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A Role for *O*-Fucosylation in the Folding of Thrombospondin Type I Repeats

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Abstract of the Dissertation

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Protein *O*-fucosyltransferase 2 (Pofut2) is a soluble, ER localized enzyme that adds a fucose residue to specific Serines and Threonines found in Thrombospondin type I repeats (TSRs). TSRs are small, cysteine-rich motifs usually found as tandem repeats. The current consensus sequence for *O*-fucosylation is CXX(S/T)CXXG. Database searches with the consensus sequence predict fifty TSR-containing protein targets for Pofut2. The *O*-fucose on TSRs is extended by the addition of a β1,3-glucose, catalyzed by β3-glucosyltransferase (B3GALTL). Pofut2 knockout mice are early embryonic lethal while B3GALTL mutations in humans cause a development disorder called Peters plus syndrome. To understand Pofut2 and B3GALTL phenotypes, it is important to deduce the molecular role of *O*-fucosylation.

Pofut2 can distinguish between properly folded and unfolded TSRs *in vitro*. Taken together with its localization to the ER, a protein-folding compartment, we have hypothesized that Pofut2 plays a role in quality control. Eliminating the donor substrate, GDP-fucose, or Pofut2, results in loss of secretion of two targets – ADAMTS13 and ADAMTSL1. In this thesis, I extend these observations to other Pofut2 targets and demonstrate that Pofut2 has a dual role as a chaperone and fucosyltransferase. I show that both the

number of tandem TSRs and the primary amino acid sequence influence fucose-dependent secretion. I demonstrate that *O*-fucosylation is both co-translational and post-translational. I show that in the absence of GDP-fucose, Pofut2 binds more tightly to its substrates, providing a potential explanation for why elimination of GDP-fucose results in decreased secretion of target proteins. I also identify several ER-resident proteins that are in complex with Pofut2, potentially assisting in the folding of TSRs and retaining Pofut2 in the ER. Mature TSRs from target proteins show high stoichiometries of *O*-fucosylation, whereas most cell-associated proteins are aggregated, partially folded and poorly fucosylated. A small portion of cell-associated protein is mostly folded and is nearly fully fucosylated, suggesting that *O*-fucosylation is a marker of properly folded TSRs in the cell. Finally, I establish a direct role for Pofut2 in the folding of TSRs *in vitro* and determine that both GDP-fucose and enzymatic activity are required for this process.

For my friends, without who I would have never found myself

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List of Abbreviations

6AF	6-Alkynyl Fucose
His6	Six Histidine protein tag
ADAMTS	A Disintegrin-like And Metalloprotease Domain with Thrombospondin type -I repeats
ADAMTSL	ADAMTS-Like
α3SiaT	α2-6Sialyltransferase
α-MEM	Alpha Minimum Essential Medium
Arg	Arginine
Asp	Asparagine
β3GlcT	β3-Glucosyltransferase
β4GalT-1	β4-Galatosyltransferase
BAI	Brain specific Angiogenesis Inhibitor
BCS	Bovine Calf Serum
BiP	Binding Immunoglobulin Protein
C	Cysteine
C-	Carboxy
CCN	Cyr61 CTGF Nov
CID	Collision Induced Dissociation
CHO	Chinese hamster ovary
CHX	Cycloheximide
CILP	Cartilage intermediate layer protein
COMP	Cartilage oligimeric matrix protein
CNX	Calnexin
CRT	Calreticulin
CTGF	Connective Tissue Growth Factor
Cys	Cysteine
D	Aspartic acid
Da	Dalton
dHex	Deoxyhexose
DMEM	Dulbecco's Modified Eagle's Medium
dPEI	Deacetylated Polyethylenimine
DSP	Dithiobis[Succinimidyl Propionate]
E	Glutamic acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor-like repeat
EIC	Extracted Ion Chromatogram
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial to Mesenchymal Transition
EndoH	Endoglycosidase H

ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
ERGIC	ER-Golgi Intermediate Compartment
EV	Empty Vector
Fuc	Fucose
Fut	Fucosyltransferase
FX	GDP-4-keto-6deoxy-D-mannose-3,5-epimerase-4-reductase
G	Glycine
g	Gravity
GalNAc	N-Acetylgalactosamine
GDP	Guanosine Diphospho
Glc	Glucose
GlcNAc	N-Acetylglucosamine
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
Glu	Glutamine
GMD	GDP-mannose 4,6-dehydratase
Gxylt	Glucoside α3-xylosyltransferase
H	Histidine
HEK293T	Human Embryonic Kidney 293T Cells
Hex	Hexose
His	Histidine
Hsp	Heat-shock Protein
HPLC	High Pressure Liquid Chromatography
I	Isoleucine
IgG	Immunoglobulin G
IP	Immunoprecipitation
K	Lysine
L	Leucine
Lac	Lactacystin
LADII	Leukocyte Adhesion Deficiency type II
LC	Liquid Chromatography
Man	Mannose
m/z	Mass to charge ratio
MMP	Matrix Metalloprotease
MS	Mass Spectra
N	Asparagine
N-	Amino
Ni-NTA	Nickel-nitrilotriacetic acid
O	Oxygen
OFUT	*O*-Fucosyltransferase
OptiMEM	Optimum Minimal Essential Medium
P	Proline

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDI	Protein disulfide isomerase
PLAC	Protease And Lacunin domain
PNGaseF	Peptide N-glycosidase F
POMT	Protein *O*-Mannoysltransferase
Pofut	Protein *O*-Fucosyltransferase
Poglut	Protein *O*-Glucosyltransferase
RIPA	Radioimmunoprecipitation assay
Q	Glutamine
S	Serine
SEMA	Semaphorin
Ser	Serine
SLC	Solute carrier family
strep	Streptavidin
T	Threonine
TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TFA	Trifluoroacetic acid
Thr	Threonine
THSD	Thrombospondin type-1 domain-containing protein
TSP	Thrombospondin
TSR	Thrombospondin type 1 Repeat
Trp	Tryptophan
TTP	Thrombotic Thrombocytopenic Purpura
UGGT	UDP-Glucose:Glucosyltransferase
V	Valine
vWF	von Willebrand Factor
W	Tryptophan
WISP	Wnt-inducible signaling pathway protein
X	Any amino acid
Xxylt	Xyloside α3-Xylosyltransferase

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I am eternally grateful to Bob Haltiwanger for investing countless hours in mentoring me, for teaching me to think inside and outside the box, for believing in me at a time when no one else seemed to, and for showing me by example how to be a wonderful person and scientist.

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Chapter 1. Introduction

The structure of a protein is key to its identity and activity. This three-dimensional structure can be modified, regulated and modulated not only by the folding of the primary amino-acid chain, but also the addition of a variety of moieties ranging from inorganic phosphates to sugars. Some modifications are reversible and are used as switches to regulate protein function while some others irreversibly alter the structure, and hence function, of proteins. Most types of glycosylation are irreversible and hence have structural influences on their target protein. Glycosylation can occur on side chains of several different amino acids and can be categorized based on the type of linkage between the sugar and amino acid. There are four types of glycosylation: *N*-linked, which commonly occurs on side chains of asparagines; *O*-linked, which occurs on hydroxyl groups of serines and threonines; *C*-linked, which occur on tryptophans, and *S*-linked, which occur on cysteines. The first two types, *N*- and *O*-linked, are the most common modifications found on proteins (1). *N*-linked glycosylation occurs on a large percentage of secreted proteins and in addition to altering the structure and function, it has been demonstrated to have a role in folding and quality control of these proteins (see Section 1.8 below) (2).

The most common type of *O*-linked glycosylation is the addition of *O*-GalNAc, which forms the core structure for what is commonly called mucin-type glycosylation (3). However, there are several less common *O*-linked additions, which form the basis for structural modifications on proteins. These occur less frequently in comparison to *O*-GalNAc but nonetheless have significant and interesting roles in glycoprotein function. *O*-Linked GlcNAc, catalyzed by *O*-GlcNAc transferase (OGT), is different from other *O*-glycosylation

modifications in that it is reversible type and is found on nuclear and cytoplasmic proteins (and some secreted proteins) (4). Addition of *O*-GlcNAc to some proteins serves as a switch in signaling cascades while the role of the modification in secreted proteins is yet unknown. *O*-Linked mannose is added in the ER by protein *O*-mannosyltransferase 1 and 2 (POMT1/2) and can be elongated in multiple ways by other glycosyltransferases (5). Defects in the *O*-mannosylation pathway have been linked to many types of muscular dystrophies, primarily because of lack of *O*-mannose on alpha-dystroglycan, an extracellular matrix protein (5). Recently, another role for *O*-mannosylation was discovered in the termination of futile protein folding cycles in the ER (6). *O*-Linked glucose and fucose are found in specific protein motifs and are important structural modifications for the functioning of their target protein (see Section 1.1 below) (7). *O*-Glucosylation has been mostly famously examined on the signaling molecule, Notch, and is necessary for Notch activity (see Section 1.2 below) (8).

1.1. *O*-Fucosylation

O-Fucosylation is the addition of a fucose moiety to the hydroxyl side chain of a serine or threonine residue. It was originally identified in 1975 as an amino acid glycoside, Glcβ1-3Fuc-*O*-Ser/Thr, isolated from human urine (9). However, it was not until 1990 that the first protein modified with an *O*-linked fucose was identified: urokinase (10). In the following years, *O*-fucose was observed on several proteins, including Factor VII, IX and XII (11,12). On human Factor IX, the *O*-fucose was elongated to a tetrasaccharide structure: NeuAcα2-6Galβ1-4GlcNAcβ1-3Fuc. Since all these proteins were modified on a common cysteine-rich motif, called Epidermal Growth Factor-like repeats (EGF repeats), the idea of a consensus sequence for *O*-fucosylation was proposed, where the *O*-fucose could be elongated to the Glcβ1-3Fuc disaccharide or the NeuAcα2-6Galβ1-4GlcNAcβ1-3Fuc tetrasacchardie (13). The subsequent discovery of the Glcβ1-3Fuc disaccharide on a different cysteine-rich motif, Thrombospondin Type 1 Repeats (TSRs) (14), suggested there may be two independent *O*-fucosylation pathways. Identification of the enzymes involved in addition of these sugars to EGF repeats and TSRs confirmed that there are two distinct pathways for *O*-fucosylation (15) (Fig. 1.1).

In mammals, *O*-fucosylation on EGF repeats is initiated by Protein *O*-fucosyltransferase 1 (Pofut1) (16,17). This modification can be extended to a tetrasaccharide by the addition of a N-acetylglucosamine, galactose and sialic acid (in that order) by the enzymes Fringe, β4-Galactosyltransferase1 and α2-6-sialytransferase respectively (15). The *O*-fucose on TSRs is added by Protein *O*-fucosyltransferase 2 (Pofut2), and the modification can be extended to a disaccharide by the addition of glucose by the enzyme β3-Glucosyltransferase (β3GlcT) (15). These enzymes are discussed in detail in Sections 1.4 and 1.6 of this chapter.

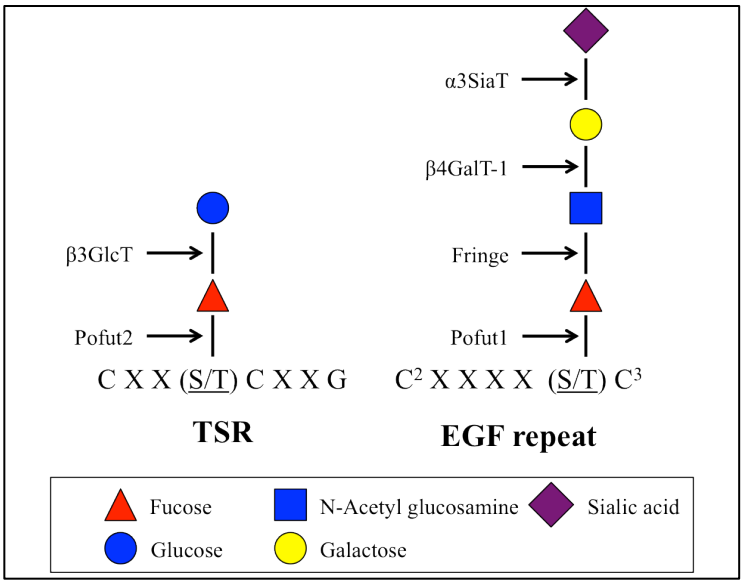


Fig. 1.1. Schematic showing *O*-fucosylation on TSRs and EGFs: Thrombospondin type I repeats (TSRs) and Epidermal growth factor-like repeats (EGF repeat) are *O*-fucosylated by distinct enzymes (15). The consensus sequences for each motif is as shown. C^2 and C^3 indicate the second and the third cysteine of the EGF repeat. Protein *O*-fucosyltransferase (Pofut) 1 and 2 add *O*-fucose to EGF repeats and TSRs respectively. The modification on TSRs is elongated by β3-glucosyltransferase (β3GlcT). *O*-Fucose on EGF repeats is extended by Fringe, β4-galactosyltransferase 1 (β4GalT-1) and α2-6-sialytransferase (α3SiaT).

Both EGF repeats and TSRs are small cysteine-rich modules characterized by three disulfide bonds (7). The fucose is added to the hydroxyl side chain of serine or threonine (Fig. 1.1) present within a consensus sequence. Based on previously mapped sites of fucosylation on EGF repeats and TSRs, consensus sequences have been derived that can predict other sites of modification. The current consensus sequence for EGF repeats and TSRs is as indicated in Figure 1.1 (15). Over one hundred proteins have at least one EGF repeat with a consensus sequence and fifty proteins have at least one TSR with a consensus sequences (16,18). Most of these proteins have known biological roles and several have pathological associations.

1.2. Epidermal Growth Factor-like (EGF) repeats

EGF repeats are cysteine-rich motifs around 40 amino acids long and were first identified in Epidermal Growth Factor (EGF) (19). They are widely found modules seen in about 198 proteins in the Uniprot database. However, only a subset of these proteins (~100) contains a consensus sequence for *O*-fucosylation by Pofut1 (20). EGF repeats have six conserved cysteines that form 3 disulfide bonds, and the consensus sequence for *O*-fucosylation is found between the second and third cysteine (Fig 1.1). This modification is known to exist on EGF repeats in several proteins including Notch, a transmembrane receptor, extracellular matrix proteins such as laminin and soluble factors like Factor VIII and XI.

The *O*-fucosylation sites on the EGF repeats of Notch extracellular domain have been extensively studied (21). Elongation of the *O*-fucose by Fringe modulates Notch signaling by altering ligand preference (22,23). Elimination of Pofut1 in mice results in embryonic lethality with some similarities to a Notch knockout phenotypes (24). In addition to *O*-fucosylation, EGF repeats on Notch can be modified with *N*-glycans, *O*-glucose and *O*-GlcNAc (25-27). *O*-Glucosylation on EGF repeats also occurs within a consensus sequence (C^1XSXPC^2), and is catalyzed by Protein *O*-glucosyltransferase (Poglut) (28). This modification can also be extended by the addition of two xyloses. Two Glucoside α3-xylosyltransferases, 1 and 2, (Gxylt1/2) add an α3-xylose to *O*-glucose, Xyloside α3-xylosyltransferase (Xxylt) adds the second xylose residue (29,30). Some EGF repeats also contain consensus sequences for Calcium binding, and are important for the structural integrity of the domain (19). Mutations in the *O*-glycosylation sites, calcium-binding regions or in the enzymes involved in synthesis lead to defects in Notch signaling to various degrees (27,31).

1.3. Thrombospondin type I repeat (TSR)

Thrombospondin type I repeats were originally named as one of the domains in the multi-domain matricellular protein Thrombospondin 1 (TSP1). They are found in many secreted proteins (~65 in humans) and are typically found as C-terminal tandem repeats. TSRs are about 60 amino acids in length and are defined by a number of conserved residues including six cysteines that form three disulfide bonds. Depending on the disulfide-bonding pattern of Cys1-6, TSRs can be classified as either group 1 or group 2 (32,33). Group 1 TSRs have the pattern: 1-5, 2-6, 3-4 while Group 2 repeats have the pattern: 1-4, 2-5, 3-6 (Fig. 1.2). Structural analysis of TSR1-3 from TSP1 revealed that TSRs consist of three anti-parallel strands that are held together by disulfide bonds (Fig. 1.3) (34).

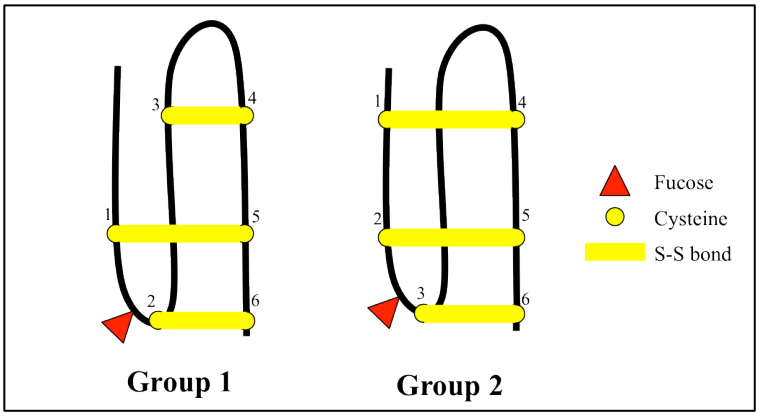


Fig. 1.2. Schematic showing the different disulfide bonding patterns of Group 1 and 2 TSRs: Group 1 TSRs have a 1-5, 2-6, 3-4 disulfide bonding pattern with the fucosylation site between Cys^1 and Cys^2. Group 2 TSRs have a 1-4, 2-5, 3-6 bonding pattern. The fucosylation site is between Cys^2 and Cys^3 (32).

The consensus sequence for *O*-fucosylation in both groups is Cys-X-X-(Ser/Thr)-Cys-X-X-Gly (35). The modified serine and threonine is present between Cys^1 and Cys^2 in Group I repeats while in Group 2 repeats, it is present between Cys^2 and Cys^3. In addition to the presence of the consensus sequence, accurate disulfide bond formation between the cysteines is also crucial for fucosylation (36). Database searches with the consensus sequence in the context of a TSR reveal 50 proteins predicted to be *O*-fucosylated (Table 1). *O*-Fucosylation has been

confirmed on several of these (TSP1 (14), TSP2 (35), F-spondin (14), Properdin (37), ADAMTS13 (38), ADAMTSL1 (39)) indicating that the consensus sequence can be used to accurately predict the presence of *O*-fucosylation. Database searches with the consensus sequence outside the context of TSRs predict an additional 185 proteins in humans. It remains to be examined if consensus sequences outside of TSRs can be modified by Pofut2.

In addition to *O*-fucosylation, TSRs may be modified with a *C*-linked mannose (Fig. 1.4) (14). *C*-mannosylation occurs on tryptophan residues present within the consensus sequence Trp-X-X-Cys. Not much is known about the function of *C*-mannose residue, since the enzyme catalyzing the modification was not known until recently (40). In *Drosophila,* DPY19 is the only mannosyltransferase that adds a *C*-linked mannose to TSRs. Based on sequence homology, there are four human genes related to DPY19: DPY19L1-4(41). *Drosophila* DPY19 and mouse DPY19L1 play roles in neuronal migration (34). Human DPY19L2 expression is linked to globozoospermia while deletions lead to infertility (34).

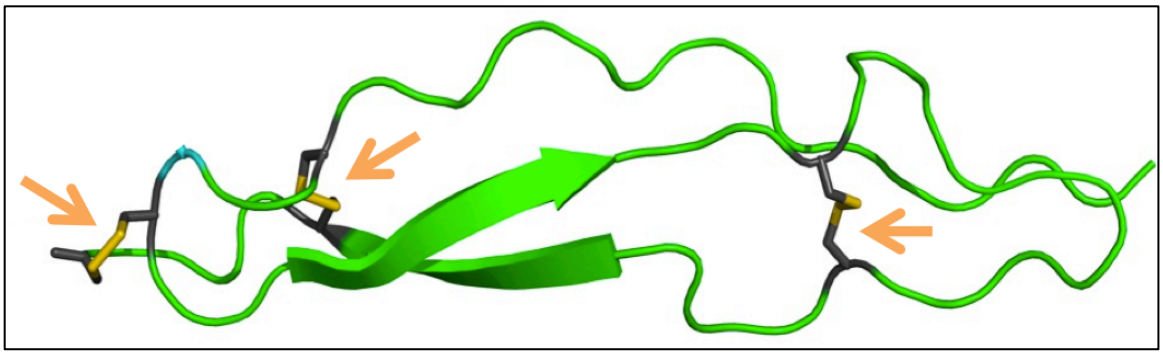


Fig. 1.3. Structure of TSP1-TSR3: Crystal structure of TSP1-TSR3 (adapted from (32)) showing two anti-parallel strands held together by three disulfide bonds (indicated by orange arrows). Formation of the disulfide bond is critical for the folding of the TSR and its subsequent fucosylation by Pofut2 (36).

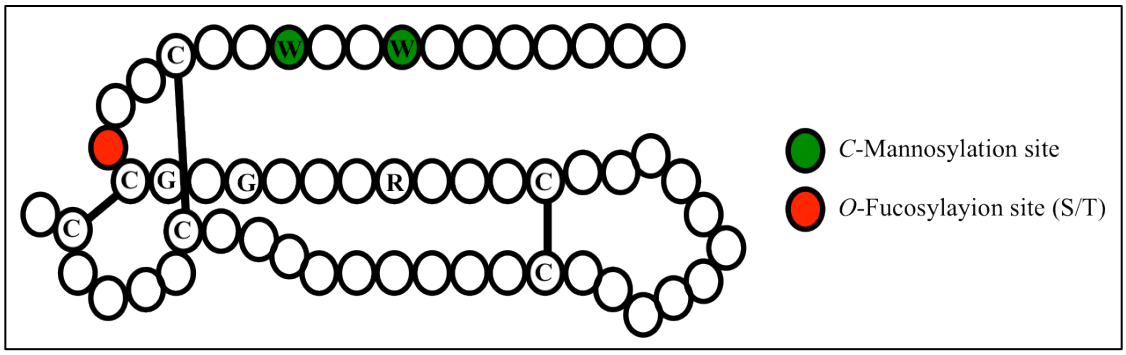


Fig. 1.4. Schematic of a group 1 TSR showing glycosylation sites and conserved elements: Each circle represents an amino acid and completely conserved amino acids are indicated within the circle. Sites of *C*-mannosylation and *O*-fucosylation are indicated as green and red circles. The TSR is oriented similar to the crystal structure in Fig 1.3. (32,42).

1.4. Protein *O*-fucosyltransferase 2 (Pofut2)

Pofut2 was identified based on sequence homology with Pofut1 and was shown to specifically modify TSRs but not EGF repeats (36). The enzyme is a soluble protein that localizes to the ER, but it is not known how it is retained since Pofut2 lacks a KDEL domain characteristic of soluble ER-resident proteins. When originally characterized, it was demonstrated that Pofut2 does not modify a fully unfolded TSR but efficiently adds an *O*-fucose to properly folded substrate containing the consensus sequence (36). Elimination of Pofut2 in mice results in an early embryonic lethal phenotype (18). Pofut2 mutant embryos show defects in cell differentiation and tissue morphology including an enhanced epithelial-to-mesenchymal transition (EMT) and a proliferation of endothelial cells. Many of the fifty predicted targets of Pofut2 are known to have roles in the extracellular matrix (ECM) (Table 1). While some have been demonstrated to synthesize and modify the ECM, some others influence cell migration and adhesion. It was hypothesized that the defects observed in the Pofut2 mutant embryos was a result of lack of *O*-fucose on one or more of these targets (18). Two of these targets, ADAMTS13 and ADAMSL1, require *O*-fucosylation for secretion (38,39).

ADAMTS13 is a blood-associated protein responsible for cleavage of the von Willebrand factor (43,44). ADAMTS13 has eight total TSRs, seven of which have consensus sequences for *O*-fucosylation (38). In mature ADAMTS13, all seven of these are known to be modified in high stoichiometries with the fucose-glucose disaccharide. Eliminating the O-fucosylation site in one or more of the tandem TSRs in ADAMTS13, resulted in the loss of secretion of the protein (38). Eliminating Pofut2 by siRNA knockdown or making GDP-fucose, the donor substrate for Pofut2, unavailable, had similar effects on ADAMTS13 secretion. Similar observations were made for ADAMTSL1, a secreted protein with unknown function (39). Thus, the lack of Pofut2

may result in loss of secretion of many Pofut2 targets leading to the severe embryonic lethal phenotype.

A recently published crystal structure of Pofut2 offered more insight into substrate recognition, specificity, and catalytic activity (45). Chen *et al*. were able to obtain crystal structures of the enzyme alone and the enzyme in a complex with GDP-fucose. While the crystal structures themselves provide a lot of information about Pofut2 residues involved in donor and substrate binding, they were able to use the structure as guide to make a series of point mutations in Pofut2. Experiments with the mutants demonstrated roles for three important residues in GDP-fucose binding and catalysis. The Asp297 and Arg294 close to the C-terminus are involved in the binding of GDP-fucose, while the Glu54 residue is important for the actual hydrolysis of the donor substrate. Mutations of all these residues resulted in at least partial loss of fucosyltransferase activity.

In order to understand how Pofut2 recognizes and binds to its substrates, key elements in a TSR that interacted with the enzyme were also identified. Chen *et al*. were able to do this by constructing a truncated TSR molecule with sufficient structural resemblance to the full TSR, and using it as a substrate for Pofut2. This synthetic molecule (dubbed mini-TSR) has only two disulfide bonds and mimics the half of a full TSR molecule that would bind to the substrate pocket of Pofut2 (Fig. 1.5A). Of the thirty residues that build the enzyme binding face of a TSR, ten are conserved (including the consensus sequence for fucosylation), nine are solvent exposed (hence do not interact with Pofut2) and the rest show great variability across known TSRs (Fig. 1.5B) (45). They hypothesize that the substrate binding pocket accommodates the expected features of a TSR, but also has "large cavities" to make room for variable residues. This observation loosens the substrate criteria and could possibly increase the number of putative

targets of Pofut2 but confirmed the need for a folded structure to be recognized. Perhaps the most interesting feature of the Pofut2 structure is the presence of a second substrate-binding patch, which suggests the ability of the enzyme to interact with an adjacent TSR. Since TSRs usually occur as tandem repeats, this may be especially important in the functioning of Pofut2.

