A disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif 9 (ADAMTS9) regulates fibronectin fibrillogenesis and turnover

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Running title: Fibronectin proteolysis by ADAMTS9

ABSTRACT

The secreted metalloprotease ADAMTS9 has dual roles in extracellular matrix (ECM) turnover and biogenesis of the primary cilium during mouse embryogenesis. Its gene locus is associated with several human traits and disorders, but ADAMTS9 has few known interacting partners or confirmed substrates. Here, using a yeast two-hybrid screen for proteins interacting with its C-terminal Gon1 domain, we identified three putative ADAMTS9-binding regions in the ECM glycoprotein fibronectin. Using solid-phase binding assays and surface plasmon resonance experiments with purified proteins, we demonstrate that ADAMTS9 and fibronectin interact. ADAMTS9 constructs, including those lacking Gon1, co-localized with fibronectin fibrils formed by cultured fibroblasts lacking fibrillin-1, which co-localizes with fibronectin and binds several ADAMTSs. We observed no fibrillar ADAMTS9 staining after blockade of fibroblast fibronectin fibrillogenesis with a peptide based on the functional upstream domain of a Staphylococcus aureus adhesin. These findings indicate that ADAMTS9 binds fibronectin dimers and fibrils directly through multiple sites in both molecules. Proteolytically active ADAMTS9, but not a catalytically inactive variant, disrupted fibronectin fibril networks formed by fibroblasts in vitro, and ADAMTS9-deficient RPE1 cells assembled a robust fibronectin fibril network, unlike wild type cells. Targeted LC–MS analysis of fibronectin digested by ADAMTS9-expressing cells identified a semi-tryptic peptide arising from cleavage at Gly-2196–Leu-2197. We noted that this scissile bond is in the linker between fibronectin modules III17 and I10, a region targeted also by other proteases. These findings, along with stronger fibronectin staining previously observed in Adamts9 mutant embryos, suggest that ADAMTS9 contributes to fibronectin turnover during ECM remodeling.

Extracellular matrix (ECM) is a complex network of proteins, glycoproteins, proteoglycans and glycosaminoglycans that ensures tissue structural integrity. It also provides positional information and mechanical signals to cells and sequesters growth factors and cytokines (1, 2). Since nearly all signals perceived by cells are potentially modifiable by the ECM, a highly regulated ECM composition and organization are necessary for correct cell and organ function.
ECM is continuously remodeled by coordinated biosynthesis and proteolysis of its components (3). Among several enzymes participating in ECM proteolysis are ADAMTS proteases, a family of 19 secreted zinc proteases with diverse and essential roles in mammalian development and homeostatic processes [reviewed in (4-6)]. Naturally occurring mutations affecting ADAMTS proteases cause a variety of Mendelian disorders in humans and other species (4) and ADAMTS proteolytic activity may drive the pathology of acquired disorders such as osteoarthritis and coronary artery disease (7, 8).

ADAMTS proteases comprise an N-terminal pro-protease domain upstream of a series of canonical modules constituting an ancillary domain, whose hallmark is the presence of one or several thrombospondin type I repeats (TSRs) (9). ADAMTS9 and its homolog ADAMTS20 each contain 15 TSRs and a unique C-terminal module named Gon1 after their *C.elegans* ortholog Gon-1 (10, 11). They have high domain and sequence homology with Gon-1, which is necessary for gonadal morphogenesis (10) as well as with the *D. melanogaster* metalloprotease ADAMTS-A, which is essential for tracheal development (12). Thus, these proteases are evolutionarily conserved and likely to have significant functions in mammals. Indeed, genetically engineered mouse mutants has shown that ADAMTS9 is indispensable for several aspects of mammalian embryogenesis. *Adams*9 null mouse embryos fail to undergo gastrulation, a crucial early morphogenetic event during which the definitive germ layers are formed, and die by 7 days of gestation (13). This mouse allele, and others, including a hypomorphic *Adams*9 allele (*Adams*9Gt), chemically-induced point mutations, and conditional *Adams*9 deletion have identified crucial functions in craniofacial, neural, vascular, cardiac, eye, pigment and limb development (14-19). In adult mice, ADAMTS9 has a role in parturition via regulation of ECM control of uterine smooth muscle cell differentiation (20). ADAMTS9 and ADAMTS20 were recently found to be essential for biogenesis of the primary cilium, providing an unexpected role for these secreted proteases in formation of a cellular organelle (6, 21). Genome-wide analysis has linked the *ADAMTS9* locus to diverse genetic traits, disease susceptibility markers and common disorders of complex etiology, including type 2 diabetes mellitus, obesity and age-related macular degeneration (22-26). *ADAMTS9* was identified as a tumor suppressor gene in gastric, nasopharyngeal and esophageal squamous cell carcinoma, where it may act via suppression of angiogenesis (27-29). Despite this considerable biological and disease relevance, the only known ADAMTS9 substrates to date are the ECM proteoglycans aggrecan and versican (11).

