 DNA was a kind gift from Dr. Laurie Parker, from the ORF kinase library (Addgene). The remaining constructs were ordered as mammalian codon optimized gBlocks from IDT with In-Fusion ends.

Cell culture

All cell lines were cultured in DMEM (Corning) supplemented with 10% FBS (Gibco) and 0.5% penicillin/streptomycin (Gibco). Cells were incubated at 37 °C in 5% CO_2.

Notch signaling assay

The Notch signaling assay was performed as described (Gordon et al. 2015). For co-culture assays, 0.1, 1, 2, or 10 ng chimera constructs were reverse transfected with reporter plasmids (50 ng Gal4 reporter plasmid and 1 ng PRL-TK reporter plasmid) in triplicate into U2OS cells in a 96-well plate. 24 hours post-transfection, MS5 cells or MS5 cells stably expressing DLL4 were plated on top of the U2OS cells with DMSO or drug (40 μM BB-94 or 1 μM GSI). 48 hours post-transfection, cells were lysed in passive lysis buffer (Promega). Lysate was added to a white 96 well half volume plate, and Dual-Luciferase Reporter Assay (Promega) was performed according to manufacturer's recommendation and read out on a Molecular Devices LMaxII^{384} plate reader.

In assays using recombinant MMP-2 and MMP-9, activated MMP-2 or MMP-9 was diluted to 0.46 μg/mL in D10 media and added 36 hours post-transfection. Media was swapped out 38 hours post-transfection. Cells were lysed in passive lysis buffer 50 hours post-transfection and read out as previously described. For signaling assays using antibody, DECMA-1, Herceptin, or Sheep IgG control was added during the co-culture step 24 hours post-transfection.

Cell surface ELISA

100 ng of Notch chimera constructs were transfected into U2OS cells in a sterile opaque tissue culture-treated 96 well plate (Corning 353296) in triplicate. 24 hours post-transfection, cells were washed once with PBS and fixed using 4% PFA (Thermo Fisher 28906) for 20 minutes, then washed three times with PBS. Cells were blocked in TBS+5% milk for 1 hour. Then, Flag primary antibody (Sigma-Aldrich F1804) was added 1:250 in TBS+5% milk for 2 hours. Cells were washed 3 times for 5 minutes each with TBS+5% milk. The cells were then incubated 1:10000 with an HRP secondary antibody for 1 hour before being washed 5 times for 5 minutes each with TBS. Chemiluminescent substrate was added for 1 minute before reading out on a luminescence plate reader.