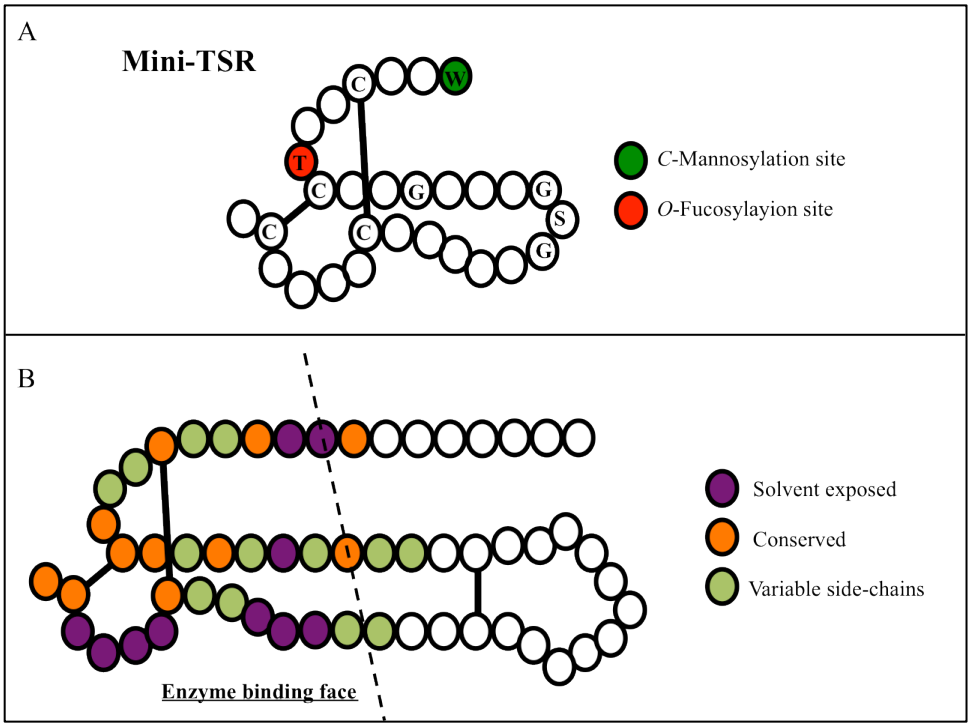


Fig. 1.5. Schematic of the part of TSR that interacts with Pofut2: A) Truncated TSR4 from F-spondin used in experiments to demonstrate essential structural elements required for substrate recognition by Pofut2. B) Distribution of various residues of a TSR involved in enzyme-substrate reaction. Of about 30 sites involved in enzyme interaction, 10 are conserved (amongst known Pofut2 substrates), 9 are solvent exposed and the rest have highly variable side-chains. While specific residues in the active site interact with the conserved regions of the TSR, large cavities in the substrate-binding pocket accommodate the variable side chains(45).

1.5. Pofut2 targets and significance of *O*-fucosylation on TSRs

As mentioned previously, Pofut2 has fifty predicted targets, majority of which are secreted ECM proteins. Mutations in several of these targets have known pathologies in humans (Table 1). Deletions of some targets also display embryonic lethality in mice while some others have interesting phenotypes (Table 1). The Pofut2 phenotype is not identical to any individual substrate mutant reported to date, but maybe a result of compound effects caused by lack of *O*-fucose on more than one target.

The ADAMTS (A Disintegrin and Metalloprotease containing Thrombospondin type repeats) superfamily contains 19 ADAMTS proteases (including ADAMTS13) and 6 ADAMTS-like members (including ADAMTSL1) (46). Mutations in ADAMTS13, a validated Pofut2 target that requires *O*-fucosylation for secretion, causes Thrombotic Thrombocytopenic purpurea (TTP) (47). TTP is characterized by localized blood clots and high blood pressure. Although several of the ADAMTS family members are redundant with each other in terms of their function, dysregulation of each of them has been implicated in several cancers (48). ADAMTS family members are typically involved in remodeling ECM. Table 1 below lists a result of all known human and murine phenotypes associated with ADAMTS family proteins.

The ADAMTSL family differs from the ADAMTS family in that they lack a metalloproteinase domain. Most ADAMTS family members, except ADAMTS13, have roles in the ECM. While most of the ADAMTSL family members have unknown functions, mutations in ADAMTSL2 cause a developmental disorder called Geleophysic dysplasia (49).

TSP1 and 2, both members of the Thrombospondin family, are secreted matricellular proteins with anti-angiogenic properties (50). All TSRs in TSP1 and TSP2 are modified with *O*-fucose (14,35). TSP1 levels are elevated in malignant breast secretions (51). Thrombospondins

interact with various integrins, CD36 and preoteoglycans. Peptides from TSR1 of TSP1 including the fucosylated site have been examined for their therapeutic value as inhibitors of angiogenesis in cancer. The Brain specific Angiogenesis Inhibitor (BAI) family has three members, all of which are G-protein coupled receptors. Each of the BAI proteins has four predicted sites of *O*-fucosylation. Not much is known about BAI2 and 3, but studies suggest that BAI1 is linked to recognition of apoptotic cells by phagocytes and also promotes myoblast fusion (52).

Six members of the CCN family, each with only one TSR, are also potential substrates of Pofut2. CCN family members have been shown to play roles in the development of the cardiovascular system and tumor angiogenesis (53,54). Several experimental findings suggest CCN2 as a prognostic marker for heart failure, making it an important pharmacological molecule (55).THSD7a and 7b are single pass type I membrane protein with more than 14 TSRs each. While THSD7a is known to have an inhibitory effect on endothelial cell migration and tube formation, there is no known function for THSD7b (56).

A few Pofut2 targets also play roles in neurological contexts. Semaphorin proteins 5a and B (SEMA5a and b) contain seven and five TSRs respectively and are important in axonal guidance (57-59). UNC5a with just one TSR is a receptor for netrin and is required for axon repulsion of neuronal growth cones (60). SCO-spondin is a large glycoprotein, containing 25 TSRs (only about half have consensus sites) and is required for neurogenesis during early brain development in mice (61). F-spondin is required for embryonic axons to find accurate paths, implicating it in in inflammatory processes in the nervous system (62).

Mutations in Hemicentin-1 are linked with age-related macular degeneration, although the exact mechanism of disease progression is not known (63). Properdin, with four tandem

TSRs, is a positive regulator of the alternative complement pathway (64). Mutations in properdin have been linked to higher bacterial infections, especially meningococcal infections (65).

The fact that *O*-fucosylation is required for the secretion of two TSR-containing proteins and that Pofut2 can distinguish between folded and unfolded forms of its substrates, strongly argues in favor of a quality control role for *O*-fucosylation. Understanding the function of the fucose will also help elucidate the role of glucosylation (Section 1.6).

1.6. β3-Glucosyltransferase (β3GlcT)

β3-Glucosyltransferase (β3GlcT) is an unusual enzyme in that it is one of only a few glycosyltransferase in mammals known to modify fucose (66). The others are members of the Fringe family, which are fucose-specific b3-N-acetylglucosaminyltransferases (22). β3GlcT adds a β-linked glucose to the 3'-OH of *O*-fucose on TSRs (66-68). β3GlcT is a soluble ER protein with a canonical KDEL-like sequence. β3GlcT has the gene name B3GALTL (β3-galactosyltransferase-like) because mutations in the gene were identified as the cause of a rare congenital disorder of glycosylation (CDG) called Peters plus syndrome (69,70). The gene has sequence similarity to β3-galactosyltransferases, so was called B3GALTL. Subsequently, the gene was demonstrated to encode the β3GlcT enzymatic activity (67). Glucosylation of TSRs appears to be completely abrogated in Peters plus patients (27). Peters plus is characterized by anterior eye chamber defects, cleft palate/lip, and other developmental delays/abnormalities.

Thus far, all mapped *O*-fucose sites are elongated by the addition of glucose, albeit in variable stoichiometries. For instance, TSRs from mature ADAMTS13 are mainly modified with the Glucose-Fucose disaccharide (38), while TSRs from mature ADAMTSL1 were seen as mixed populations of mono- and di-saccharide modifications (39). The reason and significance for this variable stoichiometry of glucosylation is known. Hence, the phenotypes observed in Peters plus patients are probably the cumulative effect of the lack of glucose on more than one TSR-containing protein.

When β3GlcT was originally demonstrated to have a role in Peters plus, it was shown via mass spectrometry that glycopeptides from Properdin, a soluble blood protein that positive regulates the alternative complement pathway, were not extended past the *O*-fucose. Interestingly, Peters plus patients also showed a mild secretion defect for Properdin in

comparison to control samples (Fig. 1.6) (71). The substrate selectivity of β3GlcT is an important area of study so as to be able to understand the Peters plus phenotype better.

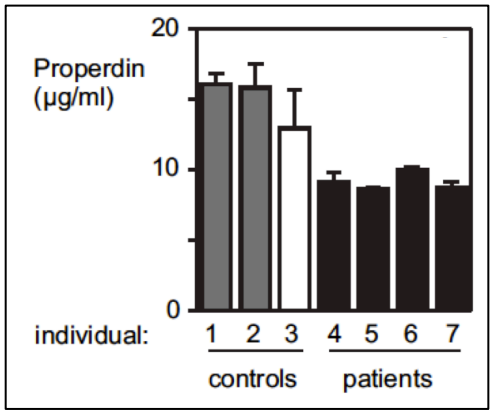


Fig. 1.6. Properdin levels in Peters plus patients: ELISA measurements of serum Properdin from control and Peters plus patients: Patients with Peters plus showed lower levels of properdin than control patients (71).

1.7. GDP-fucose: biosynthesis, transport and bioorthogonal analogs

All known fucosyltransferases utilize GDP-fucose as the donor substrate. There are two pathways of GDP-fucose biosynthesis: *de novo* pathway and salvage pathway (72) (Fig. 5). The *de novo* pathway, like the name suggests, utilizes commonly available hexoses (such as mannose and glucose) and converts them to GDP-mannose in a multi-step pathway. This GDP-mannose is then utilized by GDP-mannose 4,6-dehydaratase (GMD) to make GDP-4-keto-6-deoxy-mannose, which is finally converted to GDP-fucose by the enzyme FX. Alternatively, fucose can be salvaged from breakdown of glycoproteins and converted to GDP-fucose through the salvage pathway.

There are a total of eleven Golgi-associated fucosyltransferases (Fut) in mammals that add fucose to various *N*-glycan or mucin-type *O*-glycan structures. Pofut1 and Pofut2 are the only known fucosyltransferases in the ER. The GDP-fucose synthesized in the cytoplasm is transported to the Golgi by a nucleotide sugar transporter, Slc35C1 (73). Mutations in Slc35C1 cause a congenital disorder of glycosylation called leukocyte adhesion deficiency type II (LADII) (73). LADII patients show neurological and developmental abnormalities because of decreased fucosylation on selectin ligands that mediate leukocyte adhesion during immune responses, but this defect does not appear to affect fucosylation of Notch, a substrate of Pofut1 (74). Until recently, Slc35C1 was the only known GDP-fucose transporter, so the source of GDP-fucose in the ER was not known.

Ishikawa *et al.* demonstrated the presence of a *Drosophila* ER-localized GDP-fucose transporter, *Efr* (75). However, the mammalian homologue of this gene, Slc35B4 has been previously examined and does not show GDP-fucose transporter activity (76). Soon after, Lu *et al.* showed that the expression levels of Slc35C2 (a close homologue of Slc35C1) proportionally

affected fucosylation on Notch (77). While they were not able to directly show Slc35C2 has GDP-fucose transporter activity, immunofluorescence experiments led them to conclude that the transporter localized to the ER-Golgi intermediate compartment (ERGIC). They hypothesize that Pofuts shuttle between the ERGIC and the ER, thus allowing them access to GDP-fucose (Fig. 1.7). Prior to the discovery of Slc35C2, it was thought that GDP-fucose could be retrograde transported to the ER from the Golgi. Neither of these hypotheses exclude the possibility of an unknown dedicated ER GDP-fucose transporter.

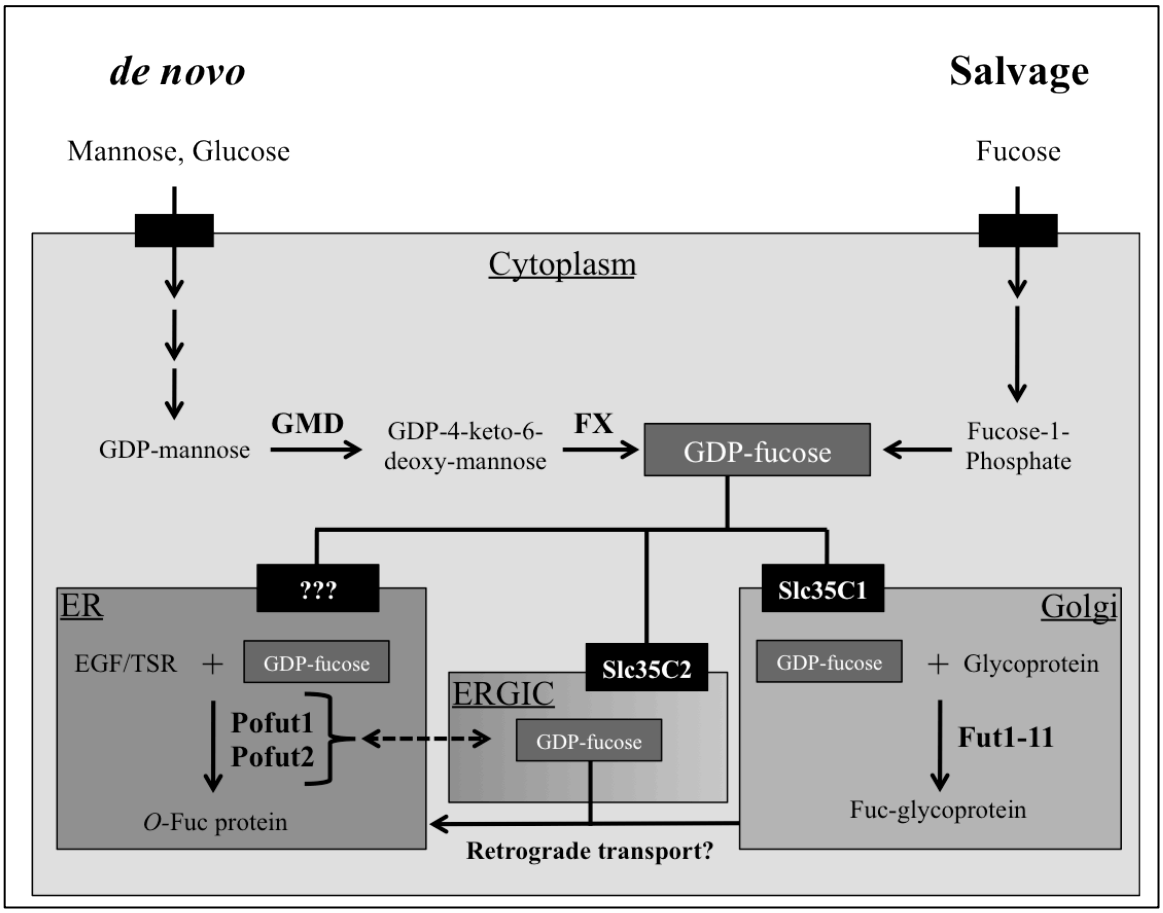


Fig. 1.7. Schematic showing the two pathways of GDP-fucose biosynthesis: GDP-fucose can be made *de novo* from other hexoses or from L-fucose obtained via the salvage pathway. This GDP-fucose is then transported to various cellular compartment for use by fucosyltransferases.

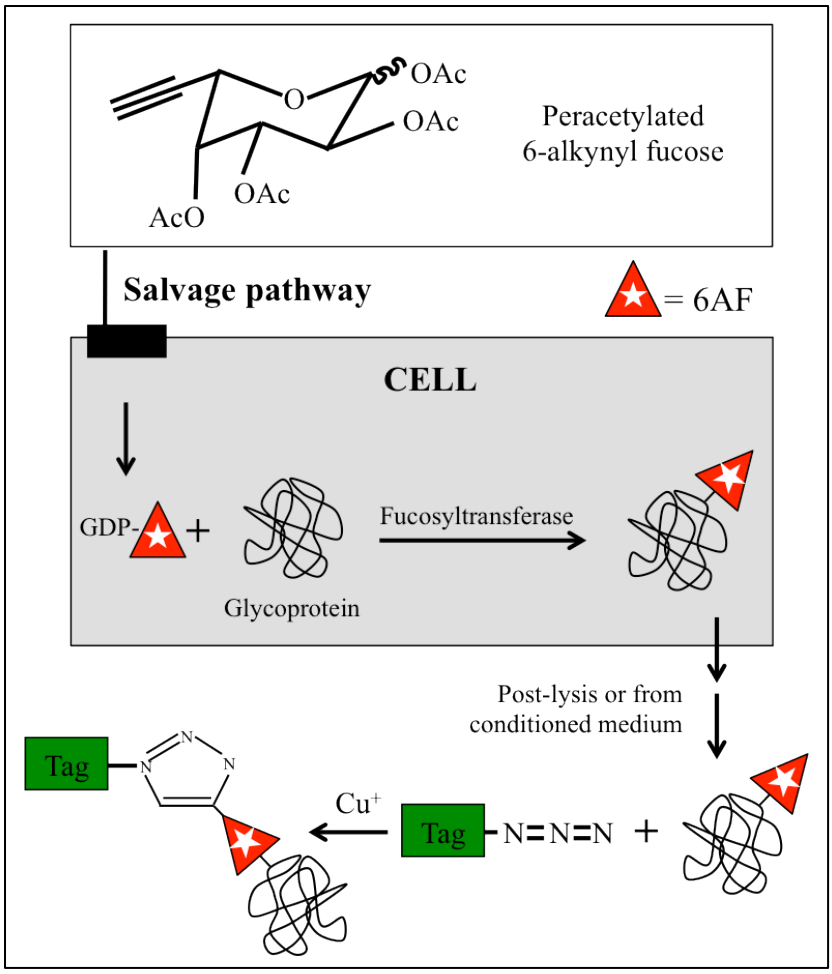


Fig. 1.8. Metabolic labeling with 6-Alkynyl fucose (6AF): Top Panel shows the structure of 6AF in its peracetylated form. Peracetylation enables the molecule to be membrane permeable. Once inside the cell, the acyl groups are removed by non-specific esterases. The enzymes of the salvage pathway then convert it to GDP-6AF. This GDP-6AF is then utilized by fucosyltransferases to modify glycoproteins (middle panel). Post-lysis or in the conditioned medium, the metabolically labeled glycoprotein can be reacted with an azide-tag (e.g. azido-biotin) to undergo azido-alkyne cycloaddition reaction in the presence of a Cu^{+} catalyst (bottom panel). The 6AF now conjugated with a tag can be detected by a suitable reagent (e.g. streptavidin).

The fact the fucose is a metabolic dead end allows for the use of GDP-fucose analogs to be used in the detection of *O*-fucosylation (72). [^{3}H]-Fucose has been long used to follow *O*-fucosylation, but recently a bioorthogonal analog of *O*-fucose was shown to be tolerated by both Pofuts (78). 6-Alkynyl fucose (6AF) is structurally similar enough to be converted to GDP-6AF in cells and incorporated into EGF repeats and TSRs. The alkyne group allows 6AF to participate in azide-alkyne cyclo-addition reactions. In the presence of a copper catalyst, 6AF

can be reacted with azido-biotin (or any other azide conjugated tag) (Fig. 1.8). This biotinylated fucose can then be detected with labeled Streptavidin. 6AF is more sensitive and can be used for more applications than [^{3}H]-fucose. The use of fucose analogs is limited to mammalian systems since lower organisms do not have a salvage pathway.

1.8. Glycosylation and protein folding

In addition to having specific functions on mature proteins, *N*-glycans have an important role in folding of proteins in the ER. *N*-glycans are added co- and post-translationally to the asparagine residues in the sequence N-X-S/T (where X cannot be proline). The multi-enzyme complex Oligosaccaryltransferase (OST) transfers the entire oligosaccharide $Glc_3Man_9GlcNAc_2$ to the asparagine (2) (Fig. 1.9). The terminal glucose residue is immediately removed by glucosidases I and II (GI/II) to generate a $Glc_1Man_9GlcNAc_2$ glycan. This monoglucosylated form is recognized by an ER chaperones, calnexin (CNX, membrane associated) and careticulin (CRT, soluble), that attempt to fold the protein. At the end of the folding cycle, glucosidase II (GII) removes the remaining glucose residue to generate a $Man_9GlcNAc_2$ glycan. If the protein is properly folded, it exits the ER. If the protein is not properly folded, it is recognized by the enzyme UDP-glucose: glucosyltransferase (UGGT), which adds the glucose back to recreate the $Glc_1Man_9GlcNAc_2$ glycan, allowing the protein to enter another folding cycle with CNX and/or CRT. UGGT has the unique property of being able to distinguish between properly and improperly folded proteins, a key characteristic for an enzyme involved in quality control. This cycle is iterated until the protein is properly folded. Of course, there are several other chaperones, including disulfide isomerases, that are involved in this process and that intimately interact with the components of the *N*-glycan pathway.

The idea of *O*-fucosylation being a quality control mechanism has been discussed in several publications (18,38,39,79,80). Pofut1 was recently suggested to have a chaperone function in the folding of Notch (80). Okajima *et al.* observed that eliminating OFUT1 (*Drosophila* homolog of Pofut1) resulted in accumulation of Notch protein in the ER. They also demonstrated that this phenotype was alleviated by introducing an enzymatically inactive form

of OFUT1 (R245A mutant). However, it is not clear whether Pofut1 participates directly in the folding of EGF repeats. Since both Pofut1 and Pofut2 require properly folded acceptor substrates (EGF repeats or TSRs, respectively) to transfer fucose, these observations raise the question of whether Pofut2 may play a role in quality control/protein folding as well.

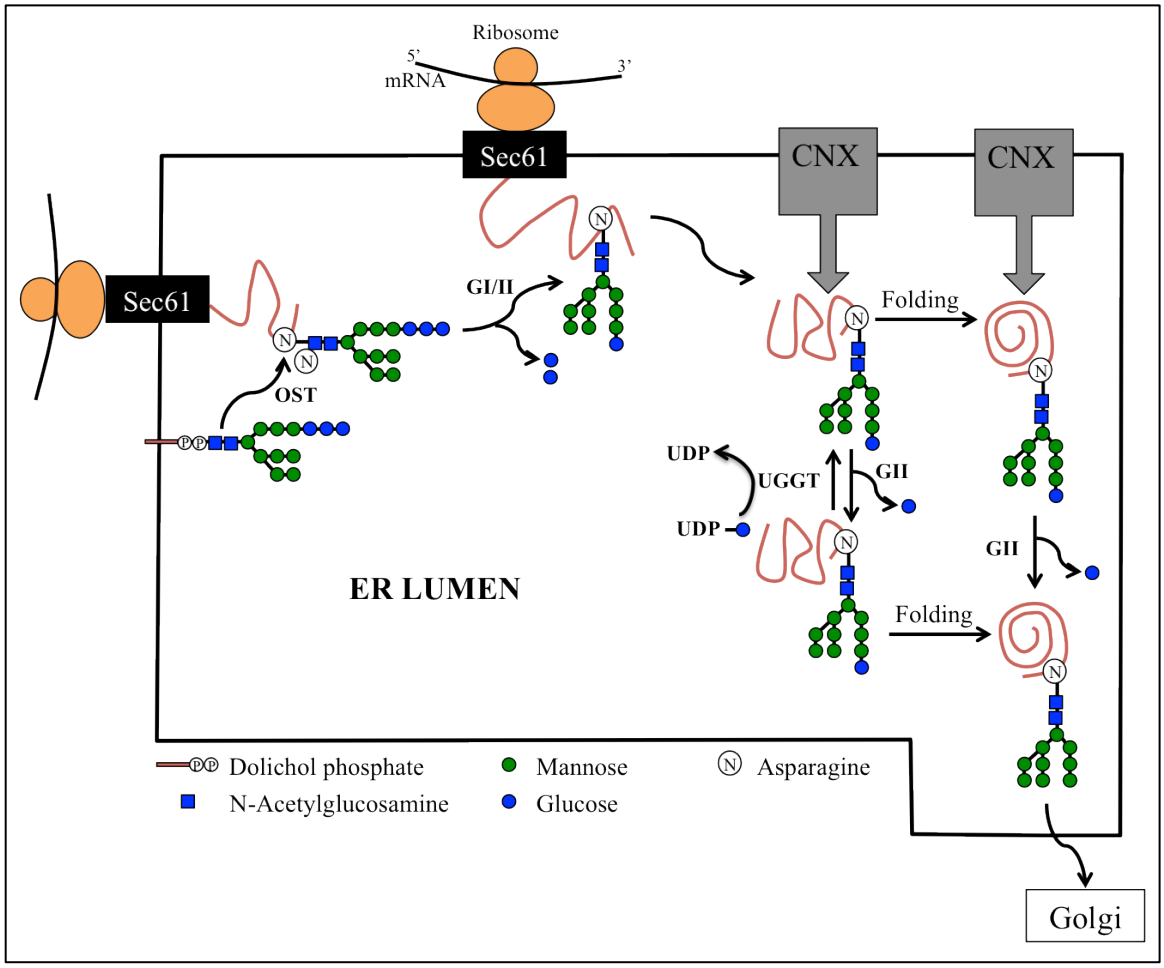


Fig. 1.9. Proposed scheme for the role of *N*-glycans in the folding of glycoproteins: The *N*-glycan is co-translationally added en-bloc to the nascent peptide being transported into the ER lumen by the Sec61 complex. The reaction is catalyzed by OST using Dol-P-P-$Glc_3Man_9GlcNAc_2$ as the donor. The terminal glucoses are immediately cleaved by GI/II, making the glycoprotein a substrate for CNX. If CNX successfully helps the protein fold, GII removes the final glucose allowing the protein to progress to the Golgi. If the protein is not properly folded, then UGGT utilizes UDP-glucose to add a terminal glucose, making the misfolded glycoprotein a substrate for CNX again (adapted from (2)).