Previous work has demonstrated that ADAMTS9 enters the secretory pathway, where it undergoes obligate glycosylation, binds therein to several chaperones, and arrives at the cell surface, where its propeptide is cleaved by furin (11, 13, 14, 30-32). Recent work shows that furin-processed, catalytically active ADAMTS9 is endocytosed and trafficked to Rab11 vesicles that form a girdle around the basal body of the primary cilium (33). There, through an as yet, unknown mechanism, ADAMTS9 mediates ciliary growth. Here, adding to the growing understanding of this important protease, we report identification of the ECM and circulating glycoprotein fibronectin as a novel ADAMTS9 binding partner and show that its assembly and abundance are reduced in the presence of ADAMTS9. These findings are consistent with stronger fibronectin staining previously observed in mouse embryos homozygous for the loss-of-function *Adams*9Gt mutation (14, 19).

**Results**

**Fibronectin is a novel binding binding partner of the ADAMTS9 Gon1 domain**

A yeast two-hybrid screen of a human placenta library of *Gal4* fusion proteins was undertaken using the human ADAMTS9 Gon1 module (residues 1733-1935, SwissProt accession no. Q9P2N4) (Fig. 1A) as the “bait”, fused in-frame and downstream of the *Gal4* DNA-activating domain. In addition to several other secreted or cell-surface proteins, which could be putative binding partners, eight independent ADAMTS9 Gon1 interacting cDNA clones, each encoding part of fibronectin (Fig. 1B) were identified in-frame with the *Gal4* DNA-activating domain. The selected interacting domains (SIDs), of fibronectin, which are minimum interacting regions determined by the overlap between these clones (34), are shown in Fig. 1B. The 3 SIDs,
SID1, SID2 and SID3, spanned three non-overlapping regions of fibronectin comprising both type I and type III repeats (Fig. 1B).

Yeast two-hybrid interactions occur in a reducing environment between proteins that are synthesized without post-translational modifications typical for secreted mammalian proteins. Such modifications include introduction of disulfide bonds and N-glycosylation. Two disulfide bonds stabilize fibronectin type I modules (35). The human ADAMTS9 Gon1 module contains 10 cysteines (11), which are conserved in other Gon1 modules (http://pfam.xfam.org/family/PF08685) and very likely form disulfides, and 2 sites of potential N-glycosylation. Therefore, experiments were performed in follow-up of the yeast two-hybrid results asking if the ADAMTS9-fibronectin interactions could be replicated with proteins that had been processed and undergone modification in a eukaryotic secretion pathway. Since we were unable to purify full-length ADAMTS9, myc-His6-tagged ADAMTS9 Gon1 module and longer C-terminal ADAMTS9 constructs with exogenous signal peptides were expressed and purified from the medium of mammalian cells (Fig. 1A,C). Recombinant ADAMTS9 Gon1 bound to plasma fibronectin, a disulfide-linked dimer of 220-kDa subunits in a solid phase binding assay (Fig. 2A), as well as to N- and C-terminally truncated fibronectin constructs N-1F(III) and 1F(III)-C respectively (Fig. 2B). These overlapping constructs were expressed as secreted proteins using recombinant baculoviruses to ensure proper disulfide bonds and represent approximately the N-terminal 1/3rd and the C-terminal 2/3rd of fibronectin respectively (Fig. 1B).

In solid phase binding assays, ADAMTS9 Gon-1 showed stronger binding to fibronectin than ADAMTS9 TSR9-15 (Fig. 2C), whereas ADAMTS9 TSR2-8 did not bind (data not shown). Purified ADAMTS9 TSR9-Gon1 construct containing both binding regions was unstable and therefore could not be used in the binding assays. ADAMTS9 Gon-1 and ADAMTS9 TSR9-15 binding to fibronectin was validated and compared using surface plasmon resonance (Fig. 2D). These constructs bound fibronectin with an estimated $K_D$ of 2.8 nM ($\chi^2/R_{max}=4\%$) and 104 nM ($\chi^2/R_{max}=6\%$) respectively. Thus, binding of the Gon1 module to different parts of fibronectin as identified in the yeast-two-hybrid screen was replicated using proteins purified after secretion from eukaryotic cells, and the TSR9-15 region of ADAMTS9 was also found to contain one or more binding sites for fibronectin.

**ADAMTS9 constructs bind fibronectin fibrils**

Many cell types that secrete fibronectin or are provided exogenous fibronectin assemble it into fibrils, starting within a few hours of attachment if plated at sufficiently high density (36). Fibronectin fibrils provide a template for assembly of fibrillin-1 microfibrils, which appear soon after initial fibronectin assembly and colocalize extensively with them (37, 38). Furthermore fibrillin-1, the major fibrillin produced by most cells, is a binding partner for several ADAMTS proteins (39). Therefore, to eliminate fibrillin-1 as a potential confounder in the analysis, we used Fbn1-/- mouse embryonic fibroblasts. In the short-term cultures used for these experiments, Fbn1-/- mouse embryo fibroblasts do not assemble fibrillin-2, an ADAMTS ligand and substrate (40-42).

