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Abstract of the Thesis

_N_- and _O_-linked Glycosylation of TSR6 Promotes Efficient Secretion of ADAMTSL2

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Mutations in ADAMTSL2 (a disintegrin and metalloproteinase with thrombospondin repeats–like 2) are known to cause secretion defects, leading to geleophysic dysplasia. One mutation, G811R, is within the consensus sequence for *O*-fucosylation of TSR6. Interestingly, this site also contains an *N*-glycosylation site. It is not known whether TSR6 is modified with *O*-fucose or *N*-glycans. We hypothesized that the secretion defect of the G811R mutation is caused by an alteration in glycosylation of TSR6. Using site directed mutagenesis and secretion assays, we demonstrated that mutations in the predicted *N*-linked glycosylation site or *O*-fucosylation site resulted in a significant decrease in protein secretion. Interestingly, when both glycosylation sites were eliminated,

secretion levels were similar to N807Q or T809V. Together these findings suggest that amino acid substitutions in the *N*-linked glycosylation site or *O*-fucoylsation site, predicted to interfere with glycosylationin TSR6, could contribute to the Geleophysic dysplasia phenotype.

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LIST OF ABBREVIATIONS

9E10	Anti c-myc antibody
°C	Degrees Celcius
µL	Microliter
µM	Micromolar
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ADAMTSL2	A Disintegrin And Metalloproteinase with Thrombospondin Motifs like 2
amp	Ampicillin
Asn	Asparagine
Asp	Aspartic Acid
B3Glc-T	β1,3-glucosyltransferase
C	Cysteine
cm	Centimeter
ddH2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DpnI	Type II restriction enzyme DpnI
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
G	Glycine
g	Gravity
GD	Geleophysic dysplasia
GDP	Guanosine diphosphate
GlcNAc	N-Acetylglucosamine
GluC	Endoproteinase Glu-C
HCL	Hydrochloric acid
HEK293T	Human embryo kidney 293T
hIgG	Human immunoglobulin G
I	Isoleucine
kDa	Kilodalton
L	Leucine
LB	Lysogeny broth

LTBP-1	Latent transforming growth factor beta binding protein 1.
M	Molar
m	Mouse
mL	milliliter
N	Asparagine
NaCl	Sodium chloride
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
O	Oxygen
OPTI-MEM	Reduced serum media
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PEN/STREP	Penicillin/streptomycin
PhD	Doctor of philosophy
PNGase F	N-Glycosidase F
POFUT2	Protein *O*-fucosyltransferase 2
Q	Glutamine
R	Arginine
RIPA	Radio-Immunoprecipitation Assay
S	Serine
SDS	Sodium dodecyl sulfate
T	Threonine
TBST	Tris-buffered saline with Tween 20
TGF-β	Transforming growth factor *beta*
Tris	Tris(hydroxymethyl)aminomethane
TSL2	ADAMTSL2
TSP	Thrombospondin
TSR6	Thrombospondin type 1 repeat 6
U/μL	Units per microliter
UDP	Uridine diphosphate
V	Valine
V	Volts

VWR	VWR International
W	Tryptophan
X	Any amino acid
β	*Beta*

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INTRODUCTION

Thrombospondin Family

The thrombospondins (TSP) are a family of calcium binding glycoproteins associated with the extracellular matrix (ECM) (for review, see Adams and Tucker 2000). These multi-domain proteins consist of a carboxy terminus, three or four epidermal growth factor-like repeats, seven thrombospondin type 3 repeats, and an N-terminal domain which is the site for multimerization (Adams and Tucker 2000). Two thrombospondins, TSP-1 and TSP-2, contain three thrombospondin type 1 repeats (TSRs) within their protein structure. These TSRs are known to play an important role in cell adhesion and migration, cell-motility, and protease activity (Adams and Lawler 1993). TSRs are approximately 60 amino acids in length and consist of six conserved cysteines, forming 3 disulfide bonds (Figure 1A, Appendix A) (Lawler and Hynes 1986). They are found as tandem repeats in many secreted and transmembrane proteins.

TSR containing proteins can be post-translationally modified with several types of sugars, a process known as glycosylation. *N*-Linked glycosylation is a co-translational modification, in which an oligosaccharide is covalently attached to an asparagine (Asn or N) residue in the consensus sequence N-X-S/T (for review, see Imperiali and O'Connor 1999). Once the protein is transported to the

Golgi apparatus, the *N*-linked oligosaccharide undergoes further modifications, including the removal of mannose residues and the addition of *N*-acetylglucosamines (GlcNAc), sialic acid and galactose, to form a complex oligosaccharide (for review, see Imperiali and O'Connor 1999). *N*-Linked glycosylation plays a key role in protein folding and transport through the ER (for review, see Helenius and Aebi 2001). Mutations in various components of the glycosylation pathway lead to the development of a group of human diseases known as the congenital disorders of glycosylation(CDG) (Freeze and Schachter, 2009).

In addition to being *N*-glycosylated, TSRs can specifically be modified with *O*-fucose, an unusual modification (Luo *et al*., 2006) added by protein *O*-fucosyltransferase 2 (POFUT2). POFUT2 is a soluble protein located in the endoplasmic reticulum (Luo *et al*., 2006), and modifies properly folded TSRs with the consensus sequence $CX_{2\text{-}3}(\underline{S/T})CX_2G$ (X=any amino acid) (Figure 1B, Appendix A) (Luo *et al*., 2006; Hofsteenge *et al.,* 2001). POFUT2 uses GDP-fucose to *O*-fucosylate the serine or threonine within the consensus sequence (Luo *et al*., 2006). POFUT2 is essential for maintaining tissue organization and modulating expression levels of important growth factors in the pre-gastrula mouse embryo (Du *et al*., 2009). Loss of POFUT2 results in epithelial disorganization, unrestricted epithelial to mesenchymal transition, and abnormal organization of the parietal and visceral endoderm. Mutant cells preferentially

differentiate into vascular endothelial cells, preventing progression of development beyond gastrulation (Du *et al*., 2009). *O*-Fucose on TSRs can be further modified by a β1,3-glucosyltransferase (B3Glc-T), which uses UDP-glucose (Kozma *et al.,* 2006). Failure to add β1,3-linked glucose leads to a disorder known as Peter's Plus Syndrome (Lesnik Oberstein *et al*., 2006). Common features of Peter's Plus Syndrome include developmental delay, eye chamber defects and a short stature (Hess *et al*., 2008). There are 52 predicted targets of mouse POFUT2 (Leonhard-Melief and Haltiwanger 2011; Du *et al*., 2010). A number of these proteins are matrix metalloproteases that modify the ECM, or matricellular proteins that influence adhesion/migration, and cell signaling (Tucker 2004).

