Conformational regulation of proteolysis in the extracellular matrix receptor dystroglycan

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Abstract:

The adhesion receptor dystroglycan provides a critical mechanical link between the extracellular matrix (ECM) and the actin cytoskeleton to help muscle cells withstand contraction and neural cells maintain the blood brain barrier. Disrupting the link results in diseases such as cancer and muscular dystrophy. Proteolysis of dystroglycan by Matrix Metalloproteases (MMPs) also breaks the mechanical link and is amplified in several pathogenic states. We use a combination of biochemical, cellular and, signaling assays to show that dystroglycan proteolysis is conformationally regulated by an extracellular, juxtamembrane "proteolysis domain", comprised of tandem Ig-like and SEA-like domains. The intact proteolysis domain is resistant to MMP cleavage, but structurally-disruptive disease-related mutations or domain truncations sensitize dystroglycan to proteolysis. Moreover, increased dystroglycan proteolysis correlates with faster cell migration, suggesting a role for abnormal proteolysis in disease pathogenesis. Finally, we find that the dystroglycan proteolysis domain can functionally substitute for the Notch proteolytic switch in the context of a Notch signaling assay, hinting at a role for mechanical force in regulating dystroglycan proteolysis.

Introduction:

Dystroglycan is an extracellular matrix (ECM) receptor that is typically expressed in the membrane of cells that adjoin basement membranes, such as epithelial, neural, and muscle cells and plays important roles during development and adult homeostasis (Barresi and Campbell 2006). Dystroglycan's most well-understood function in cells is to provide a mechanical link between the extracellular matrix and the cytoskeleton (Michele et al. 2003). In its function as a core member of the dystrophin-glycoprotein-complex (DGC) in muscle cells, dystroglycan provides a critical anchor between the ECM and the actin cytoskeleton to protect the sarcolemma of muscle cells from the forces of muscle contraction (Petrof et al. 1993; Ervasti 2007). Similarly, dystroglycan expressed on astrocyte endfeet in the brain links to the basement membrane of endothelial cells lining blood vessels to help maintain the blood brain barrier (Agrawal 2006). Thus, loss of functional dystroglycan or breaking of the mechanical link it provides is generally detrimental to cells. Whole animal dystroglycan deletion leads to embryonic lethality (Williamson et al. 1997), and conditional knockouts in muscle and brain tissues result in drastic tissue disorganization (Cohn et al. 2002; Moore et al. 2002). Moreover, mutation or deletion of glycosyltransferases that post-translationally modify dystroglycan breaks the link between dystroglycan and the ECM, leading to subtypes of muscular dystrophy, cancer, and brain disorders (Mercuri and Muntoni 2012; Bonnemann et al. 2014). Similarly, mutations and truncations of dystrophin, the adaptor protein that connects the intracellular portion of dystroglycan to actin, lead to Duchenne Muscular Dystrophy (Hoffman, Brown, and Kunkel 1987; Cohn and Campbell 1997; Straub and Campbell 1997).

However, dystroglycan's link to the ECM must be dynamically modulated in contexts where cellular plasticity is required, such as cell migration, wound repair, or function of neural

synapses. One such mechanism of modulation of dystroglycan's adhesion to the ECM is post-translational cleavage by matrix metalloproteases (MMPs), which has been shown to normally be involved in processes such as synaptic plasticity (Pribiag et al. 2014;Michaluk et al. 2007; Meighan et al. 2006; Nagy et al. 2006). MMPs are Zn^{2+}-dependent endopeptidases with many known physiological substrates. Therefore, a delicate balance between proteolysis and adhesion must exist to avoid the negative outcomes associated with breaking the mechanical link important for muscle cell and blood brain barrier integrity. However, enhanced proteolysis of dystroglycan has been associated with many pathogenic states, such as cancer (Singh et al, 2004, Losasso et al, 2000, Jing et al, 2004), muscle diseases (Yamada et al. 2001, Matsumura et al. 2005), and autoimmune disorders (Agrawal et al. 2006), suggesting that aberrant proteolysis may contribute to pathogenic mechanisms. Indeed, treatment of mice exhibiting muscular dystrophy phenotypes with MMP inhibitors ameliorates symptoms(Kumar, Bhatnagar, and Kumar 2010; Li et al. 2009, Hindi et al. 2013). Moreover, MMP2/MMP9 double-knockout mice in which MMP cleavage of dystroglycan is blocked are resistant to the onset of autoimmune encephalomyelitis, underscoring the relevance of this cleavage in disease pathogenesis (Agrawal et al. 2006).

Dystroglycan (DG) is encoded by the *Dag1* gene and translated as a single polypeptide precursor that undergoes post-translational processing to generate two non-covalently interacting subunits at the cell-surface: the extracellular and highly glycosylated α-DG that binds laminin and other ligands in the ECM and the transmembrane β-DG that binds to the cytoskeleton via adaptor proteins such as dystrophin (Ervasti and Campbell 1991; Ibraghimov-Beskrovnaya et al. 1992). The non-covalent association of α- and β-DG occurs within a putative Sea urchin-Enterokinase-Agrin-like (SEA-like) domain, located extracellularly between the ECM-binding and the transmembrane regions (Akhavan et al. 2008). Importantly, this SEA-like domain also houses the predicted MMP cleavage site(s) and harbors one of the two patient-

derived muscular dystrophy mutations (C669F) identified in dystroglycan thus far (Geis et al. 2013). Intriguingly, the SEA-like domain bears striking structural homology to the heterodimerization domain (HD) of Notch receptors, which houses Notch's critical ADAM protease site that is responsible for Notch activation. In Notch, the neighboring N-terminal Lin-12-Notch (LNR) domain forms a structural unit with the HD domain to conformationally protect the cleavage site (Gordon et al. 2007) from its protease until ligand engagement(Kovall et al. 2017, Gordon et al. 2015). Motivated by the incidence of abnormal proteolysis of dystroglycan in pathogenic contexts, the putative structural homology to the proteolytic switch domain in Notch, and the presence of disease-related mutations in this domain (Geis et al. 2013, Hara et al. 2011, Jayanisha et al. 2003), we present here a study of the regulation of proteolysis in the extracellular juxtamembrane region of dystroglycan. Through a series of biochemical, cellular, and signaling assays, we show that dystroglycan proteolysis is conformationally regulated, that tuning levels of dystroglycan proteolysis has substantial effects on cell migration phenotypes, and that the proteolysis domain of dystroglycan can functionally substitute for Notch's proteolytic switch in the context of intercellular forces.