Protein, other names	Consensus /total	Human pathology	Murine phenotypes, known functions
ADAMTS1, METH1	3/3	-	**KO** mice show growth retardation, urologic abnormalities, impaired female infertility, renal lesions (81,82)
ADAMTS2, NPI	2/4	Ehlers-Danlos syndrome (83)	**KO** mice show fragile skin, male infertility, reduced fibrosis; expression in monocytes stimulated by glucocorticoids (84-86)
ADAMTS3	2/4	-	Processes procollagen II, expressed in coronary lesions in acute myocardial infarctions (87,88)
ADAMTS4, Aggrecanase 1	1/1	-	**KO** mice viable with no evident phenotype; Cleaves aggrecan and brevican, possible involvement in osteoarthritis (89-91)
ADAMTS5, Aggrecanase 2	2/2	-	**KO** mice viable with no evident phenotype; cleaves aggrecan; regulates interdigital web regression; abrogates cartilage destruction in osteoarthritis (92,93)
ADAMTS6	3/5	-	Expressed in placenta in mice, upregulated in breast carcinomas in humans (94,95)
ADAMTS7, COMPase	4/8	-	Expressed ubiquitously in mouse development, binds and degrades cartilage oligomeric matrix protein (COMP) (95,96)
ADAMTS8, METH2	1/2	-	Expressed in human adult and fetal lung tissue, inhibits endothelial cell migration (anti-angiogenic), expressed in atherosclerotic plaques (97,98)
ADAMTS9	12/15	-	**KO** embryonic lethal, expressed highly throughout mouse development, versican cleaving protease, regulates interdigital web regression; required for cardiac development and homeostasis, down-regulated in esophageal cancer in humans (99-102)
ADAMTS10	3/5	Weill-Marchesani syndrome (103)	Expressed throughout mouse development, Involved in sperm adhesion to zona pellucida in mice, microfibril biogenesis (*in vitro)* (104-106)
ADAMTS12	6/8	-	**KO** mice viable with no evident phenotype; Binds and

			degrades COMP, possible role in arthritis; Anti-angiogenic and tumor suppressor, necessary for normal inflammatory response (107-110)
ADAMTS13, vWFCP (38)	7/8	Congenital Thrombotic thrombocytopenic purpurea (111)	**KO** mice viable with no TTP-like symptoms; von-Willebrand factor cleaving protease (111-113)
ADAMTS14	2/4	-	Expressed in several mouse tissues, (esp. bone, tendon and skin); processes aminoprocollagen *in-vitro*, maybe functionally redundant with ADAMTS2 and 3. (114)
ADAMTS15	3/3	-	Upregulated in mouse embryonic myogenesis, expresed only in fetal liver and kidney in humans; inactivated in some colorectal cancers, possible tumor suppressor; processes versican (115-117)
ADAMTS16	6/6	-	Targeted mutations in rats leads to lower blood pressure; expressed in fetal lung, kidney and adult ovary and prostrate in humans, expression in ovarian follicles stimulated by FSH; upregulated in some esophageal squamous cell carcinomas (117-120)
ADAMTS17	3/5	Weill-Marchesani like syndrome (121)	Recessive mutations cause isolated spherophakia with short stature; expressed in fetal lung and adult ovaries in humans (117,122)
ADAMTS18	5/5	Early onset severe retinal dystrophy (123); Microcornea, Myopic Chorioretinal Atrophy, and Telecanthus (MMCAT) (124)	Frequently inactivated in multiple carcinomas; expressed in fetal lung, liver, kidney and adult brain, prostrate, submaxillary gland, endothelial cells in humans; reduces migration of melanoma cells *in-vitro*, (117,125,126)
ADAMTS19	4/5	-	Expressed in fetal lungs; SNP in ADAMTS19 correlate with premature ovarian failure (117,127,128)
ADAMTS20	11/15	-	Mutant mice show defective neural crest-derived melanoblast

			development (belted/spotting phenotype); cleaves versican; regulates interdigital web regression; required for gonadal morphogenesis in *C.elegans* (101,129-131)
ADAMTSL1, Punctin 1 (39)	4/4, 8/9*	-	Expressed in human and mouse skeletal muscle, binds to extra-cellular matrix components; *has 2 splice variants with 4 or 9 TSRs (46,132)
ADAMTSL2	5/7	Geleophysic dysplasia (49)	Interacts with latent TGFβ-binding protein 1, fibrillin 1 (49,133)
ADAMTSL3, Punctin 2	10/12	-	Frequently mutated in colorectal tumors; expressed in most adult human tissues, late in mouse development; localizes to ECM (134,135)
ADAMTSL4, TSRC1	1/3, 2/6[#]	Bilateral ectopia lentis 2 (136)	Expressed in several tissues including ocular in humans and mice; [#]has two splice variants with 3 or 6 TSRs (136-138)
ADAMTSL5, THSD6	1/1	-	Expressed in widely during mouse development; binds to fibrillin 1/2; localizes to ECM and binds cell surface (139)
ADAMTSL6, THSD4	3/6	-	Has 2 splice variants, α and β. ADAMTSL6α is expressed ubiquitously in adult mice but β-form is seen in brain, eye, liver, spinal cord; β-form binds to fibrillin 1 and rescues microfibril disorder in Marfan syndrome mouse model by promoting fibrillin-1 assembly (140,141)
TSP1, THBS1 (14)	3/3	-	**KO** mice show lung abnormalities and high numbers of WBC in circulation; expressed in many tissues during mouse development, expression lost during malignant progression; anti-angiogenic activitiy; promote CNS synaptogenesis in mice; binds to CD36 (142-145)
TSP2, THBS2 (35)	3/3	-	**KO** mice show connective tissue abnormalities; single nucleotide polymorphism correlates with lumbar disc herniation in humans; anti-angiogenic, binds to CD36;

			promotes synaptogenesis, inhibits breast cancer growth and metastasis in mice (145-148)
CCN1, CYR61	1/1	-	**KO** embryonic lethal primarily due to vascularization defects; expressed in several tissues during mouse development; angiogenic inducer, may promote tumor growth and vascularization; binds to heparin, promotes adhesion of endothelial cells *in-vitro*; downregulated in some non-small cell lung cancers (53,149,150)
CCN2, CTGF	1/1	-	**KO** mice are perinatally lethal because of respiratory defects; overexpression in mice leads to reduced bone density, dwarfism; Polymorphism associated with systemic sclerosis; expressed in cardiovascular, gonadal, renal tissues during mouse development, abundant in adult human platelets; binds to insulin-like growth factor (54,151-153)
CCN3, NOV	1/1		**KO** mice have abnormal skeletal and cardiac development; regulates human hematopoitetic stem cells; binds to insulin-like growth factor, fibulin-1; expressed in adult human adrenal tissues; downregulated in some adrenocortical tumor malignancies (152,154-157)
CCN4, WISP1	1/1	-	Wnt-inducted, aberrant expression in colon cancers; regulates osteogenesis, vascular smooth muscle cell migration/proliferation (158-161)
CCN5, WISP2	1/1	-	**KO** mice are early embryonic lethal; aberrant expression in breast tumors, colon and pancreatic cancers; Wnt-induced; transcriptionally regulates TGF-β expression, expressed ubiquitously in mouse development and adult human adrenal gland, breast, colon, pancreas, uterus, ovary (159,162-165)
CCN6, WISP3	1/1	Progressive pseudorheumatoid arthropathy of childhood (166)	Neither **KO** nor transgenic overexpression mice have evident phenotypes; acts as a tumor suppressor in breast cancer,

			aberrant expression in colon tumors; Wnt-induced; expressed in human adult kidney and testis(159,167,168)
BAI1, GDAIF	4/5	-	**KO** mice have weak musculature, impaired muscle regeneration post-injury; engulfment receptor for apoptotic cells, binds to p53; anti-angiogenic transmembrane protein (169-171)
BAI2	2/2, 3/3, 4/4^{+}	-	Participates in ischemia induced brain angiogenesis; regulates VEGF expression; expressed in adult and fetal mouse brain; $^{+}$has alternatively spliced variants with varying expression throughout development (172-174)
BAI3	4/4	-	Low expression in malignant gliomas; binds to C1q-like proteins, BAI1; controls dendritic arborization growth, branching in cultured neurons (175-178)
THSD7a	4/15	-	Single nucleotide polymorphism correlates with osteoporosis; mediates endothelial cell migration and tube formation; expressed in all brain tissues except skeletal muscle (179-181)
THSD7b	3/18	-	Single nucleotide polymorphisms correlate with some non-small cell lung cancers (182)
SEMA5a, SEMAF	2/7		**KO** mice show no evident phenotype; expressed reduced in brains from autistic patients, high levels of expression correlates with metastasis of pancreatic cancer; expressed in adult muscle, heart, lung, spleen; binds to plexin B3 (183-188)
SEMA5b, SEMG	1/5	-	**KO** mice are d12.5 emrbyonic lethal because of impaired cranial vascularization, **KO** mice in a different background are viable and show neurite mistargeting in the retina; regulates development and maintenance of synapse size in hippocampal neurons; proteolyrically processed by ADAM17 (59,189,190)
ISM1	1/1	-	Overexpression in mice suppresses melanoma tumor growth; expressed throughout *Xenopus* development; knockdown in

			zebrafish results in abnormal intersegmental vessel formation; Anti-angiogenic; co-expresses with Fgf-8 (191-193)
THSD1, TMTSP	1/1	-	Downregulated in all esophageal squamous cell carcinomas, potential tumor suppressor; expressed predominantly in hematopoetic stem and progenitor cells (194,195)
F-spondin, Spondin-1(14)	5/6	-	Reduces Aβ-plaque deposition in mouse models of Alzheimer's, inhibits cleavage of apolipoprotein; promotes neural cell adhesion and neurite extension, secreted and attached to the ECM; expressed predominantly in lungs amongst other tissues (196-198)
SCO-spondin, SSPO	9/25	-	Required for neurogenesis in early brain development in chicks; expressed specifically in the subcommisural organ (SCO) in the brain, secreted to CSF, expression downregulated by serotonin (61,199,200)
UNC5a, UNC5h1	1/1	-	**KO** mice show decreased apoptosis and increased no. of neurons in the spinal cord; netrin receptor; expression regulated by p53, regulates apoptosis; (201-203)
Hemicentin-1, Fibulin 6	6/6	Age related macular degeneration 1 (63)	Maybe involved in the pathology of Sjogren's syndrome; downregulation leads to cell detachment, reduced viability, increased anoikis cell death *in-vitro* (204)
Properdin, PFC(37)	4/6	Properdin deficiency (65,205)	**KO** mice showed properdin to be required for AP complement activation in some contexts; deficiency in humans increases disposition to lethal meningococcal infections; positive regulator of the alternative pathway, facilitates complement activation; secreted to plasma (206,207)
Papilin	4/5	-	Deletion in *Drosophila* and *C.elegans* are lethal, overexpression in *Drosophila* causes lethal abnormalities in muscle; tissue-specific splice-variant expression in *Drosophila* (208-210)

CILP2	1/1	-	Single nucleotide polymorphism associated with blood LDL and HDL cholesterol in humans; downregulated in experimental osteoarthritis in mice; expressed in cartilaginous tissues (211,212)
C6	1/3	Complement component 6 deficiency (213-216)	Deficiency in humans is associated with recurrent meningitis; Rat and mice deficient in C6 show coagulation defects, reduced platelet aggregation; constituent of the lytic membrane attack complex (217-219)

Table 1. List of potential human Pofut2 targets: Putative targets of Pofut2 generated using the consensus sequence C-X(2,3)-[ST]-C-X(2)-G in ScanProsite from the Uniprot database with their alternative names. Table also shows the number of TSRs with consensus sequence/total number of TSRs, known pathologies in humans, mouse models and known functions. Confirmed targets of Pofut2 are underlined. **Published** data is underlined and bolded with corresponding reference indicated. Unpublished data is from the thesis work of Dr. Christina Melief. *ADAMTSL1, #ADAMTSL4 and +BAI2 have alternate spliced forms with variable numbers of TSRs.

Chapter 2. Aims of this dissertation

The primary aim of my study is to understand the role of *O*-fucosylation and Pofut2. My hypothesis is that in addition to the role *O*-fucose may play in the functioning of mature protein, Pofut2 is intrinsically involved in the folding and secretion of TSR containing proteins in general. This hypothesis can be broken down into the following parts that I attempt to answer empirically.

1. Impact of *O*-fucosylation on secretion of TSR containing proteins
    a. Can the secretion defect phenotype in the absence of fucose be generalized to Pofut2 targets other than ADAMTSL1 and ADAMTS13?
    b. Does the number or the primary amino acid sequence of TSRs influence the influence *O*-fucose dependent secretion?
    c. What is the fate of unfucosylated protein?
2. Subcellular location of fucosylation
    a. Where does *O*-fucosylation occur?
    b. Is *O*-fucosylation a co-translational process?
    c. Does expression of TSR containing proteins induce unfolded protein response?
3. Molecular role of *O*-fucose
    a. How does Pofut2 react with its substrate ±GDP-fucose?
    b. What is the stoichiometry of *O*-fucosylation on TSRs on mature, secreted protein compared to protein while folding?
    c. Is *O*-fucosylation of TSRs a marker of folding within cells?

4. Chaperone activity of Pofut2
    a. Does Pofut2 directly participate in the folding of TSRs?
    b. Does Pofut2 require enzymatic activity for its function?
5. ER-localization and regulation of Pofut2 (in collaboration with Artem Serganov, and Hideyuki Takeuchi)
    a. What are Pofut2's interaction partners?
    b. Does expression of TSR containing proteins result in upregulation of Pofut2?
6. A role for β3GlcT
    a. How does eliminating glucosylation affect TSR containing proteins?

Using the results from my experiments, I attempt to draw a model summarizing the role of *O*-fucosylation on Pofut2 targets.

Chapter 3. Impact of *O*-fucosylation on the secretion of TSR-containing proteins

In order to assess a general role for *O*-fucosylation on TSR-containing proteins, I did experiments with several predicted Pofut2 targets. In this study, I use five different model proteins: TSP1, ADAMTS9, ADAMTS13, ADAMTSL1 and ADAMTSL2. I chose these particular proteins because they differ in the number of TSRs in their C-termini (Fig. 3.1). The effects of *O*-fucosylation on ADAMTS13 and ADAMTSL1 have already been established so I primarily use them as positive controls in my assays (38,39).

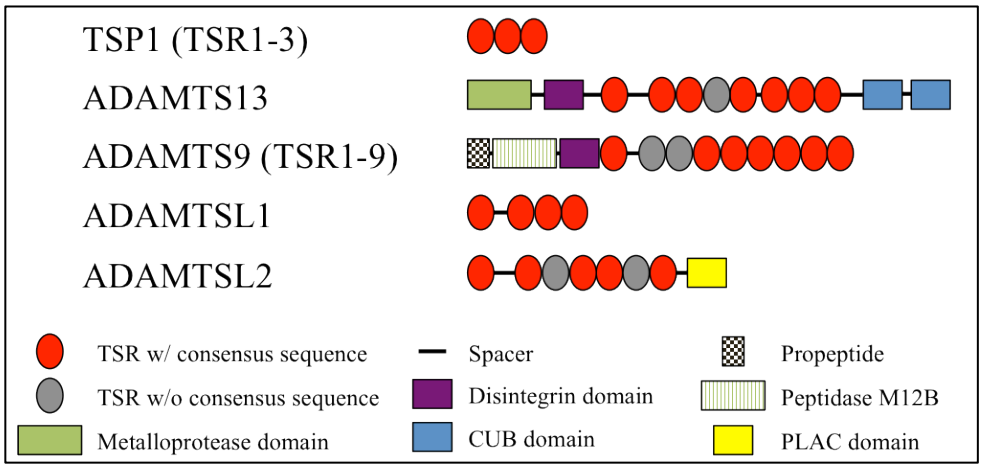


Fig. 3.1. Schematic showing the different domains of Pofut2 substrates used in this study: The ADAMTS13, ADAMTSL1 and ADAMTSL2 constructs contain full-length protein whereas TSP1 and ADAMTS9 are truncated constructs.

The effect of *O*-fucosylation on secretion can be examined in two different ways: by eliminating GDP-fucose or by siRNA knockdown Pofut2. To test the effect of depleting GDP-fucose on TSR containing proteins, I used a mutant variety of Chinese Hamster Ovary (CHO) cells called Lec13. Lec13 CHO cells bear a point mutation in one of the enzymes in the *de novo* GDP-fucose biosynthesis pathway, GMD (Section 1.7, Fig. 1.7) (220). By culturing these cells in

low-fucose medium, GDP-fucose levels in cells can be reduced or even eliminated. The phenotypes resulting from this GDP-fucose elimination can be rescued by the addition of fucose to the culture media and relying on the salvage pathway for fucose uptake.

3.1. *O*-Fucosylation is required for the secretion of TSP1, ADAMTS9, ADAMTS13, ADAMTSL1 and ADAMTSL2

Transient transfections in Lec13 CHO cells with constructs encoding several proteins from Figure 3.1 showed that in the absence of fucose, TSR-containing proteins were not secreted efficiently (Fig. 3.2). Human IgG was used here as both a loading and a transfection control since its secretion is not affected by fucosylation. When 1 mM fucose was added to the culture medium, the secretion defect was rescued. The extent of the secretion defect varies from substrate to substrate, with TSP1(TSR1-3) showing the least dependence on fucose for secretion.

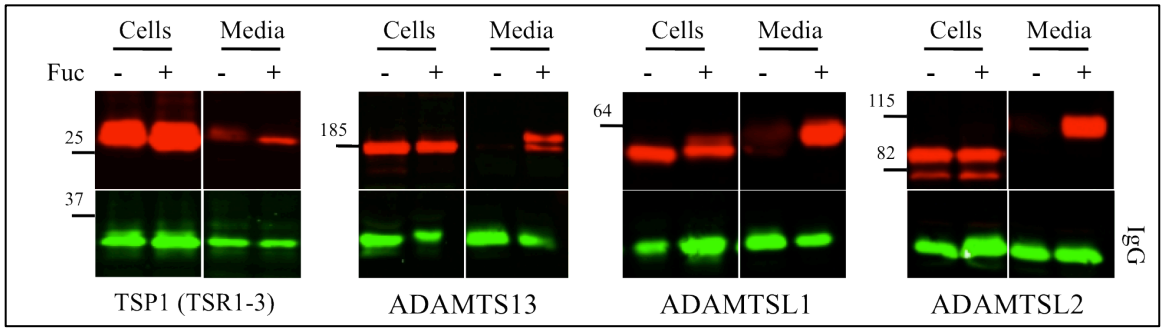


Fig. 3.2. Secretion defect of TSP1, ADAMTS13, ADAMTSL1 and ADAMTSL2: Various Pofut2 substrates were transiently transfected in Lec13 CHO cells ±fucose. IgG was cotransfected as a loading control. Equal fractions of cell and media were analyzed. All substrates tested localized to the ECM. In the absence of fucose, the substrates were less efficiently secreted into the media.

Similar results were seen when Pofut2 levels were reduced using targeted siRNA in HEK293T cells (Fig. 3.3). The extent of the secretion defect varied between several proteins but correlated with the results seen in Lec13 CHO cells. The most striking defect was seen in ADAMTS9 (9 TSRs) and the mildest effect was seen in TSP1 (3 TSRs). This suggests that the number of tandem TSRs may influence secretion of the protein.

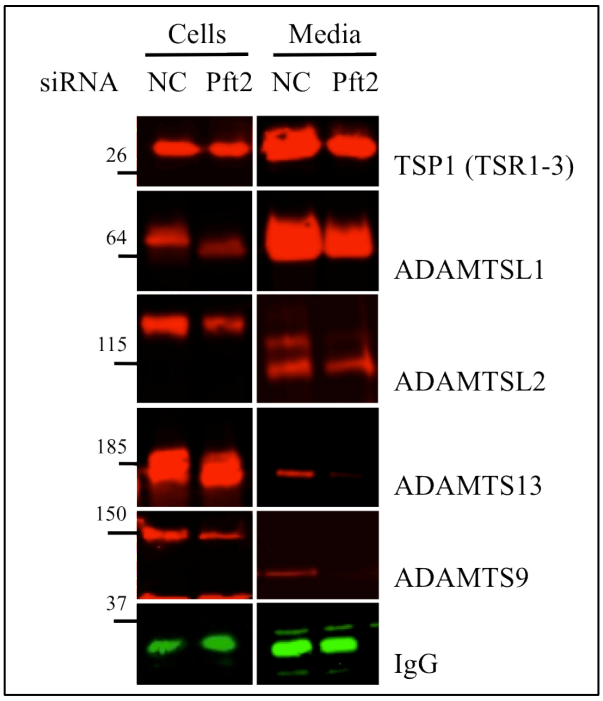


Fig. 3.3. RNAi knockdown of Pofut2: HEK293T cells were co-transfected with siRNA targeting Pofut2 (Pft2) and various TSR containing proteins. All tested substrates showed loss of secretion to the media when Pofut2 was knocked down. IgG was used as a negative control. ADAMTS9 is processed at the cell surface by furin and hence the secreted form is smaller than the cell-associated form. (221).

3.2. Number of tandem TSRs influences fucose dependent secretion of proteins

Results from Section 3.1 indicate that the more tandem TSRs in a protein, the more the secretion defect in the absence of fucose or Pofut2. To address this possibility, truncation mutants of ADAMTS13 were used in Lec13 CHO cell secretion assays. Of the eight TSRs in ADAMTS13, seven are *O*-fucosylated (Fig. 3.4A) (38) C-terminal TSRs were sequentially eliminated to generate constructs containing from 1-8 TSRs. The absence of fucose had almost no effect on the secretion of proteins containing 1-2 TSRs (Fig. 3.4B). All truncation mutants with three or more TSRs showed secretion defects to varying degrees. These results suggest that the more tandem TSRs in a protein, the more likely it requires *O*-fucosylation for secretion. Alternatively, a specific TSR from ADAMTS13 could require fucosylation (*e.g.* TSR3) and inclusion of this TSR leads to fucose dependence on secretion.

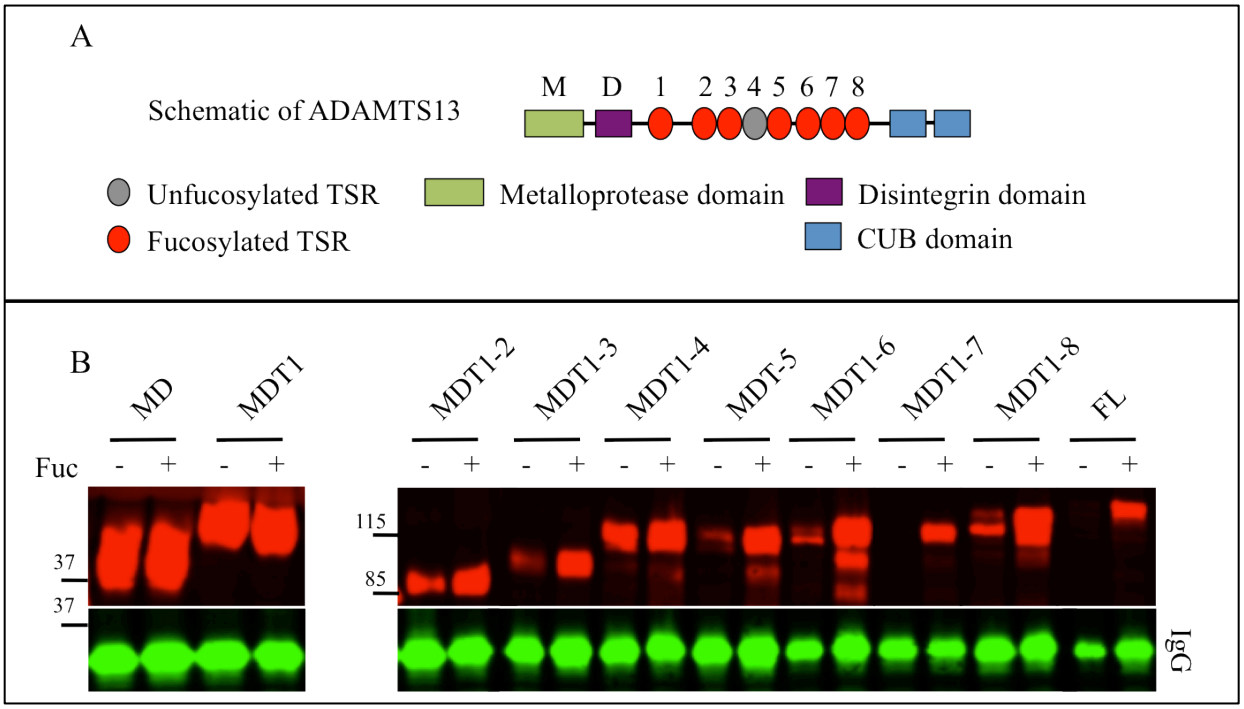


Fig. 3.4. Fucose dependent secretion varies based on number of tandem TSRs: A) Schematic diagram showing the various domains of ADAMTS13. All TSRs except TSR4 (which does not have a consensus sequence) are fucosylated. B) C-terminal truncation mutants of ADAMTS13 transfected in Lec13 CHO cells ±fucose. MDT1 has only the metalloprotease (M) and disintegrin (D) domains with the first TSR. MDT2 has M, D and the first 2 TSRs and so on. Constructs with more TSRs show greater dependence on fucosylation for secretion. Constructs with only one or two TSRs show almost no defect at all.

3.3. Primary amino acid sequence of a TSR influences fucose dependent secretion

Although TSRs have some conserved features, majority of the module is composed of diverse amino acids (33). This variation could influence the ease of folding and hence fucosylation by Pofut2. To test this hypothesis, constructs expressing single TSRs (1, 2, 3, 5 and 6) from ADAMTS13 were expressed in Lec13 CHO cells ±fucose. Of the TSRs transfected, only TSR3-ADAMTS13 showed a secretion defect in the absence of fucose (Fig. 3.5). These results suggest that the sequence of the TSR influences its efficient folding and requirement for fucosylation. Nonetheless, the results in Figure 3.4 also suggest that multiple, tandem TSRs enhance the need for fucosylation to efficiently secrete ADAMTS13.

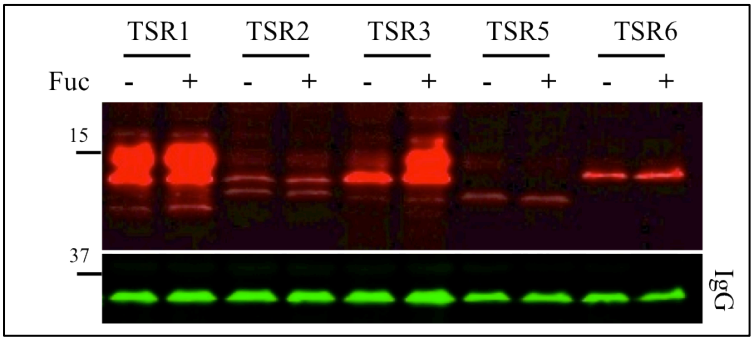


Fig. 3.5. Fucose dependence varies based on primary amino acid sequence of the TSR: Media fractions from Lec13 CHO cells ±fucose expressing single TSR1, 2, 3, 5 and 6 from ADAMTS13. Only TSR3 shows a strong fucose effect for efficient secretion.

3.4. Unfucosylated protein is rapidly turned over in the cell via ERAD

Even though TSR containing proteins are not secreted in the absence of fucose, they do not accumulate in the cell (Fig 3.2). This unfucosylated protein is presumably turned over by a proteosomal mechanism. Misfolded proteins in the cell are retro-transported to the cytoplasm, where they are ubiquintinated and targeted for degradation. This process is called ER-associated degradation (ERAD) (222). To examine if unfucosylated protein is being degraded via this pathway, a proteosomal inhibitor was used to block degradation and examine whether the unfucosylated protein accumulates. Lec13 CHO cells grown ±fucose were transfected with ADAMTS13-V5-His_6 and treated with lactacystin, a proteosomal inhibitor (Fig 3.6A). Samples that were treated with lactacystin showed higher molecular weight species of ADAMTS13. An anti-ubiquitin blot showed that the lactacystin treatment was successful since there was an overall increase in the ubiquitin signal. To confirm that the higher molecular weight species seen are ubiquitinated forms of ADAMTS13, the cell fraction was immunoprecipitated with anti-V5 (Fig 3.6B). The eluates were probed with anti-Ub to confirm the presence of ubiquitin on ADAMTS13.

Interestingly, these ubiquitinated forms occur both in the presence or absence of fucose. Quantitation of the western blot shows that the level of ubiquitinated protein in the absence of fucose is slightly higher (Fig. 3.6C) indicating that a greater percentage of the protein is ubiquitinated in the absence of fucose. Nonetheless, this data suggests that even under normal conditions (in the presence of fucose), proper folding of ADAMTS13 is complex (48 cysteines in the TSRs alone that must find their proper partners). The protein is often ubiquitinated and turned over rapidly by ERAD. Later results (see chapter 5) support the idea that the majority of ADAMTS13 in cells is unfolded and may be degraded by ERAD.