ADAMTS family

One specific family of POFUT2 target proteins is A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS). This superfamily is comprised of 19 secreted metalloproteinases and seven ADAMTS-like (ADAMTSL) proteins (for review, see Porter *et al*., 2005). The structure of the ADAMTS family is well conserved, consisting of a signal peptide, pre-prometalloprotease domain, disintegrin-like domain, cysteine rich domain, spacer domain, and one or more TSRs (Figure 2A, Appendix A, for review, see Apte

2004). The ADAMTS-like (ADAMTSL) proteins resemble ADAMTS proteases but lack the pro-metalloprotease and disintegrin-like domains (Figure 2B, Appendix A) (Hirohata *et al*., 2002). The ADAMTS C-terminal ancillary domain includes the cysteine rich domain, spacer domain and TSRs (for review, see Apte 2004). Processing of this C-terminal domain regulates enzyme localization and its substrate specificity (for review, see Porter *et al*., 2005). The protease domains are involved in a variety of biological functions including angiogenesis, molecular maturation of proteins, and morphogenesis (for review see Apte 2009). For example, ADAMTS1, a versicanase, is critical for versican proteolysis during mouse cardiac development (Stankunas *et al*., 2008). ADAMTS2 is essential in removing the *N*-propeptide of fibrillar collagens (Li *et al*., 2001). ADAMTS13 is required for maturation of the von Willebrand factor (Zheng *et al*., 2001).

Because of their lack of protease domain, ADAMTSL proteins are predicted to play a more structural or regulatory role in the extracellular matrix (Le Goff and Cormier-Daire, 2011). ADAMTSL2 is a secreted glycoprotein consisting of seven TSRs, six of which contain the consensus sequence for *O*-fucosylation by POFUT2. Site mapping confirmed that TSR1, 2, 3, 5 and 7 are modified with a fucose-glucose disaccharide (Figure 2B, Appendix A) (unpublished, C. Leonhard-Melief). There are no data for mapping TSR6 modifications. *O*-Fucosylation is required for ADAMTSL2 secretion (unpublished, R.S. Haltiwanger). ADAMTSL2 is found most abundantly in the

liver, but is also expressed in the kidney, heart, brain, skeletal muscle and lungs in both mice and humans (Koo *et al*., 2007). Although the function of ADAMTSL2 in these tissues is unknown, mutations in *ADAMTSL2* lead to development of disease in both canines and humans.

Musladin-Leuke Syndrome is a canine disorder characterized by short stature, taught skin, and restricted joint mobility (Bader *et al*., 2010). A missense mutation, resulting in R221C, in *ADAMTSL2* causes aberrant disulfide bond formation, leading to a decrease in secretion and insufficient extra-cellular ADAMTSL2 (Bader *et al*.,2010). In humans, Geleophysic dysplasia (GD) is an autosomal recessive disorder belonging to the acromelic dysplasia group of diseases, causing delayed bone ossification, shortened tubular bones, restricted joint mobility and often lethality before age 5 (Le Goff *et al*., 2008). Other characteristic features include a "happy" face with full cheeks, skin thickening, muscular hypertrophy, respiratory failure, and cardiac disease (Le Goff *et al*., 2008). ADAMTSL2 directly interacts with latent TGF-β-binding protein 1 (LTBP-1) (Le Goff *et al*., 2008). In cultured medium of fibroblasts from GD patients, there was a tenfold increase in total TGF-β, suggesting that ADAMTSL2 plays a role in regulating TGF-β availability (Le Goff *et al*., 2008). Sequence analysis of 33 GD patients led to the identification of 18 mutations in the ADAMTSL2 gene (Le Goff *et al*., 2008; Allali *et al.,* 2011). Almost half of these mutations are within TSRs. The mutations W50C (TSR1) and R593C (TSR2) are

in regions essential to TSR folding, and may lead to incorrect disulfide bonding. In addition, the R72Q (TSR1) mutation is predicted to be structurally important for the protein. Two mutations, S635L (TSR3) and G811R (TSR6) are within the consensus sequence for *O*-fucosylation by POFUT2 (Figure 2C, Appendix A). G811R has been demonstrated to cause secretion defects, leading to the development of GD (Le Goff *et al*., 2008).

G811R is close to both the predicted *N*-linked glycosylation and *O*-fucosylation consensus sequence in TSR6. This overlapping configuration of *N*- and *O*-linked consensus sites is highly unusual and it is not known whether both of these sites are modified *in vivo,* or whether these mutations interfere with modification. As previously stated, *O*-fucosylation is necessary for secretion of ADAMTSL2, and it is possible that the G811R secretion defect arises from loss of either or both *N*-linked glycosylation and *O*-fucosylation (unpublished, R.S. Haltiwanger). We propose that the secretion defect of the disease causing mutation, G811R, in the ADAMTSL2 TSR6, results from an alteration in glycosylation. The goal of this study is to determine whether TSR6 from ADAMTSL2 is modified with an *N*-linked glycan at N807, and *O*-fucose at T809, and determine whether elimination of either the site of *N*-linked glycosylation or *O*-fucosylation will cause the same secretion defect as G811R. In addition, we want to determine whether missense mutation G811R in the TSR6 region of

ADAMTSL2 interfere with either *N*-glycosylation or *O*-fucosylation and/or secretion.

MATERIALS AND METHODS

Site Directed Mutagenesis

Primers used to generate ADAMTSL2 (in pcDNAmTSL2) (Koo *et al.,* 2007) substitutions: G811R, S635L, T809V, N807Q and N807Q_T809V are listed in Table 1 (Appendix B). PCR using 40ng ADAMTSL2 plasmid was performed in 20 µL volume of 10nM of primer, 2µM dNTPs, 1x Herculase buffer, and 5 U/µL Herculase DNA Polymerase (Stratagene cat #600262). For the double mutant, N807Q_T809V, 63ng of ADAMTSL2 T809V plasmid was used as the template. PCR conditions were 30 seconds at 95°C, 1 minute at 55°C, 15 minutes at 68°C, and repeated for 18 cycles. Following amplification, the PCR reaction was treated with 20,000 U DpnI (New England Bio Labs cat# R0176L) and incubated for 1 hour at 37°C. DpnI was inactivated at 65°C for 10 minutes. 10 µL of the PCR reaction was transformed into 50 µL DH5α competent cells. After incubating on ice for 30 minutes, the competent cell/DNA mixture was heat shocked for 45s at 42°C. 500 µL of LB was added to the cells,

and placed on a shaker at 230 rpm for 1 hour at 37°C. 100 µL of cell suspension were plated on LB + 100 µg/mL ampicillin (amp) plates, and incubated overnight at 37°C. Bacterial colonies were selected and grown in 5 mL LB+ 100 µg/mL amp for 18 hours at 37°C. Plasmid DNA was extracted using QIAGEN QIAprep Spin Mini Prep Kit (Cat # 2176) and sent for sequencing using the primer 5'-GTGGGAGCTGTGGACAGGGC-3' (Table 3, Appendix B).