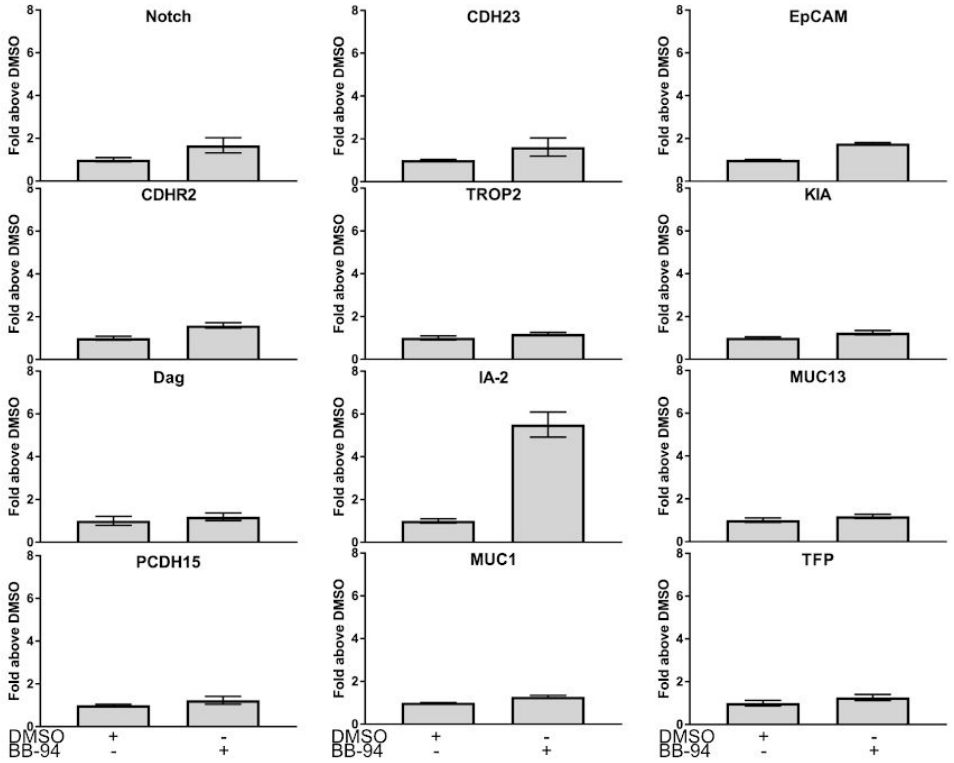
Western blot

48 hours post-transfection of dystroglycan constructs, Cos7 cells were lysed with RIPA buffer containing protease inhibitor cocktail. Lysates were run on a 4-20% SDS-PAGE gel with 2 mM sodium thioglycolate in the running buffer. The protein was

then transferred to a nitrocellulose membrane using a Genie Blotter (Idea Scientific) and blocked with 5% milk in TBS. β-dystroglycan antibody was diluted 1:1000 in TBS with 5% bovine serum albumin (BSA) added. A goat-anti mouse HRP conjugated antibody (Invitrogen) was used as a secondary antibody. Western blots were imaged using chemiluminescent buffer (Perkin Elmer Western Lightning Plus ECL) and the Amersham 600UV (GE) with staff support at the University of Minnesota-University Imaging Center.