To test whether the ADAMTS9 Gon1 module bound fibronectin fibrils, Fbn1-/- fibroblast monolayer cultures were co-stained with anti-myc (for the exogenous myc-His6-tagged Gon1 domain) and an anti-fibronectin antibody. Despite interacting with full-length fibronectin dimer and fragments of fibronectin monomer (Fig. 2A,C,D), recombinant ADAMTS9 Gon1 module did not co-localize with fibronectin fibrils (data not shown). ADAMTS9 constructs containing upstream regions of the ancillary domain were subsequently tested by adding conditioned medium containing them to the fibroblast cultures. ADAMTS9 TSR9-Gon1 bound to fibronectin fibrils, indicated by co-staining of fibrillar structures by anti-myc and fibronectin antibodies (Fig. 3A). We similarly tested binding of an ADAMTS9 TSR9-15 construct to ask whether the TSRs could have a role in fibronectin fibril binding independent of the Gon1 module. This construct co-localized with fibrils in a comparable manner as ADAMTS9 TSR9-Gon1 (Fig. 3A). Because these co-staining experiments were done after addition of exogenous ADAMTS9, i.e., via conditioned medium, they suggest that the ADAMTS9 constructs were incorporated into forming fibrils or bound to the surface of fibronectin fibrils. The experimental approach thus
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indicated that it was not necessary for ADAMTS9 and fibronectin to undergo initial association in the secretory pathway. Therefore, fibronectin fibrils assembled by one cell, or previously deposited in the ECM could bind to ADAMTS9 produced by another cell. Specificity of the ADAMTS9 binding for fibronectin fibrils was demonstrated by absence of fibrillar myc-staining in cultures treated with 100 nM functional upstream domain (FUD) (43) which specifically prevents fibronectin fibril assembly (Fig. 3B). Elimination of the possibility of fibrillin assembly by these MEFs provided additional evidence that ADAMTS9 constructs bound specifically to fibronectin fibrils and not to fibrillin-1 and -2 microfibrils.

Fibronectin fibrils are fragmented in the presence of catalytically active ADAMTS9

Fibronectin binding by the C-terminal ADAMTS9 constructs suggested that it could be an ADAMTS9 substrate. We therefore investigated the ability of full-length ADAMTS9 and the N-terminal construct ADAMTS9 N-L2, each containing the protease domain, to bind fibronectin fibrils and specifically, to modify fibronectin dimers or fibronectin fibrils proteolytically. Full-length ADAMTS9 is a large and unstable enzyme (>200 kDa), which is expressed at low levels by transfected mammalian cells and no high-production natural sources are known. Despite multiple attempts, we have been unable to obtain substantially pure recombinant full-length ADAMTS9 or ADAMTS9 N-L2. As an alternative approach, we co-cultured mouse NIH-3T3 cells with HEK293T cells expressing ADAMTS9 or an inactive mutant construct in which the catalytic residue, Glu\textsuperscript{435} was replaced by Ala. Similar experiments were conducted using the ADAMTS9 N-L2 construct, which excludes the TSR9-Gon1 region (Fig. 1A) and its corresponding inactive mutant construct (ADAMTS9 N-L2 Glu\textsuperscript{435}Ala) and demonstrated that they co-localized with fibronectin fibrils (Fig. 4A).

The fibronectin networks showed considerable discontinuity or fragmentation in the presence of catalytically active ADAMTS9 constructs compared to the inactive mutants (Fig. 4A,B). These observations suggested impaired fibronectin assembly or fibronectin fibril degradation in the presence of catalytically active ADAMTS9. We also studied the human retinal pigment epithelium-derived cell line hTERT RPE-1, which expresses endogenous ADAMTS9, and an RPE-1 clone, D12, in which ADAMTS9 was mutated using CRISPR-Cas9 (33). Fibronectin fibril networks formed by these two lines were compared after culture for 48h in the presence of serum, or 24h or 48h culture subsequent to this period without serum. Under both conditions, a more robust fibril network was observed in D12 cultures (Fig. 5A,B). qRT-PCR showed no significant difference in FN1 mRNA levels between the two lines (Fig. 5C), strongly suggesting that ADAMTS9 either hindered the assembly of or proteolytically eliminated fibronectin fibrils in hTERT RPE1 cells.

ADAMTS9 activity leads to fibronectin cleavage at a specific site

To identify fibronectin cleavages generated in the presence of ADAMTS9, we coated tissue culture wells with human fibronectin on which we re-cultured ADAMTS9 N-L2- or ADAMTS9 N-L2 EA-expressing HEK293T cells (Fig. 6A). The rationale for this experimental design was that surface-attached and fibrillar fibronectin may have an extended conformation, which would expose cryptic cleavage sites, in contrast to the globular conformation of fibronectin in solution (35, 36). HEK293T cells do not assemble fibronectin fibrils, hence the experiment tested proteolysis of exogenous fibronectin adherent to the plates. The ADAMTS9 N-L2 and ADAMTS9 N-L2 EA constructs were expressed at comparable levels by HEK293T cells (Fig. 6B). Gelatin zymography indicated comparable levels of MMP2 in the medium of the respective HEK293T cells, whereas MMP9 was not detected (Fig. 6C). Conditioned medium from these experiments was obtained after 24h of culture for targeted analysis of human fibronectin by LC-ESI-MS/MS. Fibronectin was identified by 44 and 23 tryptic peptides covering 26% and 15% of the fibronectin sequence from the medium of cultures expressing wild-type and inactive ADAMTS9 N-L2 respectively. The MS\textsuperscript{1} spectrum identified a unique peptide ion of 1034 Da in digests of fibronectin with active ADAMTS9 N-L2 but not ADAMTS9 N-L2 EA (Fig. 6D, inset). The sequence of this peptide was determined in the MS\textsuperscript{2} spectrum by assignment of fragment ions (Fig. 6D). The C-terminal Arg residue of this peptide was consistent with tryptic
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cleavage, but its N-terminus was preceded by Gly in full-length fibronectin (Fig. 6E); hence the peptide did not arise by tryptic digestion during sample preparation and represents a ‘semi-tryptic peptide” indicative of fibronectin proteolysis at the Gly