Cell Culture and Transfections

HEK293T cells were routinely grown on 10 cm tissue culture plates in 10 mL Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) supplemented with 10% Bovine serum and 1% pen/strep. Cells were seeded at a 1:3 split ratio into a six well dish, and transfected 24 hours later. Transfections contained 0.8 µg/µL of plasmid DNA (wild type ADAMTSL2, an empty vector pSecTag, N807Q, T809V, or N807Q_T809V), and 0.2 µg/µL hIgG, to bring the total DNA to 1 µg/µL. 100 µL of 0.15M NaCl was added to DNA in a 1.5 mL Eppendorf tube. 6 µL of ice cold polyethylenimine (PEI) was added to the DNA solution, vortexed immediately, and incubated for 20 minutes at room temperature. The DNA/PEI mixture was added drop wise, using a 100 µL pipette, and incubated for 4 hours at 37 ºC. To reduce the toxicity of PEI to the cells, the transfected cells were washed with 1 mL 1x phosphate buffered saline (PBS) and the media was

replaced with 1 mL of the reduced serum OPTI-MEM (GIBCO). At 100% confluency (48 hours later) 1 mL of media was collected and transferred to a labeled 1.5 mL Eppendorf tube. To solubilize the cells, 1 mL of ice cold lysis buffer (10mL RIPA/EDTA and one complete protease inhibitor cocktail tablet (Roche)) was added to the wells, pipetted up and down 4x, and transferred to a labeled 1.5 mL Eppendorf tube. The cell lysates and media samples were stored at -20 ºC.

Western Analysis

Cell lysate and media samples from the transfection were thawed in a water bath for 5 minutes at 37 ºC. The samples were spun down at 16,000 x *g* for 1 minute. 100 µL of sample was added to 500 µL ice cold acetone and kept at -20 ºC for 4 hours. The samples were spun down at 16,000 x *g* for 1 minute at 4 ºC. The supernatant was discarded and the pellet allowed to air dry for 10 minutes. The pellet was re-suspended in 10 µL 2x Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125M Tris HCL pH 6.8) and boiled for 5 minutes. Samples were loaded onto an 8% sodium dodecyl sulfate-polyacrylamide gel and electrophoresed at 150 V for approximately 1.5 hours. Proteins from the polyacrylamide gel were transferred onto 0.45 µm nitrocellulose paper (VWR) at 0.4 Amps for 1 hour. The membrane

was blocked with 20 mL 1% milk/1x phosphate buffered saline with tween-20 (PBST) for 1 hour at room temperature, then washed 3x15 minutes with 1x PBST. The membrane was incubated with anti myc antibody (9E10) (Sigma) diluted 1:2000 in 1% milk/1x PBST for 1 hour, then washed 3x15 minutes with 1x PBST. Goat anti-mouse Alexa Fluor 680 (Invitrogen) and anti hIgG IRDye 800 (LI-COR Biosciences) antibodies were diluted 1:10,000 in 1% milk/ 1x PBST, and added to the membrane for 1 hour. The membrane was washed 6x5 minutes in 1x PBST, then 1x with ddH2O to remove any remaining PBST before being scanned by the Odyssey-Infrared Imaging System (LI-COR Biosciences).

Protein Purification

HEK293T cells were grown on five 10 cm cell culture plates, each containing 10 mL DMEM (GIBCO) supplemented with 10% Bovine serum and 1% pen/strep, for 48 hours. For each plate, 600 µL of 0.15M NaCl was added to 6 µL ADAMTSL2 DNA. 36 µL of ice cold PEI was added to the DNA solution, vortexed immediately, and incubated for 20 minutes at room temperature. HEK293T cells at 80% confluency, were transiently transfected with the DNA/PEI mixture in 10 mL DMEM, and incubated for 4 hours at 37 ºC. To decrease PEI toxicity, transfected cells were washed with 10 mL 1x PBS and the media was replaced with 10 mL of the reduced serum OPTI-MEM (GIBCO).

After 72 hours, 10 mL of media was collected from each plate, bringing the total volume to 50 mL ADAMTSL2 media. 200 µL Ni-NTA superflow beads (QIAGEN cat # 30430) were added to two 1.5 mL Eppendorf tubes. The slurry was centrifuged for 1 minute at 1,500 x *g*. The supernatant was removed and the beads were washed in 1 mL tris buffered saline (TBS), followed by spinning down for 1 minute at 1,500 x *g* 3x. 200 µL of beads was added to 50 mL ADAMTSL2 medium and incubated overnight at 4°C while rotating. The media was centrifuged for 10 minutes at 1,500 x *g*. The beads were washed 3x 1 mL TBST + 0.5M NaCl followed by spinning down for 3 minutes at 1,500 x *g*. The protein was eluted in 300 µL of 250mM imidazole for 1 hour at 4°C. Equal fractions of sample were collected from the washes, ADAMTSL2 medium, flow through, and elution. To ensure that the protein was purified, samples were run on an 8% sodium dodecyl sulfate-polyacrylamide gel and analyzed by both Western blot and Coomassie blue staining.

PNGase F digest

Approximately 2 µg of nickel purified ADAMTSL2 (in 100 µL 250mM imidazole) was precipitated with 400 µL of ice cold acetone at -20°C for 4 hours. The protein was pelleted by centrifugation at 16,000 x *g* for 10 minutes. Acetone was aspirated and the pellet was air dried for 10 minutes, and then re-suspended

in 40 μL of 1% SDS/1 % β-Mercaptoethanol. The sample was boiled for 5 minutes at 100°C. The sample was split into two 1.5 mL Eppendorf tubes, each with 450 μL of 50mM Tris-HCL pH 8.6, 10mM EDTA, 0.7% NP-40 (complete mini EDTA-free protease inhibitor, Roche). 5 μL peptide: *N*-glycosidase F (PNGase F) was added to one of the tubes, while 5 μL ddH2O was added to the other as a negative control. Both were incubated at room temperature for 16 hours. To stop the reaction, 4 volumes of ice cold acetone was added to the samples, and incubated overnight at -20°C. The sample was pelleted and air dried as before, and re-suspended in 2x Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125M Tris HCL pH 6.8). To verify that *N*-glycans were successfully removed, the sample was run on an 8% SDS-PAGE gel and stained with GelCode Blue (Pierce Net).

RESULTS

Site directed mutagenesis of ADAMTSL2

Allali *et al.* (2011) identified several mutations in ADAMTSL2 associated with Geleophysic dysplasia. G811R is in close proximity to the *N*-linked glycosylation and *O*-fucosylation sites of TSR6, while S635L is within the *O*-

fucosylation site of TSR3 (Figure 2C, Appendix A). G811R causes secretion defects proposed to lead to the development of this disorder (Le Goff *et al.,* 2009). Unlike the other TSRs of ADAMTSL2, TSR6 has a highly unusual configuration of overlapping *N*-linked glycosylation and *O*-fucosylation consensus sites. While *N*-linked glycosylation of TSR6 in both wild type and mutant proteins has not been studied, it has been demonstrated that *O*-fucosylation is essential for ADAMTSL2 secretion (unpublished, R.S Haltiwanger). We wanted to determine whether eliminating the *N*-linked glycosylation site or *O*-fucosylation site of TSR6 of ADAMTSL2 causes a secretion defect.