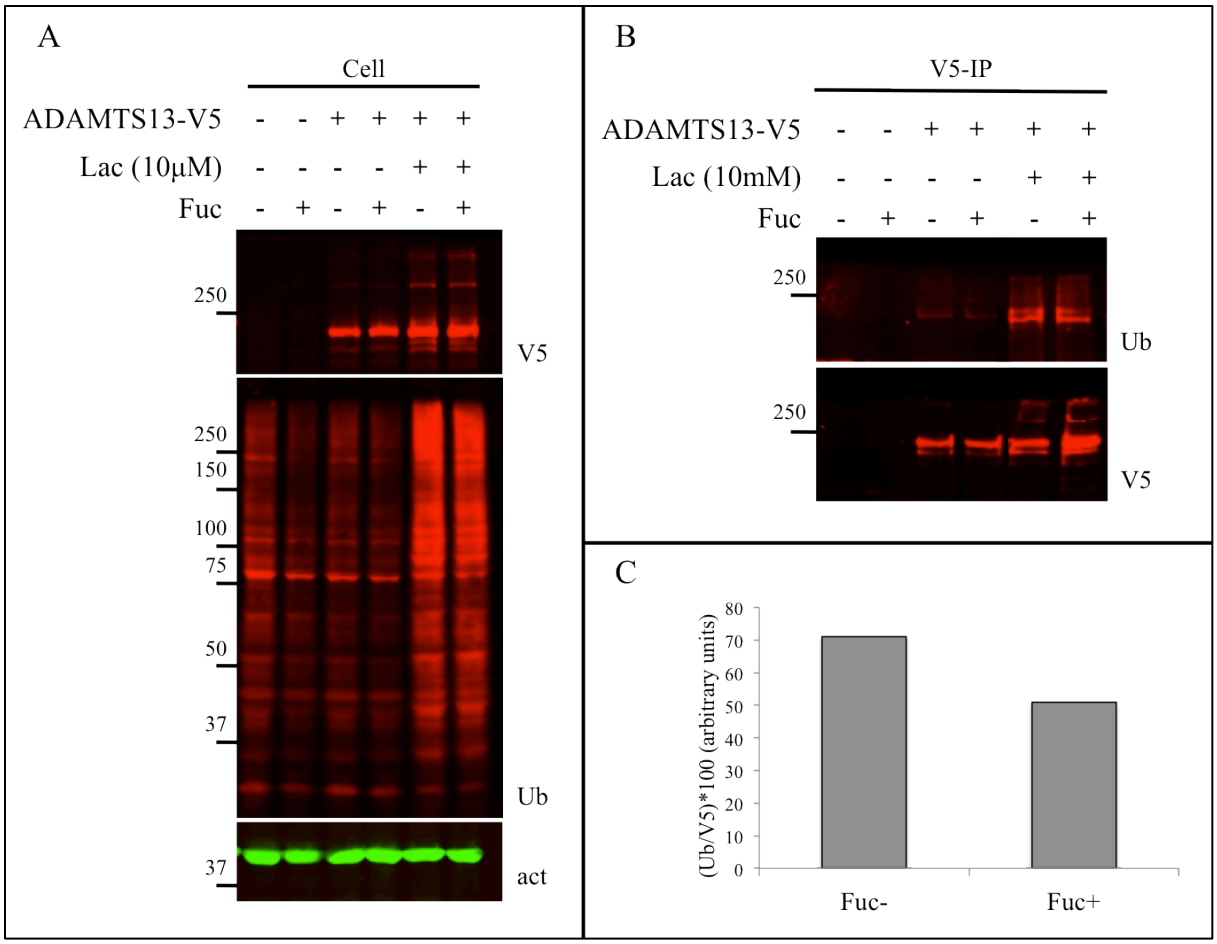


Fig. 3.6. Unfucosylated protein is turned over by ERAD: A) Western blot showing the accumulation of higher molecular weight forms of ADAMTS13 in samples treated with lactacystin (lac). Anti-ubiquitin (Ub) blots show overall increased ubiquitination in these samples demonstrating effectiveness of the lactacystin treatment. B) Immunoprecipitation of ADAMTS13 using the V5 tag shows that the higher molecular weight forms are ubiquitinated. C) Quantitation of the blots from panel B showing ratio of ubiquitinated ADAMTS13 to total ADAMTS13. In the absence of fucose there is more ADAMTS13 being turned over via ERAD.

3.5. Conclusions

In this section, I extend the secretion defect phenotype for ADAMTS13 and ADAMTSL1 seen in the absence of fucose cells to three other proteins: TSP1, ADAMTSL2 and ADAMTS9. The extent of the secretion defect varies depending on the number of tandem TSRs a substrate contains. I also demonstrate this using truncation mutants of ADAMTS13 with varying number of C-terminal TSRs. The dependence on fucose may also vary depending on the primary amino acid sequence of the TSR. In the absence of fucose or Pofut2, TSRs-containing proteins do not accumulate in cells and appear to be degraded via ERAD. Much of the proteins appears to be degraded even in the presence of Pofut2 and fucose, suggesting that like many proteins, only a small portion of the protein in the ER is ultimately secreted.

3.6. Methods

Plasmids used in this study: The preparation of full-length pcDNA4.0 (human) ADAMTS13-V5-His_6 construct was described previously (38). Truncation mutants were prepared using this plasmid as a template to generate necessary fragments. The fragments were then ligated into pcDNA4.0 V5-His_6 (Invitrogen). Single TSRs from ADAMTS13 were similarly amplified using the full length ADAMTS13-V5-6His as a template and ligated into pSecTag2 Hygro C Myc-His_6 (Invitrogen). The human ADAMTS9-myc-His_6 and human ADAMTSL2-myc-His_6 construct were generous gifts from Dr. Suneel Apte (Cleveland Clinic). Human IgG, human TSP1 (TSR1-3)-myc-His_6 and ADAMTSL1-myc-His_6 used are as described in (223), (78) and (39) respectively.

Antibodies used in this study: TSP1, ADAMTS9, ADAMTSL1 and ADAMTSL2 were detected using mouse anti-myc (9E10) antibody (Hybridoma facility, Stony Brook) in a 1:2000 dilution (5% non-fat dry milk, PBS-tween). ADAMTS13 was detected using mouse anti-V5 antibody (Life Technologies) 1:5000 dilution (5% non-fat dry milk, PBS-tween). The secondary antibody used was rabbit anti-mouse Alexafluor 680 (Life Technologies) in 1:10,000 dilution (in 1% non-fat dry milk, PBS-tween). hIgG was detected using goat anti-hIgG IRDye 800 (Rockland immunochemicals) in 1:10,000 dilution (1% non-fat dry milk, PBS-tween). Anti-Ubiquitin (Santa Cruz) was used in a 1:1000 dilution (PBS-tween).

Lec13 CHO secretion assays: Lec13 CHO cells were cultured for at least 3 passages ± 1mM fucose (final) in alphaMEM (GIBCO) containing 10% BCS (Life Technologies). For transfections, $1x10^6$ cells were seeded per well of a 6-well dish and allowed to grow overnight. The cells were then transfected with 1µg of DNA (total) using dPEI. The ratio of IgG:TSR-containing protein was kept at 1:4. After 6 hours, the media was changed to OptiMEM (Life

Technologies) and the cells were incubated 24 hours. Cells were then lysed in RIPA buffer containing protease inhibitor cocktail (Roche). Equal fractions of cell and media samples were analyzed by western blotting.

RNAi experiments: $1x10^6$ HEK293T cells were seeded in a 6-well dish in DMEM (Life Technologies) supplemented with 10% BCS (Life Technologies) and allowed to grow overnight. The cells were then transfected with 1μg of DNA and 25 pmoles of siRNA (Life Technologies) using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. The sequence of the siRNA targeting Pofut2 is as described previously (38). Six hours post-transfection, the cells were rinsed with PBS and the media was replaced with OptiMEM. Cell and media fractions were harvested 48 hours later and analyzed by western blotting.

Lactacystin experiments: Lec13 CHO cells were grown and transfected as described above. Twenty hours post-transfection, the cells were treated with 10 μM lactacystin (Sigma Aldrich) for 8 hours and then lysed in RIPA. An aliquot of the lysate was saved for western blotting and the rest was subjected to immunoprecipitation with anti-V5 antibody bound to Protein-G agarose (Roche). Samples were eluted by boiling in SDS sample buffer. One half was analyzed on an anti-Ub blot and the other on an anti-V5 blot. The blots were quantitated using ImageJ as a ratio of the ubiquitin signal to the V5 signal to obtain a ratio of ubiquitinated protein to total protein. The average of the untreated samples were subtracted prior to graphing to eliminate background signal.

Chapter 4. Subcellular location of *O*-fucosylation

To understand the cell biology of *O*-fucosylation better, I decided to investigate where in the cell the process takes place. Immunofluorescence experiments showed that Pofut2 localizes to the ER (15). Two curious but related facts are that Pofut2 does not have a KDEL sequence (which is characteristic of soluble ER resident proteins) and there is no known source of GDP-fucose in the ER (Sections 1.4 and 1.7). There are two possible mechanisms by which Pofut2 can be retained in the ER: 1) by a novel retention signal, or 2) by piggybacking onto a KDEL-protein. Either of these mechanisms allows for a model where Pofut2 binds to its substrates and travels to the ERGIC or Golgi, where GDP-fucose is available, and then is recycled back to the ER. Experiments in this section address these issues and provide insight about the subcellular location of the *O*-fucosylation process. To follow fucosylation, I use a bioorthogonal analog of fucose called 6-alkynyl fucose (6AF), which is described in Section 1.7. Post lysis of cells, I use azide-alkyne cycloaddition to tag 6AF with a biotin moiety, thus enabling detection of fucosylation using streptavidin. This azide-alkyne cycloaddition is also called ‘click reaction’ (78).

4.1. *O*-Fucosylation occurs in the ER and/or ERGIC compartments

The location of cell-associated fucosylated protein gives preliminary insight to the subceullar location of the *O*-fucosylation process. In addition to *O*-fucosylation sites, several of Pofut2's substrates have *N*-glycosylation sites. *N*-Glycans are added as high-mannose type structures in the ER and are further processed in the *cis*- and *trans*-Golgi into hybrid or complex-type structures. Hence, the structure of the *N*-glycan on a protein provides information about its subcellular location. *N*-glycan structures can be differentiated using glycosidases that specifically cleave particular types of *N*-glycans. Endoglycosidase H (EndoH) cleaves only high-mannose type glycans and hence eliminates glycans on proteins that are still in the ER or ERGIC. Peptide *N*-glycosidase F (PNGaseF) is a more indiscriminate enzyme that cleaves both complex and high-mannose structures (Fig. 4.1) (224). In combination with 6AF to detect fucosylation, the progress of a glycoprotein through the secretory pathway can be tracked using these enzymes.

Secretory pathway

	N-glycan type	EndoH	PNGaseF
ECM	Complex	-	+
Golgi	Complex	-	+
ERGIC	High-mannose	+	+
ER	High-mannose	+	+

Fig. 4.1. Schematic showing *N*-glycan processing in the secretory pathway: *N*-glycans are added in the ER as high-mannose structures but processed further in the *cis*- and *trans*-Golgi to be complex type structures. Specific glycosidases can be used to monitor the type of *N*-glycosylatio and hence the subcellular location of the protein.

293T cells transfected with ADAMTSL1 or ADAMTS13 were grown in the presence of 6AF. ADAMTSL1 has 1 *N*-glycosylation site in addition to its 4 *O*-fucosylation sites and ADAMTS13 has 10 *N*-glycosylation sites in addition to 7 *O*-fucosylation sites. Post lysis, the media and cell fractions were clicked to biotinylate the 6AF and purified. The samples were then subjected to EndoH or PNGaseF treatment and analyzed by western blotting to look for a

molecular weight change because of *N*-glycan cleavage (Fig. 4.2). Sufficient amounts of 6AF-labeled ADAMTS13 could not be purified from the media fraction for this analysis. Media associated ADAMTSL1 is Endo-H resistant but PNGaseF sensitive, demonstrating the presence of complex-type *N*-glycans on the mature, secreted protein. The PNGaseF digested protein also labeled with 6AF indicating the presence of *O*-fucose on the protein, since any fucose on *N*-glycans would have been removed by PNGaseF. In contrast, analysis of the ADAMTSL1 and ADAMTS13 from cell lysates showed EndoH sensitive, *O*-fucosylated TSR-contaning protein, suggesting that *O*-fucose is added to TSRs before N-glycans become resistant to EndoH (which occurs in the *cis*-Golgi). While these data suggest that *O*-fucosylation of TSRs occurs in the ER, they do not rule out the possibility that the protein is *O*-fucosylated in the ERGIC.

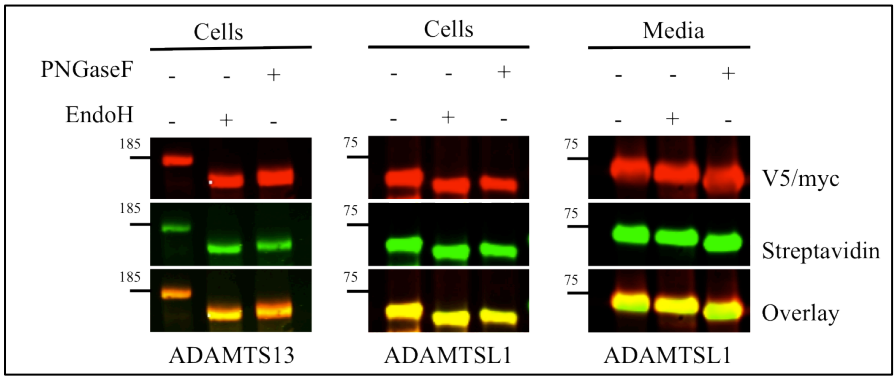


Fig. 4.2. *O*-Fucosylation occurs in the ER: EndoH and PNGaseF digests on cell and media associated TSR-containing proteins labeled with 6AF. ADAMTSL1 purified from media shows EndoH resistance indicating that the *N*-glycans are processed to complex type structures. Cell-associated ADAMTS13 and ADAMTSL1 are expectedly EndoH sensitive but are also labeled with 6AF.

4.2. *O*-Fucosylation is at least partly co-translational

An alternative way of examining whether *O*-fucosylation occurs before the ERGIC is to test if the process is co-translational. This can examined by purifying ribosome-associated nascent TSR-containing proteins and examining their *O*-fucosylation status. One approach to achieve this is by taking advantage of puromycin, a translation terminator (225). Puromycin inhibits translation by covalently attaching itself to the growing end of the polypeptide chain (226).

HEK293T cells cultured in the presence of 6AF were transfected with N-terminal 3XFlag-ADAMTS13 and treated with puromycin. Following puromycin treatment, the cells were lysed, reacted with azido-bioin, and ADAMTS13 was immunoprecipitated with anti-Flag-antibody-agarose. The eluate after immunoprecipitation was analyzed on a Western blot using anti-puromycin antibody and streptavidin (Fig. 4.3). In the 6AF-labeled, puromycin-treated samples, distinct bands of nascent polypeptides labeled with 6AF were seen indicating the presence of *O*-fucose. The bands that labeled with 6AF were above 50kDa, which corresponds to the size of a polypeptide translated past the first TSR on ADAMTS13, even though bands smaller than 50 kDa reacted with the anti-puromycin antibody. The 6AF bands also reacted with the anti-puromycin antibody (see overlay) suggesting incorporation of fucose and puromycin into the same polypeptide chains. The puromycin labeling on the polypeptides decreased with size as expected since the full-length protein would have been synthesized before translation was interrupted. The existence of 6AF on the same proteins labeled with puromycin strongly argue that *O*-fucosylation is at least partially co-translational and occurs in the ER.

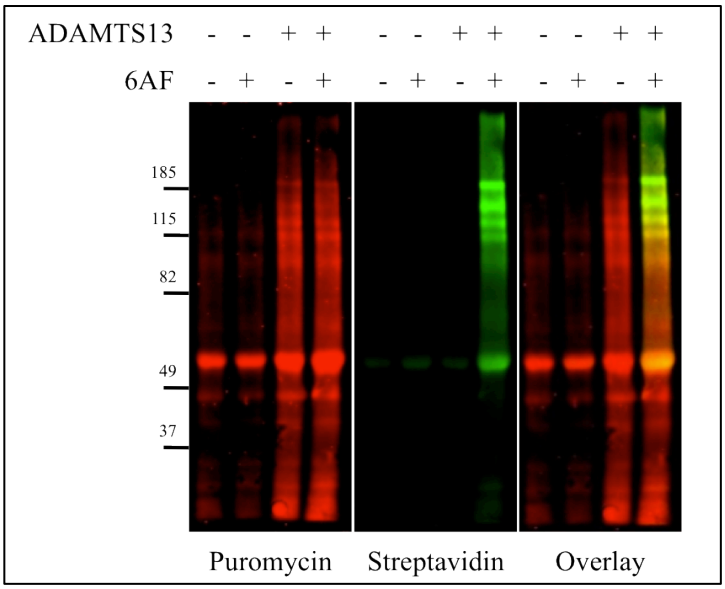


Fig. 4.3. Puromycin-tagged ADAMTS13 is *O*-fucosylated: Flag-immunoprecipitation of 6AF-labeled ADAMTS13 from 293T cells treated with puromycin. The western blot was probed with anti-Puromycin (red) and Streptavidin (green) to detect truncated polypeptides and O-fucose respectively. The overlay of the two blots showed the presence of fucosylated, puromycin-labeled truncated polypeptides.

An alternative approach to test the co-translation hypothesis is to purify ribosomes from cells expressing N-terminal 3XFlag-ADAMTS13 and examining their fucosylation status. HEK293T cells transfected with N-terminal 3XFlag-ADAMTS13 and grown in 6AF were treated with 100 µM cycloheximide (CHX) for 15 minutes prior to lysis. Cycloheximide is a translational inhibitor that affects the displacement of tRNA molecules and thus blocks elongation of the protein. Ribosomes were pelleted from the lysate by using sucrose cushion ultracentrifugation. Western blot analysis of the ribosome pellets showed a range of fragments of ADAMTS13 with a Flag-tag with some of the peptides labeled with Streptavidin (Fig. 4.4). To eliminate the possibility that fucosylation might occur while sample processing, the ribosomal fraction was demonstrated to lack Pofut2 activity using an *in vitro* assay (Fig. 4.5). This result in combination with the puromycin experiments strongly suggests that *O*-fucosylation is a co-translational process.

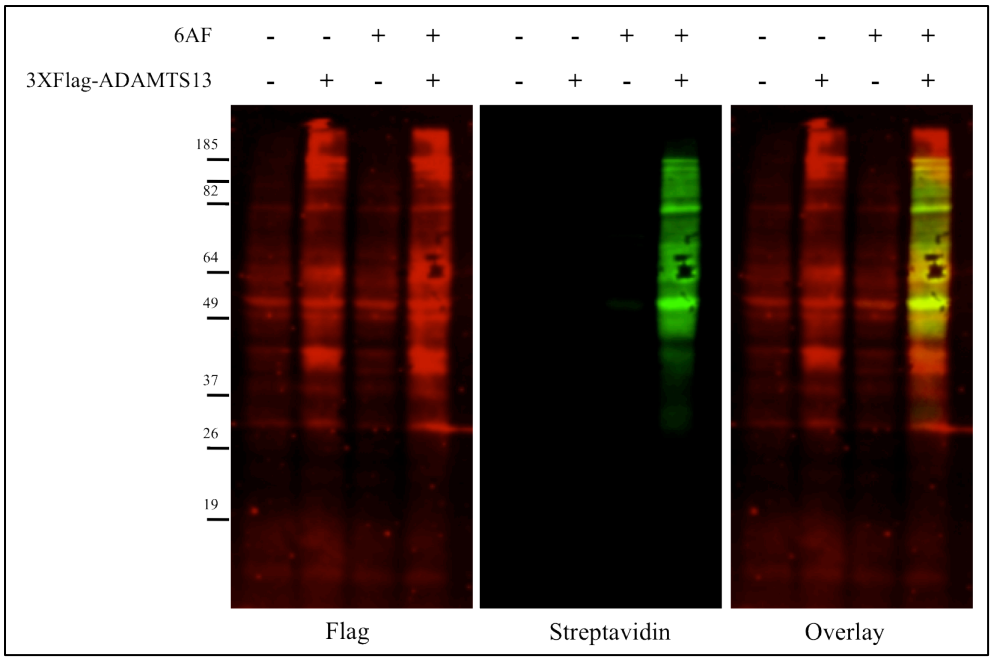


Fig. 4.4. Ribosome associated ADAMTS13 is *O*-fucosylated: Western blot on ribosomal fraction of 293T cells expressing 3XFlag-ADAMTS13 shows 6AF labeling suggesting that *O*-fucosylation is co-translational.

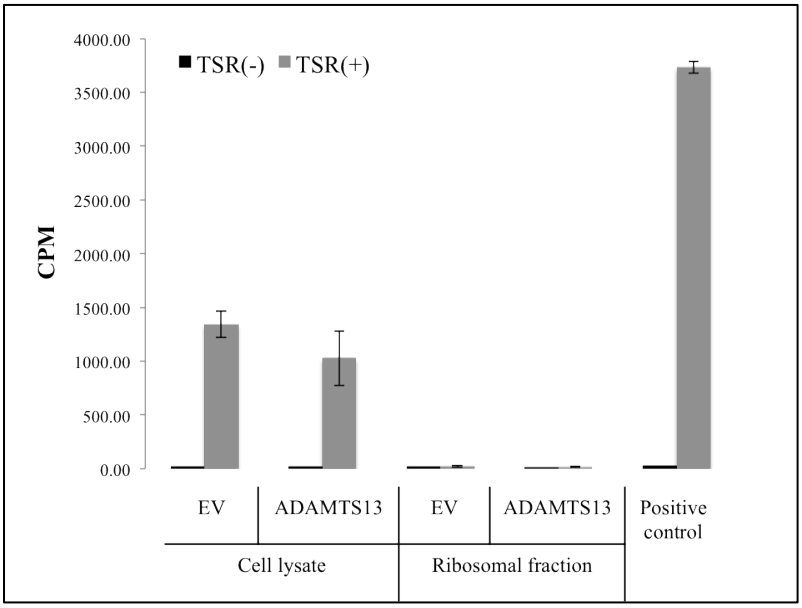


Fig. 4.5. Ribosome fraction does not show Pofut2 activity: *In* vitro fucosyltransferase assay using [^{3}H]-GDP-fucose on total cell lysates and ribosomal fractions to measure Pofut2 activity. TSR3 from TSP1 is used as an acceptor substrate and various cell fractions are used as the enzyme source.

4.3. Large TSR-containing proteins take a long time to be folded and secreted

Results from the sections 4.1 and 4.2 suggest that *O*-fucosylation occurs early in protein synthesis. To further understand the timing of *O*-fucosylation, the rate of protein synthesis and secretion needs to be determined. To determine this, I performed pulse-chase experiments with [^{35}S]-Methionine on *wt* CHO cells (Pro5) expressing ADAMTS13. CHO cells transiently transfected with N-3XFlag-ADAMTS13 were pulsed with [^{35}S]-Methionine for 10 minutes. As a control, cells transfected with the heavy chain of human IgG, were used. The cells were then chased in the presence of unlabeled methionine for several time points. ADAMTS13 from media and cell fractions were subjected to immunopurification using Flag M2 agarose. IgG was similarly purified using Protein G agarose. The eluates were analyzed on a reducing gel and autoradiographed as a measure of total ADAMTS13 (Fig. 4.6).

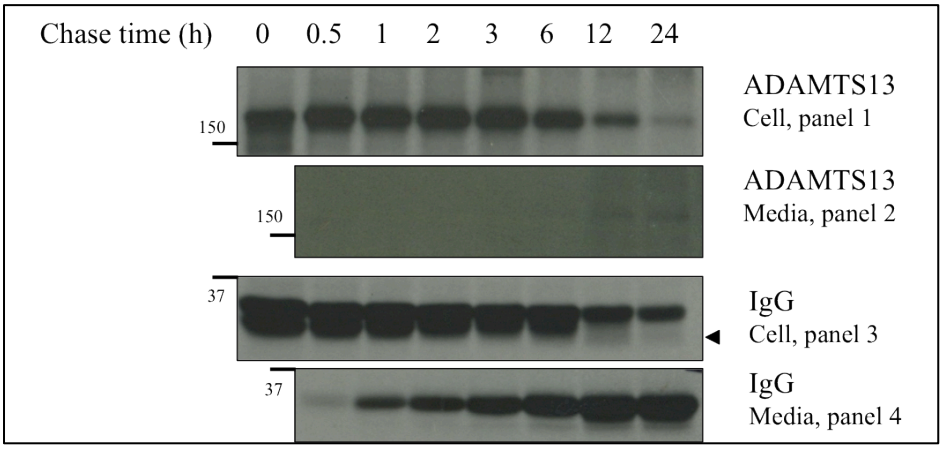


Fig. 4.6. ADAMTS13 takes a long time to be folded and secreted: ^{35}S-metabolic labeling of 3XFlag-ADAMTS13 in Pro5 cells. After pulse-chase, cell and media fractions were subjected to immunoprecipitation. Labeled ADAMTS13 appears in the media only at 12 h (panel 2). Similar studies on human IgG (panel 3, 4), an unrelated protein with no TSRs, showed the protein to be much more rapidly secreted. (Panels 1, 3 and 4 represent 1.5 day film exposures but panel 4 represents a week long exposure)

As expected, the signal corresponding to cell-associated ADAMTS13 showed similar signals in increasing time points, indicating that the pulse efficiently labeled the protein. The

earliest signal in the media fraction was seen only at 12 h and is much weaker than the 0 h signal in the cell. In comparison, IgG was secreted much more rapidly with the earliest signal in the media appearing at 30 minutes and the signal saturating at 12 h. This suggests that ADAMTS13 takes a long time to be folded and secreted, and that not all ADAMTS13 synthesized makes it out of the cell. Since ADAMTS13 (~185 KDa) is a much larger protein that IgG (~40 KDa), similar studies were performed on ADAMTSL1 (~55 KDa), which is closer to IgG in size. [^{35}S]-Methionine labeled cell-associated ADAMTSL1 was immunoprecipitated with anti-myc and media-associated ADAMTSL1 was affinity purified with NiNTA agarose. The autoradiograph revealed that ADAMTSL1 also takes much longer to be secreted into the media in comparison to IgG, with the earliest signal in the media appearing at 1 h (Fig. 4.7). Unlike ADAMTS13, most of ADAMTSL1 labeled at 0 h in the cell appears to be secreted into the media at 24 h. However, this needs to be rigorously tested since the current experimental set up utilizes different methods of purification for cell and media associated proteins and cannot be compared directly.