\[ \text{Gly}^{2196}\text{-Leu}^{2197} \]

peptide bond (sequence enumeration after fibronectin isoform with UniProt identifier P02751)(Fig. 6E). This peptide bond lies within a short linker connecting fibronectin III\(_{17}\) and I\(_{10}\) modules that may comprise an unstructured region susceptible to proteolytic processing (Fig. 6E). The semi-tryptic peptide was not observed in analysis of fibronectin exposed to catalytically inactive ADAMTS9 N-L2. We undertook another targeted LC-ESI-MS/MS analysis, i.e., selective reaction monitoring, for the semi-tryptic peptide and other fibronectin ions, and confirmed its presence exclusively in digests using the active ADAMTS9 N-L2 (Fig. 6F). Indeed, relative quantification of fibronectin peptides detected by LC-MS/MS of the medium of cells expressing wild type or inactive ADAMTS9 N-L2 demonstrated that several tryptic fibronectin peptides were more abundant in the medium of cells expressing wild-type ADAMTS9 N-L2 than ADAMTS9 N-L2 EA (Fig. 6F). The relative abundance of fibronectin was determined in relation to enolase, detected at comparable levels in the samples, which was thus used as a normalization factor (examples of extracted ion chromatograms are shown in Fig. 6G). Thus, in addition to identification of a unique semi-tryptic peptide, the greater fibronectin sequence coverage and quantitative fibronectin peptide excess upon digestion of adherent fibronectin with active ADAMTS9 N-L2 suggested an overall greater abundance of fibronectin released in these experiments, also supporting fibronectin proteolysis by ADAMTS9. In the absence of an antibody suitable for recognition of the C-terminus of fibronectin, we have not been able to obtain additional evidence of cleavage by a western blot.

Discussion

Observations from complementary approaches, including the yeast two-hybrid assay, solid-phase binding and surface plasmon resonance using purified proteins collectively support direct interaction of ADAMTS9 with fibronectin. ADAMTS9 localized specifically to fibronectin fibrils, since all ADAMTS9 fibrillar ECM staining was abolished by FUD, which prevents fibronectin polymerization, and these cells could not assemble fibrillin-1, a known ADAMTS ligand. ADAMTS9 produced by HEK293 cells localizes to their cell-surface and is released into medium (11), indicative of binding in trans to fibronectin fibrils formed in the co-cultures, and demonstrating that the interaction can occur extracellularly. Thus, fibronectin and ADAMTS9 could be co-assembled during fibril assembly or ADAMTS9 may interact with preformed fibronectin fibrils. Distinguishing between these possibilities is challenging, since fibronectin assembly in cultured cells is a continuous, rapid, and extremely dynamic process involving binding of integrins to the secreted fibronectin dimer, extension of the bound fibronectin by cell movements, and polymerization via exposed binding sites on fibronectin dimers (36). Although the Gon1 module bound to purified fibronectin dimer in solid-phase assays, it did not co-localize with fibronectin fibrils. In contrast, upstream non-overlapping ADAMTS9 constructs such as TSR9-15 and ADAMTS9 N-L2, both lacking the Gon1 module, consistently bound to fibrils. Taken together with three non-overlapping fibronectin SIDs identified in the yeast two-hybrid screen, the findings suggest multiple points of contact between the two molecules and distinct modes of interaction of different ADAMTS9 regions with fibronectin dimer or fibrils.

The observed binding of non-catalytic regions of ADAMTS9, i.e., Gon1 and TSR9-15 constructs, logically led to consideration of fibronectin as a potential ADAMTS9 substrate. A semi-tryptic peptide identified by mass spectrometry was determined to be specific for immobilized fibronectin digested by active ADAMTS9, since it was not generated in the presence of the catalytically inactive mutant. Detection of higher levels of fibronectin tryptic peptides overall in the presence of active ADAMTS9 suggests additional ADAMTS9 cleavage sites that liberate fibronectin fragments or that cleavage at the Gly

\[ \text{Gly}^{2196}\text{-Leu}^{2197} \]

bond may promote fibronectin instability and/or depolymerization. One caveat of this study is that it has not been possible to purify ADAMTS9; hence, we cannot exclude the possibility that ADAMTS9 is not the terminal protease that...
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degrades fibronectin, but instead activates another fibronectinase. Fibronectin was previously identified as a substrate of several proteases, including the metalloproteases ADAM12 (44), MMP2, MMP8 and MMP14 (45, 46), which may cleave it at the Ala\(^{1078}\)-Thr\(^{1079}\) bond (47). The ADAMTS9 processing site identified here, Gly\(^{2196}\)-Leu\(^{2197}\), was previously identified as a cleavage site for MMP2 and MMP8 (48), but no change in MMP-2 was seen on gelatin zymography in our experimental system. Furthermore, direct binding of ADAMTS9 to both the fibronectin dimer and fibronectin fibrils suggests that it likely cleaves fibronectin directly. The data presented here using either addition of ADAMTS9 conditioned medium or knockout of endogenous ADAMTS9 each show that fewer fibrils are present in the presence of catalytically active ADAMTS9, supporting its role in turnover of fibronectin fibrils. The observed ADAMTS9 incorporation in trans from conditioned medium or during co-culture experiments suggests that ADAMTS9 is integrated into fibrils as they form, potentially providing an innate mechanism for their physiological turnover, i.e., a “Trojan horse” effect. The cell-surface localization of ADAMTS9 (11, 31, 33), suggests it may access fibronectin fibrils quite early in the fibril assembly process.