I used site-directed PCR mediated mutagenesis (described in Materials and Methods), to make substitutions in mouse ADAMTSL2 (Koo *et al*., 2007). To increase the efficiency of primer-template base pairing, primers were designed to be up to 45 bases in length with a melting temperature above 70°C (Table 1, Appendix B). I generated *ADAMTSL2* clones containing mutations predicted to block *N*-linked glycosylation (N809V), *O*-fucosylation (T809V) and both *N*-linked glycosylation and *O*-fucosylation (N807Q_T809V) (Figure 2D, Appendix A). In addition, to determine the effects of the Human Geleophysic dysplasia mutations on *O*-fucosylation, I generated the mouse *ADAMTSL2* G811R mutation and designed primers to generate the S635L mutation. Due to time constraints I was unable to complete the S635L mutagenesis. Mutations in *ADAMTSL2* clones

N807Q, T809V, N807Q_T809V, and G811R were verified by DNA sequencing using TSL2_seq1 primer (Table 3, Appendix B, Figure 3, Appendix A). Sequencing data from the N807Q, T809V, and N807Q_T809V, and G811R mutants were analyzed using 4peaks software (http://www.mekentosj.com/science/4peaks) (Figure 4, Appendix A). I performed a translated blast (tblastn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparing the cloned sequence to the wild type mADAMTSL2 protein (NP_084257), to verify that the clones contained the expected amino acid substitution (Figure 5,Appendix A). For the G811R clone, Dr. Holdener and I also performed a nucleotide blast, comparing the G811R mutant reference ESTs from the database. While reviewing the G811R sequencing data using Sequencher (http://genecodes.com) we noticed a G to A nucleotide substitution which resulted in a V to I amino acid change at position 903 (Figure 5, Appendix A). This nucleotide substitution is present in all our mutant clones, as well as the original ADAMTSL2 plasmid that was obtained from Dr. Suneel Apte's lab. We performed a multiple sequence alignment using Clustalw (http://www.genome.jp/tools-bin/clustalw) and found that valine is highly conserved (Figure 6, Appendix A). Due to time constraints, I was only able to sequence part of the N807Q, T809V, N807Q_T809V, and G811R clones. For wild type ADAMTSL2, I sequenced the beginning, mutation site, and end of the clone (Figure 3, Appendix A). Since there is a possibility of PCR induced

random mutations, all clones need to be fully sequenced before being used for future secretion assays and mass spectral analysis of TSR6 modification.

Loss of predicted N-linked glycosylation and/or O-fucosylation disrupts ADAMTSL2 secretion

To evaluate whether mutations N807Q, T809V and N807Q_T809V in mADAMTSL2 cause a secretion defect, we transiently co-transfected HEK293T cells with *ADAMTSL2* clones and *hIgG* control plasmid, and collected both the culture media and cell lysates 72 hours post transfection to use for Western analysis. ADAMTSL2 protein was visualized by western blot with 9E10 anti myc antibody, and visualized using goat anti-mouse Alexa Fluor 680 antibody, and visualized hIgG with anti hIgG IRDye 800 antibody (Figure 7A, Appendix A). Protein expression was quantified using Odyssey-Infrared Imaging System (LI-COR Biosciences). ADAMTSL2 levels in the media and cell lysates were normalized to hIgG, and are represented as percentages of wild type ADAMTSL2 (Figure 7 B and C, Appendix A). Mutant T809V displayed a similar secretion defect as N807Q. Interestingly, when both the predicted *N*-linked glycosylation and *O*-fucosylation sites are removed (N807Q_T809V) the protein is still secreted at levels comparable to the single mutants (Figure 7, Appendix A). In the N807Q

mutant, there is a slight shift downward in both the cell and culture media. This observation provides correlative evidence that this site is modified by *N*-glycans.

Purification of ADAMTSL2 for Mass Spectrometry analysis of TSR6 glycosylation

ADAMTSL2 consists of seven TSRs, 6 of which contain the consensus sequence for *O*-fucosylation. Site mapping confirmed that TSR1, 2, 3, 5, and 7 are modified with a fucose-glucose disaccharide (unpublished C. Leonhard-Melief). TSR6 has not yet been demonstrated to by *O*-fucosylated. As previously stated, TSR6 is highly unusual in that it contains an *N*-linked glycosylation site within the *O*-fucosylation consensus sequence. We predicted that TSR6 is modified with *O*-fucose or *N*-glycans, and that these two modifications may compete with one another. To evaluate this hypothesis, we wanted to use mass spectrometry to characterize modifications on purified mADAMTSL2. To collect protein for analysis, we expressed mADAMTSL2 plasmid in HEK293T cells and enriched ADAMTSL2 from media fractions using Ni-NTA purification. Because *N*-linked glycans complicate mass spectral analysis, we removed all the *N*-linked glycans from the purified protein with PNGase F digestion. As PNGase F removes *N*-glycans, Asn is converted to Asp, providing an indication that the amino acid was modified by an *N*-glycan. To determine whether the PNGase F

digest was successful, we ran the compared untreated and digested samples on an 8% sodium dodecyl sulfate-polyacrylamide gel and stained with Code Blue (Figure 8, Appendix A). Purified ADAMTSL2 isolated from culture media normally runs just below 180 kDa. When treated with PNGase F, the ADAMTSL2 band shifted down to just below 115 kDa, demonstrating that the *N*-glycans were successfully removed. These data indicated that ADAMTSL2 is modified with *N*-glycans, but since there are multiple *N*-glycosylation sites (N87, N364, N428, N475, N511, N524, N533, N544, N731, and N807); we cannot conclude from this data that N807 is modified. Due to time constraints, Christina, a PhD student in the Haltiwanger Lab, excised the protein bands and subjected them to in gel digest with either trypsin or GluC. Attempts to visualize the resulting peptides from TSR6 were unsuccessful (data not shown). Our results suggest that some *N*-linked glycans are present on ADAMTSL2. However, we were unable to successfully map TSR6 of ADAMTSL2, and therefore do not know whether there is an *N*-glycan at position N807, and/or an *O*-fucose at position T809.

DISCUSSION

We predicted that the *N*-linked glycosylation and/or *O*-fucosylation sites of TSR6 of ADAMTSL2 were necessary for efficient protein secretion. To test this hypothesis, we generated ADAMTSL2 mutations predicted to disrupt *N*-linked glycosylation, *O*-fucosylation, or both *N*-linked glycosylation and *O*-fucosylation, analyzed the effects of these mutations on ADAMTSL2 secretion, and attempted to visualize glycosylation (or lack of) on ADAMTSL2 TSR6. In addition to these mutants, we generated a G811R substitution in the mouse ADAMTSL2 TSR6 similar to that observed in GD patients (and designed primers for generation of a second GD mutation S635L). Future studies will determine whether these substitutions interfere with ADAMTSL2 secretion and/or *N*-glycosylation or *O*-fucosylation.