Results

Dystroglycan α/β-processing requires both Ig-like and SEA-like domains

In order to understand how dystroglycan proteolytic cleavage is regulated, we first aimed to map the boundaries of the extracellular juxtamembrane domain that houses both the putative MMP cleavage site(s) ~25 amino acids from the membrane and the α/β-processing site defining the α/β dystroglycan transition located ~50 amino acids from the membrane (Bozzi et al. 2009, Yamada et al 2001). Previous studies of mucin SEA domains have revealed that the post-translational autoproteolytic α/β-processing event can only occur if the domain is correctly folded, due to the necessity of a precisely strained loop to permit the serine in the cleavage

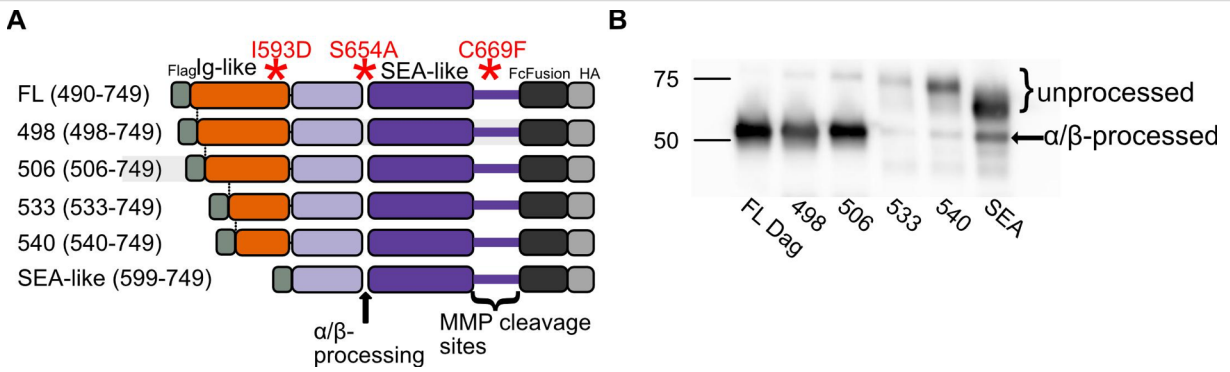


Figure 1. Dystroglycan α/β-processing requires both Ig-like and SEA-like domains (a) Infographic of constructs used to map dystroglycan proteolysis domain. Constructs comprised of Ig-like (orange) and SEA-like (purple) domains contain N-terminal FLAG and C-terminal HA epitope tags as well as a C-terminal Fc-fusion. Sites of α/β-processing, MMP cleavage, and disease-related mutations (red asterisks) are depicted. **(b)** α-HA western blot comparing unprocessed and α/β-processed protein levels in dystroglycan truncation constructs from (a) secreted into conditioned media and immunoprecipitated with Protein A beads.

sequence to nucleophilically attack the neighboring glycine (Macao et al 2006) . Thus, we aimed to use SEA-domain α/β-processing as an indication of correct protein folding to map the minimal functional unit of dystroglycan's proteolysis domain and devised a series of constructs including the SEA-like domain and its neighboring Ig-like domain. Dual-epitope tagged full-length and truncation constructs of dystroglycan's proteolysis domain were designed using predicted secondary structure as a guide and secreted into conditioned media of mammalian cells. The construct also included a C-terminal Fc-Fusion tag to enable immunoprecipitation with Protein A beads. (Fig 1a)

The protein was enriched from the conditioned media with protein A beads and Western blotted for the C-terminal HA tag. Two potential α-HA-tagged protein species were expected: a band of ~50 kDa corresponding to the correctly α/β-processed C-terminus for all of the constructs and a variable-sized larger band corresponding to the full-length unprocessed protein. The construct containing the entire Ig-like domain in addition to the SEA-like domain showed a dominant 50 kDa band and faint 75 kDa band, reflecting >90% α/β-processing. (Fig 1b). However, the N-terminal truncation constructs exhibited an increasing ratio of unprocessed to processed

dystroglycan, and the construct containing solely the SEA-like domain showed the lowest degree of α/β-processing. These data suggest that the Ig-like domain folds in concert with the SEA-like domain to support dystroglycan α/β-processing. We will term the portion of dystroglycan comprised of the full Ig-like and SEA-like domains the dystroglycan "proteolysis domain."

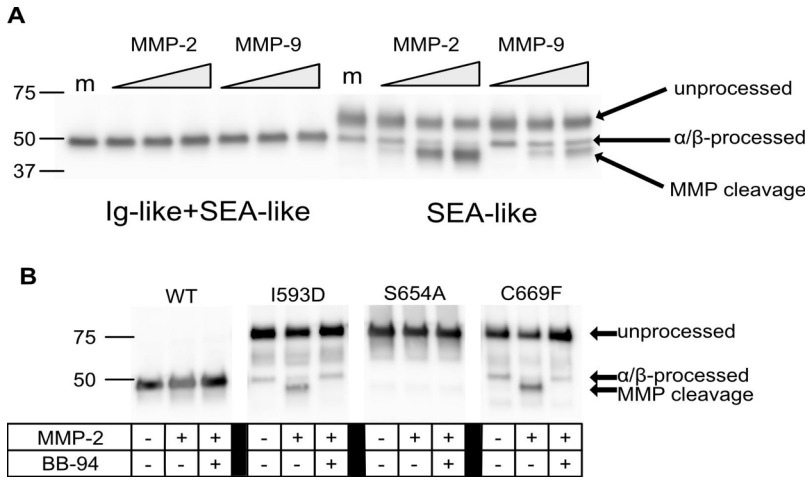


Figure 2. MMP cleavage of the dystroglycan proteolysis domain is conformationally regulated. (**a**) α-HA western blot of full length dystroglycan proteolysis domain (Ig-like and SEA-like domains) versus SEA-like domain only. Increasing concentrations of activated MMP-2 and MMP-9 were reacted in MMP buffer with protein secreted from conditioned media and immunoprecipitated with Protein A beads. m= MMP buffer only. (**a**) α-HA western blot of secreted dystroglycan proteolysis domains harboring disease mutations. Reactions were performed in MMP buffer and 0.73 μg/ml activated MMP-2 and 40 μM BB-94 were added as noted.

MMP cleavage of the dystroglycan proteolysis domain is conformationally regulated

We next wanted to ask whether the MMP cleavage, predicted to occur ~20 amino acids external to the transmembrane domain (Bozzi, M et al. 2009; Sbardella et al. 2012), was conformationally regulated in the context of the isolated proteolysis domain, as in Notch receptors. To study the accessibility of dystroglycan's proteolytic site(s) to exogenous MMPs, full-length and SEA-like domain constructs were expressed in HEK-293T cells as dual-epitope tagged secreted proteins as in the mapping studies above. The immunoprecipitated dystroglycan constructs were incubated with increasing amounts of activated MMP-2 and MMP-9 for 1 hour, eluted from the beads, and Western blotted against the HA tag. Upon addition of exogenous MMPs, the full-length dystroglycan proteolysis domain remains a single band on the α-HA western blot while a faster-migrating species appears for the SEA-like domain alone construct (Fig 2a). This suggests that the MMP site is conformationally

exposed when the Ig-like domain is not present, likely due to incorrect folding of the domain, which is consistent with the truncation studies in Figure 1b.

Since protection of the MMP site in the context of isolated proteolysis domains relies on the correct conformation of the domain, we next wanted to test whether mutations associated with muscle disease found in humans and model organisms alter the conformation of the domain and expose the protease site. To date, three disease-related mutations within the dystroglycan proteolysis domain are associated with muscular dystrophy, but how they contribute to disease pathogenesis is unclear. I593D, a mutation located in the Ig-like domain, leads to a muscular dystrophy phenotype in zebrafish (Gupta et al. 2011). The S654A mutation blocks α/β-processing and has been observed to cause a muscular dystrophy phenotype in mice (Jayasinha et al. 2003). Finally, the C669F mutation located near the C-terminus of the SEA-like domain, which disrupts a putative disulfide linkage, was discovered in patients with a severe form of muscular dystrophy (Geis et al. 2013). It should first be noted that decreased α/β-processing is observed for the three mutations (Fig. 2b). While expected for the S654A mutant in which the cleavage site is abolished, the incorrect processing of C669F and I593D suggests that they disrupt the structure of the domain, much like the truncation constructs described in Figures 1b and 2a. Indeed, addition of exogenous MMPs to proteolysis domains harboring the I593D and C669F mutations resulted in the appearance of an MMP-cleaved species that was abrogated by the addition of batimastat (BB-94), a global metalloprotease inhibitor (Fig 2b). In contrast, previous structural work of mucin SEA domains has shown that mutating the α/β-processing cleavage site as in S654A does not impact the overall fold of the SEA domain (Macao et al. 2006). Thus, the S654A cleavage mutant is not cleaved by MMPs, suggesting an intact proteolysis domain. This effect was further observed in double mutants where α/β-processing was fully blocked by creating I593D/S654A and C669F/S654A constructs,

suggesting that the metalloprotease sensitivity is not caused solely by decreased α/β-processing in these disease mutations (Supp Fig 1).