Previously, endocytosis of MMP-cleaved fibronectin bound to integrins was implicated as a major degradation pathway operational in cultured cells (46). Like ADAMTS9, MMP14 also attenuates fibronectin matrix assembled by cultured cells. Among ADAMTS proteases, it was recently shown that ADAMTS2, ADAMTS3 and ADAMTS14, which are evolutionarily distinct from ADAMTS9, cleaved the fibronectin Ala\(^{292}\)-Val\(^{293}\) bond within a linker between domains I\(_5\) and I\(_6\) to release an N-terminal 30 kDa fragment that is crucial for fibronectin assembly (49). In addition, this region contains an ADAMTS16 cleavage site just upstream of the ADAMTS2/3/14 cleavage site, i.e., at the Ser\(^{285}\)-Phe\(^{286}\) peptide (50), Putative ADAMTS7 cleavage sites, Thr\(^{279}\)-Ser\(^{280}\) and Ser\(^{281}\)-Gly\(^{282}\) in the linker between I\(_5\) and I\(_6\) were also recently identified (51). Also like ADAMTS9, ADAMTS16 processing at this site led to reduced fibronectin fibril assembly (50), but the effect of ADAMTS2, ADAMTS3, ADAMTS7 and ADAMTS14 on fibronectin assembly and turnover is yet to be investigated. Like ADAMTS9 processing in the III\(_{17}\) I\(_{10}\) linker, Thr\(^{279}\)-Ser\(^{280}\), Ser\(^{281}\)-Gly\(^{282}\), Ser\(^{285}\)-Phe\(^{286}\) and Ala\(^{292}\)-Val\(^{293}\) are present within a linker that may present an exposed, unstructured region susceptible to proteolysis.

Fibronectin is present in the circulation and in many matrices where it is a critical cell adhesion substrate. ADAMTS9 is constitutively produced by vascular endothelium, suggesting a potential physiological role in fibronectin turnover during wound healing or a potential mechanism of its potent anti-angiogenic effect (28). Fibronectin is a foundational matrix molecule that promotes assembly of fibrillin microfibrils, elastic fibers, collagen fibrils, and LTBPs (36) and is an essential component of embryonic extracellular matrix (52). Thus, fibronectin assembly and turnover have considerable physiological relevance to embryogenesis and adult processes. The present work, together with a recent publication (50), emphasizes that fibronectin proteolysis, and not only its transcriptional and assembly mechanisms are crucial for controlling its levels in ECM. Most work on fibronectin proteolysis has utilized cultured cells, and thus few relevant fibronectin-degrading proteases operating at the tissue level are known. ADAMTS9 and MMP14 are among the few for which combined biochemical, in vitro and in vivo evidence as fibronectinases is available. ADAMTS9 appears to be essential for embryonic fibronectin turnover as evidenced by stronger fibronectin staining in several tissues of Adamts9\(^{Gt/Gt}\) embryos. In contrast to Adamts9 null embryos, Adamts9\(^{Gt/Gt}\) embryos survive past gastrulation to undertake organogenesis, during which fibronectin-rich ECMs are produced. In previous analysis, Adamts9\(^{Gt/Gt}\) umbilical cord was noted to have stronger fibronectin staining than wild-type littermate umbilical cord, and immunostaining of Adamts9\(^{De/+}\) eyes demonstrated stronger fibronectin staining (14, 19). More intense fibronectin staining was also seen in the pregnant mouse uterus upon conditional Adamts9 deletion in smooth muscle cells (20). Taken together with these in situ observations, the present data suggest that fibronectin turnover is a widespread morphogenetic role of ADAMTS9.

In contrast, Mmp14 appears not to have as crucial a role in fibronectin turnover during early
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development, since it is most highly expressed
during skeletal development (53), and Mmp14
deficient mice survive to birth without dramatic
morphogenetic defects (54, 55). However,
Mmp14 appears to be necessary for fibronectin
turnover during formation of tendons (47), a tissue
where it is highly expressed (53), but Adamts9 is
not (56). Thus, diverse proteases may regulate
fibronectin content of matrix in specific spatial
and temporal contexts, with this abundant, large
dimeric molecule and its supramolecular structures
being targeted by a plethora of proteases at
different sites.