TSR6 is unique in that it is the only TSR in ADAMTSL2 with an *N*-linked glycosylation site within the *O*-fucosylation consensus sequence. We predicted that *N*-linked glycosylation and *O*-fucosylation act as signals for secretion, and are able to compensate for one another when either is removed. However, our results demonstrated that elimination of both the *N*-linked glycosylation site and *O*-fucosylation site (N807Q_T809V) resulted in secretion levels similar to N807Q or T809V. If these glycosylation sites compensated for one another, we would

expect to see a more dramatic secretion defect in the N807Q_T809V mutant. One possibility is that eliminating the *N*-linked glycosylation site, and/or the *O*-fucosylation site is disrupting the complex folding of TSR6. While quantifying ADAMTSL2 expression, we also noticed a slight accumulation of mutant ADAMTSL2 in the cell. If the loss of glycosylation caused a defect in folding, we expect to see degradation of the misfolded protein. Previous results demonstrated that *O*-fucosylation of ADAMTSL2 is essential for efficient protein secretion (unpublished, R.S Haltiwanger). Since ADAMTSL2 has seven TSRs, six of which contain an *O*-fucosylation consensus site, loss of glycosylation on a single TSR may not be enough to completely disrupt protein secretion. In patients with GD, mutations in ADAMTSL2 cause secretion defects (Le Goff *et al*., 2009). However, it may be possible that the disease is not entirely related to the secretion defects, but could also result from altered functions in the extracellular matrix. Additional experiments should be performed to determine the function of wild type ADAMTSL2, and ADAMTSL2 mutants that have been identified in GD patients.

One specific disease causing mutation, G811R, is found within TSR6 of ADAMTSL2. We sought to identify whether the G811R mutation, located within the *O*-fucosylation consensus sequence is disrupting glycosylation. For this study, we did not have enough time to map the G811R clone. Given the fact that G811R mutants display dramatic secretion defects, and our glycosylation mutants

were still being secreted, I propose that this mutation is causing structural changes to ADAMTSL2, therefore disrupting proper protein folding since this change substitutes a hydrophobic amino acid (glycine) for a positively charged amino acid (arginine).

While reviewing the sequencing data for our G811R clone, we noticed a G to A nucleotide change resulting in a valine to isoleucine amino acid substitution. This was present in all of our mutant clones, as well as our wild type ADAMTSL2 plasmid from Dr. Suneel Apte's lab. To determine whether this substitution could be critical to protein function, we performed a multiple sequence alignment, and found that valine was highly conserved among 11 species (Figure 5, Appendix A). Although valine and isoleucine vary in length, both have β branched carbons, which may be useful for stabilizing secondary structure or giving points of rigidity to the protein. For use in future experiments, mutants N807Q, T809V, N807Q_T809V, and G811R should be fully sequenced to verify that no additional mutations were introduced during site directed PCR mutagenesis. Future studies should focus on mapping glycosylation sites in TSR6 of ADAMTSL2. Due to difficulties in visualizing the peptides from TSR6, we have been unable to map this region. Although the shift on our SDS-PAGE gel strongly suggests that N807 is modified with *N*-glycans (Figure 7, Appendix A), we still need site mapping data for verification. It may be useful to try a larger sample size, and try a different digestive enzyme for future experiments.

Mapping these sites will provide a better understanding of how TSR6 is modified, along with new possible explanations for the secretion defects seen in N807Q, T809V, and N807_T809V mutants. Site mapping should also be performed on the G811R clone to determine whether this mutation is disrupting the predicted glycosylation or causing a folding defect. Additionally, the full-length sequence of ADAMTSL2 needs to be checked using sequencing primers to cover every 500 bases. N807Q, T809V, N807Q_T809V and G811R mutants also need to be fully sequenced to verify that no additional mutations were introduced.

Due to time constraints, the secretion assays were not performed in triplicate. In the future, we should set up triplicate secretion assays to verify the results demonstrated in this paper. Triplicate samples with additional clones also need to be analyzed to accurately determine the disparity in mutant secretion and whether it is statistically significant. In summary, knowledge of the regulatory roles of *N*-linked glycosylation and *O*-fucosylation that are necessary for protein secretion, has important implications for understanding how the secretion defects in ADAMTSL2 cause Geleophysic dysplasia.

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APPENDIX A

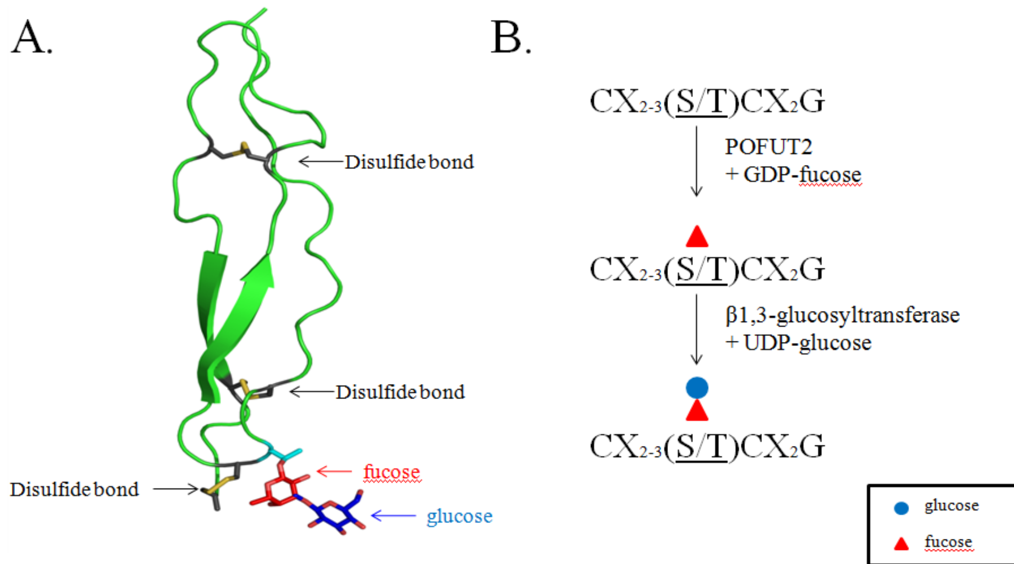

Figure 1. Structure and glycosylation of TSRs. **A.** TSRs are approximately 60 amino acids in length and consist of six conserved cysteines, forming 3 disulfide bonds (Lawler and Hynes 1986). C. Leonhard-Melief modeled the glycans onto TSR2 from human TSP1 (Tan *et al*., 2002) using Sweet Software (www.glycosciences.de/modeling/sweet2/doc/index.php). **B.** POFUT2 uses GDP-fucose to *O*-fucosylate TSRs at a S or T in the consensus sequence CX_{2-3}(S/T)CX_2G. The fucose (▲) can be elongated with glucose (●) by the enzyme β1,3-glucosyltransferase using UDP-glucose.