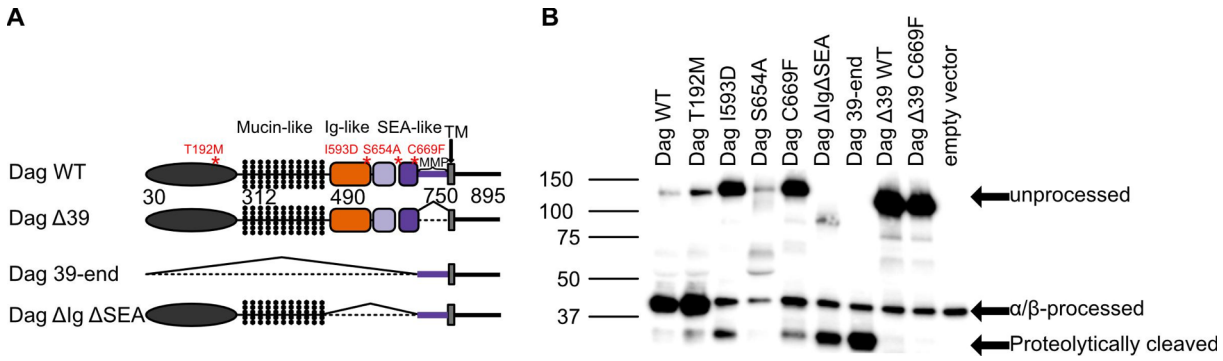


Figure 3: Presence of the 31-kDa B-dystroglycan proteolytic fragment depends on the structural integrity of the proteolysis domain. (a) Schematic of full-length dystroglycan constructs. Red asterisks denote location of disease mutations. Dashed lines denote domain deletions **(b**) β-dystroglycan western blot of full-length dystroglycan constructs transiently transfected in Cos7 cells. Full-length β-dystroglycan migrates at 42 kDa while proteolytically-cleaved β-dystroglycan migrates at 31 kDa. T192M, I593D, and C669F disease-related mutations and deletion of the entire proteolysis domain save putative protease sites (ΔIgΔSEA show a 31 kDa proteolysis product. Deletion of protease sites Δ39 in the context of C669F abolishes 31 kDa band. The 39-end construct provides an approximate size marker for the expected proteolytic fragment.

The 31-kDa β-dystroglycan proteolytic fragment also depends on the structural integrity of the proteolysis domain

We next asked whether the MMP resistance phenotype of the wild type proteolysis domain and the proteolysis sensitive phenotype observed in truncation and muscular dystrophy disease mutants was recapitulated in the full-length receptors. Full-length dystroglycan constructs (Fig 3A) were transiently transfected into Cos7 cells (Fig 3B), HEK293T, and U2OS cells (Supp Fig 2). Cell lysates were Western blotted for β-dystroglycan. Full-length wild-type dystroglycan undergoes normal α/β-processing and no appreciable lower molecular weight species are observed, as expected. In contrast, the I593D and C669F mutations both disrupt α/β-processing and a new 31 kDa fragment appears, which corresponds to the size of β-dystroglycan that is cleaved by MMPs. To confirm that this band corresponds to a proteolytic fragment of the correct

size, we expressed a construct (Dag 39-end) beginning 39 amino acids outside of the transmembrane approximately corresponding to the expected MMP-cleaved species that migrated similarly to the observed 31 kDa fragment. To further test that the new species corresponds to proteolytic events near the C-terminus of the SEA-like domain and is not just an artifact of overexpression, we created a construct DagΔ39 in which the putative MMP cleavage sites were deleted (removal of amino acids 711-749 directly before the transmembrane domain). When this deletion is present in the context of the C669F mutation, the 31 kDa species is abolished (Fig 3B). Moreover, in the context of the construct DagΔIgΔSEA that removes all of the proteolysis domain except amino acid 711-749 in which one might expect constitutively exposed proteolysis sites, a large fraction of β-dystroglycan is further proteolyzed into the 31 kDa species. A laminin-overlay assay confirms that the C669F mutant exhibits similar glycosylation levels to wildtype (Supp Fig2B).

Modulation of Dystroglycan proteolysis Alters Cell Migration

Thus far, we have observed proteolysis of dystroglycan that depends on the structural integrity of the "proteolysis domain." Wild-type receptors and receptors where putative cleavage sites have been deleted are resistant to cleavage while domain truncations and disease-related mutations sensitize dystroglycan to proteolytic cleavage. To determine if this proteolysis is relevant to dystroglycan's critical roles in the cell, we next asked whether modulating dystroglycan proteolysis results in cellular phenotype changes. Dystroglycan mutations/truncations used above were used to modulate levels of proteolysis and determine effects on cell migration in a wound-healing assay. Constructs were transiently transfected into 3T3-L1 fibroblast cells and U251 glioblastoma cells (Fig. 4 and Supp Fig 3), and a scratch was introduced after 24 hours. Migrating cells were imaged at different timepoints up to 18 hours post-scratch, and the wound area was calculated. Analysis of three areas of the wound in

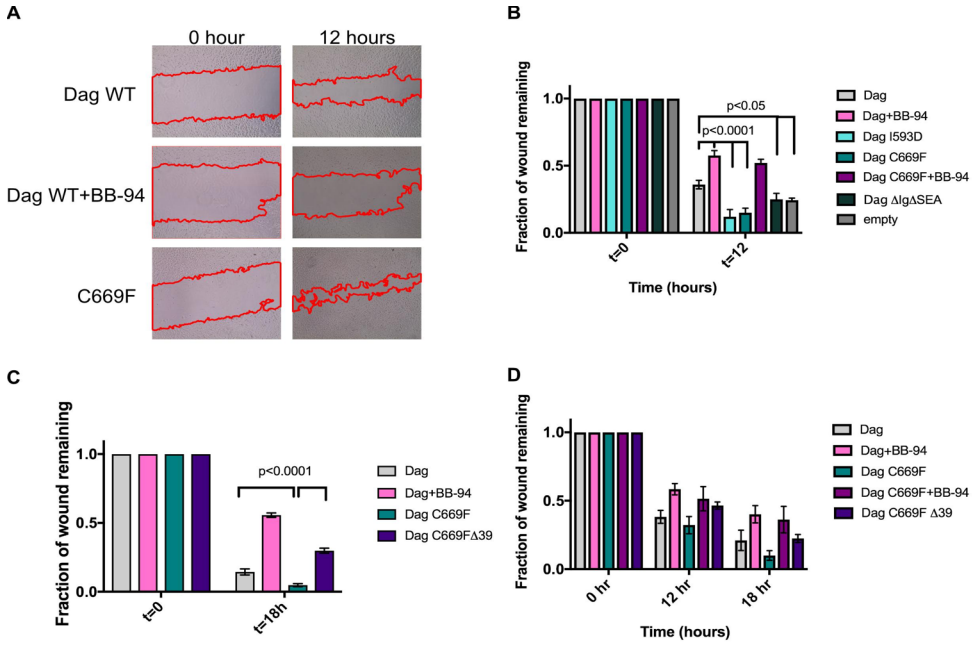


Figure 4. Modulation of dystroglycan proteolysis alters cell migration. (**a**) Representative brightfield images of wounds scratched in 3T3 cells transiently transfected with dystroglycan constructs at t=0 hours and t=12 hours. Red outline denotes wound edges. (**b-d**) Bar graphs denoting fraction of wounds remaining for dystroglycan constructs or empty vector at different timepoints. Wound areas were calculated for 8-12 images for each construct at each timepoint, averaged, and normalized to wound area at t=0. Error bars represent the SEM of 8-12 measurements, and statistical significance was determined with a two-way ANOVA followed by a post hoc Bonferroni test in Graphpad Prism. (**b,c**) Wound healing assays performed on different days in 3T3 cells. (**d**) Representative wound healing assay performed in U251 cells.