Experimental procedures

Yeast two-hybrid screen— A two-hybrid screen of
the human placenta RP5 library (Hybrigenics,
Cambridge, MA) was undertaken using the human
ADAMTS9 Gon1 module (residues 1734-1935)
fused in-frame and C-terminal of a Gal4 DNA-
binding domain in the pB66 vector. The hybrid
protein was determined to be neither auto-
activating nor toxic in yeast, hence screening was
done without inclusion of 3-amino-1,2,4-triazole.
81.4 X 10^6 yeast clones were screened. A
proprietary Predicted Biological Score (34) was
computed by Hybrigenics to assess interaction
reliability of isolated fibronectin clones and
suggested that the probability of the interaction
being nonspecific was low. Selected interaction
domains representing the minimal region
corresponding to all the isolated clones were
identified using PIMRider® (Hybrigenics).

ADAMTS9 expression plasmids and protein
purification— The human ADAMTS9 TSR9-15
expression plasmid was previously described (14).
Human ADAMTS9 TSR2-8 (residues Phe^879-
Thr^1299), primers 5'AAAGCGCGCCTTTTACTGGAACAGTCAT
GGGCCAT3', AscI site underlined and
5'AACTCGAGGGTCTTTGAGGGCATGGT
GACA3', XhoI site underlined, and Gon1
expression plasmids (residues - ); primers and
5'AACTCGAGTTAAAACTCGCACCTCCAGG
CCAG3', XhoI site underlined) were generated
by PCR amplification, sequenced-verified in
totality and cloned into pSecTagA (Thermo Fisher
Scientific, Waltham, MA) for in-frame expression
with an N-terminal signal peptide and C-terminal
myc-His6 tag in mammalian cells. Plasmids were
transfected into CHO cells (ATCC, Manassas,
VA), and stably expressing clones were isolated as
previously described. hADAMTS9 TSR2-8,
TSR9-15, and Gon1 were purified from the
conditioned medium using Ni^2+-Sepharose
affinity chromatography (Invitrogen, Carlsbad,
CA) essentially as previously described (14). The
purified proteins were analyzed by Coomassie
Blue Stain (Thermo, Rockford, IL) and western
blotting using anti-myc mouse monoclonal
antibody (clone 9E10, Lerner Research Institute
Hybridoma Core). Plasma fibronectin was purified
as previously described (57). Purification of
fibronectin constructs N-1F(III) and 1F(III)-C was
previously described (58). The 1F(III)-C construct
lacks alternatively spliced 8F(III) and 13F(III)
modules found in tissue fibronectin and absent in
plasma fibronectin.

Surface plasmon resonance (SPR) analysis—
Purified ADAMTS9 Gon1 or ADAMTS9 TSR9-
15 in 10 mM acetate, pH 4.0 were immobilized on
a BIAcore CM5 sensor chip (research grade) with
the amine coupling kit following the
manufacturer's instructions (GE Healthcare). 900
and 1600 resonance units of ADAMTS9 Gon1 or
ADAMTS9 TSR9-15 respectively were coupled to
the chip for analysis in a BIAcore 3000 instrument
(GE Healthcare, Chicago, IL). The single cycle
kinetic analysis was performed at 25 °C in 10 mM
Hepes buffer, pH 7.4 with 0.15 M NaCl, 1mM
CaCl2, and 0.005% (v/v) surfactant P20 at a flow
rate of 30 µl/min. Human plasma fibronectin (57)
diluted in the above buffer at different
concentrations and injected through an uncoupled
control flow cell in series with a flow cell coupled
to ADAMTS9 constructs. The sample injection
time was two minutes and was followed by a one
minute pause before the next injection. The
dissociation time was 15 minutes. 50 mM glycine,
PHe 3.0 was used for chip surface regeneration at a
flow rate of 100 µl/min for 30-60 s after Gon-1
binding analysis, whereas 50 mM NaOH was
required for regeneration of the ADAMTS9 TSR9-
15 chip surface. All data were corrected with
reference to the background binding in the control
flow cell. The kinetic constants were calculated
assuming a 1:1 (Langmuir) binding model using
the BIAevaluation software (version 4.0.1; GE
Healthcare).
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*Solid phase binding assay*—96-well plates were coated with either fibronectin or BSA (control) at 50 nM/well. After washing and blocking the wells with 2.5% milk, 20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 2 mM CaCl₂, 0.05% Tween 20 (TBST Ca⁺), 50 µL of purified ADAMTS9 Gon1 in 2.5% milk/TBST Ca⁺ at a concentration between 0.3 to 10 µM was added to the coated wells and incubated on a shaker (300 Hz) for 2 hrs at RT. Anti-myc mouse monoclonal antibody was used to detect bound Gon1. For fibronectin sub-construct binding experiments, 10 µg/mL fibronectin solution was used per well for coating, and 2.5 µM ADAMTS9 Gon1 was used as ligand. To compare binding of different ADAMTS9 constructs, 10 mg/mL of fibronectin was used for coating the wells, and 80 nM of purified ADAMTS9 Gon1, TSR9-15 or TSR2-8 constructs were added to the coated wells. Absorbance was measured at 492 nm.