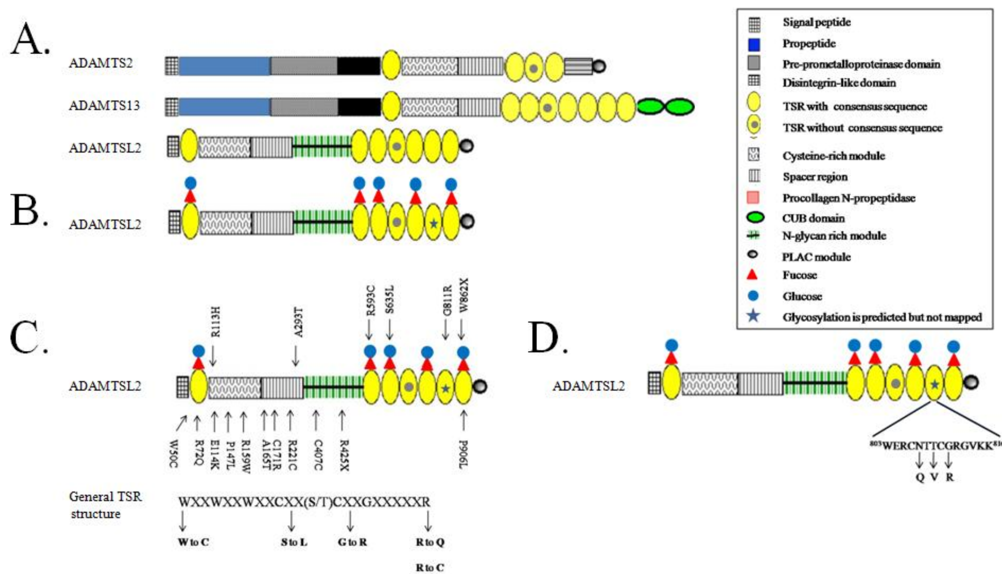

Figure 2. ADAMTS and ADAMTSL structure, ADAMTSL2 mapped *O*-fucosylation sites, location of GD causing mutations, and glycosylation mutations. This Figure is adapted from Apte 2009 and Allali *et al.* 2011. **A.** The ADAMTS proteases are structurally similar, consisting of a signal peptide, pre-prometalloproteinase domain, disintegrin-like domain, one or more TSRs, a spacer region and cysteine rich module. ADAMTSL proteins lack the prometalloproteinase and disintegrin like domain. **B.** Six of the seven TSRs of ADAMTSL2 contain the consensus sequence for *O*-fucosylation by POFUT2. Of these six, TSR1, TSR2, TSR3, TSR5 and TSR7 have been shown to be modified with a fucose-glucose disaccharide (unpublished, C. Leonhard-Melief). There is no mapping data for TSR6. **C.** Allali *et al.* (2011) identified several mutations in patients with GD located throughout the ADAMTSL2 gene. The mutations G811R and P906L were labeled incorrectly in the Allali et al. (2011) paper. This figure was modified with G811R in TSR6, and P906L in TSR7, as verified by the ADAMTSL2 reference protein: Q86TH1. Seven mutations are located within TSRs. A single TSR is represented below with the amino acid substitutions seen in the mutations W50C, R72Q, R593C, S635L, and G811R. The mutation W862X causes TSR7 truncation. **D.** TSR6 of ADAMTSL2 with mutations made to block the *N*-linked glycosylation site (N807Q), *O*-fucosylation site (T809V) or both glycosylation sites (N807Q_T809V), and the GD causing mutation, G811R.

```
T7 ──>
   1 tgggctgagagcccctcctgggaacccccctcccttgaatgctctttgaagtgggagagggaggcagctggggaagcaggaccagagacaggcagagcccgcacatctctgcactcacctt
 121 cctgcaccgtgcacctggcagtagctctcaccctttctctaaaaggaggacaacctgctgaccctgggtgaggatggatggcagacggcagcatccacactgggcttggtccttgctgg
 241 ctgtagcagttgtggctggggtgcggcacctacggaggcttccgacaacagccccacatccaacagcctagagggaggtgctgacaccactgcctactggtgggggagtggaccaagt
 361 ggacagcatgttcccgcagttgtgggggcggggtgacatcccaggagcgccactgccttcaacagaggaggaagtctgttccaggcacagggaacaggacctgtgtgggcacgtccaaac
 481 ggtaccagctctgcagagtgcaggagtgtccaccggatgggaggagcttccgtgaagagcagtgtgtctccttcaactcccgtgtgtatgacggccgggcgtaccagtggaagcccctttt
 601 acccggatgactacgtgcacatctccagtaagccatgcgacctacactgcagcaccgtggacggccaacggcagctcacggtgccggcccgagacggcacttcctgcaagctcaccgacc
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 961 ttgcagatgaagctggtttctacttcttcaatggcaactacaaagtggacagccccaagaacttcaacattgctggcacagtggtcaagtaccgacggcccatggatgtctatgagactg
1081 gcatcgagtatatagtggcacaggggcccaccaaccagggcctgaatgtcatggtgtggaaccagaacggcaaaagcccctctatcaccttcgagtacaccttgctgcagtcacctcaca
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1921 tctcatcccatgagccctgcagtgccacatgcaccacaggagtcatgtccacctatgccatgtgtgttcgctatgatggtgttgaagtggatgacagctactgcgatgccctcacccgtc
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                                                       TSL2_seq1 ──>
2401 agaactgcactgggcctccctgtgaccgccagtggactgtctcggactggggaccgtgcagtgggagctgtggacagggccgcactatcaggcatgtgtactgtaagaccagcgatggac
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2881 gtgacccactggtgaagcctgttggccgacaggcttgtgacttgcagccatgccccacagagcccccagatgacagctgccaggaccagccaggcaccaactgtgcactggcaatcaaag
                                    TSL2_seq2 ──>