duplicate wells at each time point were performed and averaged, and consstent results were obtained over at least four separate experiments performed on different days. Representative images of wounds for Dag WT, Dag + BB-94 and Dag C669F in U251 cells and 3T3-L1 are shown in in Figure 4A and supplementary figure 3, respectively. Over-expression of wild-type dystroglycan in 3T3 cells slightly slowed down cell migration (Fig. 4B). Treating the wild type-overexpressing cells with BB-94 significantly slowed down wound-healing, suggesting that broadly blocking proteolytic cleavage in cells alters the balance of adhesion versus migration to favor adhesion. On the other hand, cells transfected with disease-related mutations C669F, I593D, and the truncation mutant ΔIgΔSEA consistently migrated at a faster rate than wild-type, suggesting that the alterations of the proteolysis domain that enhance proteolysis favor cellular

migration at the expense of adhesion. Treating C669F expressing cells with BB-94 rescued the enhanced migration phenotype.

Since global metalloprotease inhibitors are not specific and are likely blocking proteolysis of other receptors in the cells, we also tested Dag Δ39 constructs in which the putative MMP sites are deleted. The deletion of the putative proteolytic cleavage sites rescues the effect of the C669F mutation on migration, as cells migrate similarly to wild-type (Fig 4C). This result suggests that proteolytic cleavage of C669F near the C-terminus of the proteolysis domain alters a cell's migration phenotype to favor faster migration/decreased adhesion and furthermore suggests that abnormal proteolysis of dystroglycan in the context of disease mutations or other alterations in the cellular microenvironment may present a relevant mechanism of disease pathogenesis. The migration phenotypes were consistent in U251 glioblastoma cells (Fig 4D).

Dystroglycan functions as a Notch-like proteolytic switch in the context of intercellular signaling assays

We have thus far observed that dystroglycan is proteolytically cleaved near the C-terminus of the proteolysis domain when mutations or truncations disrupt the integrity of the domain. However, in the majority of studies reporting enhanced cleavage in pathogenic states, dystroglycan is not mutated and the stimuli in the cellular microenvironment that induce abnormal proteolysis are unknown. Potential factors are missing components of the DGC, altered chemical or mechanical properties of the extracellular matrix, or increased concentrations of MMPs. Thus, we next developed a synthetic system to ask if wild-type dystroglycan proteolysis could be switched on in an assay with a known stimulus and a sensitive readout. Such an assay would provide first steps toward teasing apart cellular stimuli which might act to reveal dystroglycan's conformationally-occluded proteolysis site. We turned

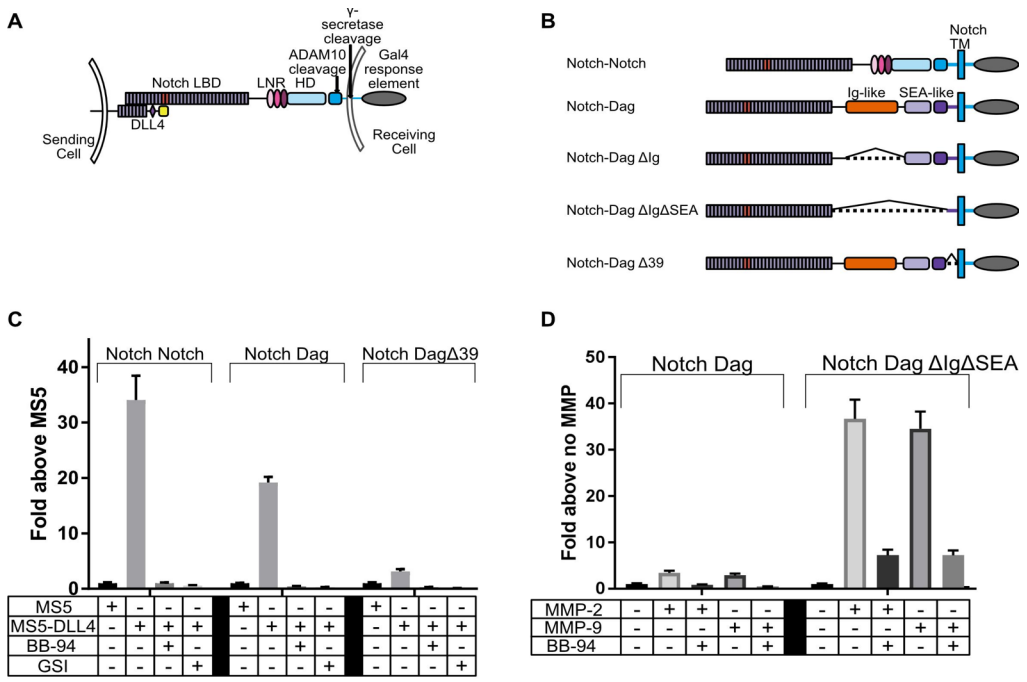


Figure 5. Dystroglycan proteolysis domain functions as a Notch-like proteolytic switch in the context of intercellular signaling assays. (**a**). Schematic of standard Notch-signaling luciferase assay. Cells co-expressing a Notch-Gal4 chimera and luciferase reporter constructs are co-cultured with DLL4 ligand-expressing cells. Luciferase emission correlates with Notch activation. DLL-4=Delta-like ligand 4. LBD=Ligand binding domain. LNR: Lin-12 Notch-like repeats. HD=heterodimerization domain (**b**) Schematic of chimeric constructs utilized in the signaling assay. Dashed lines denote domain deletion. (**c**) Luciferase reporter gene activity profile of Notch in comparison to the Notch-Dag and Notch-DagΔ39 chimeras co-cultured with ligands BB-94=pan-metalloprotease inhibitor GSI=gamma-secretase inhibitor (**d**) Ligand-independent luciferase reporter gene activity data for Notch-Dag and Notch- Dag ΔIg ΔSEA in the presence of activated MMP-2 and MMP-9 showing partial protection of the intact dystroglycan proteolysis domain in the presence of exogenous MMPs.

to the Notch signaling pathway as a well-known example of conformationally controlled proteolysis to develop our assay. Exposure of Notch's occluded proteolytic site within its NRR proteolysis domain is induced by mechanical forces derived from contact with ligand on a neighboring cell (Gordon et al. 2015). Standard Notch signaling assays utilize cells co-expressing Notch receptors containing a C-terminal Gal4 domain and luciferase reporter constructs (Fig. 4A). These are then co-cultured with cells expressing Notch ligands. When the Notch proteolytic cascade is triggered by ligand engagement, the Gal4-containing intracellular domain translocates to the nucleus, binds to Gal4-response elements upstream of luciferase,

and turns on a luciferase signal. Signaling is routinely blocked using BB-94 and an inhibitor of the subsequent intramembrane gamma-secretase cleavage event (GSI).