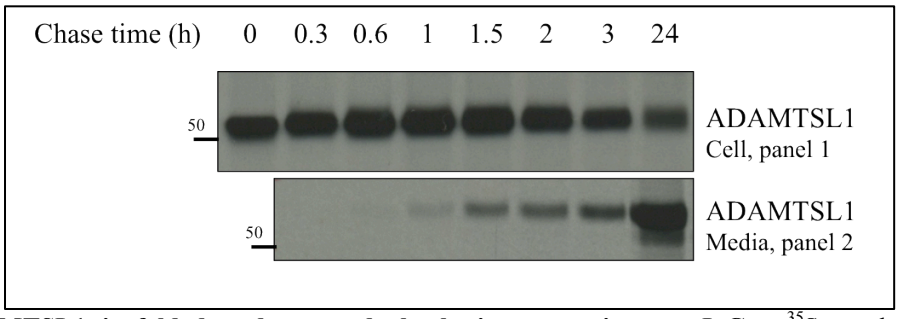


Fig. 4.7. ADAMTSL1 is folded and secreted slowly in comparison to IgG: ^{35}S-metabolic labeling of ADAMTSL1-myc-His$_6$ in Pro5 cells. After pulse-chase, ADAMTSL1 was immunopurified with anti-myc form the cell and affinity purified with NiNTA agarose from the media. Labeled ADAMTSL1 appears in the media only at 1 h in comparison to IgG (Fig. 4.6) which is secreted within 20 min (panel 2).

4.4. Does Pofut2 shuttle to the Golgi?

The results so far in this section strongly suggest that *O*-fucosylation is an ER-associated event. This conclusion raises the question of where the GDP-fucose necessary for fucosylation reactions comes from. As described in earlier sections, it is possible that Pofut2 shuttles between the ER, ERGIC and Golgi where it is "loaded" with GDP-fucose. This donor-substrate bearing Pofut2 could then interact with TSRs being folded in the ER. Pofut2 has 3 *N*-glycosylation sites that may be processed into complex structures if Pofut2 travels to the Golgi. This can be tested using EndoH and PNGaseF sensitivity, similar to experiments in Section 4.1. Flag-tagged hPofut2 was immunopurified from 293T cells and subjected to EndoH and PNGaseF digests (Fig. 4.8). All cell-associated Pofut2 was seen to be EndoH sensitive showing that the glycans on the fucosyltransferase were still high mannose type structures. This indicates that at least majority of cell-associated Pofut2 had not travelled to the cis-Golgi.

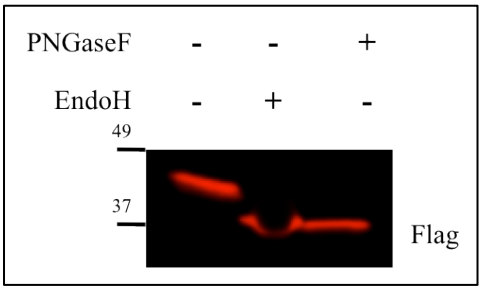


Fig. 4.8. Pofut2 does not shuttle between the ER and Golgi: EndoH and PNGaseF digestion on Pofut-Flag purified from 293T cells shows that all *N*-glycans on Pofut2 are high-mannose type structures.

4.5. Conclusions

Results in this section demonstrate that *O*-fucosylation is an ER-associated event. I used several approaches to show that the process is at least partly co-translational, though since proteins with tandem TSRs fold slowly and hence *O*-fucosylation may also occur post-translationally. The source of GDP-fucose in the ER still remains unknown. Using a simple assay, I eliminated the possibility that Pofut2 may shuttle between the ER and Golgi. However, Pofut2 may still shuttle between the ER and the ERGIC.

4.6. Methods

Plasmids used in this section: The ADAMTSL1-myc-His_6 and ADAMTS13-V5-His_6 constructs used is the same as described in Section 3.6. Using p3XFlag-CMV (SIGMA) as a template, a 3XFlag fragment with HindIII and BamHI sites was generated. This fragment was ligated in to pSecTag2 HygroC to generate new backbone with an N-terminal flag tag following the IgK leader sequence. Full length ADAMTS13 with XhoI overhangs were amplified from an existing construct and ligated into the pSecTag2 N-3XFlag backbone. Flag tagged human Pofut2 constructs were a generous gift from Heinz Gut (45).

Antibodies used in this section: The anti-myc and V5 antibodies used are described in Section 3.5. Streptavidin IRDye 800 (Rockland immunochemicals) was used to detect biotinylated 6AF in a dilution of 1:10,000 (1% NFDM, PBST). Anti-Flag (Sigma) was used in a 1:2000 dilution (5% NFDM, PBST). The anti-puromycin antibody was generous gift from Dr. Ari Helenius and was used in a 1:1000 dilution in PBST.

Azide-alkyne cycloaddition (click reaction): Reactions were performed as described in (78). Media and cell lysates metabolically labeled with 200μM 6AF were tagged with Biotin (0.1 mM Azido-Biotin (CC Tools), 0.1 mM tris-(benzyltriazolylmethyl)amine catalyst (AnaSpec), 1 mM copper sulfate, 2 mM sodium ascorbate, at room temperature for 1 hour.

EndoH and PNGaseF digests: HEK293T cells were transiently transfected with ADAMTSL1-mycHis$_6$ or ADAMTS13-V5-His_6 and grown in the presence of 6AF. Media samples were purified using Ni-NTA agarose (Qiagen) and lysates were immunopurified as described in

Section 3.5. Samples were "clicked" as described above and treated with PNGaseF or EndoH as described in (227).

Puromycin experiments: HEK293T cells were transiently transfected with N-3XFlag-ADAMTS13. Following transfection, the cells were incubated in Opti-MEM containing 200 μM 6AF overnight. The cells were then treated with 3 μM puromycin (Sigma) for 20 minutes at 37°C. Cell lysate was prepared in RIPA containing 100mM iodoacetamide (IoA), clicked as described above, and immunoprecipitated with Flag-M2 agarose (Sigma) according to manufacturer's instructions. The flag eluates were then analyzed by western blotting.

Ribosome experiments: HEK293T cells transiently transfected with N-3XFlag-ADAMTS13 incubated in 6AF, were treated with 100 μM CHX (Sigma) for 15 minutes at 37°C. The cells were then lysed in 50mM NH_4HCl, 12mM $MgCl_2$, 50mM Tris acetate and 0.5% NP-40. The lysates were passed through a 27.5 gauge syringe twice to thoroughly free any adherent ribosomes and debris is removed by centrifuging at 14000 rpm (20 minutes at 4°C). The lysate was loaded on 1 ml of 34% sucrose cushion containing 50mM NH_4HCl, 12mM $MgCl_2$, 50mM Tris acetate and ultracentrifuged at 100,000g for 3 hours. The pellet was rinsed 3X with lysis buffer and resuspended in sample buffer for western blotting. This experiment was done in collaboration with Dr. Preeti Mehta and Dr. Perry Woo in Dr. Wali Karzai's laboratory at Stony Brook University.

Fucosyltransferase assays: Reactions were performed as described in (36). 100μM TSR3 from TSP1 (purified from BL21 cells) was incubated with the enzyme source and 10μM of [^{3}H]-GDP-

fucose in HEPES-$MnCl_2$ buffer for 20 minutes at 37°C. The reaction was stopped by adding 1000 reaction volumes of 0.1M EDTA. TSRs were then purified from the mixture using Sephadex columns (Pierce) and counted on a Beckman scintillation unit.

Pulse chase assay: Pro5 CHO cells were transiently transfected with 3XFlag-ADAMTS13 or hIgG. Following methionine-starvation for 2 h, the cells were pulsed with EXPRES^{35}S (Perkin Elmer) for 10 min to a final specific activity of 250 μCi/ml and chased with OptiMEM for indicated periods of time. ADAMTS13 was immunopurified with Flag M2 agarose (Sigma) and IgG was purified with Protein A agarose (Roche). Cell-ADAMTSL1 was purified with anti-myc (9E10) and Protein A agarose (Roche) and media-ADAMTSL1 was purified with NiNTA agarose (Qiagen). Equal fractions of eluates were analyzed using SDS-PAGE and autoradiography. Equal fractions of eluates were analyzed using SDS-PAGE and autoradiography.

Chapter 5. Molecular role of *O*-fucose on TSRs

The results from Section 4.3 suggest *O*-fucosylation occurs early in protein synthesis in the ER and ADAMTS13 takes a long time to be secreted relative to proteins without tandem TSRs like IgG. One prediction resulting from this observation is that cell-associated protein in the ER should have a mixture of fucosylated and unfucosylated TSRs. Previously publications from our laboratory have examined *O*-fucosylation on secreted (mature) protein using mass spectral approaches and found that the TSRs from ADAMTS13 are modified with the fucose-glucose disaccharide (38). In this study, I use the same mass spectral approaches to compare the stoichiometries of *O*-fucosylation on TSRs from mature ADAMTS13 purified from media with immature ADAMTS13 protein isolated from cell lysates. To do so, I purify ADAMTS13 from HEK293T cells, digest them with trypsin (after reduction and alkylation) and analyze them on an ESI-Ion Trap LC MS system (35). I then find and confirm the identity of glycopeptides and compare their relative abundances using extracted ion chromatograms (EIC). I also compare the stoichiometry of fucosylation on unfolded and mostly folded ADAMTS13 from cell lysates, separated based on their migration on a non-reducing gel.

5.1. Mature ADAMTS13 is fucosylated at high stoichiomitries, but cell-associated ADAMTS13 is not

Our laboratory has previously mapped sites of *O*-fucosylation on mature ADAMTS13 using nano LC-MS/MS methods (38). To examine the stoichiometry of fucosylation I took advantage of a semi-quantitive method to examine the relative amounts of the glycoforms of peptides known to be modified, searching for unmodified, monosaccharide fucose, and disaccharide Glucose-Fucose forms using EIC. HEK293T cells were transfected with ADAMTS13-V5-His_6 and the protein was purified from the media using Ni-NTA agarose. Peptides from TSRs 5, 7 and 8 were analyzed in detail. Table 5.1 lists the charge states, mass and sequence of the specific glycopeptides.

EIC of the different glycoforms of these peptides showed that the mature protein isolated from the media is efficiently modified with the major glycoform being the disaccharide (Fig. 5.1A). Very little unmodified peptide was detected at any of the sites, indicating that the efficiency of glycosylation is very high on mature protein. Figure 5.1B shows MS/MS spectra for each of the peptides confirming their identity. In contrast, a mixture of unmodified and glycosylated peptides were detected on the same peptides isolated from cell lysates, with a majority of unmodified peptide (Fig. 5.2). Interestingly, the peptide from the most C-terminal site (TSR8) was more poorly modified than peptides from N-terminal sites (TSR5 or 7) suggesting that fucosylation may be processive. These results confirm that the cell-associated protein is partially modified, supporting the observations made in Section 4.3.

TSR#	Sequence	Charge (n)	$[M+nH]^{n+}$ (calc.)	$[M+nH]^{n+}$ (obs.)	$[M+nH+Hex]^{n+}$ (obs.)	$[M+3H+dHex+Hex]^{3+}$ (obs.)
5	^{899}TGAQAAHVWTPAAG SCSV<u>S</u>CGR^{910}	3	744.8	744.9	793.5	847.5
7	^{1018}VMSLGPCSA<u>S</u>CGLGT AR^{1034}	2	862.4	862.8	935.9	1017.1
8	^{1076}WHVGTWMECSV<u>S</u>C GDGIQR^{1094}	3	756.1	756.1	805.1	859.1

Table 5.1. Peptides identified in the MS analysis of ADAMTS13: List of peptides used for preparing extracted ion chromatograms (EICs) in the analysis of TSRs from various ADAMTS13 fractions and their corresponding calculated (calc.) and observed (obs.) masses. The *O*-fucosylation site is underlined.

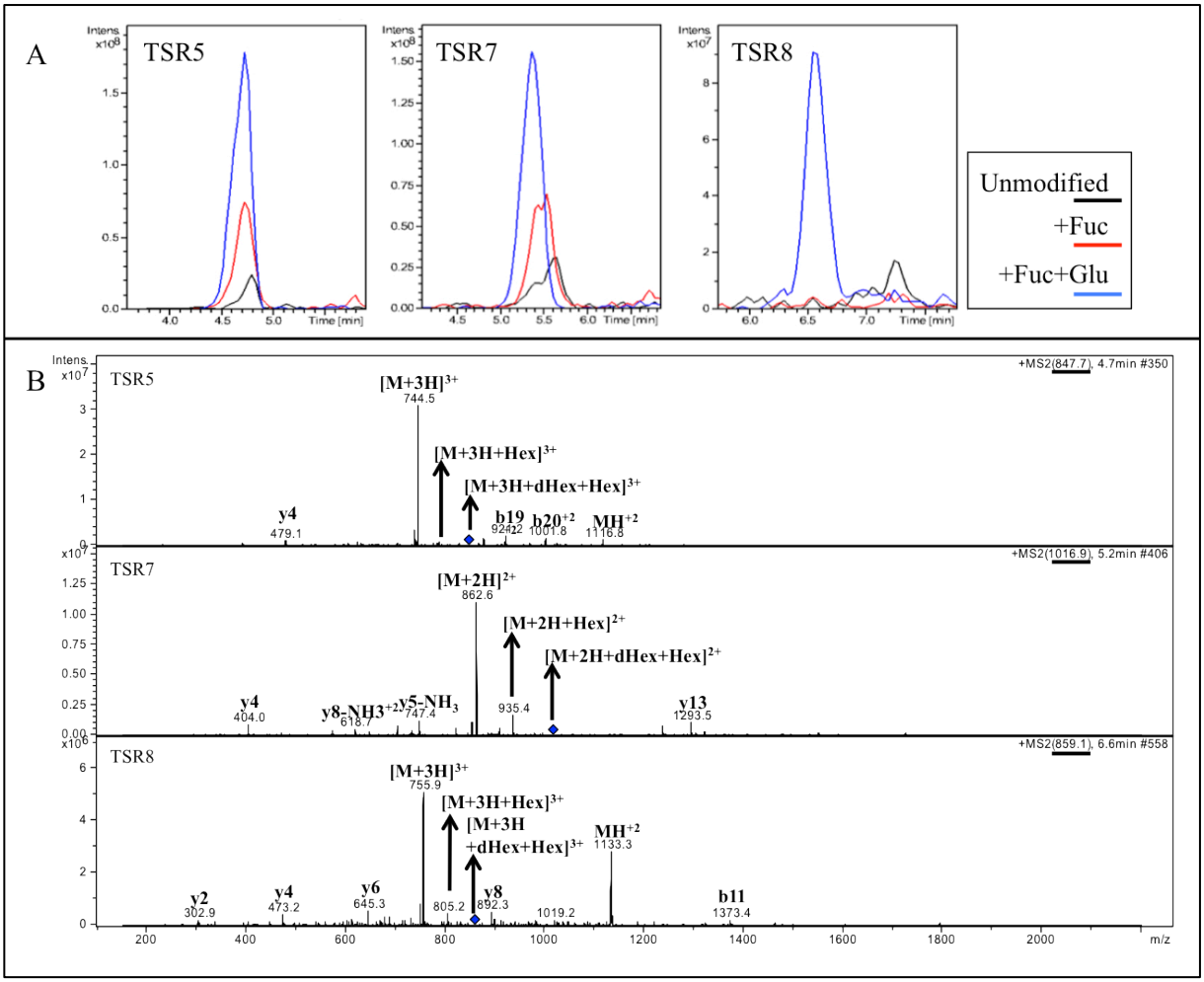


Fig. 5.1. Mature ADAMTS13 is fully fucosylated: A) EICs of unmodified, fucosylated and fuc-glucosylated peptides from TSRs 5, 7 and 8 of media associated ADAMTS13 purified from HEK293T cells showing that most of the TSRs are fucosylated. B) MS/MS spectra of fully modified peptides from A showing constant neutral loss of glucose and fucose and some major b and y ions.

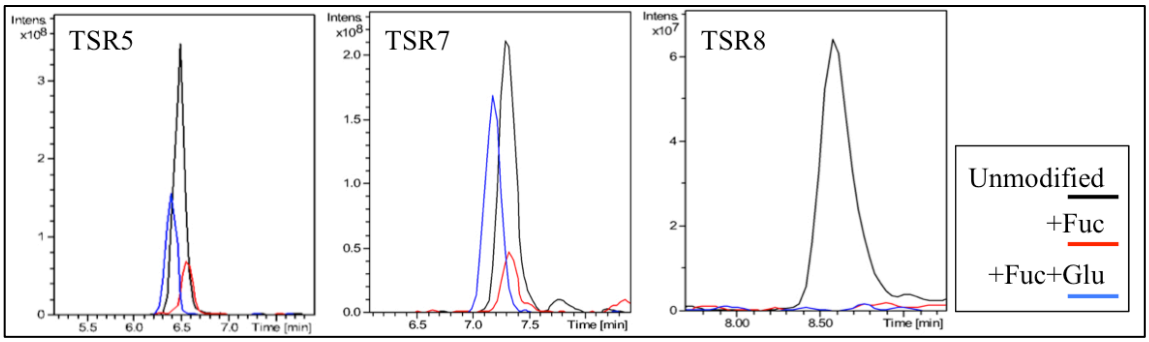


Fig. 5.2. Cell-associated ADAMTS13 is a mixture of various glycoforms: EICs of peptides from TSR5, 7 and 8 of ADAMTS13 purified from HEK293T cell lysates showing mixtures of unmodified, mono- and disaccharide forms in contrast to media associated ADAMTS13 (Fig. 5.1A) that is fully modified.

5.2. *O*-Fucosylation is a marker for properly folded TSRs in cells

Since Pofut2 only modifies properly folded TSRs *in vitro* (36), I hypothesized that the unmodified peptides within cells would be from incompletely folded TSRs, whereas the *O*-fucosylated peptides would be from folded TSRs. Each fully folded TSR has three disulfide bonds so it is likely that unfolded or improperly folded TSRs would have unpaired cysteines whereas folded TSRs would not. To separate folded and unfolded forms of ADAMTS13, I lysed cells in the presence or absence of excess iodoacetamide (to prevent aberrant disulfide bond formation after lysis), immunopurified ADAMTS13 and analyzed the eluates on reducing and non-reducing SDS-PAGE (Fig. 5.3A). The protein runs at the predicted size on the reducing gel regardless of the presence or absence of iodoacetamide. In contrast, all of the protein runs as an aggregate at the top of the non-reducing gel in the absence of iodoacetamide suggesting that all ADAMTS13 molecules in the cell have at least one unpaired cysteine. The mature protein from the medium, however, runs at its predicted size on the non-reducing gel, showing it no longer has unpaired cysteines.

When cells are lysed in the presence of iodoacetamide, unpaired cysteines in ADAMTS13 are alkylated. This results in some of the protein running at the size of the mature protein on the non-reducing gel, while the majority still runs as an aggregate at the top of the gel. This suggests that the ADAMTS13 that enters the non-reducing gel has fewer unpaired cysteines (*i.e.* is more completely folded) than that at the top of the gel. When peptides derived from the mostly unfolded population at the top of the gel were analyzed by mass spectrometry, the majority of them were not fucosylated (Fig. 5.3B), consistent with the TSRs being partly or mostly unfolded in this protein population. When peptides from the band corresponding to the mature protein were analyzed, it showed almost completely glycosylated TSR5 and 7, but TSR8

was still predominantly unmodified (Fig. 5.3C). This suggests that the TSRs in this protein population are mostly folded and will exit the cell upon the folding and fucosylation of TSR8. These data demonstrate that *O*-fucosylation serves as a marker for the folded TSRs within the cell.

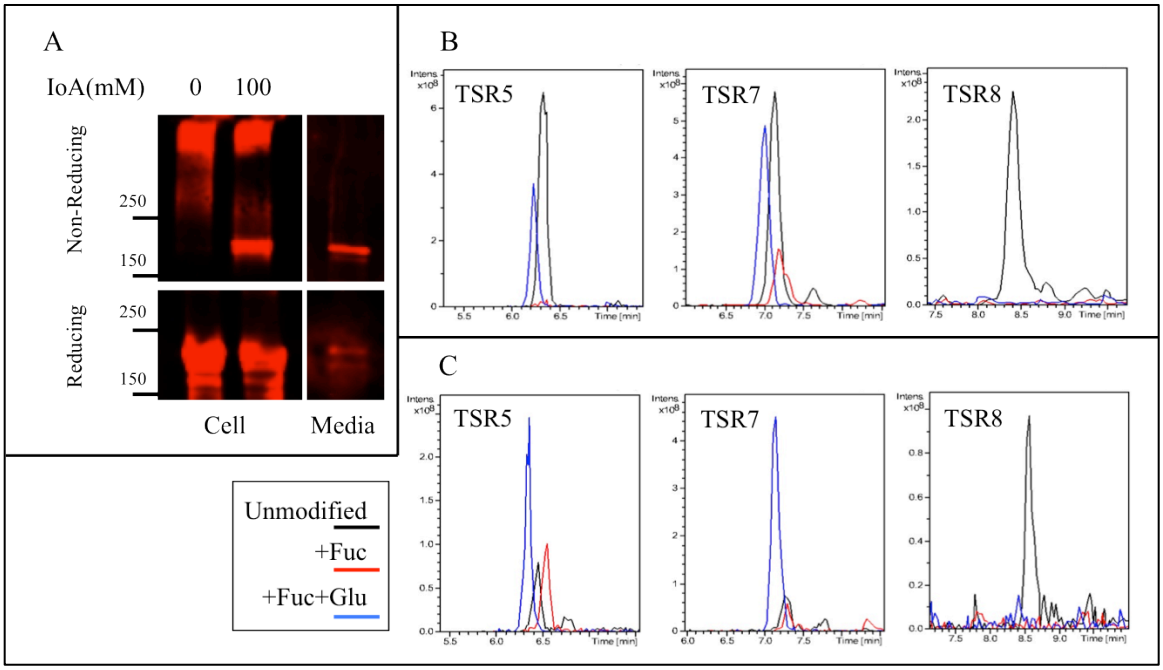


Fig. 5.3. *O*-Fucosylation is a marker for properly folded TSRs: A) Western blot showing the segregation of unfolded aggregates and mostly folded forms of ADAMTS13. The two populations were separated by using iodoacetamide during lysis and analyzed on a non-reducing gel (top panel). Treating the samples with BME led to complete reduction of ADAMTS13, making the two populations indistinguishable. B) Extracted ion chromatograms of peptides from unfolded aggregates showing profiles similar to that of total cell lysates (Fig. 5.2). C) EICs of the peptides from mostly folded cell-associated ADAMTS13 (as separated in A) showing predominantly modified peptides from TSR5 and 7 and completely unmodified peptide from TSR8.

5.3. Conclusions

In this section, I demonstrate that fully folded (*i.e.* mature) TSRs are completely modified with *O*-fucose. Total cell-associated TSRs are a mixture of different glycoforms, with the unmodified form being predominant. TSR8, the last TSR in the tandem sequence, is completely unmodified. Since Pofut2 only modifies properly folded TSRs, I used Iodoacetamide to alkylate free cysteines at the time of lysis. Despite adding molar excess of iodoacetamide, I see that most cell-associated protein migrates as an aggregate at the top of a non-reducing gel. However, I was able to separate misfolded protein (with unpaired disulfide bonds) from mostly folded proteins (most of whose cysteines are correctly paired) using this method. Mass spectrometric analysis on the misfolded aggregates show similar profiles to total cell lysate, but the mostly folded fraction shows all TSRs to be fully modified except TSR8. I reason that this population of ADAMTS13 is still in the cell because of this unfolded terminal TSR.

5.4. Methods

Plasmids and antibodies used in this study: The 3XFlag-ADAMTS13 used in the site mapping experiments is as described in Section 4.6. The protocols for western blotting and immunopurification using anti-Flag antibody are also described in the same section.

MS Analysis of ADAMTS13: HEK293T cells were transiently transfected with N3XFlag-ADAMTS13 and allowed to grow for 48 hours before being lysed in RIPA buffer containing 100mM Iodoacetamide (Sigma). The lysates were incubated on ice for 30 minutes to allow alkylation, followed by centrifugation at 14,000 rpm for 10 minutes to clear insolubles. The lysates were immunoprecipitated using Flag-M2 agarose (Sigma) as described by the manufacturer. ADAMTS13 was eluted off using 3XFlag peptide as described by the manufacturer (Sigma). The eluate was divided and run on reducing and non-reducing gels. Relevant bands were subjected to in-gel protease digestions and mass spectral analysis as described in (35). EICs were prepared using DataAnalysis software (Agilent) and chromatograms were smoothed using Gaussian methods.

Chapter 6. Chaperone activity of Pofut2

The experiments from Chapter 5 showed that Pofut2 marks properly folded TSRs in cells with an *O*-linked fucose, consistent with its ability to distinguish between folded and unfolded TSRs *in vitro* (36). Together with the demonstration that fucosylation by Pofut2 is required for efficient secretion of TSR-containing proteins (Chapter 3), this data suggests that Pofut2 may play a direct role in the folding of TSRs. To test if this is the case, I performed a series of *in-vitro* and *in-vivo* experiments to examine the interaction between Pofut2 and its substrates. The *in vitro* assays take advantage of the fact that different folding isomers of a given TSR have different hydrophobicities. Based on these differences, a mixture of unfolded, partially folded and fully folded TSR can be separated using reverse phase HPLC on a C18 column.

6.1. Pofut2 remains bound to its substrates in the absence of GDP-fucose

One possible explanation for the secretion defect observed for TSR containing proteins in Lec13 cells grown in the absence of fucose (Fig. 3.2) is that Pofut2 may bind to but not release the substrates in the absence of GDP-fucose. To examine this possibility, I did co-immunoprecipitation studies in the presence of a cell-permeable, thiol-cleavable cross-linker, DSP, in Lec13 CHO cells. Flag-tagged Pofut2 was co-transfected with several of its substrates and treated with DSP before lysis. Post-lysis, the cell fraction was subject to a Flag immunoprecipitation and the eluates were run on a reducing gel. The resulting Western blot showed that not only does Pofut2 interact with its substrates, it does so in a fucose-dependent manner (Fig. 6.1). More substrate co-precipitated with Pofut2 in the absence of fucose than in the presence. This suggests that in the absence of GDP-fucose, Pofut2 cannot release the substrate and remains bound, trapping it inside the cell. This provides a potential explanation for at least part of the secretion defect of TSR-containing proteins in Lec13 CHO cells.