3001 tgaacctctgtggccactggtattacagcaaagcctgctgtcgctcctgcaggccccccattcctagctgctgctgctgcagcccctgagatataagcacgctatcctggggcttcctt
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Figure 3. Sequencing primers for verification of mutations. Sequencing primers were designed to cover the beginning, mutation site (highlighted), and end of mADAMTSL2 (Koo et al., 2007) to verify that only the appropriate mutations were introduced. Primer names are listed in red. The arrows represent primer direction. Additional primers need to be designed to cover the entire ADAMTSL2 sequence to verify that no other mutations were introduced during site directed PCR mutagenesis.

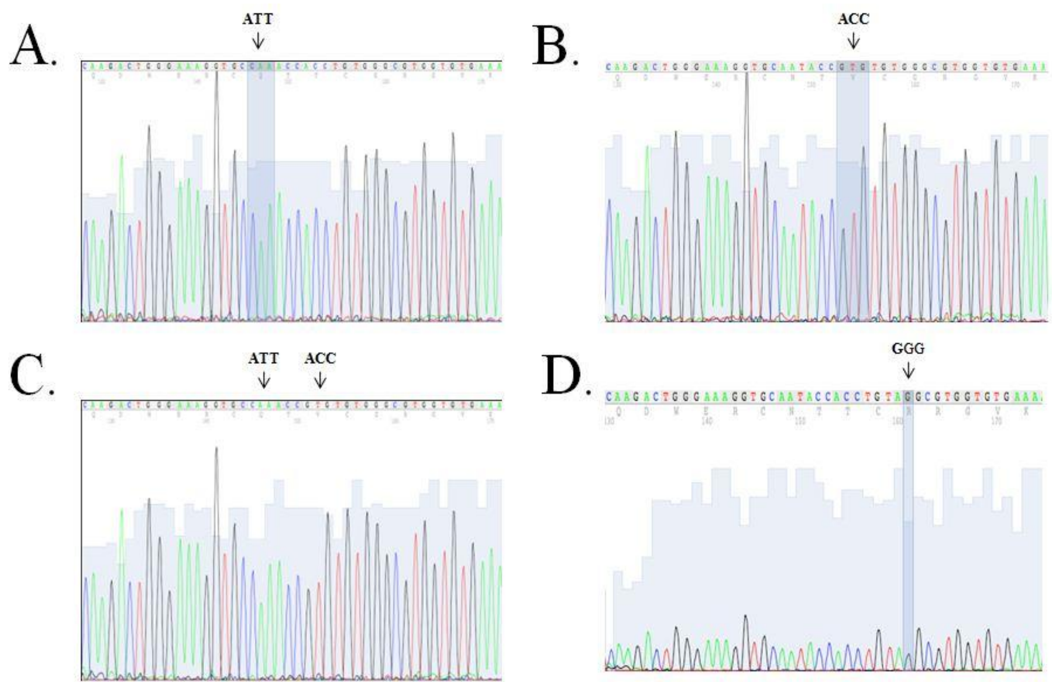

Figure 4. DNA sequence analysis of ADAMTSL2 clones N807Q, T809V, N807Q_T809V, and G811R using 4peaks software (http://www.mekentosj.com/science/4peaks). Chromatograms demonstrate desired nucleotide substitutions (bold) in **A.** N807Q **B.** T809V **C.** N807Q_T809V and **D.** G811R mutants.

Figure 5. Amino acid comparison of ADAMTSL2 (NP_084257) and mutants N807Q, T809V, N807Q_T809V, and G811R. ADAMTSL2 protein sequence (NP_084257) was compared to translated partial DNA sequence from ADAMTSL2 mutants: **A.** N807Q, **B.** T809V, **C.** N807Q_T809V, and **D.** G811R mutants using translated blast (tblastn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The correct amino acid change is boxed in blue. The valine to isoleucine amino acid change is boxed in red.

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               843                                                                                              940
DOG            CGLAKKPPEESTCFERPCFKWYTSPWSE----------------------------------------------------------------------
GIANT PANDA    CGLAKKPPEESTCFERPCFKWYTSPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVGRQACDLQACPTEPPDDSCQDQPGTNCGLAIKVSLCGHW
HORSE          CGLAKKPPEESTCFERPCFKWYTSPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVGRQACDLQPCPTEPPDDSCQDQPGTNCALAIKVNLCGHW
CATTLE         CGLAKKPPEESTCFERPCFKWYTSPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVGRQACDLQPCPTEPPDDSCQDQPGTNCALAIKVNLCGHW
HUMAN          CGLAKKPPEESTCFERPCFKWYTSPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVGRQACDLQPCPTEPPDDSCQDQPGTNCALAIKVNLCGHW
MOUSE          CGLARKPPEESTCFERPCFKWYTSPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVGRQACDLQPCPTEPPDDSCQDQPGTNCALAIKVNLCGHW
HAMSTER        CGLSRKPPEESTCFERPCFKWYTSPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVGRQACDLQPCPTEPPDDSCQDQPGTNCALAIKVNLCSHW
OPOSSUM        CDITKKPTEESTCFERPCFKWYTTPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVAKQTCDLQPCPTEPPDDSCQDQPGTNCALAIKVNLCSHW
GIBBON         CGLAKKPAEESTCFERPCFKWYTSPWSECTKTCGVGVRMRDVKCYQGTDIVRGCDPLVKFVGRQACDLQPCPTEPPDDSCQDQPGTNCALAIKVNLCGHW
ANOLE          CDVTKKPIEETTCFERPCFKWYTTPWSECTKTCGIGVRMRDVKCYQGKDIVRGCDPLVKFVGKQTCDLQPCPTEPPDDSCQDQAGTNCALAIKVNLCSHW
ZEBRAFISH      CDASKRPADEDTCFERPCFKWYTTPWSECTKTCGVGVRMRDVKCYQGREIVRGCDPLTKFVNKQTCALQPCPTEPPDENCQDRPTTNCSLALKVNLCSHW
               *.  :::*  :* ************:****
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Figure 6. Multiple sequence alignment demonstrates that valine is highly conserved among species. Segment of ADAMTSL2 multiple sequence alignment using Clustalw (http://www.genome.jp/tools-bin/clustalw). All species depicted above have the amino acid valine at position 903 (boxed in blue).