To test whether wild-type dystroglycan's proteolysis domain can act like a proteolytic switch akin to the Notch NRR, we engineered chimeras in which Notch's negative regulatory region (NRR) is replaced with dystroglycan's proteolysis domain (Fig 4B). In the Notch signaling assay, luciferase activity is stimulated in the Notch-Dag chimera in the only in presence of MS5 cells expressing the Notch ligand DLL4, not in MS5 control cells (Fig. 4C). As in Notch, the signal is abrogated by the protease inhibitors BB-94 and GSI. These results suggest that dystroglycan proteolysis can be “turned-on” in the context of the Notch signaling assay, and that the dystroglycan proteolysis domain can functionally replace the Notch NRR. Replacing the Notch NRR with a control domain mTFP did not exhibit proteolytic switch behavior. (Supplementary Figure 4) When 39 amino acids containing the putative MMP cleavage sites were deleted in Notch-DagΔ39, the luciferase signal was drastically decreased, suggesting that proteolysis in this region is responsible for the effect, though we cannot say whether it is MMPs or one of the Notch proteases that is cleaving in this context.

We next wanted to take advantage of the sensitivity of the luciferase readout in this assay to begin to determine how variables associated with pathogenic states affect conformational switching. Since MMPs are known to be upregulated in many disease contexts including muscular dystrophy and EAE, we asked whether adding increasing concentrations of MMPs in the absence of ligand caused increased proteolysis. We tested the Notch-Dag chimera as well as the Notch-DagΔIgΔSEA mutant, in which the protease site(s) are expected to be constitutively exposed. While the chimera containing the wild-type dystroglycan proteolysis domain showed ~2-3 fold increase in signal when various concentrations of MMP-2/MMP-9 were added, the chimera with putative exposed protease sites exhibited up to 30-fold higher

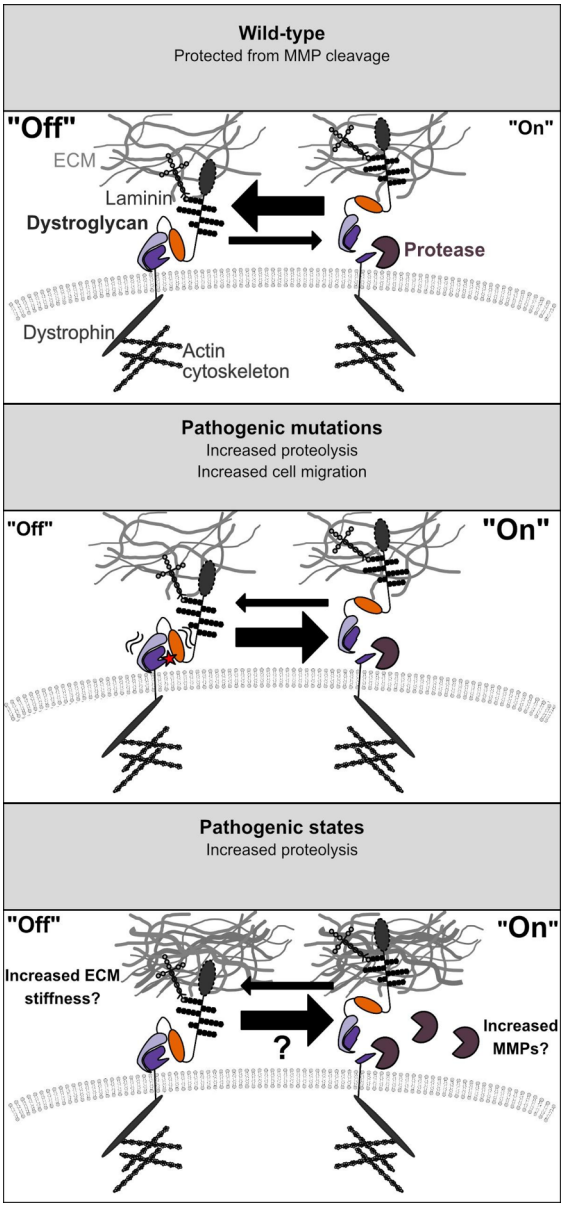


Figure 6: Model for conformational control of dystroglycan proteolysis in wild-type and mutated dystroglycan. Wild-type dystroglycan provides an important mechanical connection between the ECM and actin cytoskeleton. Mutations, such as C669F, destabilize the proteolysis domain, rendering it sensitive to proteolysis and increasing cell migration rates. Cellular factors characterizing pathogenic states, such as increased ECM stiffness and increased MMPs, may also increase dystroglycan proteolysis rates and contribute to pathogenicity.

protease sensitivity (Fig. 5D). This result reinforces that the wild-type dystroglycan protease sites are conformationally protected and that increased MMP concentrations in the environment do not have a drastic effect on its protease sensitivity. We can now use this assay to make additional chimeras e.g. adding in the native Dag ECM binding domains to begin to explore how the ECM composition may affect proteolytic switching .

DISCUSSION:

The adhesion receptor dystroglycan provides a critical mechanical link between the ECM and the actin cytoskeleton, most notably in muscle and brain cell types. In muscle, dystroglycan is at the core of the multi-protein DGC, acting as a shock-absorber to protect muscle cells from contractile forces. In the brain, dystroglycan organizes laminin in the basal lamina to maintain the blood brain barrier as well as provide the cortical infrastructure necessary for neuronal migration. When dystroglycan binding to the ECM or to the actin-adaptor dystrophin is

impaired, the mechanical link is disrupted and diseases such as muscular dystrophy and cancer arise(Barresi and Campbell 2006).

The ECM to cytoskeleton anchor that dystroglycan provides can also be disrupted by proteolytic cleavage of dystroglycan by MMPs just outside of the transmembrane domain, and several lines of evidence suggest a role for abnormal dystroglycan proteolysis in disease pathogenesis. While observed at low levels in normal contexts, proteolytic cleavage of β-dystroglycan to a 31 kDa species is enhanced in several pathogenic states, such as muscle diseases (Yamada et al. 2001, Matsumura et al, 2005), cancer (Singh et al, 2004, Losasso et al, 2000, Jing et al, 2004), and experimental autoimmune encephalomyelitis(Agrawal et al. 2006). The cleavage has been shown to be blocked by metalloprotease inhibitors (Singh et al, 2004). Moreover, double knockout MMP-2/MMP-9 mice are protected from the stimuli that induce EAE, a model for autoimmune diseases normally characterized by high levels of MMPs and increased dystroglycan proteolysis. Additionally, high levels of MMPs are found in biopsies of muscular dystrophy patients (Nadarajah et al, 2011), and muscular dystrophy phenotypes are ameliorated in mice treated with metalloprotease inhibitors(Li et al. 2009). Thus understanding how proteolysis is regulated will provide critical insights into dystroglycan's multiple functions as a mechanical link and adhesion receptor in the cell and may also provide insights disease pathogenesis.

Our results show that dystroglycan proteolysis is conformationally regulated in a manner similar to Notch proteolysis by ADAM proteases. Dystroglycan contains a Notch-like proteolytic switch sandwiched between its ECM-binding and transmembrane domains. *In vitro* studies of secreted proteins reveal that tandem predicted Ig-like and SEA-like domains cooperate to support the non-covalent complex formed from α/β-processing during receptor maturation, which we term the "proteolysis domain". This domain in isolation is resistant to exogenous

metalloproteases, but becomes susceptible to proteolysis when the domain is truncated or encodes disease-related mutations, much like in Notch receptors (Fig 6) (Sanchez-Irizarry et al. 2004; Malecki et al. 2006). We tested a series of mutations and truncations of the proteolysis domain to evaluate dystroglycan's propensity for proteolysis in the context of the full-length receptor to the frequently observed 31-kDa product and found that disease-related mutations or truncation of the majority of the proteolysis domain resulted in robust cleavage, while deletion of the putative protease sites prevented proteolysis. We next exploited the tunable proteolytic sensitivity of these constructs to interrogate effects of dystroglycan proteolysis on cellular migration phenotypes and found that mutants that lead to increased proteolysis result in faster migration phenotypes while mutants that block proteolysis are linked to slower migration phenotypes similar to effects of pan-metalloprotase inhibitors. This effect was observed in both human fibroblast and glioblastoma cells.