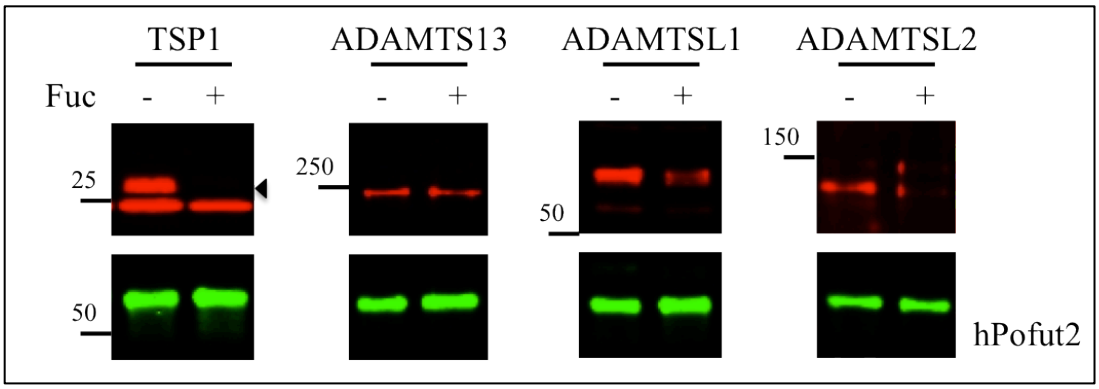
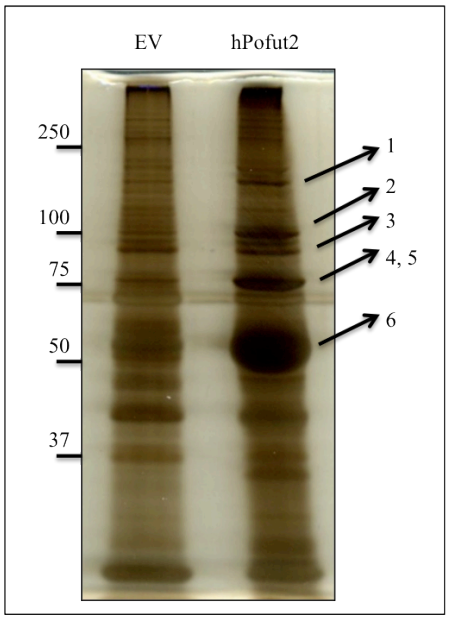


Fig. 6.1. Pofut2 does not release its substrate in the absence of fucose: Flag immunoprecipitation of DSP- treated Lec13 CHO cells (±fucose) co-transfected with Pofut2 (green) and various TSR-containing substrates (red). More substrate co-immunoprecipitated with Pofut2 in the absence of fucose than in the presence of fucose.

6.2. Pofut2 interacts with several chaperones in the ER

Pofut2 does not have a KDEL-like ER-retention sequence found in most soluble ER proteins, suggesting that Pofut2 piggybacks on other ER proteins to remain in the ER (36). To examine this possibility, 293T cells expressing Pofut2-Flag were treated with DSP. After DSP-treatment, the cells were lysed, Pofut2 immunoprecipiated with anti-Flag antibodies, and the eluates were analyzed on a silver-stained acrylamide gel (Fig. 6.2). Bands in the negative control lane were compared with the Pofut2-transfected lane and distinctive bands in the latter were cut out and identified using mass spectrometry. This analysis revealed several proteins (Table 6.1) that potentially interact with Pofut2 and aid in its ER-retention. Most of these proteins are soluble ER proteins with KDEL-like sequences (α-Glucosidase II, Binding immunoglobulin Protein (BiP), Protein Disulfide Isomerase A4 (PDIA4)), and several are known to play a role in protein folding (Heat shock protein 90 (Hsp90), BiP, PDIA4). These results suggest that a large protein-folding complex is possibly involved in the ER retention of Pofut2 and also in the folding of TSRs.



Protein name	GPM score	# unique peptides found
1.Hypoxia upregulated protein 1	-307.6	26
2.Alpha glucosidase II	-197.4	19
3.Heat shock protein 90	-274.6	22
4.Binding immunoglobulin protein	-335.7	25
5.Protein disulfide isomerase A4	-231.6	22½
6.Protein *O*-fucosyl transferase 2	-208.7	17

Fig. 6.2. Pofut2 has several interaction partners: Silver stain gel showing distinct bands that coimmunoprecipitated with Pofut2. Empty vector (EV) control and Pofut2 were transfected in 293T cells and treated with a crosslinker prior to lysis and Flag-IP;
Table 6.1. Putative Pofut2 interaction partners: List of proteins identified from 6.2 with corresponding GPM scores and number of unique peptides found for each protein

6.3. Pofut2 is directly involved in the folding of TSRs

The Lec13 CHO cell Pofut2 retention model described above provides a potential explanation for why there is a secretion defect for TSR-containing proteins in Lec 13 cells grown in the absence of fucose, but does not explain why there is a secretion defect when Pofut2 levels are knocked down by RNAi (Fig. 3.3). Thus, Pofut2 may play a more direct role in TSR folding. In order to determine whether Pofut2 directly participates in the folding of TSRs, I designed an *in-vitro* folding assay. The assay utilizes bacterially expressed TSR3 from TSP1 (TSP1-TSR3) (36) that is unfucosylated because of lack of glycosylation machinery in *E.coli.* A fraction of the TSRs purified from bacteria are properly folded and function as acceptor substrates for Pofut2 in *in-vitro* assays. This properly folded TSR can be denatured using 6 M Guanidine hydrochloride and 10 mM Dithiothreitol. The folded and denatured TSR have different hydrophobic properties and hence have different retention times when analyzed on an analytical reverse phase C_{18} HPLC column (Fig. 6.3).

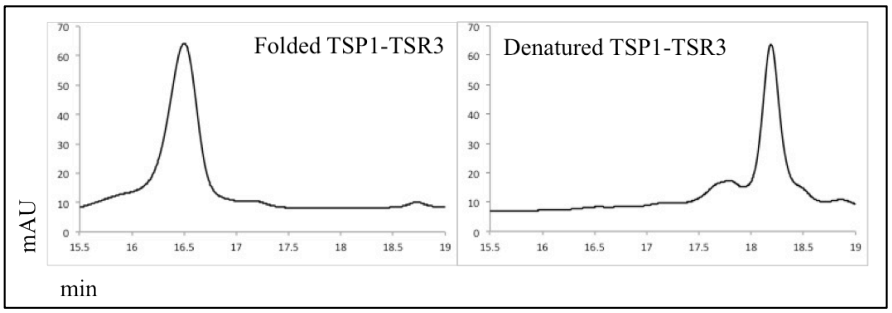


Fig. 6.3. Unfolded and folded TSRs have different hydrophobicities: HPLC traces of folded (left panel) and unfolded (right panel) TSP1-TSR3 analyzed on reverse phase C18 column showing different retention times for different states of the protein.

The unfolded TSR can be refolded *in vitro* in the presence of redox agents (mixture of oxidized and reduced glutathione) at room temperature and neutral pH. Aliquots were removed at various times points and the folding intermediates from this reaction were acid-trapped by the

addition of trifluoroacetic acid (TFA) to prevent shuffling of disulfide bonds (228). The reaction mixture was then analyzed by reverse phase HPLC. Since the hydrophobicity of the protein decreases as it folds (see Fig. 6.3), the elution time decreases as the TSR moves between unfolded, intermediary and folded forms (Fig. 6.4).

To evaluate the effects of Pofut2 on folding, Pofut2 with or without GDP-fucose was added to the *in vitro* folding reaction. Addition of Pofut2 alone to the redox system did not have a significant effect on the rate of folding. However, when the reaction was supplemented with both Pofut2 and GDP-fucose, the rate of formation of folded TSR greatly increased (Fig. 6.4). When the reaction mixture products from the 90 min time point were analyzed by mass spectrometry, the majority of the TSP1-TSR3 population was fucosylated in the presence of Pofut2 and GDP-fucose (Fig. 6.5). These results suggest that Pofut2 accelerates the rate of TSR folding in the presence of GDP-fucose.

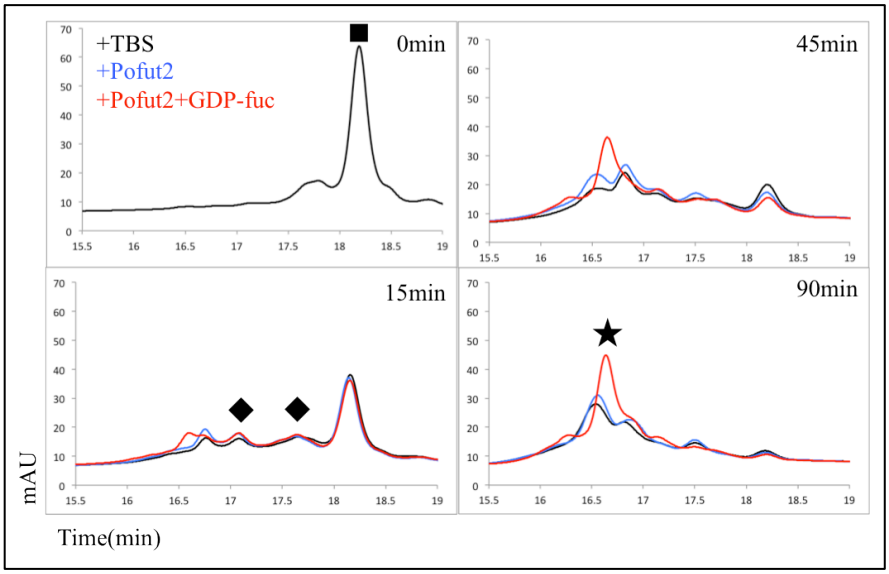
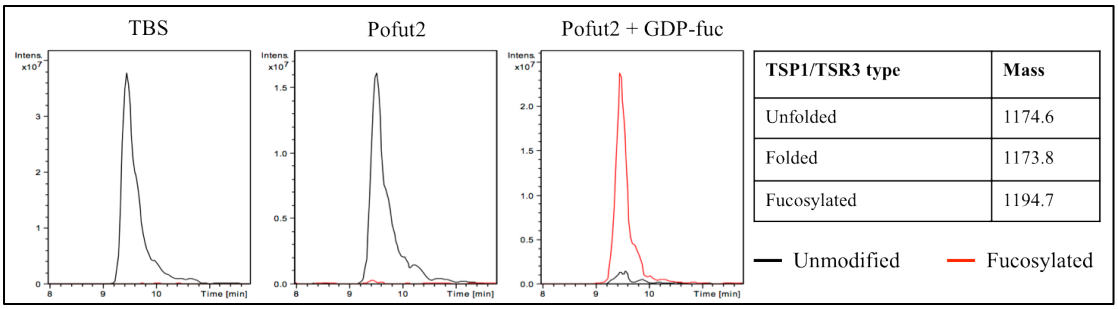


Fig. 6.4. Pofut2 accelerates the rate of refolding of TSRs: *In vitro* folding assay on TSP1-TSR3. Bacterially purified TSP1-TSR3 was denatured and refolded *in vitro* in the presence of redox agents. Aliquots of the refolding reaction were acid trapped and analyzed using reverse-phase HPLC. The retention time of the denatured protein (■, 0min time point) is greater than that of the fully folded TSR (★, 90min time point). Folding intermediates (◆, 15min time point) have retention times between the folded and unfolded forms. Addition of just Pofut2 did not affect the rate of formation of folded TSR. However, in the presence of GDP-fucose Pofut2 accelerates the rate of folding of the TSR



TSP1/TSR3 type	Mass
Unfolded	1174.6
Folded	1173.8
Fucosylated	1194.7

Fig. 6.5. Majority of TSRs are fucosylated during the folding reaction: Extracted ion chromatograms of unfolded, folded and fucosylated TSP1-TSR3 from mass spectrometric of folding intermediates from Fig. 6.4 (90min time point) showing the relative abundance of each population in the reaction.

Table 6.2. Masses used to prepare EICs of TSP1-TSR3: EICs in Fig. 6.5 were prepared of the +7 charge state of TSP1-TSR3. Similar patters were seen in other charge states of the peptide as well.

6.4. Pofut2 requires enzymatic activity and GDP-fucose hydrolysis for its chaperone effect

GDP-fucose appears to be a requirement in Pofut2-mediated refolding of TSP1-TSR3. It was necessary to distinguish between two possibilities: 1) that binding GDP-fucose was a structural necessity in substrate binding and/or recognition, and 2) fucosyltransferase activity was intrinsically involved in the refolding of TSP1-TSR3. The Pofut2-E54A mutant has previously been shown to have no fucosyltransferase activity (45). This was confirmed by using the mutant enzyme in an overnight fucosyltransferase assay with TSP1-TSR3 as the acceptor substrate, followed by analysis using LC-MS/MS (Fig. 6.5).

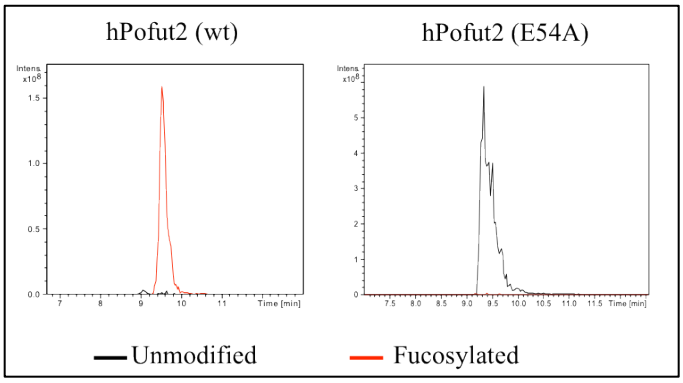


Fig. 6.6. The Pofut2-E54A mutant does not have fucosyltransferase activity: Extracted ion chromatograms for TSP1-TSR3 subjected to overnight fucosyltransferase assays using wt and E54A mutant of Pofut2

In-vitro folding assays were performed using WT and catalytically inactive Pofut2 in the presence of GDP-fucose (Fig. 6.6A). The E54A mutant did not affect the rate of folding of TSR. This data suggests that both the catalytic activity and GDP-fucose are required for Pofut2 chaperone activity. Pofut1, which modifies EGF repeats, also did not accelerate the folding of the TSR in the presence of GDP-fucose, demonstrating the specificity of this reaction. Quantitation of the unfolded TSR (18.2' peak) and the folded TSR (16.7' peak) from Figure 6.6A across the different conditions and time points showed while the amount of unfolded protein decreased at roughly the same rate, the rate of appearance of the folded TSR was much higher in the presence of Pofut2 and GDP-fucose (Fig. 6.6B). These data suggest that in the

absence of Pofut2 and GDP-fucose, the TSR is trapped in folding intermediates that take time to resolve. Addition of GDP (not GDP-fucose) to wild-type Pofut2 also had no effect on folding (Fig. 6.7). These data suggests that the energy derived from hydrolyzing GDP-fucose during the fucosylation reaction is necessary for accelerating the rate of TSR folding.

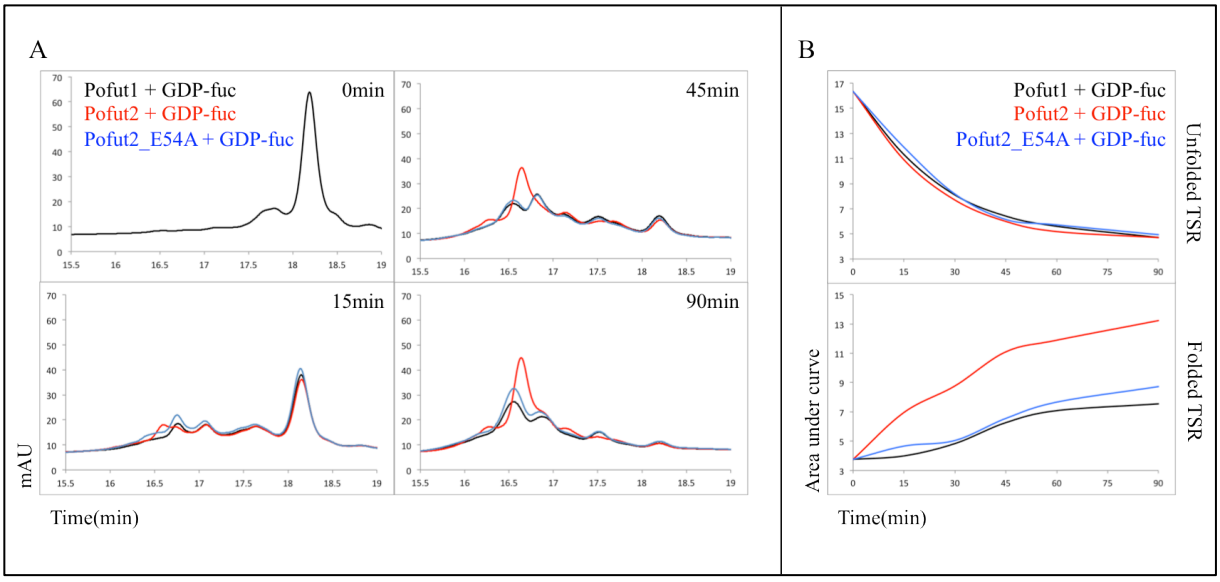


Fig. 6.7. Pofut2 requires enzymatic activity for its chaperone function: A) *In vitro* refolding assay in the presence of GDP-fucose using Pofut1, a closely related *O*-fucosyltransferase, as a negative control, *wt* Pofut2 and the catalytically inactive Pofut2-E54A. Pofut1 and Pofut2-E54A have no effect on TSR folding; B) Quantitation of unfolded (18.2') and folded product (16.7') by computing area under the curves in 5B shows the rate of disappearance of unfolded protein to be similar for Pofut1, Pofut2 and Pofut2-E54A but the rate of formation of folded protein to be elevated only for Pofut2.

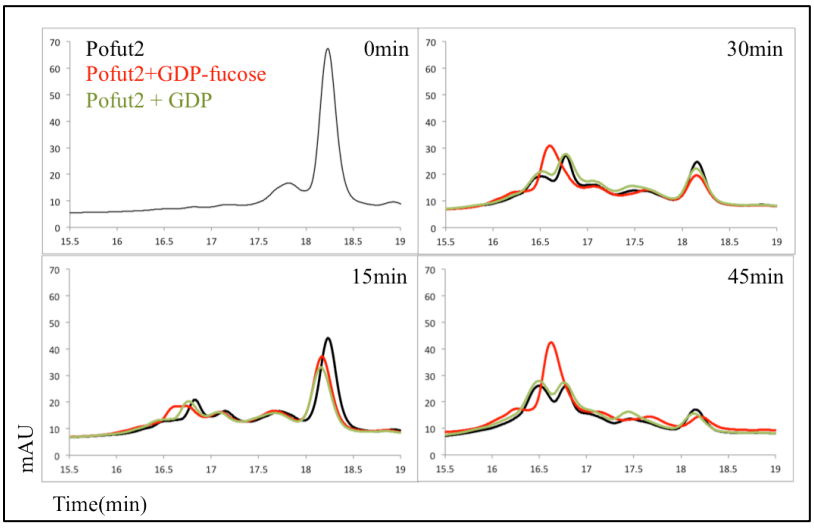


Fig. 6.8. Pofut2 requires GDP-fucose hydrolysis for its chaperone activity: In-vitro folding assay with *wt* Pofut2 supplemented with either GDP or GDP-fucose showing that GDP-fucose hydrolysis is necessary for Pofut2 chaperone function.

6.5. Conclusions

In this section, I demonstrate a direct role for Pofut2 in the folding of TSRs. In the absence of fucose, Pofut2 binds its substrates but does not release them. This is demonstrated by more substrate coimmunoprecipitating with Pofut2 in the absence of GDP-fucose than in the presence. This observation also provides an explanation for the secretion defect phenotype seen in Lec13 CHO cells. I also propose several candidate proteins that may assist in the folding of TSRs and help keep Pofut2 in the ER. Pofut2 does not have a KDEL sequence so it may piggy-back on the KDEL-sequence containing proteins that it co-immunoprecipitates with (BiP, HSP90, PDIA4).

I use *in vitro* folding assays to examine the effect of Pofut2 on TSR refolding. I show that Pofut2, in the presence of GDP-fucose, accelerates the folding of a TSR. Both GDP-fucose and enzymatic activity are required to accelerate the folding of the TSR. The fact that enzymatically active Pofut2 and that GDP-fucose cannot be substituted with GDP suggests that the energy derived from GDP-fucose hydrolysis is important for driving the folding reaction forward.

6.6. Methods

Plasmids used in this study: The TSP1-TSR3-His_6 construct in the pet20b backbone used for purifying single TSRs from *E.coli* is as described in (36). Flag tagged Human Pofut2 *wt* and E54A mutant constructs used in this section are gifts from Dr. Heinz Gut and are described in (45). Human Pofut1-His_6 construct used is as described in (227).

Co-immunoprecipitation studies: $1x10^6$ Lec13 CHO cells/well in a 6-well dish were transiently co-transfected with Pofut2 and various substrates (TSP1, ADAMTS13, ADAMTSL1 or ADAMTSL2). The cells were washed with ice-cold 1X PBS and treated with 2 mM Dithiobis [succinimidyl propionate] (DSP) (Pierce) in PBS as directed by the manufacturer, for 2 hours at 4°C. The DSP treatment was stopped using 20 mM Tris, pH 7.5 and the cells were lysed in RIPA. The lysates were incubated with 10µl Flag-agarose (Sigma) overnight at 4°C. The beads were washed 3X in RIPA before eluting with 3XFlag-peptide (Sigma). The eluates were analyzed on a western blot using mouse anti-Flag (Sigma), anti-V5 (Invitrogen), 9E10 anti-myc and Rabbit anti-mouse Alexafluor 680 (Invitrogen) secondary antibodies as described in Section 3.6.

Identifying interaction partners of Pofut2: hPofut2-Flag was transfected in HEK293T cells using dPEI. Prior to lysis, the cells were treated with DSP as described above. Both empty vector control and Pofut-transfected samples were immunopurified and eluates were run on 10%SDS gel and silver-stained (Biorad). Distinct bands were cut out, destained and subjected to in-gel tryptic digests as described in (35). Compound lists were generated using DataAnalysis software (Agilent) and queried in the Global proteome machine (GPM) database.

In-vitro folding assay: TSP1-TSR3 was purified in BL21 cells as described before (36). Pofut2 (*wt* and E54A) were expressed in HEK293T cells and purified from medium as described in (45). hPofut1-His_6 was purified as described in (227). TSP1-TSR3 was denatured in 100mM Tris pH 7.5, 6M GnHCl and 10mM DTT at room temperature for 1.5 hours. The sample was then desalted to remove the denaturants using a Sephadex G-25 column (Pharmacia). The desalted protein was then allowed to refold in the presence of 25 mM GSSG, 50 mM GSH and combinations of equimolar Pofut2 and 200 µM GDP-fucose (final). At specified time points, aliquots of the reaction were taken and acid-trapped in 4% Trifluoroacetic acid (final). The samples were then analyzed on a reverse phase HPLC as described in (228). For the quantitation in Fig. 6.6B, the area under the curve for folded and unfolded peaks were calculated and plotted.

Chapter 7. A role for β3GlcT

In mass spectrometric analyses of TSRs from various Pofut2 substrates, some TSRs show stoichiometric addition of glucose to the fucose, while some show mixtures of monosaccharide and disaccharide populations (Fig. 5.1A) (35,38,39). This variable stoichiometry of glucosylation suggests that the significance of elongation varies from site to site. Since eliminating fucosylation from a protein results in loss of its secretion, I reasoned that a similar outcome maybe possible when glucosylation is eliminated. As described in Section 1.6, mutations in β3GlcT cause a developmental disorder called Peters plus syndrome. Eliminating glucosylation by β3GlcT in these patients shows reduced levels of Properdin, a Pofut2 and β3GlcT target, in serum (Fig. 1.6). Since UDP-glucose, the donor substrate for β3-glucosyltransferase is difficult to eliminate, I decided to use RNAi methods to see the effect of glucosylation on secretion of TSR containing proteins.

7.1. RNAi knockdown of β3GlcT does not affect secretion of ADAMTS13

Of the substrates I have used in this study, ADAMTS13 contains the largest number of TSRs. Also, most TSRs from ADAMTS13 are modified at least partially with the fucose-glucose disaccharide. Hence, I decided to first test the effect of eliminating glucosylation on ADAMTS13. Three different siRNA molecules (#146, 147 and 373) targeting different regions of the β3GlcT transcript were tested for their knockdown efficiency by using qPCR (Fig. 7.1). The transcript levels were normalized to an internal loading control, GAPDH and are expressed as a percentage of the negative control siRNA. β3GlcT-147 had the highest knockdown efficiency.

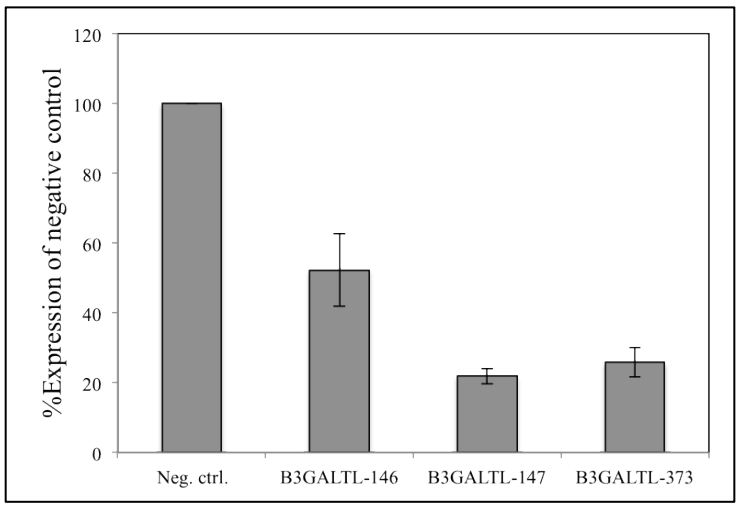


Fig. 7.1. β3GlcT message levels can be effectively reduced using RNAi: β3GlcT transcript levels (normalized to negative control) in HEK293T cells treated with various siRNA targeting different regions of β3GlcT. β3GlcT-147 shows the highest efficiency of knockdown.

All three siRNAs were tested in a secretion assay in HEK293T cells expressing ADAMTS13 (Fig. 7.2). Pofut2 siRNA was used as a positive control for this assay. None of the tested siRNA had an effect suggesting that glucosylation might not be required for the secretion of ADAMTS13.

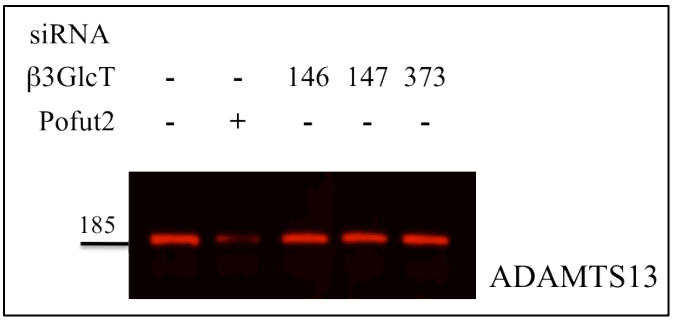


Fig. 7.2. Knocking down β3GlcT does not affect ADAMTS13 secretion: Media fractions of HEK293T cells transfected with ADAMTS13 and siRNA against either Pofut2 or β3GlcT. Pofut2 serves as a positive control for the assay. β3GlcT knockdown with any of the tested siRNA does not appear to affect secretion of ADAMTS13.