Supplementary Information

A.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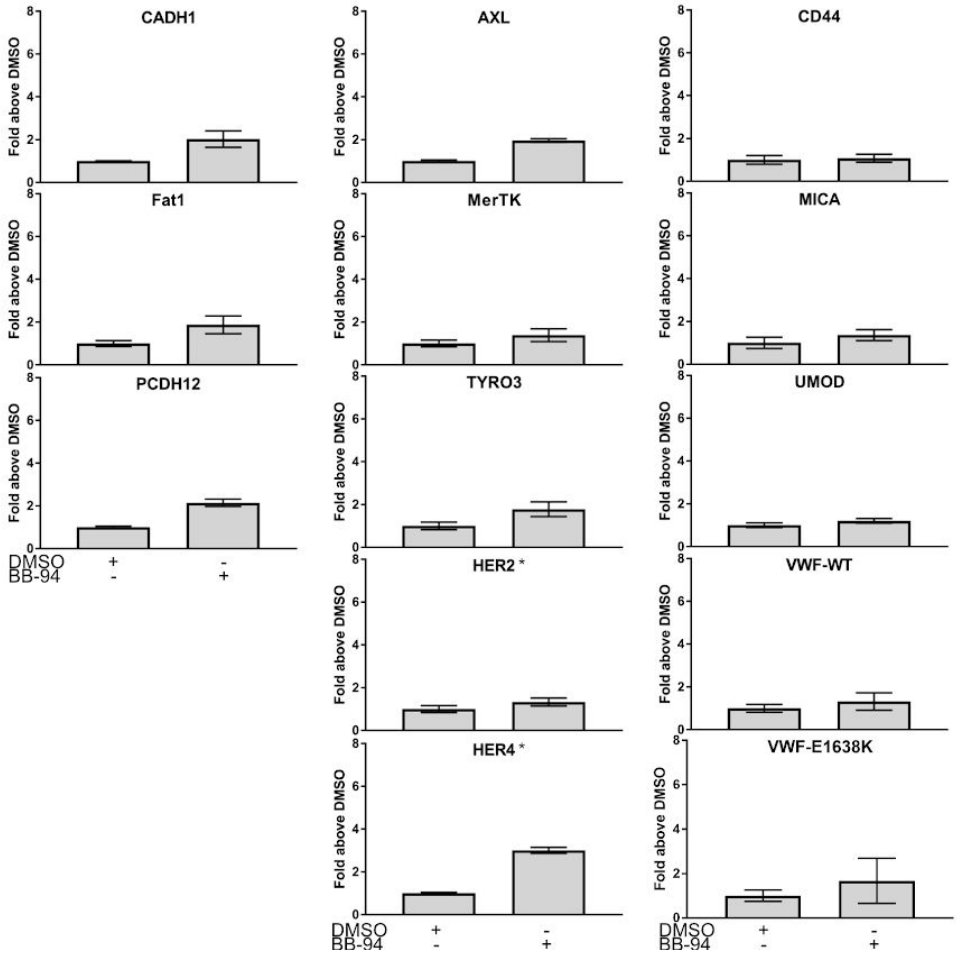
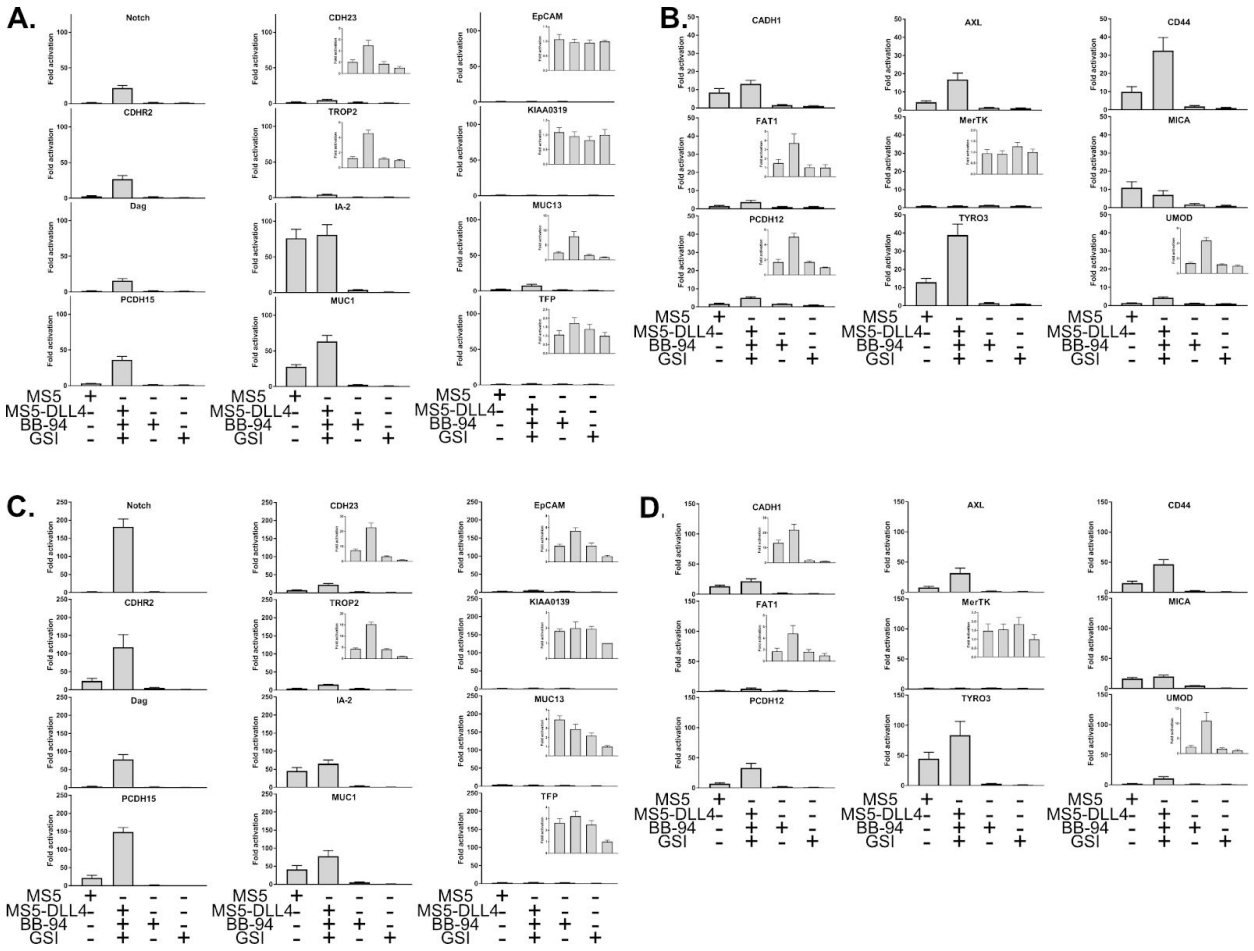
CDH23
EpCAM
CDHR2
TROP2
KIA
Dag
IA-2
MUC13
PCDH15
MUC1
TFP
Fold above DMSO
DMSO + -
BB-94 - +

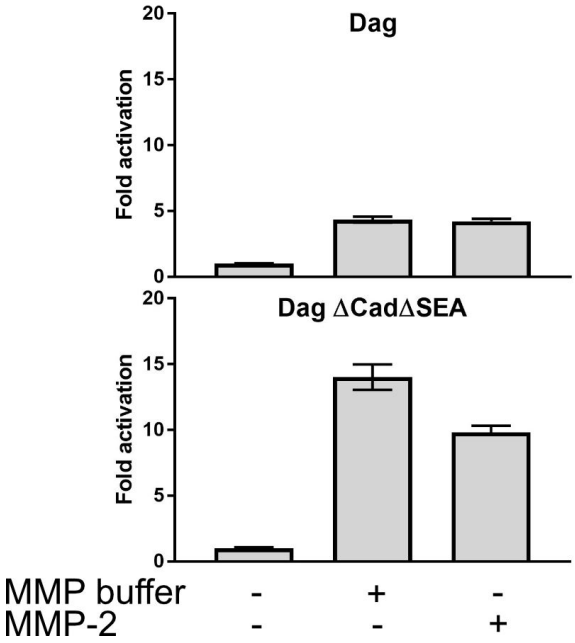
B.