*Cell culture, immunofluorescence and quantitative reverse transcriptase-PCR (qRT-PCR)*—Fbn1-/- mouse embryonic fibroblasts were made from Fbn1-/- mice (Fbn1<sup>mgN</sup>) (59) essentially as previously described (60). hTERT RPE-1 cells were purchased from ATCC (Manassas, VA; catalog no. CRL-4000). D12, an RPE-1 clone, in which ADAMTS9 was mutated using CRISPR-Cas9 was previously described (33). 5 x 10⁴ Fbn1-/- MEFs were seeded on glass coverslips placed in individual wells of a 24-well plate for overnight culture. The medium was replaced with conditioned medium from CHO cells stably expressing ADAMTS9 constructs or untransfected CHO cells and cells were maintained for a further 48 hrs. For co-culture experiments of NIH-3T3 cells (ATCC) with transfected HEK293T cells, a ratio of 3:5 was used. The cells were fixed in 4% PFA (v/v) containing 4% (w/v) sucrose, and quenched with 1% (w/v) glycine. Anti-fibronectin polyclonal antibody (AB2033, Millipore) and anti-myc mouse monoclonal antibody 9E10 (LRI Hybridoma Core, Cleveland, OH) were used to detect fibronectin and ADAMTS9 respectively. hTERT RPE-1 and D12 cells were plated at 60% confluence, reaching confluence after 48h and were subsequently stained for fibronectin as described above. Imaging was performed on a Leica TCS5 SP11 laser confocal microscope (Fig. 3B, 4B). qRT-PCR was used to compare *FN1* mRNA levels in RPE-1 and D12 cells using primers 5′GTAAACCTGAAGCTGAAGAGAC 3′ and 5′TCACCAATCTTGTAGGACTG 3′ essentially as previously described (33).

*Proteomics determination of fibronectin processing by ADAMTS9*—2 x 10⁶ HEK293T cells transiently transfected with ADAMTS9 N-L2 plasmids were washed and resuspended in serum-free DMEM, and seeded onto wells coated with fibronectin (5 µg/well) in 6-well plates. The conditioned medium was collected 24 h later, proteins were precipitated using trichloroacetic acid, dried in a Speedvac and reconstituted with 50µl Tris-HCl buffer containing 6M urea. The samples were reduced with DTT and alkylated with iodoacetamide, further diluted in 100µl 50 mM ammonium bicarbonate and trypsin was added at a 1:20 (trypsin:protein) ratio. A first round of digestion was carried out overnight, a second aliquot of trypsin was added and the sample was incubated for an additional 6 hours. The protein digest was desalted using the C18 SPE method and reconstituted in 50µL 1% acetic acid for LC-MS analysis. The LC-MS system was a Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system. The HPLC column was a Dionex 15 cm x 75 µm id Acclaim Pepmap C18, 2µm, 100 Å reversed phase capillary chromatography column. 5 µL volumes of the tryptic digest were injected. The peptides were eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.3 µL/min for introduction into the source of the mass spectrometer on-line. The nano-electrospray ion source was operated at 1.9 kV. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. The data was analyzed by using all CID spectra collected in the experiment to search the human UniProtKB sequence databases using Mascot (false discovery rate 0.01%).

Additional samples were analyzed using a targeted selective reaction monitoring (SRM)(61) experiment, which involves the fragmentation of specific ions over the course of the LC-
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experiment, including two peptides from enolase and several FN peptides. The relative abundance of the FN peptides was determined by normalizing the FN peptide peak areas to the peak areas of the enolase peptides; PA(FN)/PA(Enolase).

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
L.W.W. designed, performed and analyzed ADAMTS9-fibronectin binding, cell culture experiments, immunolocalization and proteolysis experiments. D.A. and D.M. generated fibronectin reagents and designed experiments. S.N. generated ADAMTS9 constructs, cell lines and RPE lines and performed confocal imaging. J.D. generated ADAMTS9 constructs. B.B.W. designed, performed and analyzed proteomics experiments. S.A. designed experiments and analyzed the data with other authors. L.W.W. and S.A. wrote the manuscript. All authors have read, edited and approved the manuscript.