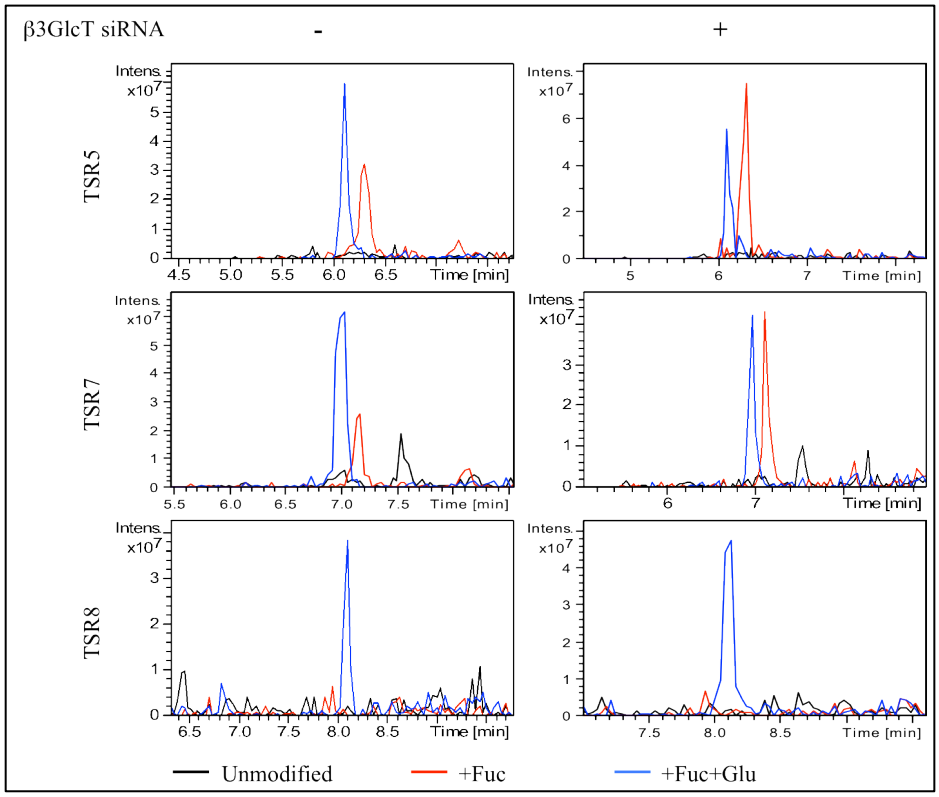


Fig. 7.3. β3GlcT knockdown reduces glucosylation on mature ADAMTS13: Extracted ion chromatograms (EIC) of peptides from TSR5, 7, and 8 of media associated ADAMTS13 expressed in HEK293T cells treated with either negative control (left panels) or β3GlcT -147 (right panels) siRNA. β3GlcT knockdown with the most efficient siRNA (#147) did result in reduction of glucosylation on TSR5 and 7. The EICs were prepared as described in chapter 5.

I attempted to validate loss of glucosylation using mass spectrometric methods. ADAMTS13 purified from HEK293T cells treated with β3GlcT-147 (the siRNA with highest knockdown efficiency) showed a reduction, but not complete abrogation of glucosylation (Fig. 7.3). This could be because the β3GlcT enzyme is stable and not turned over much in cells, or it

could be that only the glucosylated protein is efficiently secreted. The efficiency of knockdown can be further improved for treating with siRNA for longer periods of time or by dosing the cells with a second siRNA treatment.

7.2. β3GlcT is required for the secretion of TSP1, ADAMTSL1 and ADAMTSL2

The Peters plus phenotype in human beings is evidence that glucosylation is important on at least a subset of Pofut2 targets (69,70). Results from the previous section show that ADAMTS13 may not be one of these substrates. I decided to test other TSR-containing proteins (TSP1, ADAMTSL1 and ADAMTSL2) in a similar assay to assess if eliminating glucosylation affected their secretion. I used β3GlcT-147 siRNA since it showed the best knockdown efficiency (Fig. 7.1) of the ones tested. Eliminating glucosylation on TSP1, ADAMTSL1 and ADAMTSL2 resulted in loss of secretion of these substrates (Fig. 7.4).

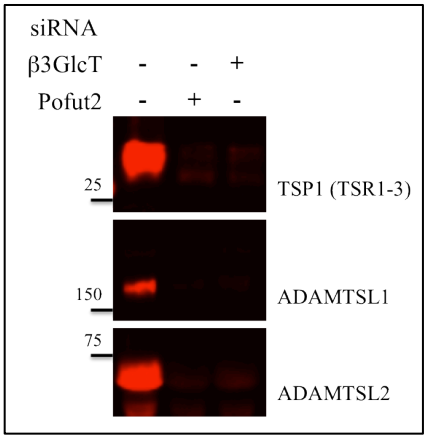


Fig. 7.4. β3GlcT knockdown affects secretion of TSP1, ADAMTSL1 and ADAMTSL2: Media fractions of HEK293T cells expressing TSP1, ADAMTSL1 and ADAMTSL2 with β3GlcT knockdown. Pofut2 siRNA is used here as a positive control. All three tested Pofut2 substrates show a secretion defect in the absence of glucosylation.

Interestingly, EIC analyses of TSRs from mature ADAMTSL1 (purified from HEK293T cells) show that they are not completely modified with glucose (Fig. 7.5). This substrate selectivity and variable effect of β3GlcT needs to be further investigated to be able to understand the molecular basis for the Peters plus phenotype.

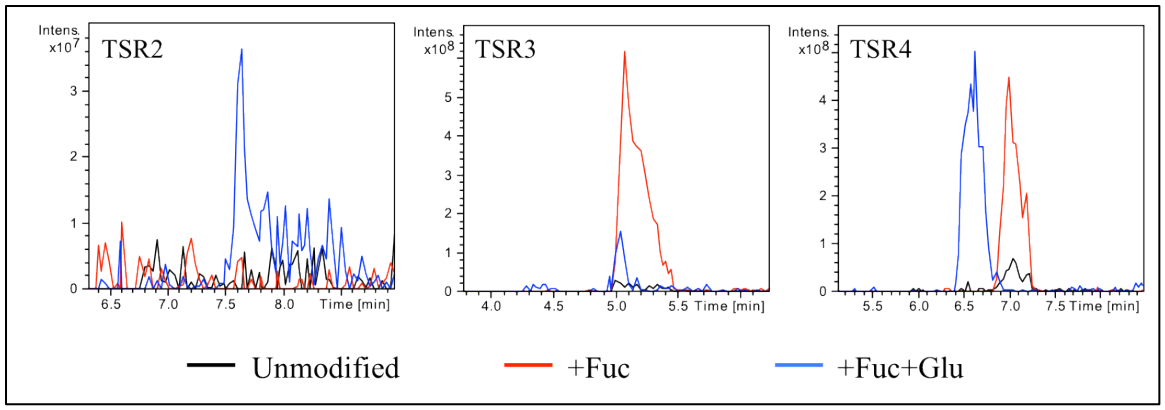


Fig. 7.5. The fucose-glucose disaccharide on TSRs in ADAMTSL2 occurs in varying stoichiometries: EICs of peptides from TSR2, 3 and 4 of media associated ADAMTSL1 showing variable stoichiometries of fucosylation and glucosylation. Peptides used to prepare these EICs are listed below in Table 6.3.

TSR#	Peptide sequence	Charge (n)	$[M+nH]^{n+}$ (calc.)	$[M+nH]^{n+}$ (obs.)	$[M+nH+Hex]^{n+}$ (obs.)	$[M+3H+dHex+Hex]^{3+}$ (obs.)
2	^{301}WRETDFFPCSATCGGGYQLTSAECYDLR^{328}	3	1117.2	1117.2	1165.3	1219.6
3	^{380}WEATPWTACSSSCGGGIQSR^{399}	2	1100.2	1100.0	1172.6	1254.3
4	^{440}WLAQEWSPCTVTCGQGLR^{457}	2	1075.7	1075.5	1148.6	1229.8

Table 7.1. Peptides identified in the MS analysis of ADAMTSL1: List of peptides and corresponding observed and calculated masses used in the preparation of EICs in Figure 7.5. The modified amino acid is underlined.

7.3. Conclusions

In this section, I propose that β3GlcT displays substrate selectivity amongst TSRs. While data from Chapter 5 shows that most TSRs from ADAMTS13 are modified with the fucose-glucose disaccharide, not all TSRs from ADAMTSL1 are elongated. Yet, β3GlcT knockdown affects the secretion of ADAMTSL1 but not ADAMTS13. A secretion defect is also seen for TSP1 and ADAMTSL2 when β3GlcT is knocked down. This suggests that glucosylation plays different roles on different proteins. These results provide preliminary insight into understanding the role of the glucose. Further experiments need to be conducted to understand the importance of glucosylation and the pathology of Peters plus syndrome.

7.4. Methods

Plasmids and antibodies used in this study: The TSP1-myc-His_6, ADAMTS13-V5-His_6, ADAMTSL1-myc-His_6 and ADAMTSL2-myc-His_6 constructs are as described in section 3.6. The anti-myc (9E10) and anti-V5 antibodies are also described in the same section.

RNAi experiments: The Pofut2 siRNA used and the protocol followed for cotransfecting DNA and siRNA are as described in 3.6. Stealth siRNA targeting different regions of β3GlcT were obtained from Life Technologies.

qPCR: HEK293T cells were transfected with negative control siRNA (Life Technologies) or β3GlcT siRNA using Lipofectamine 2000 (Life technologies) according to manufacturer's protocol. After 6 hours of transfection, the media was changed to OptiMEM and the cells were allowed to grow for 48 hours. Total RNA was prepared from by lysing the cells using Trizol (Life Technologies) according to manufacturer's protocol. The RNA pellet was resuspended in clean, RNase-free water and used in preparing cDNA. cDNA was made using SuperScript II reverse transcriptase (Life Technologies) and random primers (Life Technologies). The cDNA was quantitated and used in a quantitative PCR reaction with LightCycler 480 SYBR Green I Master (Roche) and primers (Stony Brook OSA) specific for GAPDH and β3GlcT with 100bps amplicons. The reactions were set up in a 384-well plate format and run on the Lightcycler 480 system (Roche). The system software was used to analyze the data and obtain number of cycles per gene per well required for threshold fluorescence. The β3GlcT data was normalized to the GAPDH data using the $\Delta\Delta C_p$ method. The graph showing percentage of β3GlcT expression was obtained by normalizing to negative control siRNA.

Mass spectrometric analysis: ADAMTS13-V5-His_6 was cotransfected with β3GlcT-147 using Lipofectamine 2000 as described above. Post transfection, the cells were allowed to grow for 48 hours before media was harvested and purified. ADAMTSL1-myc-His_6 was expressed in HEK293T cells and purified from conditioned medium on a NiNTA column (Qiagen). After in-gel reduction and alkylation, the samples were digested with trypsin and analysed using LC-MS/MS as described in Section 5.4.

Chapter 8. Discussion

Nearly thirty percent of all known proteins have disulfide bonds (229). The appropriate pairing of the cysteines in a crowded environ like the ER poses a challenge to the folding and quality control machinery. In this complex melee, it makes strategic sense for the cell to have dedicated chaperones for different types of cysteine-rich motifs in addition to the generic network of enzymes and chaperones. Pofut2 precisely falls into this category. Each TSR has six cysteines and most of Pofut2's targets have multiple tandem TSRs. A protein such as ADAMTS13 has 48 cysteines just in the TSRs that must find their correct partners to fold correctly. This greatly increases the complexity of the folding problem. Hence, the more TSRs a protein has, the more the dependence on Pofut2 and fucose.

I hypothesize that the main function of Pofut2 is to accelerate the rate of folding for individual TSRs while preventing aggregation with adjacent TSRs, most likely in a somewhat processive manner (from N- to C-terminal) (Fig. 8.1A). Since the most N-terminal TSR will begin folding when it arrives in the lumen of the ER, it is most likely to finish folding first. For a string of TSRs entering the ER (Fig. 8.1A-i), the first folded TSR would attract Pofut2 (previously loaded with GDP-fucose) (Fig. 8.1A-ii). This step occurs early in protein synthesis, probably co-translationally. Pofut2 recruits other chaperones to the site to help fold the chain of TSRs. While the other chaperones attempt to fold TSR2 (or other TSRs), Pofut2 fucosylates TSR1, leading to release of TSR1 from the chaperones and Pofut2 (Fig. 8.1A-iii). This process is iterated for TSR2 (Fig. 8.1A-iv) and so on until a chain of fully folded and fucosylated TSRs emerge (Fig. 8.1A-v).

The siRNA experiments (Section 3.1) show that in the absence of Pofut2, a secretion defect is observed. This observation suggests a direct role for Pofut2 in the folding of TSRs. Based on my *in vitro* folding data (Section 6.3), I propose that Pofut2 accelerates the folding of nascent TSRs, reducing the probability that the cysteines will form non-productive disulfide bonds leading to protein aggregation. In the absence of Pofut2, the TSRs fold too slowly resulting in aberrant disulfide bond formation (Fig. 8.1B-i) and are rapidly degraded in the cell (Fig. 8.1B-ii).

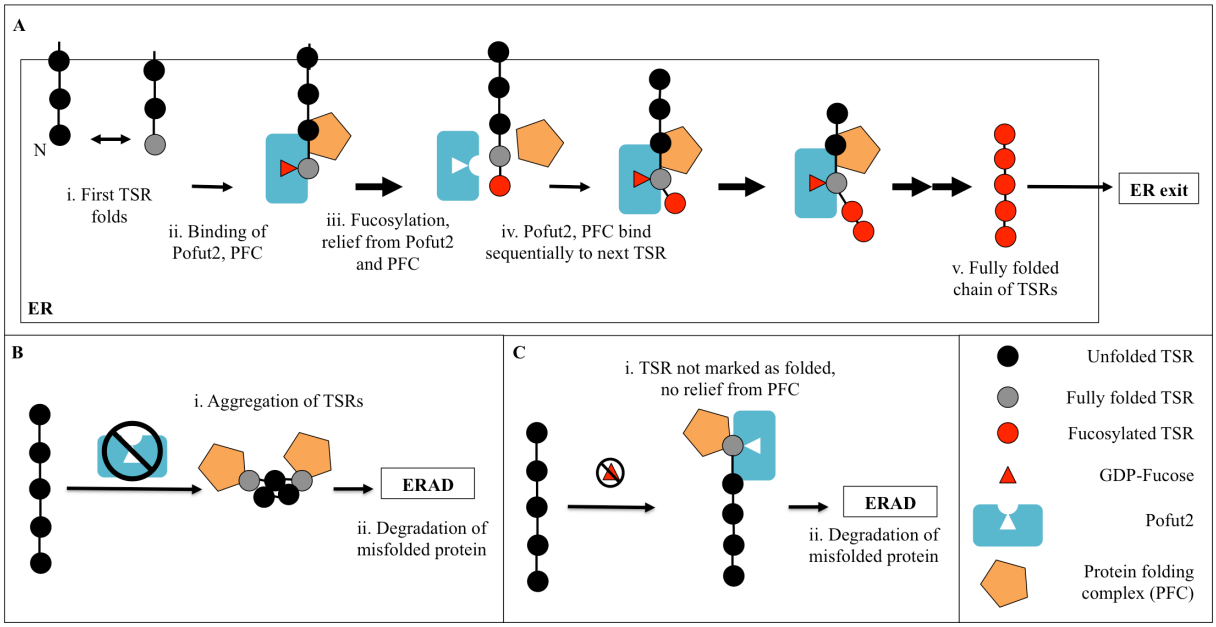


Fig. 8.1. A model for Pofut2 in the folding of TSRs: A) When a chain of TSRs enters the ER, the first TSR enters a folding equilibrium with intermediates similar to those shown during folding of TSP1-TSR3 in vitro (Figure 5A, indicated here with double headed arrow, i). When a correctly folded TSR is formed (gray circle), it is recognized by Pofut2 (ii) and *O*-fucosylated (red circle), thus pulling the folding equilibrium in the forward direction and accelerating the overall folding reaction (iii, thick arrow representing the favorable *O*-fucosylation reaction). Pofut2 is capable of recognizing two TSRs simultaneously so it serves as a spacer between adjacent motifs, preventing aggregation. Pofut2 also recruits a Protein Folding Complex (PFC), comprised of several chaperones to fold the subsequent TSRs. The PFC sequentially assists in folding the tandem TSRs while Pofut2 marks them as folded (iv). This process is iterated over the entire chain of TSRs until a folded, *O*-fucosylated chain emerges, ready for ER-exit (v). B) When Pofut2 mediation is not available (*e.g.* RNAi), the TSRs fold too slowly leading to aggregation (i). This aggregate is cleared similar to other misfolded proteins, probably via ER associated degradation (ERAD) (ii). C) When cells are depleted of fucose (Lec13 CHO model), though Pofut2 can bind a folded TSR, the reaction is not driven forward by GDP-fucose hydrolysis and Pofut2 remains bound to one TSR (i). This results in a stalemate, where the protein is eventually degraded (ii).

The fact that Pofut2 requires fucosyltransferase activity (both GDP-fucose and enzymatically active Pofut2) to accelerate TSR folding strongly suggests that the energy of GDP-fucose hydrolysis is linked to TSR folding. This is reminiscent of other chaperones that require ATP hydrolysis to complete a cycle of folding such as Hsp70, Hsp90 and Hsp100 (230). The energy from the hydrolysis of GDP-fucose could be driving the TSR folding reaction forward by mass action, pulling the TSR out of a folding equilibrium by converting it to a fucosylated product.

In Lec13 cells, TSR-containing proteins are not efficiently secreted in the absence of fucose (Section 3.1). The experiments from Section 6.1 suggest that more substrate co-immunoprecipitates with Pofut2 in the absence than in the presence of fucose (Fig. 6.1). This observation provides a partial explanation for the secretion defect in Lec13 CHO cells (Fig. 8.1C). This is most likely exacerbated by the fact that Pofut2 cannot participate in accelerating the folding of other TSRs if it remains bound to one. In the context of a cell, if *wt* Pofut2 was to be substituted by Pofut2-E54A, I predict that the effect on TSR-containing proteins would be very similar to that in Lec13 CHO cells. Pofut-E54A maybe able to bind TSRs, but since there is no GDP-hydrolysis, the protein would remain unmarked as folded and will be degraded.

Preliminary co-immunoprecipitation experiments with human Pofut2 followed by proteomics reveals that Pofut2 exists in a complex with multiple other proteins known to be involved in protein folding (Section 6.2). Thus, recruitment of Pofut2 to a TSR-containing protein will also recruit a number of other chaperones, forming a Protein folding complex (PFC) that assists in the folding of TSRs (Fig. 8.1A-ii). Since several of these proteins are also known to have KDEL sequences, association with these proteins may help retain Pofut2 in the ER.

The ability of Pofut2 to distinguish properly folded substrates comes into play when a TSR fully folds and can be marked as folded by the addition of the fucose (Fig. 8.1A-iii). Structural studies on Pofut2 have proposed that in addition to a substrate binding pocket, there is an additional hydrophobic patch that could interact with an adjacent TSR (45). This idea fits nicely with the model of Pofut2 keeping the TSRs away from one another and enabling their interactions with foldases.

The model also predicts that TSRs fold and get fucosylated from the N- to the C-terminus, in a processive manner (Fig. 8.1A-iv). Mass spectrometric data on cell-associated protein showing C-terminal TSRs to be predominantly unmodified with *O*-fucose support this idea (Section 5.2). It is intuitive that mature ADAMTS13 would not have any unpaired cysteines. This is demonstrated by the fact that mature ADAMTS13 migrates as a single band at the appropriate place on a non-reducing gel (Section 5.2). Most ADAMTS13 within cells, however, is incompletely folded and contains a mixture of inter- and intra-TSR disulfide bonds. These TSRs are poorly fucosylated, consistent with the idea that fucosylation marks a folded TSR. Interestingly, a small fraction of cell-associated ADAMTS13 runs at the same position as mature ADAMTS13 on a non-reducing gel, suggesting it is mostly folded. While TSR5 and 7 from this fraction of ADAMTS13 are fully fucosylated, TSR8 is predominantly unfucosylated (Section 5.2). I reason that this protein is still in the cell because of this last unfolded (and hence unfucosylated) TSR. Mass spectral analysis of the fucosylation status of TSRs on other cell-associate proteins shows a similar trend of decreasing fucosylation from N- to C-terminus, with C-terminal TSRs being mostly unmodified in the cell fractions. This suggests that TSR folding and fucosylation may be processive and has directionality.

The complexity of protein folding increases with the number of secondary structures and disulfide bonds (229). By extension of the above hypothesis, the more the TSRs a protein has, the more its requirement for Pofut2. This is confirmed by the results in Section 3.2. The primary amino acid sequence of the TSR also seems to influence fucose-dependent secretion (Section 3.3). There is evidence in the literature suggesting that the positioning of *N*-glycosylation sites is crucial in the protein folding and disulfide bond formation (231). A similar theory can be proposed for *O*-fucosylation, where difficult-to-fold TSRs (with *O*-fucosylation sites) are positioned in proteins so as to be able to prolong interaction of the protein with chaperones to ensure correct cysteine pairing. This can be tested using deletion mutants, such as deleting TSR3 from full length ADAMTS13.

Results from Section 3.4 demonstrate that when proteosomal degradation is blocked, an ubiquitinated form of ADAMTS13 accumulates in the cell. While my hypothesis was that I would see this form only in the absence of fucose, this effect seems to be present even in +fucose samples, albeit at lower levels. Since ADAMTS13 is one of the larger Pofut2 substrates, it is possible that even under normal conditions, a considerable amount of the protein is misfolded and degraded. Depending on the protein, folding can often be slow and error-prone, thus leading to their degradation (*e.g.* CFTR, α-synuclein, tau) (232). This accumulation of ubiquitinated ADAMTS13 strongly suggests that misfolded Pofut2 substrates are degraded by the canonical ERAD pathway (Fig. 8.1B-ii and 8.1C-ii).

In some aspects, the chaperone activity of Pofut1 is comparable to Pofut2. For example, both enzymes are required for the secretion/localization of their respective substrates. Ofut1 lacking cells in *Drosophila* show an aggregation of Notch in the ER and this defect is rescued by the expression of an enzymatically inactive Ofut1 (79,80). Pofut2 is dissimilar to Pofut1 in that

the *in vitro* folding assays indicate that the fucosyltransferase activity is required for Pofut2's chaperone activity, while enzymatically inactive Ofut1 appears to retain it's chaperone activity (79). Detailed structural studies on Pofut2 will shed more light on this subject.

Our laboratory has previously demonstrated the *O*-fucosylation on EGF repeats occurs in the ER (38) and in Chapter 4, I similarly demonstrate that *O*-fucosylation of TSRs also occurs in the ER. The identification of an *O*-fucosylated nascent peptide (Section 4.2) strongly argues in favor of GDP-fucose presence in the ER. One possible model to explain GDP-fucose availability in the ER is retrograde transport from the ERGIC or Golgi where there are known GDP-fucose transporters (Slc35C1 and C2, (73,77)). Alternatively, Pofut2 could shuttle between these compartments to "load" itself with GDP-fucose while actually carrying out its catalytic activity in the ER. Preliminary experiments suggest that this model may not be true (Section 4.4). Of course, there still remains the possibility of an unidentified ER-associated GDP-fucose transporter.

The current model I have drawn for Pofut2 in the folding of TSRs does not include a role for β3GlcT. This is because unlike Pofut2, which has a generic effect on its substrates, the glucosyltransferase is more selective in the TSRs it modifies and the effect it has on its substrates. Most TSRs in ADAMTS13 are completely elongated to the fucose-glucose disaccharide, yet there is no secretion defect when β3GlcT is knocked down. This is in contrast with the other TSR-containing proteins tested. These results somewhat correlate with the Peters plus (PP) phenotype. PP patients do not show symptoms of Thrombotic thrombocytopenic purpurea (TTP), a blood-clotting defect seen in patients with ADAMTS13 mutations. However, PP patients and patients with Geleophysic dysplasia (GD) (mutations in ADAMTSL2) have short stature and suffer from heart malformations (49,233). One Peters plus patient was reported to

have hepatomegaly, a common symptom of GD patients (234). Some PP patients also show syndactyly, the most striking phenotype of the ADAMTS5, 9 and 20 knockout mice (101,233). Given these overlap of symptoms, it is important to understand the underlying molecular significance of glucosylation.

Mutations in chaperones that cause defects in protein folding have been shown to play a role in several diseases (235). So far, only two chaperones that also modify their substrates with sugars have been identified - UDP-glucose: glucosyltransferase (GT) and Pofut1 (236). I have added a third candidate, Pofut2, to this list. To fully understand the role of Pofut1 and 2 in a cell, we must take into consideration all of their predicted targets. Site mapping of predicted targets is an on-going project in our laboratory and will eventually help us understand the Pofut2 knockout and PP phenotype.

While a lot can be gleaned from the *in vitro* experiments described here, several important questions remain to be tested. Are any of the folding intermediates fucosylated? Does Pofut2 have a more profound effect on the *in vitro* folding of a string of tandem TSRs than on a single TSR like TSP1-TSR3 that has been tested? Does Pofut2 have the same effect on TSRs without an *O*-fucose consensus sequence? The refolding assay in combination with mass spectrometric methods could be used in the future to examine this model further.

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Appendices

Appendix A. ER stress experiments

When the protein-folding load in the ER increases, it leads to the accumulation of many misfolded proteins or aggregates (237). This results in ER stress. Ire1, a transmembrane receptor with an inactive cytoplasmic endonuclease domain, monitors ER stress via its luminal domain. When ER stress is induced, the cytoplasmic endonuclease domain of IRE1 is activated and its splices a transcription factor encoding mRNA, Xbp1. Under normal physiological conditions, unspliced Xbp1 mRNA is degraded rapidly. When spliced by Ire1, Xbp1 mRNA is translated and the resulting transcription factor travels to the nucleus where it promotes expression of several ER chaperones, notably Binding Immuglobulin Protein (BiP). These chaperones help alleviate ER stress.

Since several of the experiments described in the previous sections utilize an overexpression system, it is important to understand the extent to which overexpressing TSR containing proteins induces ER stress. A reliable marker for both these processes is the expression levels of Binding Immunoglobulin Protein (BiP) (237). In this section, I examine both message and protein levels of BiP in cells overexpressing TSR-containing proteins. If overexpression of TSR-containing proteins resulted in ER stress, the it is possible that one of the chaperones that is upregulated in response is Pofut2. So in addition to examining BiP levels, Pofut2 expression was also monitored. Since a reliable antibody is not available for the latter, quantitative PCR was used to examine mRNA levels of Pofut2. Pofut1 and Poglut, two unrelated glycosyl transferases were used as negative controls and GAPDH was used as a loading control to normalize the data.

HEK293T cells were transiently transfected with mouse Notch 2 (N2, as negative control for Pofut2 upregulation), TSP1, ADAMTS13, ADAMTSL1 and ADAMTSL2. cDNA was prepared from the total mRNA harvested from these cells and used in a qPCR reaction with primers against GAPDH, Pofut1, Pofut2 and Poglut (Fig. A1). While overexpression of TSP1 and ADAMTSL1 did not induce expression of any of the tested genes, expression of N2, ADAMTS13 and ADAMTSL2 resulted in upregulation of BiP, indicating that ER stress was induced. This correlates with the secretion defect data, where proteins with more tandem TSRs (or EGFs in the case of N2) are harder to fold and secrete. Interestingly, none of the glycosyltransferases were upregulated, suggesting that the eznymes are not a limiting factor in HEK293T cells (see Appendix B).