First, our results reveal that enhanced proteolysis may provide a potential mechanism of pathogenesis in patients harboring the C669F mutation, which has been observed in two siblings (Geis et al. 2013). These patients exhibit severe muscle-eye-brain phenotypes and neuronal migration defects, symptoms that are on the severe end of the phenotypic spectrum of congenital muscular dystrophies.

Moreover, the fact that increased proteolysis generally has drastic effects on cell migration suggests that dystroglycan proteolysis may play a role in disorders where the receptor is not mutated. The finding of an increased migration phenotype when proteolysis disrupts the dystroglycan mechanical link is similar to previous studies in cancer cell lines in which increased cell migration was observed when dystroglycan was hypo-glycosylated and thus defective in binding the ECM. (de Bernabé et al. 2009). Interestingly, hypo-glycosylated and/or reduced dystroglycan expression has been associated with more metastatic cancers (de Bernabé et al. 2009; Lossasso et al. 2000; Sgambato et al. 2003, Henry et al. 2001). Thus re-expressing

deficient glycosyltransferases, which restores dystroglycan linkage to the ECM and rescues the fast migration phenotype, is being pursued as a current therapeutic avenue (Yu et al. 2013).

Moreover, overexpression of dystroglycan in breast cancer cells reduces tumorigenicity (Muschler et al. 2002), hinting that restoring broken dystroglycan links by blocking proteolysis could be a therapeutic option. Our study provides a rationale and platform for developing specific inhibitors of dystroglycan proteolysis. Since dystroglycan proteolysis is conformationally controlled as in Notch receptors, it may be possible to develop conformation-specific antibodies that clamp the domain in a closed conformation to prevent proteolysis, a strategy which has been successfully employed in Notch receptors (Li et al. 2008; Wu et al. 2010).

These results also beg further investigations into the cellular stimuli leading to aberrant proteolysis. Many alterations in the cellular microenvironment occur in disease states. For example, a common feature in muscular dystrophy is fibrosis, which is characterized by excess deposition of ECM molecules. Moreover, the ECM of cancer cells is often stiffer than that of normal cells (Plodinec et al. 2012). Such alterations in the ECM may alter the balance of dystroglycan proteolysis and thus the adhesion/migration balance. We describe a synthetic signaling assay in which we utilize the normal stimulus and response of Notch signaling to show that the dystroglycan proteolysis domain can be converted from a protease-resistant to protease-sensitive state, analogous to Notch receptors. Our assay provides a platform for future studies to systematically test factors such as ECM alterations in the cellular microenvironment that lead to enhanced proteolysis during disease pathogenesis. The fact that the dystroglycan proteolysis domain can functionally substitute for Notch's proteolytic switch hints that mechanical force may be a factor in regulation of dystroglycan proteolysis, as it is mechanical force derived from cell to cell contact that drives Notch proteolysis (Gordon et al 2015). Finally, the finding that dystroglycan can act as a proteolytic switch could also be useful

in the context of synthetic biology where Notch proteolytic switching is currently used to enhance specificity of T-CARS (Roybal et al. 2016).

Acknowledgements:

We would like to thank the Odde lab at the University of Minnesota for the U251 cells and the Titus lab for the COS7 cells. We would like to thank Jim Ervasti and Kevin Campbell for helpful discussions and Eric Aird for useful comments on this manuscript. This study was supported by an NIH NIGMS R35GM119483 grant to WRG. ANH received salary support from AHA Fellowship 17PRE33330000 and is an ARCS Fellow. WRG is a Pew Biomedical Scholar.

Experimental Procedures

Materials

Antibodies that were used in this study- Flag antibody: Sigma, HA antibody: Covance, β-Dystroglycan antibody against 15 amino acids at the extreme C-terminus: Leica Biosystems. Restriction enzymes were purchased from New England Biolabs (NEB). Cloning was performed using In-Fusion HD Cloning Mix (Clontech). All electrophoresis supplies were purchased from Bio-Rad. Dual Luciferase kit was purchased from Promega. MMP-2 and MMP-9 were purchased from R&D Systems. Mutations within dystroglycan's proteolysis domain were created using Pfu Turbo Polymerase (Agilent) and DpnI (NEB). Laminin: from mouse Engelbreth-Holm-Swarm from Sigma (**L2020),** Anti-Laminin antibody: polyclonal rabbit antibody from Sigma (L9393)

Construct creation

Full length human dystroglycan cDNA in CMV-6 plasmid was obtained from Origene and used as a template for all of the cloning.

For expression of secreted proteolysis domain: Ig-like+SEA-like domain (amino acids 490-749) and SEA-like domain (599-749) constructs were cloned into pcDNA3 vector containing an N-terminal Notch signal sequence and Flag tag and C-terminal Fc-fusion and HA tags.

For signaling assay: A version of Notch containing an N-terminal Flag tag, restriction sites around the Notch NRR, a restriction site after the Notch transmembrane domain, and a C-terminal Gal4 was previously made (Malecki et al. 2006; Sanchez-Irizarry et al. 2004). Dystroglycan's proteolysis domain and transmembrane were cloned in using In-fusion cloning.

Mammalian cell culture

All of the cells used in this paper were cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning) supplemented with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. Cos7 cells were a kind gift from Dr. Margaret Titus. U251 cells were a kind gift from Dr. David Odde. U2OS and HEK293T cells were purchased from ATCC. MS5 and MS5-DLL4 cells were a kind gift from Steve Blacklow.

All transfections were performed in Opti-MEM (Gibco) with Lipofectamine 3000 (Invitrogen) according to manufacturer instructions.

MMP activation

MMP-2 and MMP-9 were activated using p-aminophenylmercuric acetate (APMA) in MMP assay buffer (50 mM Tris pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35 (w/v)) according to manufacturer instructions. MMP-2 was activated for 1 hour at 37°C. MMP-9 was activated for 24 hours at 37°C according to manufacturer instructions.

MMP assay

10 cm plates of HEK-293T cells were transiently transfected with dystroglycan proteolysis domain. The conditioned media was collected 48 hours post-transfection. Conditioned media was bound to magnetic protein A beads and incubated for 1 hour at 4°C. The beads were washed thoroughly with wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP-40). The beads were split into separate tubes, MMP buffer was added (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35 (w/v), pH 7.5) and activated MMP and BB-94 added as appropriate. For the experiment using increasing amounts of MMP in Figure x: 0.07 ug/mL, 0.37 ug/mL, and 0.73 ug/mL of respective MMPs were added, and the tubes were incubated for 1 hour at 37°C. The remaining protein was eluted by incubating the beads with 2x SDS sample buffer with 100 mM DTT for 5 minutes at room temperature.

Western blot

Lysates and dystroglycan proteolysis domain were run on a 4-20% SDS-PAGE gel including 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. The antibodies were diluted 1:1000 in 5% bovine serum albumin (BSA). A goat-anti mouse HRP conjugated antibody (Invitrogen). 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.