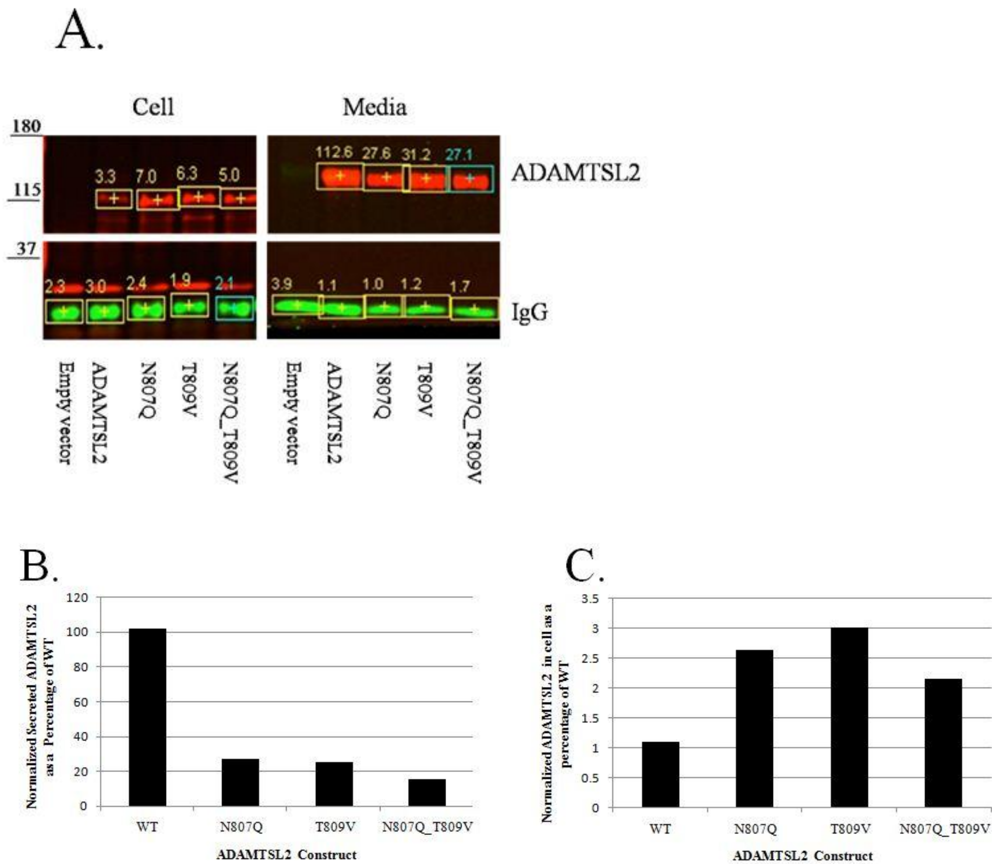

Figure 7. Elimination of one or both sites of glycosylation on mADAMTSL2 TSR6 causes decreased secretion. A. Quantitative analysis of wild type and mutant ADAMTSL2 expression using the Odyssey-Infrared Imaging System (LI-COR Biosciences). HEK293T cells were transiently transfected with wild type and mutant DNA. Media and cell lysate were collected 72 hour post transfection. B. ADAMTSL2 was normalized to IgG, and the mutants were represented as a percentage of wild type ADAMTSL2 in cultured media and C. the cell.

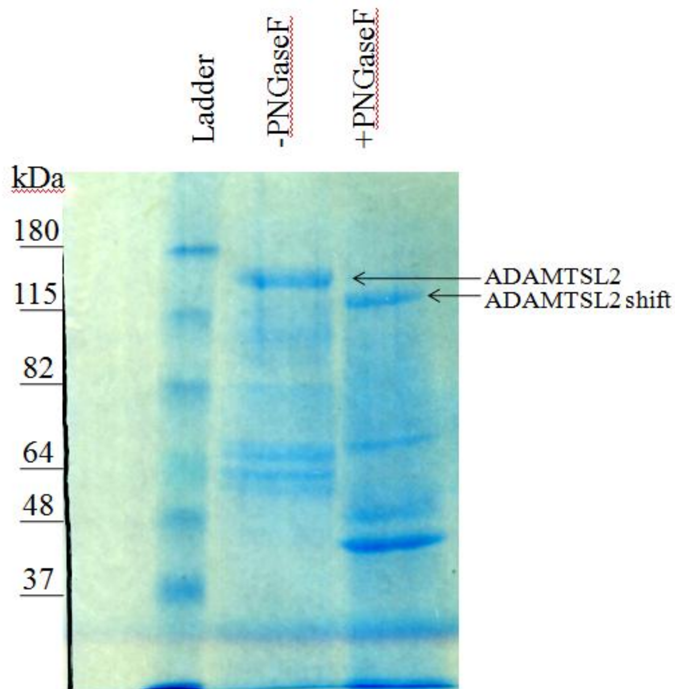

Figure 8. ***N*-glycans on Ni-NTA purified ADAMTSL2 were removed by PNGase F as demonstrated by SDS-PAGE with Code Blue staining.** Purified ADAMTSL2 was digested with PNGaseF, ran on an 8% sodium dodecyl sulfate-polyacrylamide gel and stained with Code Blue. Lane 1: Benchmark Pre-Stained Protein Ladder (Invitrogen). Lane 2: ADAMTSL2 from culture media runs just below 180kDa. Lane 3: PNGase F treated ADAMTSL2 band shifts down just above 115 kDa, demonstrating successful removal of *N*-linked glycans.

APPENDIX B

Table 1. Primer Pairs for ADAMSL2 TSR6 Site Directed Mutagenesis.

Clone	Primer Name	Sequence	Tm
N807Q	ADAMTSL2_N807Q	5’GGCTCAAGACTGGGAAAGGTGCCAAACCACCTGTGGGCGTGGTG-3’	74.77°C
	ADAMTSL2_N807Q-r	5’-CACCAGGCCCACAGGTGGTTTGGCACCTTTCCCAGTCTTGAGCC-3’	
T809V	ADAMTSL2_T809V	5’-GGCTCAAGACTGGGAAAGGTGCAATACCGTGTGTGGGCGTGGTG-3’	73.85°C
	ADAMTSL2_T809V-r	5’-CACCACGCCCACACACGGTATTGCACCTTTCCCAGTCTTGAGCC-3’	
N807Q_T809V	TSL2_N807Q_T809V	5’-GGCTCAAGACTGGGAAAGGTGCCAAACCGTGTGTGGGCGTGGTG-3’	74.77°C
	TSL2_N807Q_T809V-r	5’-CACCACGCCCACACACGGTATTGCACCTTTCCCAGTCTTGAGCC-3’	
G811R	TSL2_G811R_s2	5’-CAATACCACCTGTAGGCGTGGTGTGAAAAAGCGGTTAGTTCTCTG-3’	70°C
	TSL2_G811R_s2-r	5’-GTTATGGTGGACATCCGCACCACACTTTTTCGCCAATCAAGAGAC-3’	
S635L	TSL2_S635L	5’-GACCAGCAGCTGGAGTGAGTGCTTACGTACCTGTGGTGAGGGCC-3’	74.77°C
	TSL2_S635L-r	5’-CTGGTCGTCGACCTCACTCACGAATGCATGGACACCACTCCCGG-3’	

Primers were designed to be 44 base pairs in length with a melting temperature above 73ºC.

Table 2. Primers Used for Verification of ADAMTSL2 Mutations

Primer Name	Sequence
T7	5′-TAATACGACTCACTATAGGG-3’
TSL2_s1	5’-GTGGGAGCTGTGGACAGGGC-3’
TSL2_s2	5’-GTCGCTCCTGCAGGCCCCCC-3’

Mutations in ADAMTSL2 clones N807Q, T809V, N807Q_T809V, and G811R were verified using TSL2_s1 sequencing primer. Primers from Dr. Holdener’s Lab (T7) and TSL2_s2 were used to sequence the beginning and end of WT ADAMTSL2 (Figure 3) to confirm that no additional mutations were introduced during PCR. Additional sequencing primers need to be designed to cover the entire ADAMTSL2 sequence.

Table 3: Sequencing Records for the ADAMTSL2 TSR6 Mutants N807Q, T809V, N807Q_T809V, and G811R.

Clone	Primer name	Sequencing primer	Sequence file name	Date
N807Q	TSL2_seq1	5'-GTGGGAGCTGTGGACAGGGC-3'	E09_SDS1_Haltiwanger_Haltiwanger	09/23/11
T809V	TSL2_seq1	5'-GTGGGAGCTGTGGACAGGGC-3'	A05_SDS5_Haltiwanger_Haltiwanger	10/12/11
N807Q_T809V	TSL2_seq1	5'-GTGGGAGCTGTGGACAGGGC-3'	A08_SDS8_Haltiwanger_Haltiwanger	10/12/11
G811R	TSL2_seq1	5'-GTGGGAGCTGTGGACAGGGC-3'	C04_SDS4_Haltiwanger_Haltiwanger	11/11/11