CADH1
AXL
CD44
Fat1
MerTK
MICA
PCDH12
TYRO3
UMOD
HER2 *
VWF-WT
HER4 *
VWF-E1638K
Fold above DMSO
DMSO + -
BB-94 - +

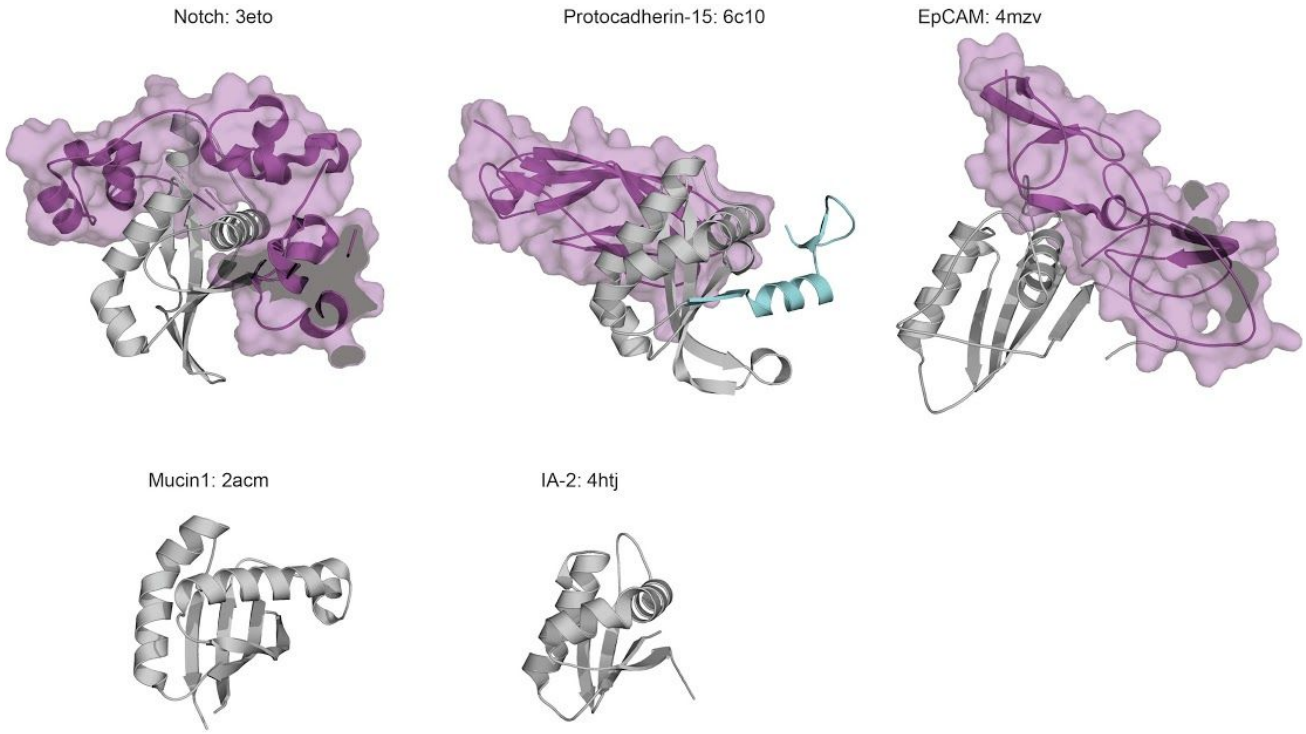
Supplementary figure 1: ELISA in the presence of BB-94 Cell surface ELISA performed with DMSO (negative control) or BB-94 (pan-metalloproteinase inhibitor). Data is normalized to the signal of DMSO condition. Error bars represent the SEM of triplicate measurements. A) SEA domains. B) Non-SEA domains. Asterisks denote cell surface ELISA was performed on a different date.