Notch signaling assay

The Notch signaling assay was performed as described (Gordon et al. 2015). Briefly, 10 ng chimera constructs were co-transfected with reporter plasmids (50 ng Gal4 reporter plasmid and 1ng PRL-TK reporter plasmid) 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. 48 hours post-transfection, cells were lysed in passive lysis buffer

(Promega). Lysate was added to a white 96 well half volume plate, and luciferase assay buffers were injected and luciferase levels were read using Molecular Devices LMaxII[384] plate reader.

For signaling assays that used MMP-2 and MMP-9: Activated MMP-2 or MMP-9 was diluted to 0.46 ug/mL in D10 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.

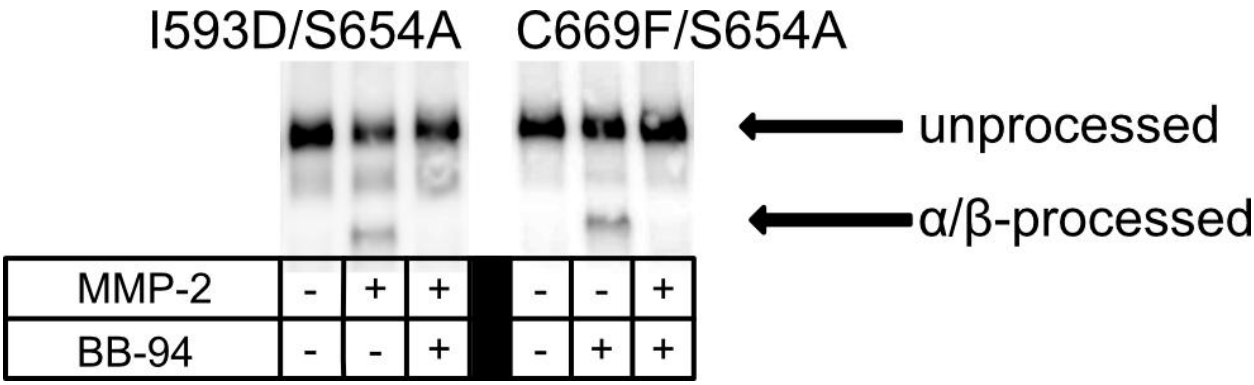
Laminin-overlay assay

Laminin overlay assay was performed as described (Michele et al. 2002). Briefly, lysates from transiently transfected U2OS, 293T, and Cos7 cells were transferred to a nitrocellulose membrane and blocked with 5% milk in laminin binding buffer (10 mM triethanolamine-HCl (pH 7.4), 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). The blot was incubated with 10 nM laminin in 3% BSA in laminin binding buffer overnight. The blot was washed, incubated with laminin primary antibody (1:1000) for 4 hours, washed, and incubated with an anti-rabbit HRP secondary antibody (1:10000) for 1 hour at room temperature.

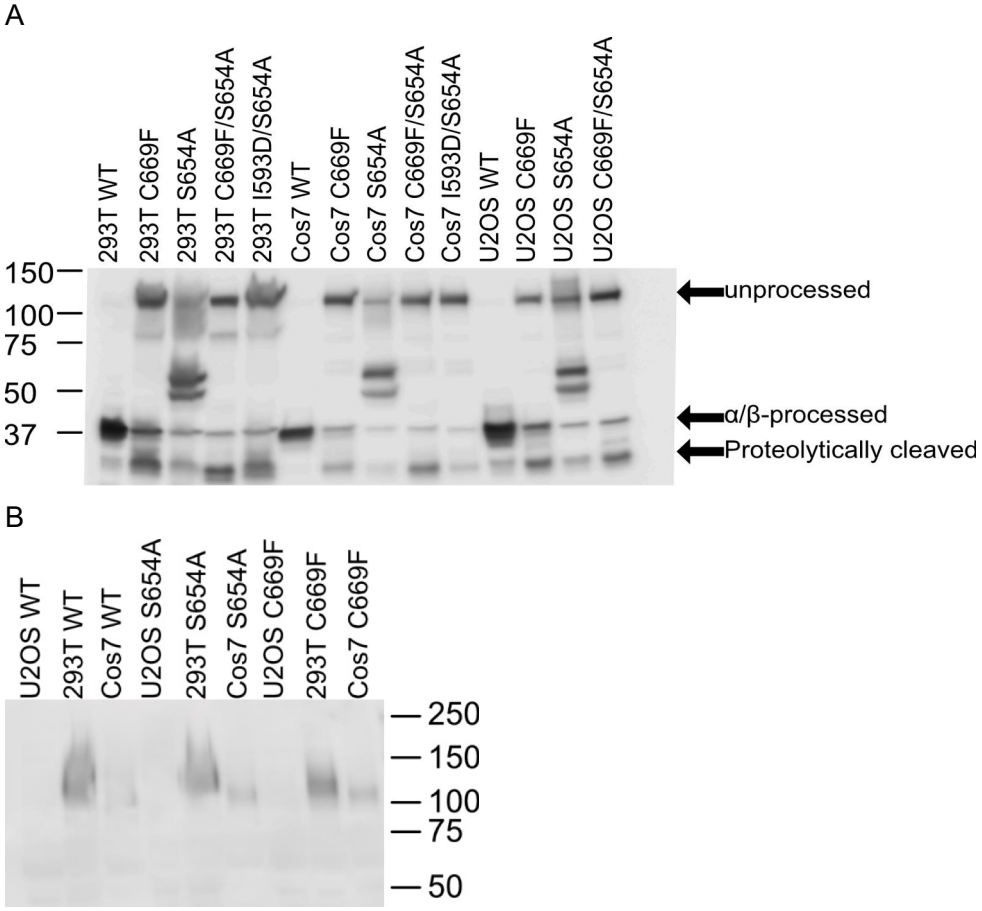
Wound healing assay

3T3 cells were reverse transfected in duplicate in a 24 well dish with dystroglycan constructs or an empty vector. After 36 hours, a scratch was made with a P200 pipette, the cells were washed once with PBS, and placed in DMEM supplemented with 5% FBS. Images were taken at respective timepoints using an EVOS FL Auto microscope. Four image areas were marked and monitored for each well, and wound area measurements were performed independently with Adobe Photoshop and the MRI Plugin for ImageJ. The data from __ independent experiments were analyzed and plotted using Prism. Each wound area was normalized where 100% of the area was at time=0. The normalized data was then used for a two-way ANOVA analysis to determine significance.