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Figure 1. ADAMTS9 constructs and interaction regions of fibronectin. A. ADAMTS9 domain structure with the various ADAMTS9 constructs indicated. B. Domain structure of fibronectin indicating the select interacting domains (SID1, SID2, SID3) identified in the yeast two-hybrid screen and recombinant FN constructs used in binding assays. C. Purified recombinant ADAMTS9 constructs (indicated by asterisks) were analyzed by reducing SDS-PAGE and Coomassie blue staining. Molecular weight markers (in kDa) are shown on the left.
Figure 2. Fibronectin binding to ADAMTS9 constructs. A. Solid phase binding assay of ADAMTS9 Gon1 module to immobilized fibronectin (red line). Bovine serum albumin (BSA, blue line) was used as a control for binding. B. ADAMTS9 Gon1 module was tested for binding to N- and C-terminally truncated fibronectin constructs N-1F(III) and 1F(III)-C respectively. Binding to these constructs was compared to binding to bovine serum albumin (BSA, negative control). ***P<0.0001, n=4. C. Solid phase binding assay comparing ADAMTS9 Gon1 and ADAMTS9 TSR9-15 binding to fibronectin. ***P<0.0001, n=4. D. Surface plasmon resonance analysis of plasma fibronectin (analyte 16.9-270 mM) binding to ADAMTS9 Gon1 module immobilized on a CM5 chip. The binding K_D was 2.8 nM, Chi^2/R_MAX=4% for ADAMTS9 Gon1 and 104 nM, (Chi^2/Rmax=6%) for ADAMTS9 TSR9-15. The purple or green line represent the sensogram and the black line is the fitted curve produced by BIAevaluation software.
Figure 3. Co-localization of ADAMTS9 ancillary domain constructs with fibronectin fibrils. A. Combined immunofluorescence using anti-myc for detection of ADAMTS9 constructs TSR9-Gon1 and TSR9-15 (see Fig. 1A) shows their co-localization with fibronectin fibrils after addition of conditioned medium containing the ADAMTS9 constructs to Fbn1−/− MEFs. In the single fluorescent images both are shown in gray-scale; in the merged image, anti-myc staining is green, and fibronectin is red. B. 100 nM FUD, which abolishes formation of fibronectin fibrils, eliminated anti-myc fibrillar staining in Fbn1−/− mouse embryo fibroblast cultures. Scale bar in A= 10 µm and B is 25 µm.
Figure 4. Constructs containing the ADAMTS9 metalloproteinase domain co-localize with fibronectin and active ADAMTS9 attenuates fibronectin fibril networks. A. Combined immunofluorescence using anti-myc for detection of the constructs ADAMTS9N-L2 and ADAMTS9N-L2 EA (green) expressed by HEK293T cells co-cultured with NIH-3T3 cells shows co-localization with fibronectin fibrils (red). In the single fluorescent images both are shown in gray-scale; in the merged image, ADAMTS9/anti-myc staining is green, and fibronectin is red. B. An interrupted and attenuated fibronectin fibril network (red) was seen in the presence of catalytically active ADAMTS9 constructs ADAMTS9 full-length (FL) wild-type (WT) and ADAMTS9 N-L2 WT compared to the corresponding ADAMTS9 E435Ala (EA) mutants. Scale bars= 10 μm in A, 25 μm in B.
Figure 5: ADAMTS9-deficient RPE-1 cells assemble a more robust fibronectin matrix than wild-type RPE-1 cells. A. Wild type RPE-1 cells and RPE-1 D12 cells in which ADAMTS9 was inactivated by CRISPR-Cas9, were cultured for 48 hours in the presence of serum. Compared to the wild-type cells, abundant fibronectin matrix was assembled by the mutant cells. B. The respective cells were cultured for 48 h, followed by 24h culture in serum-free conditions, with similar observations as in A. C. qRT-PCR for FN1 mRNA showed no significant difference between wild-type RPE-1 and D12 cells. Scale bars= 50 µm in A, upper panel, 25 µm in A, lower panel and B.
Figure 6. Fibronectin (FN) is cleaved in the presence of catalytically active ADAMTS9. A. Schematic of experimental strategy. CM and CL are conditioned medium and cell lysate respectively. B. Western blot with anti-myc monoclonal antibody of lysates of HEK293T cells expressing catalytically active ADAMTS9 N-L2 or the corresponding inactive mutant showing comparable expression. C. Gelatin zymogram showing robust detection of MMP2 and MMP9 in conditioned medium from NIH-3T3 skin fibroblasts (positive control, +) and an evident MMP2 band, but no MMP9 band in the conditioned medium of HEK293T cells expressing ADAMTS9.
D. MS/MS fragmentation spectrum of a 1034.445 Da semi-tryptic peptide generated by incubation of fibronectin with cells expressing catalytically active ADAMTS9. The MS/MS spectrum for this peptide is consistent with the semi-tryptic fibronectin peptide (2197)LNQPTDDSCFDPYTVSHYAVGDEWER(2222) (using UniProt sequence ID P02751). The parent ion mass distribution obtained by MS is shown in the inset box. E. Domain and sequence cartoon of FN showing the cleavage site location with flanking sequence and indicating the flanking domains. F. Quantitative analysis of fibronectin peptides obtained by analysis of conditioned medium of cells expressing active or catalytically inactive ADAMTS9 grown on a fibronectin substrate. The relative abundance of fibronectin in the medium obtained from cells expressing active or catalytically inactive ADAMTS9 was determined by performing a targeted SRM analysis on six fibronectin peptides and two enolase peptides. Enolase was used as a normalization factor as the amount of this protein appears to be similar in the active and inactive ADAMTS9 samples. These SRM experiments were used to determine the peak area (PA) ratios; PA(FN)/PA(enolase), for six fibronectin peptides. T indicates tryptic peptides and “semi” indicates the semi-tryptic peptide indicative of fibronectin cleavage. The relative abundance of the fibronectin peptides is lower in the mutant sample. G. Examples of extracted ion chromatograms for a tryptic fibronectin peptides (1881)IYLYTLNDNAR(1891), the semi-tryptic peptide (2197)LNQPTDDSCFDPYTVSHYAVGDEWER(2222), and one enolase peptide, (344)VNQIGSVTESLQACK(358). These chromatograms are consistent with similar abundance of enolase in these samples, lower abundance of the (1881)IYLYTLNDNAR(1891) peptide and the absence of the semi-tryptic peptide, (2197)LNQPTDDSCFDPYTVSHYAVGDEWER(2222), in the inactive ADAMTS9 sample compared to active ADAMTS9.
A disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif 9 (ADAMTS9) regulates fibronectin fibrillogenesis and turnover
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