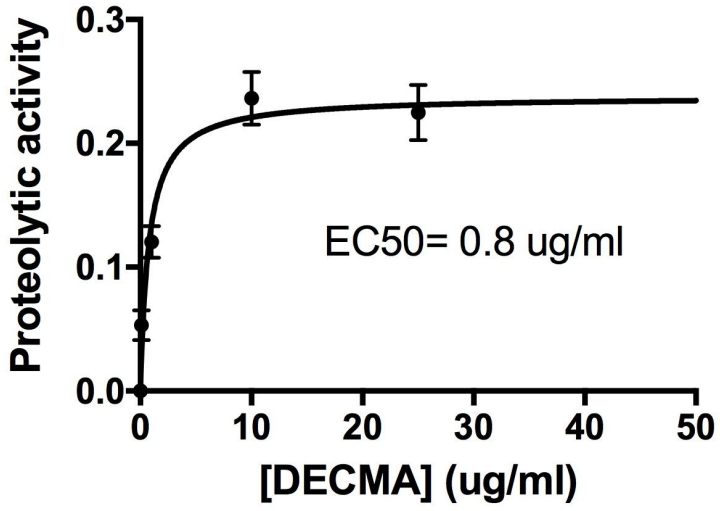
Supplementary Figure 2: Titration of DNA used in co-culture assay. Luciferase reporter gene activity profile of Notch in comparison to the Notch chimera constructs co-cultured with MS5 cells or MS5 cells stably transfected with DLL4. BB-94=pan-metalloproteinase inhibitor GSI=γ-secretase inhibitor. Error bars represent the SEM of triplicate measurements. A) and B) 0.1 ng DNA per well. C) and D) 10 ng DNA per well.



Supplementary figure 3: Comparison of MMP with MMP buffer. Notch chimera signaling assay using either activated MMP-2 or MMP buffer containing APMA. Error bars represent the SEM of triplicate measurements.



Supplementary figure 4: Comparison of known structures of SEA-like domains. Structures of SEA-like domains (grey) with any adjacent domains (purple) with their PDB IDs after their names. SEA-like domains were structurally aligned to the Notch SEA-like domain using pairwise alignment tools Dali-lite or FATCAT. Note that Mucin-1 and IA-2's SEA domains do not have any predicted adjacent domain.



Supplementary figure 5: EC50 calculation for DECMA-1. The dose-dependent proteolytic activity enhancement of DECMA-1 was calculated by first subtracting the IgG control luciferase signal from the DECMA-1 signal at each concentration. The data was fit to a single exponential using Prism, including outlier detection.

Supplementary Table 1: Sequences for all proteolysis domains used

Protein	Uniprot ID	"Proteolysis domain" residue numbers	Amino acids
AXL	P30530	223-451	VLPQQPRNLHLVSRQPTELEVAW TPGLSGIYPLTHCTLQAVLSNDGM GIQAGEPDPPEEPLTSQASVPPH QLRLGSLHPHTPYHIRVACTSSQG PSSWTHWLPVETPEGVPLGPPEN ISATRNGSQAFVHWQEPRAPLQG TLLGYRLAYQGQDTPEVLMDIGLR QEVTLELQGDGSVSNLTVCVAAY

			TAAGDGPWSLPVPLEAWRPGQA QPVHQLVKEPSTPAFSWPWW
CADH1	P12830	487-709	VPPEKRVEVSEDFGVGQEITSYTA QEPDTFMEQKITYRIWRDTANWL EINPDTGAISTRAELDREDFEHVK NSTYTALIIATDNGSPVATGTGTLL LILSDVNDNAPIPEPRTIFFCERNP KPQVINIIDADLPPNTSPFTAELTH GASANWTIQYNDPTQESIILKPKM ALEVGDYKINLKLMDNQNKDQVTT LEVSVCDCEGAAGVCRKAQPVEA GLQIPAI
CD44	P16070	170-269	PSNPTDDDVSSGSSSERSSTSGG YIFYTFSTVHPIPDEDSPWITDSTD RIPATRDQDTFHPSGGSHTTHESE SDGHSHGSQEGGANTTSGPIRTP QIPEW
CDH23	Q9H251	2729-3066	SPQYQLLTVPEHSPRGTLVGNVT GAVDADEGPNAIVYYFIAAGNEEK NFHLQPDGCLLVLRDLDREREAIF SFIVKASSNRSWTPPRGPSPTLDL VADLTLQEVRVVLEDINDQPPRFT KAEYTAGVATDAKVGSELIQVLAL DADIGNNSLVFYSILAIHYFRALAN DSEDVGQVFTMGSMDGILRTFDL FMAYSPGYFVVDIVARDLAGHNDT AIIGIYILRDDQRVKIVINEIPDRVRG FEEEFIHLLSNITGAIVNTDNVQFH VDKKGRVNFAQTELLIHVVNRDTN RILDVDRVIQMIDENKEQLRNLFRN YNVLDVQPAISVRLPDDMSALQ
CDHR2	Q9BYE9	310-1154	VNDNPPTLDVASLRGIRVAENGSQ HGQVAVVVASDVDTSAQLEIQLVN ILCTKAGVDVGSLCWGWFSVAAN GSVYINQSKAIDYEACDLVTLVVR ACDLATDPGFQAYSNNGSLLITIED