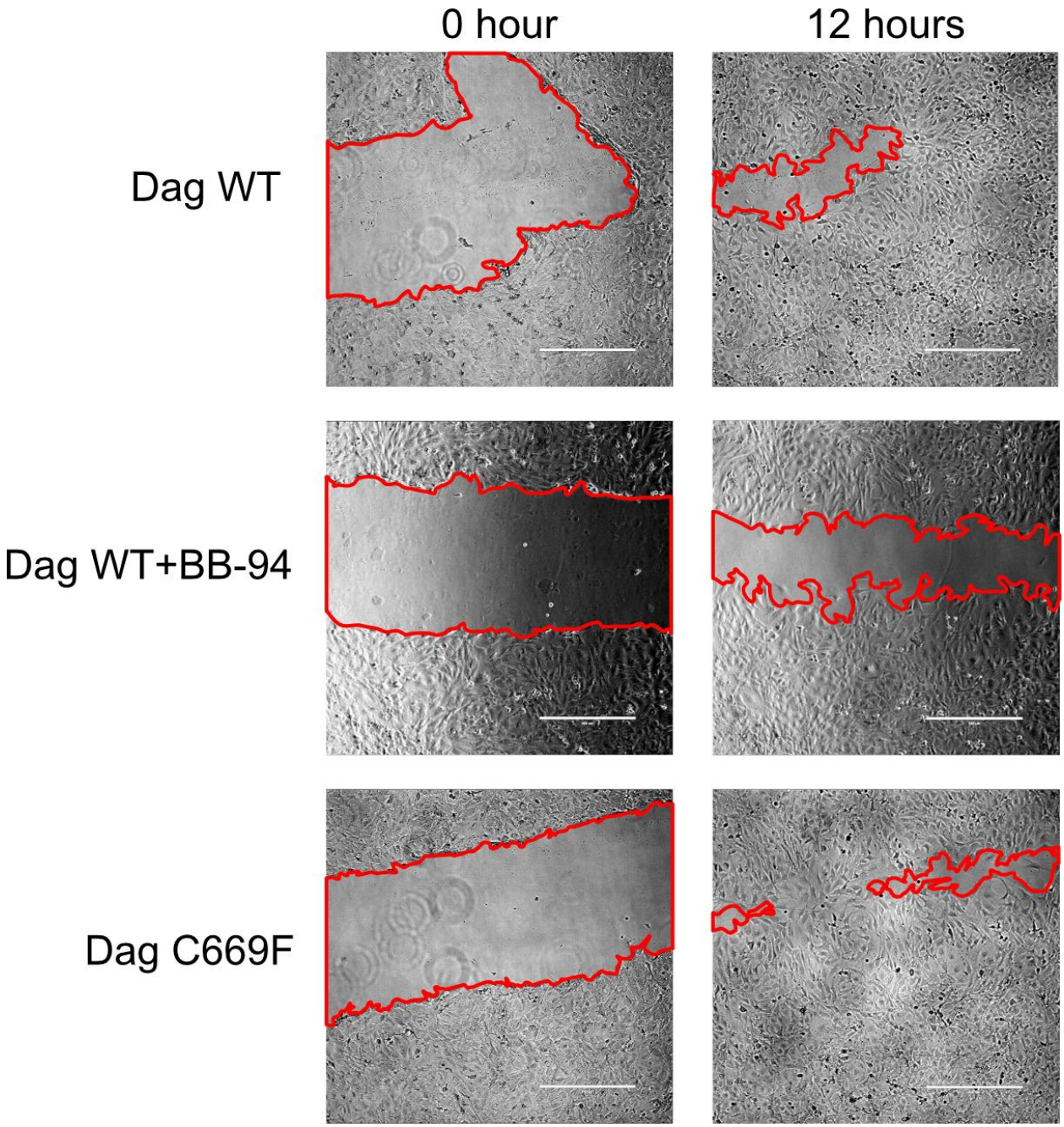
Supplementary Figures:



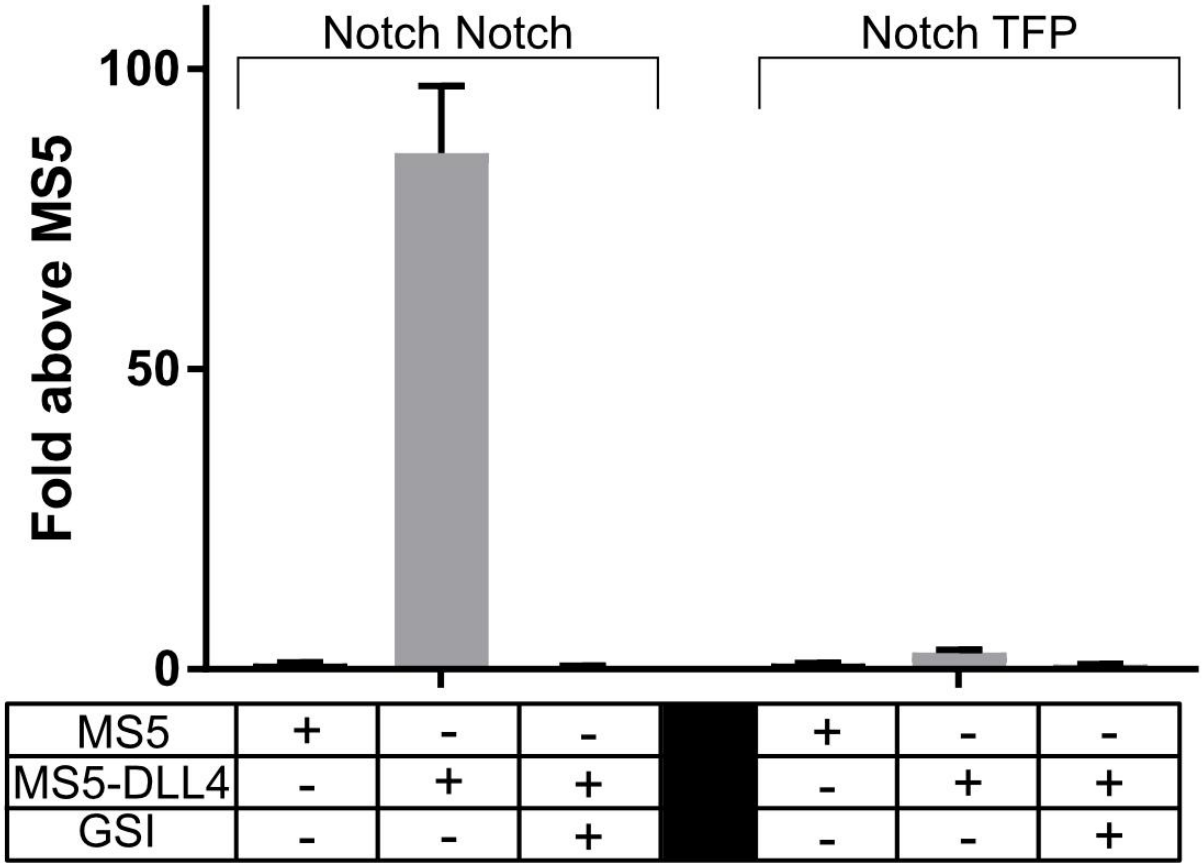
Supplementary Figure 1: Double-mutant proteolysis-domain constructs are sensitive to MMP-2. α-HA western blot of secreted dystroglycan proteolysis domains harboring disease mutations in the context of blocked α/β-processing (S654A mutation). Constructs were secreted from HEK293T cells and enriched using Protein A beads. Reactions were performed in MMP buffer and 0.73 μg/ml activated MMP-2 and 40 μM BB-94 were added as noted.



Supplementary Figure 2: A. 31 kDa proteolytic fragment of β-dystroglycan is observed in multiple cell lines. β-dystroglycan western blot of full-length dystroglycan constructs transiently transfected in HEK293T, Cos7, and U2OS cells. Full-length β-dystroglycan migrates at 42 kDa while proteolytically-cleaved β-dystroglycan migrates at 31 kDa. I593D, S654A, and C669F are disease-related mutations. **B. C669F is glycosylated to similar extent to WT dystroglycan**. Laminin-overlay assay of WT, S654A and C669F dystroglycan constructs transfected into U2OS, HEK293T, and Cos7 cells, run on SDS page, transferred to nitrocellulose, incubated with laminin, and blotted with anti-laminin antibody.



Supplementary Figure 3: C669F patient-derived mutation increases cell migration rates in 3T3 cells. Representative brightfield images of wounds scratched in 3T3 cells transiently transfected with dystroglycan constructs at t=0 hours and t=12 hours. Red outline denotes wound edges.



Supplementary Figure 4: Notch signaling assay requires a mechanosensitive domain. Luciferase reporter gene activity profile of the Notch-Notch and Notch-TFP chimera co-cultured with ligands. Luciferase reporter data was normalized to MS5 condition. BB-94=pan-metalloprotease inhibitor GSI=gamma-secretase inhibitor.

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