			VNDNAPYFLPENKTFVIIPELVLPN REVASVRARDDDSGNNGVILFSIL RVDFISKDGATIPFQGVFSIFTSSE ADVFAGSIQPVTSLDSTLQGTYQV TVQARDRPSLGPFLEATTTLNLFT VDQSYRSRLQFSTPKEEVGANRQ AINAALTQATRTTVYIVDIQDIDSAA RARPHSYLDAYFVFPNGSALTLDE LSVMIRNDQDSLTQLLQLGLVVLG SQESQESDLSKQLIS
Dag	Q14118	490-749	GGEPNQRPELKNHIDRVDAW VGTYFEVKIPSDTFYDHEDTTTDK LKLTLKLREQQLVGEKSWVQFNS NSQLMYGLPDSSHVGKHEYFMHA TDKGGLSAVDAFEIHVHRRPQGD RAPARFKAKFVGDPALVLNDIHKKI ALVKKLAFAFGDRNCSTITLQNITR GSIVVEWTNNTLPLEPCPKEQIAG LSRRIAEDDGKPRPAFSNALEPDF KATSITVTGSGSCRHLQFIPVVPP RRVPSEAPPTEVPDRDPEKSSED DV
EpCAM	P16422	24-265	QEECVCENYKLAVNCFVNNNRQC QCTSVGAQNTVICSKLAAKCLVMK AEMNGSKLGRRAKPEGALQNND GLYDPDCDESGLFKAKQCNGTST CWCVNTAGVRRTDKDTEITCSER VRTYWIIIELKHKAREKPYDSKSLR TALQKEITTRYQLDPKFITSILYENN VITIDLVQNSSQKTQNDVDIADVAY YFEKDVKGESLFHSKKMDLTVNG EQLDLDPGQTLIYYVDEKAPEFSM QGLK
Fat1	Q14517	4089-4181	LSPYCKDEPCKNGGTCFDSLDGA VCQCDSGFRGERCQSDIDECSGN PCLHGALCENTHGSYHCNCSHEY RGRHCEDAAPNQYVSTPWNIGLA E

HER2	P04626	25-652	TQVCTGTDMKLRLPASPETHLDM LRHLYQGCQVVQGNLELTYLPTN ASLSFLQDIQEVQGYVLIAHNQVR QVPLQRLRIVRGTQLFEDNYALAV LDNGDPLNNTTPVTGASPGGLRE LQLRSLTEILKGGVLIQRNPQLCYQ DTILWKDIFHKNNQLALTLIDTNRS RACHPCSPMCKGSRCWGESSED CQSLTRTVCAGGCARCKGPLPTD CCHEQCAAGCTGPKHSDCLACLH FNHSGICELHCPALVTYNTDTFES MPNPEGRYTFGASCVTACPYNYL STDVGSCTLVCPLHNQEVTAEDG TQRCEKCSKPCARVCYGLGMEHL REVRAVTSANIQEFAGCKKIFGSL AFLPESFDGDPASNTAPLQPEQL QVFETLEEITGYLYISAWPDSLPDL SVFQNLQVIRGRILHNGAYSLTLQ GLGISWLGLRSLRELGSGLALIHH NTHLCFVHTVPWDQLFRNPHQAL LHTANRPEDECVGEGLACHQLCA RGHCWGPGPTQCVNCSQFLRGQ ECVEECRVLQGLPREYVNARHCL PCHPECQPQNGSVTCFGPEADQ CVACAHYKDPPFCVARCPSGVKP DLSYMPIWKFPDEEGACQPCPINC THSCVDLDDKGCPAEQRASPLT
HER4	Q15303	26-651	QSVCAGTENKLSSLSDLEQQYRA LRKYYENCEVVMGNLEITSIEHNR DLSFLRSVREVTGYVLVALNQFRY LPLENLRIIRGTKLYEDRYALAIFLN YRKDGNFGLQELGLKNLTEILNGG VYVDQNKFLCYADTIHWQDIVRNP WPSNLTLVSTNGSSGCGRCHKSC TGRCWGPTENHCQTLTRTVCAEQ CDGRCYGPYVSDCCHRECAGGC

			SGPKDTDCFACMNFNDSGACVTQ CPQTFVYNPTTFQLEHNFNAKYTY GAFCVKKCPHNFVVDSSSCVRAC PSSKMEVEENGIKMCKPCTDICPK ACDGIGTGSLMSAQTVDSSNIDKF INCTKINGNLIFLVTGIHGDPYNAIE AIDPEKLNVFRTVREITGFLNIQSW PPNMTDFSVFSNLVTIGGRVLYSG LSLLILKQQGITSLQFQSLKEISAG NIYITDNSNLCYYHTINWTTLFSTIN QRIVIRDNRKAENCTAEGMVCNHL CSSDGCWGPGPDQCLSCRRFSR GRICIESCNLYDGEFREFENGSICV ECDPQCEKMEDGLLTCHGPGPD NCTKCSHFKDGPNCVEKCPDGLQ GANSFIFKYADPDRECHPCHPNC TQGCNGPTSHDCIYYPWTGHSTL PQHARTP
IA-2	Q16849	449-575	SPLGQSQPTVAGQPSARPAAEEY GYIVTDQKPLSLAAGVKLLEILAEH VHMSSGSFINISVVGPALTFRIRHN EQNLSLADVTQQAGLVKSELEAQ TGLQILQTGVGQREEAAAVLPQTA HSTSPMR
KIAA0319	Q5VV43	729-955	RARAGGRHVLVLPNNSITLDGSRS TDDQRIVSYLWIRDGQSPAAGDVI DGSDHSVALQLTNLVEGVYTFHLR VTDSQGASDTDTATVEVQPDPRK SGLVELTLQVGVGQLTEQRKDTLV RQLAVLLNVLDSDIKVQKIRAHSDL STVIVFYVQSRPPFKVLKAAEVAR NLHMRLSKEKADFLLFKVLRVDTA GCLLKCSGHGHCDPLTKRCICSHL WMENLIQRYIWDGESNCEWS

MerTK	Q12866	285-505	PPTEVSIRNSTAHSILISWVPGFDG YSPFRNCSIQVKEADPLSNGSVMI FNTSALPHLYQIKQLQALANYSIGV SCMNEIGWSAVSPWILASTTEGAP SVAPLNVTVFLNESSDNVDIRWMK PPTKQQDGELVGYRISHVWQSAG ISKELLEEVGQNGSRARISVQVHN ATCTVRIAAVTRGGVGPFSDPVKI FIPAHGWVDYAPSSTPAPGNADP VLII
MICA	Q29983	24-307	EPHSLRYNLTVLSWDGSVQSGFL TEVHLDGQPFLRCDRQKCRAKPQ GQWAEDVLGNKTWDRETRDLTG NGKDLRMTLAHIKDQKEGLHSLQE IRVCEIHEDNSTRSSQHFYYDGEL FLSQNLETKEWTMPQSSRAQTLA MNVRNFLKEDAMKTKTHYHAMHA DCLQELRRYLKSGVVLRRTVPPM VNVTRSEASEGNITVTCRASGFYP WNITLSWRQDGVSLSHDTQQWG DVLPDGNGTYQTWVATRICQGEE QRFTCYMEHSGNHSTHPVPSGKV LVLQSHW
MUC1	P15941	961-1158	ASGSASGSASTLVHNGTSARATT TPASKSTPFSIPSHHSDTPTTLASH STKTDASSTHHS SVPPLTSSNHSTSPQLSTGVSFFF LSFHISNLQFNSSLEDPSTDYYQE LQRDISEMFLQIYKQGGFLGLSNIK FRPGSVVVQLTLAFREGTINVHDV ETQFNQYKTEAASRYNLTISDVSV SDVPFPFSAQSGAGVPG
MUC13	Q9H3R2	173-421	PSNPCQDDPCADNSLCVKLHNTS FCLCLEGYYYNSSTCKKGKVFPG KISVTVSETFDPEEKHSMAYQDLH SEITSLFKDVFGTSVYGQTVILTVS TSLSPRSEMRADDKFVNVTIVTILA

			ETTSDNEKTVTEKINKAIRSSSSNF LNYDLTLRCDYYGCNQTADDCLN GLACDCKSDLQRPNPQSPFCVAS SLKCPDACNAQHKQCLIKKSGGA PECACVPGYQEDANGNCQKCAF GYSGLDCKDKFQL
Notch1	P46531	1449-1739	YSFGGGAGRDIPPPLIEEACELPE CQEDAGNKVCSLQCNNHACGWD GGDCSLNFNDPWKNCTQSLQCW KYFSDGHCDSQCNSAGCLFDGFD CQRAEGQCNPLYDQYCKDHFSD GHCDQGCNSAECEWDGLDCAEH VPERLAAGTLVVVVLMPPEQLRNS SFHFLRELSRVLHTNVVFKRDAHG QQMIFPYYGREEELRKHPIKRAAE GWAAPDALLGQVKASLLPGGSEG GRRRRELDPMDVRGSIVYLEIDNR QCVQASSQCFQSATDVAAFLGAL ASLGSLNIPYKIEAVQSETVEPPPP AQLH
PCDH12	Q9NPG4	461-718	EKSRYEVSTRENNLPSLHLITIKAH DADLGINGKVSYRIQDSPVAHLVAI DSNTGEVTAQRSLNYEEMAGFEF QVIAEDSGQPMLASSVSVWVSLL DANDNAPEVVQPVLSDGKASLSV LVNASTGHLLVPIETPNGLGPAGT DTPPLATHSSRPFLLTTIVARDADS GANGEPLYSIRSGNEAHLFILNPH TGQLFVNVTNASSLIGSEWELEIV VEDQGSPPLQTRALLRVMFVTSV DHLRDSARKPGALSMSMLT
PCDH15	Q96QU1	1037-1376	QEEYRPPPVSELATKGTMVGVISA AAINQSIVYSIVSGNEEDTFGINNIT GVIYVNGPLDYETRTSYVLRVQAD SLEVVLANLRVPSKSNTAKVYIEIQ DENNHPPVFQKKFYIGGVSEDAR MFTSVLRVKATDKDTGNYSVMAY RLIIPPIKEGKEGFVVETYTGLIKTA

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TFP	n/a	n/a	MVSKGEETTMGVIKPDMKIKLKME GNVNGHAFVIEGEGEGKPYDGTN TINLEVKEGAPLPFSYDILTTAFAY GNRAFTKYPDDIPNYFKQSFPEGY SWERTMTFEDKGIVKVKSDISMEE DSFIYEIHLKGENFPPNGPVMQKK TTGWDASTERMYVRDGVLKGDV KHKLLLEGGGHHRVDFKTIYRAKK AVKLPDYHFVDHRIEILNHDKDYN KVTVYESAVARNSTDGMDELYK
TROP2	Q8BGV3	27-274	HTAAQDNCTCPTNKMTVCSPDGP GGRCQCRALGSGMAVDCSTLTSK CLLLKARMSAPKNARTLVRPSEHA LVDNDGLYDPDCDPEGRFKARQC NQTSVCWCVNSVGVRRTDKGDL SLRCDELVRTHHILIDLRHRPTAGA FNHSDLDAELRRLFRERYRLHPKF VAAVHYEQPTIQIELRQNTSQKAA GDVDIGDAAYYFERDIKGESLFQG RGGLDLRVRGEPLQVERTLIYYLD EIPPKFSMKRLT
Tyro3	Q06418	227-429	APFNITVTKLSSSNASVAWMPGAD GRALLQSCTVQVTQAPGGWEVLA VVVPVPPFTCLLRDLVPATNYSLR VRCANALGPSPYADWVPFQTKGL APASAPQNLHAIRTDSGLILEWEE VIPEAPLEGPLGPYKLSWVQDNGT QDELTVEGTRANLTGWDPQKDLI VRVCVSNAVGCGPWSQPLVVSS HDRAGQQGPPHSRTS

Uromod	P07911	334-614	ECGANDMKVSLGKCQLK SLGFDKVFMYLSDSRCSGFNDRD NRDWVSVVTPARDGPCGTVLTRN ETHATYSNTLYLADEIIIRDLNIKINF ACSYPLDMKVSLKTALQPMVSAL NIRVGGTGMFTVRMALFQTPSYT QPYQGSSVTLSTEAFLYVGTMLD GGDLSRFALLMTNCYATPSSNAT DPLKYFIIQDRCPHTRDSTIQVVEN GESSQGRFSVQMFRFAGNYDLVY LHCEVYLCDTMNEKCKPTCSGTR FRSGSVIDQSRVLNLGPITRKGVQ ATVS
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