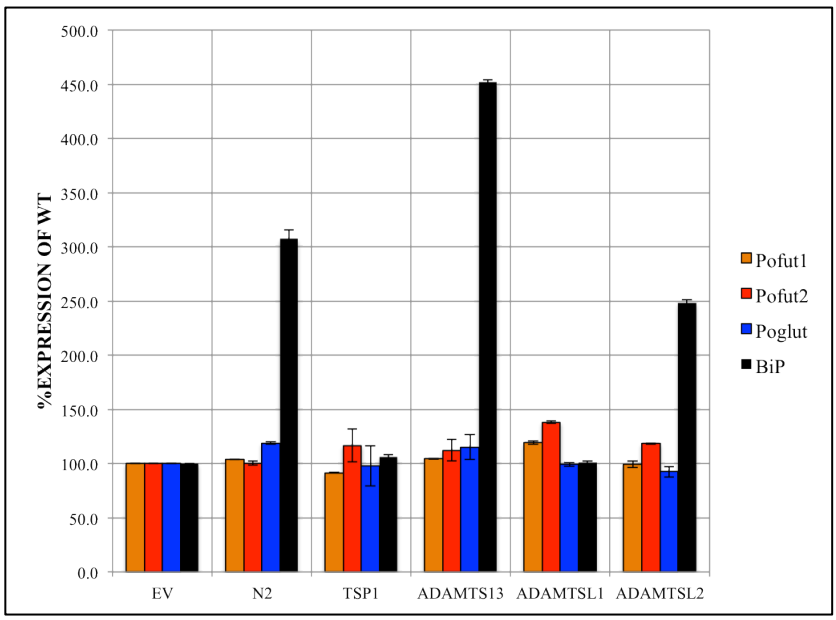

Fig. A1. Overexpression of TSR-containing proteins results in ER stress in HEK293T cells: qPCR quantitation of Pofut1, Pofut2, Poglut and BiP in 293T cells expressing either mouse Notch 2 or TSR-containing proteins shows an increase in message levels of BiP for larger proteins (N2, ADAMTS13, ADAMTSL2). This correlates with the observation that proteins with greater number of tandem TSRs are harder to fold. The data is normalized to GAPDH and is a representative of three independent experiments.

To test if depleting fucose also induces ER stress, protein levels of BiP in Lec13 CHO cells grown in the presence or absence of fucose was compared with *wt* Pro5 CHO cells (Fig.

A2). Tunicamycin (TNK), an antibiotic that blocks transfer of *N*-glycans and thus induces ER stress, was used as a positive control. In the empty vector controls, treatment with TNK for 16 h showed an increase in BiP levels in *wt* and Lec13 cells with or without fucose. In cells transfected with ADAMTS13, Lec13 cells showed slightly higher levels of BiP than *wt* CHO cells. This defect was rescued by the addition of fucose to the cells. CHO cells express much less protein than HEK293T cells, hence just the overexpression of ADAMTS13 does not appear to induce ER stress, but the combined effect of ADAMTS13 overexpression and fucose depletion may induce ER stress.

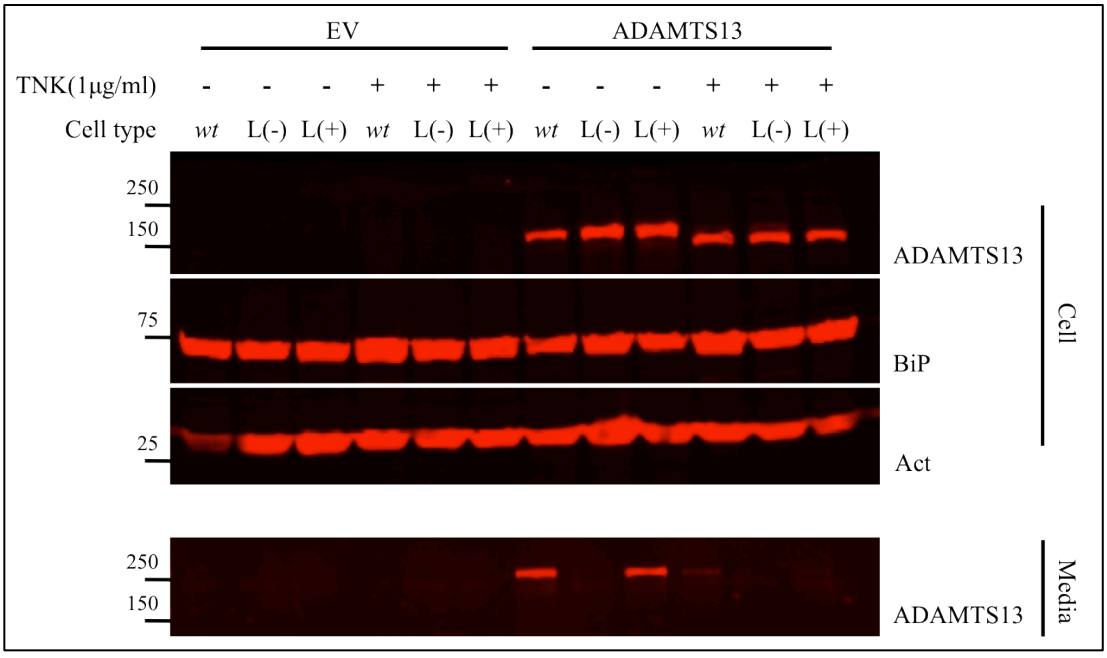

Fig. A2. Fucose depletion in combination with overexpression of ADAMTS13 causes ER stress in CHO cells: *wt* or Lec13 CHO cells (L(-) = Lec13; L(+) = Lec13 rescued with fucose) transfected with empty vector (EV) controls show no change in BiP levels. ADAMTS13 overexpression in Lec13 cells results in BiP upregulation, which is rescued by the addition of exogenous fucose. Tunicamycin (TNK) is a positive control for ER stress induction. TNK inhibits transfer of *N*-glycans, hence ADAMTS13 (which has 10 *N*-glycan sites) shows a lower molecular weight when expressed in cells treated with TNK. As expected, TNK treatment results in loss of secretion of ADAMTS13 in the *wt* and Lec13 rescue (L(+)) cells.

Appendix B. Limiting factors in ADAMTS13 secretion

Experiments from the Appendix A suggest that Pofut2 is not upregulated when TSR-containing proteins are overexpressed (Fig. A1). This could be either because Pofut2 is not a limiting factor in the secretion of TSR-containing proteins or because Pofut2 is not target of the ER stress/UPR pathways. To test whether Pofut2 is a limiting factor, ADAMTS13 was cotransfected with *wt* Pofut2 or Pofut2-E54A (catalytically inactive mutant) as a control in HEK293T cells (Fig. B1). Expression of *wt* Pofut2 resulted in a slight increase in ADAMTS13 secretion indicating that Pofut2 levels miht be limiting in HEK293T cells. These results suggest that there may not be any mechanism in the cell for Pofut2 upregulation in response to ER stress induced by expression of TSR-containing proteins.

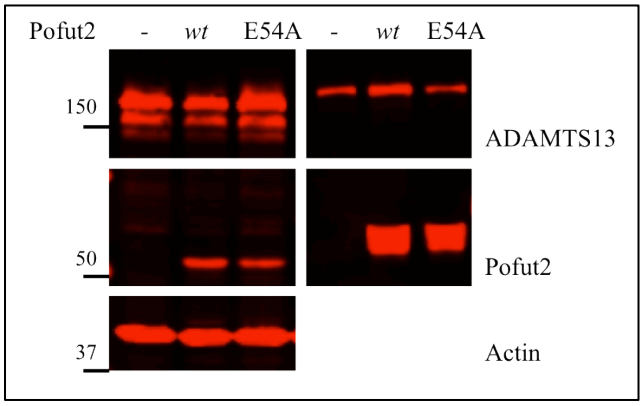

Fig. B1. Pofut2 is not limiting in HEK293T cells: Cotransfecting *wt* or E54A Pofut2 with ADAMTS13 in HEK293T cells results in increased ADAMTS13 secretion.

Secretion assays from Chapter 3 demonstrate that both Pofut2 and GDP-fucose are required for ADAMTS13 secretion. To test if GDP-fucose is limiting in cells, HEK293T cells expressing ADAMTS13 were treated with increasing amounts of GDP-fucose ranging from 0-10 μM (Fig. B2). Western blotting of cell and media fractions showed no change in ADAMTS13 expression or secretion levels suggesting that GDP-fucose is not limiting in cells. Similar

experiments in Lec13 CHO cells showed saturation of ADAMTS13 secretion at 1 μM GDP-fucose (Fig. B3).

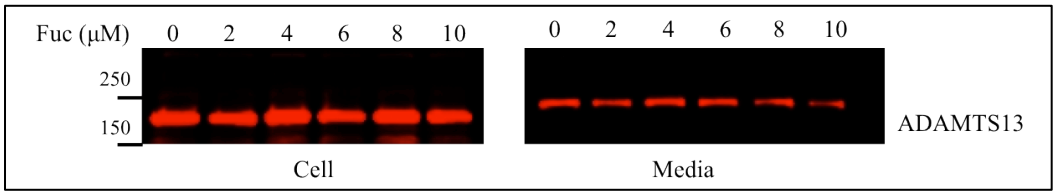

Fig. B2. GDP-fucose is not limiting in HEK293T cells: Addition of increasing amounts of L-fucose to culture media of HEK293T cells expressing ADAMTS13 does not improve secretion of ADAMTS13.

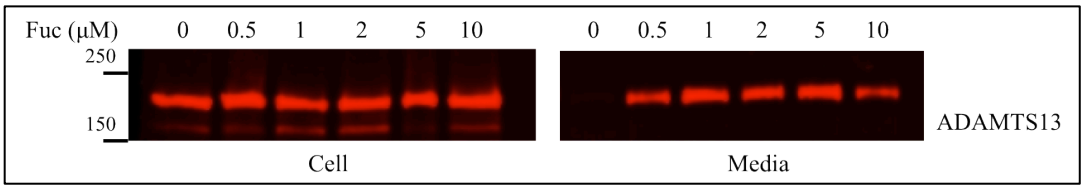

Fig. B3. ADAMTS13 secretion saturates at 1 μM L-fucose in Lec13 cells: Lec13 cells secrete ADAMTS13 poorly. This is rescued by the addition of L-fucose exogenously in concentrations as low as 0.5 μM. However, ADAMTS13 secretion is saturated at 1 μM L-fucose, which is the concentration used in secretion assays.

Appendix C. Co-immunoprecipitation studies

C.1. Pofut2 interaction partners

Two of the potential interaction partners of Pofut2 from the proteomics analysis in section 6.2 are Binding Immunoglobulin Protein (BiP) and Protein Disulfide Isomerase A4 (PDIA4). Since antibodies were available for both these proteins, co-immunoprecipitation (co-IP) studies were performed to confirm these interactions partners. HEK293T cells transiently transfected with Pofut2-Flag, treated with DSP and subjected to Flag-immunoprecipitation. In the presence of DSP, small amounts of BiP coimmunuprecipitated with Pofut2, affirming the results seen with proteomics (Fig. C1-A). However, PDIA4 was detected in the cell lysates but not in the IP fraction. It is possible that PDIA4 interaction with Pofut2 is not direct, thus introducing variability in the assay. PDIA4 is an oxido-reductase but little is known about it. It is possibly that PDIA4 is involved in the folding of TSRs since TSRs are cysteine rich. To test if the PDIA4 interacts with TSR-containing proteins, ADAMTS13-V5 was transfected in HEK293T cells, treated with DSP and subjected to immunoprecipitation with anti-V5. The eluates from the IP were blotted with BiP and PDIA4 (Fig. C1-B). Interestingly, BiP co-immunoprecipitated with ADAMTS13, suggesting that either Pofut2-BiP interaction is substrate mediated or that both Pofut2 and ADAMTS13 associate with BiP independent of each other. PDIA4 did not appear to coimmunoprecipitate with ADAMTS13.

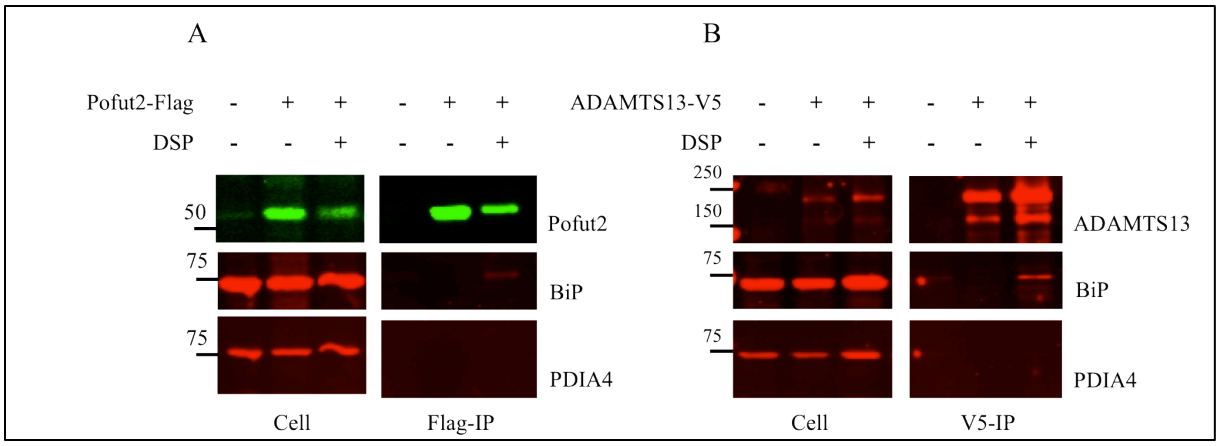

Fig. C1. Pofut2 interaction partners: A) HEK293T cells transfected with Pofut2, treated with DSP and subjected to Flag-immunoprecipitation shows that Pofut2 interacts with BiP but not PDIA4. B) Similar analysis with ADAMTS13 also shows BiP association but not with PDIA4.

C.2. BiP association with TSR-containing proteins

Results from the previous section showed ADAMTS13 to interact with BiP. Since BiP is an abundant ER-resident chaperone, it may associate with TSR-containing proteins to help their folding. Since the ADAMTS13 poorly folds in the absence of fucose, there maybe observable differences in BiP association in Lec13 cells grown with or without fucose. To test association of other TSR-containing proteins with BiP, Lec13 cells were transfected with TSP1, ADAMTS13, ADAMTSL1 or ADAMTSL2. Following treatment with DSP and cell-lysis, samples were subjected to immunoprecipitation with anti-V5 or –myc antibody (Fig. C2). BiP co-immunoprecipitated with all TSR-containing proteins tested. For larger TSR-containing proteins, ADAMTS13 and ADAMTSL2, more BiP appears to co-immunoprecipitate in the absence of fucose than in the presence of fucose. This observation supports the idea that proteins with several TSRs are more difficult to fold, and hence may require more chaperone assistance than proteins with fewer TSRs. This experiment also raises the possibility that BiP association may be dependent on Pofut2 levels. To study this, HEK293T cells expressing TSR-containing proteins were co-transfected with Pofut2 siRNA. Post-lysis, the cells immunprecipitated with anti-Myc or –V5 (depending on the TSR-containing protein) and blotted for BiP (Fig. C3). This experiment was less successful since siRNA knockdown of Pofut2 affects cellular levels of TSR-containing proteins (see Fig. 8).

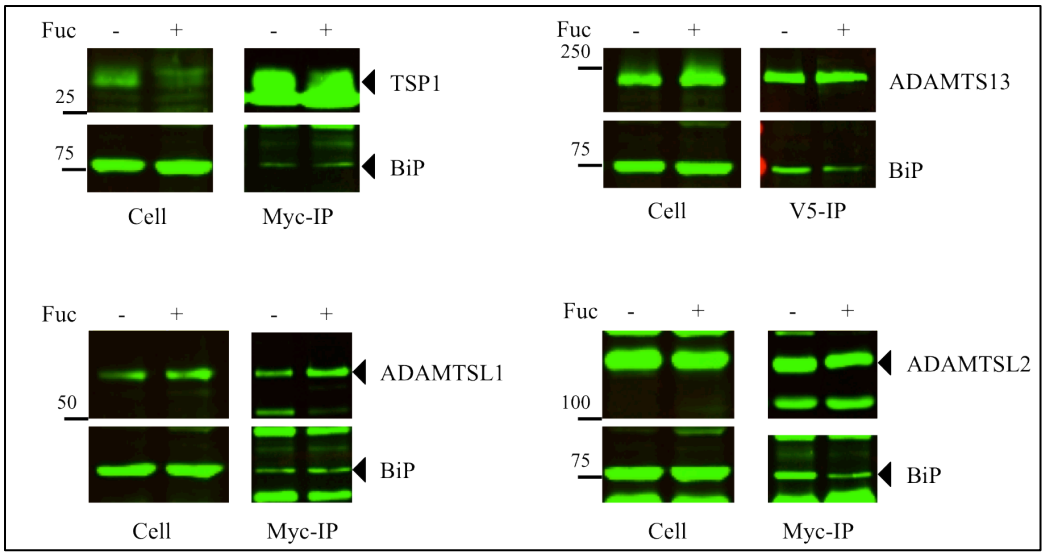

Fig. C2. BiP associates with several TSR-containing proteins: Immunoprecipitation (-IP) of TSR-containing proteins from Lec13 cells grown ±fucose shows that BiP association. For proteins with more tandem TSRs (ADAMTS13, ADAMTSL2), this association appears to be fucose-dependent. (Black arrow indicates the specific band corresponding to indicated protein for some panels).

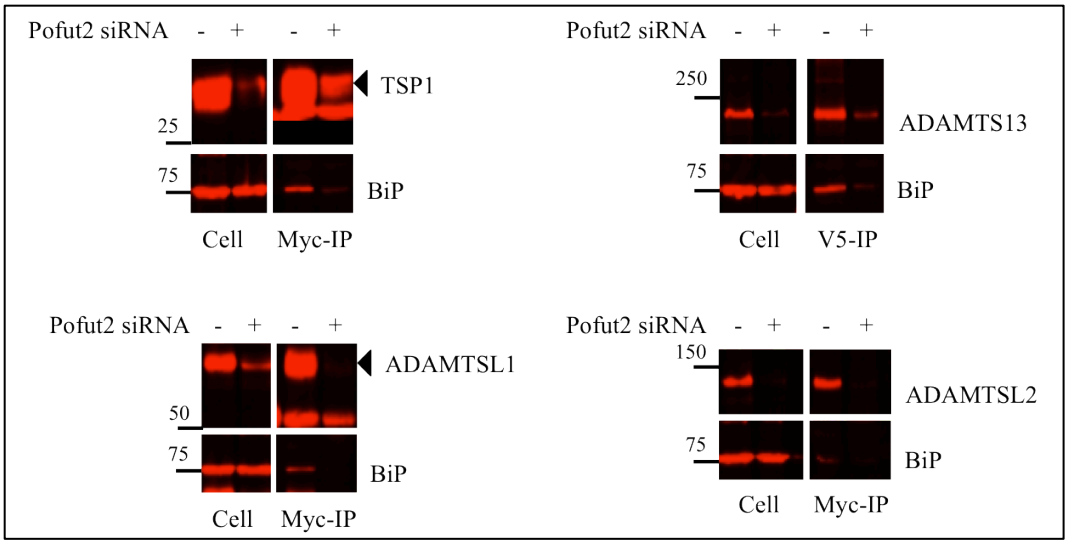

Fig. C3. BiP association with TSR-containing protein in the absence of Pofut2: When cotransfected with Pofut2 siRNA, cell assoicated TSR-contianing protein is rapidly degraded, presumably due to the lack of Pofut2's protective function. This makes the analysis of BiP association in the absence of Pofut2 more difficult in HEK293T cells.

C.3. Pofut2 association with ADAMTS13

Results from section 6.1 show that Pofut2 associates differently with its substrates in the presence or absence of fucose. Particularly for ADAMTS13, this interaction was also found to be independent of a crosslinker. The stability of the interaction directly depends on substrate recognition by Pofut2. By mutating the hydroxyl amino acid in the consensus sequence, Pofut2 substrate recognition may be altered. This hypothesis was tested by cotransfecting Pofut2-Flag with *wt* or S to A mutants of ADAMTS13 (Fig. C4). Some mutants had a single S to A change (TSR1 and 3), some were double mutants (TSR1+2, TSR5+6) and one mutant had six changes (TSR1 to 6). Post-lysis, samples were subjected to Flag-immunoprecipitation to purify Pofut2 and blotted for ADAMTS13, BiP and Pofut2. All mutants except TSR5+6 expressed well and showed reduced Pofut2 association, although the extent of the effect varied from site to site. Interestingly, BiP association with Pofut2 did not depend on Pofut2's interaction with its substrate. This suggests that Pofut2 might interact with BiP independent of its substrates.

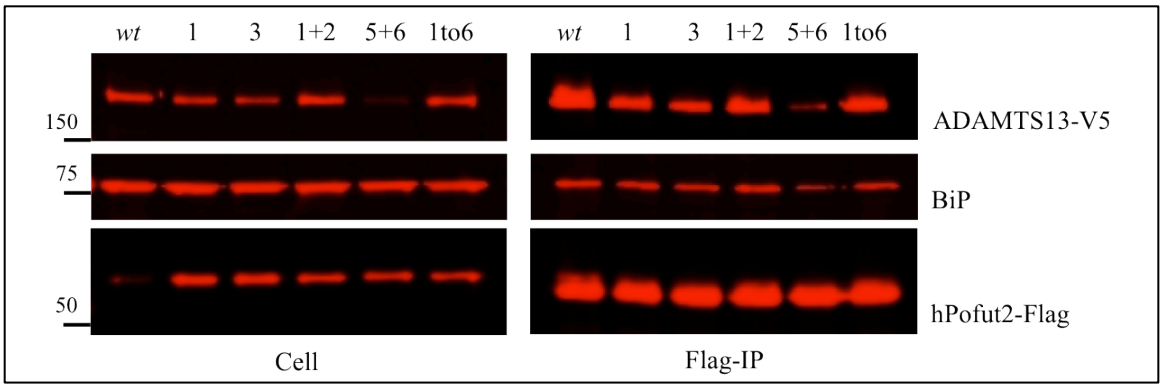

Fig. C4. Pofut2-substrate is altered by acceptor site: Co-IP of either *wt* or S to A mutants (in indicated TSRs 1, 3, 1+2, 5+6, 1 to 6) ADAMTS13 with Pofut2 in HEK293T cells shows that substrate association is reduced when the consensus sequence is altered. This effect varies from site to site, with TSR3 S to A mutant associating the least. Interestingly, BiP association with Pofut2 was not altered suggesting a substrate-independent interaction between the two proteins.

Appendix D. 6AF studies

D.1. Examining fucosylation of ADAMTS13 associated with Pofut2

Results in section 6.2 show ADAMTS13 to associate with Pofut2. Subsequent to this experiment, the fucosylation status of substrate associated with Pofut2 was examined using 6-alkynyl fucose. HEK293T cells co-transfected with Pofut2-Flag and ADAMTS13-V5 were incubated with 6AF for 24 h. Post-lysis, part of the lysate was saved as the 'input' fraction and the rest was subjected to immunopurification with Flag. Western blot analysis of the eluate showed that ADAMTS13 associated with Pofut2 was fucosylated, suggesting that this molecule is partially folded (Fig. D1). Analysis of the supernatant after immunoprecipitation showed 6AF-labeled ADAMTS13. This population of ADAMTS13 is presumably associated with untagged endogenous Pofut2. To test the if the ADAMTS13 associated with Pofut2 was properly folded, experiments similar to section 5.2 (using Iodoacetamide at the time of lysis) were performed. These experiments did not yield enough material for analysis.

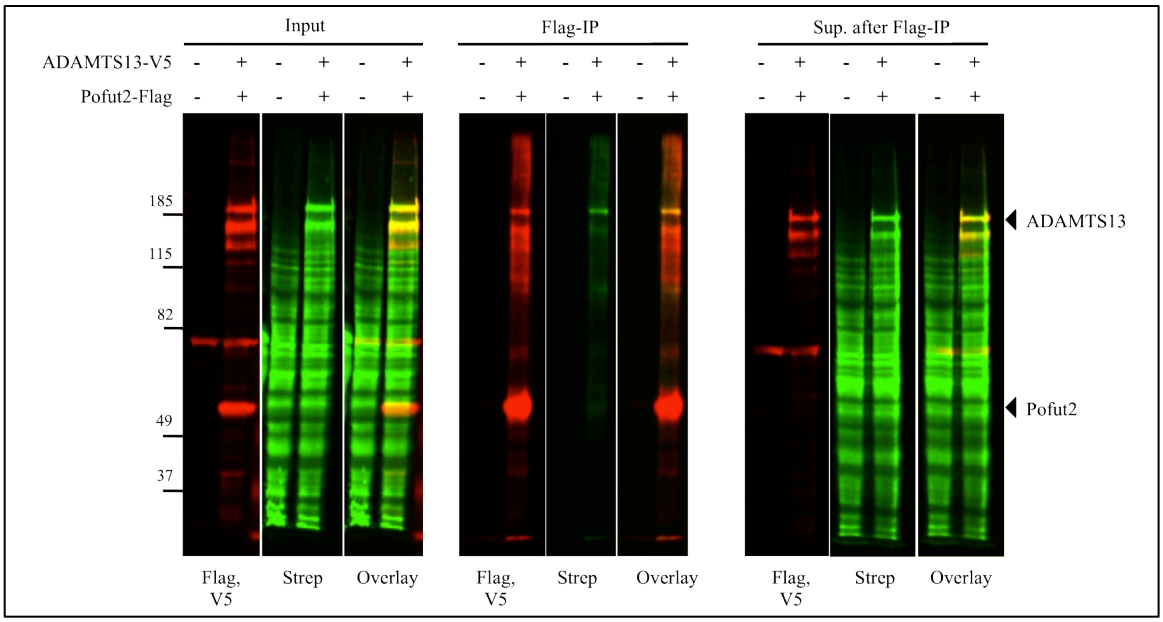

Fig. D1. ADAMTS13 associated with Pofut2 is labeled with 6AF: Western blotting analysis of three fractions- total cell lysate (input), immunopurified Pofut2 (Flag-IP) and the supernatant after immunopurification (sup. after Flag-IP)- shows that ADAMTS13 associated with Pofut2 is fucosylated. Interestingly, the third fraction shows that there is fucosylated ADAMTS13 that is not associated with tagged Pofut2.

D.2. 6AF labeling of single TSRs from ADAMTS13

Proteomics analysis of ADAMTS13 shows all TSRs to be *O*-fucosylated (38). It is possible that the individual TSRs from ADAMTS13 tested in section 3.3 behave differently than in the context of the full-length protein. To test if individual TSRs from ADAMTS13 are fucosylated, HEK293T cells were transiently transfected with various single TSRs from ADAMTS13 and grown in the presence of 6AF for 48 h. Cell and media fractions were collected and analysed using anti-Flag and streptavidin probes (Fig. D2). Western blotting with anti-Flag showed that TSRs 2 and 5 express poorly in comparison to TSRs 1 and 3. This is similar to results seen in CHO cells (Fig. 3.5). Overlay of the streptavidin and Flag signals shows that TSR1 and 3 are both fucosylated. TSR1 is secreted efficiently and very little can be detected in the cell. TSR6 is secreted, though not efficiently, but is not labeled with 6AF. This suggests that some TSRs may fold poorly and not be fucosylated when isolated from a tandem chain.

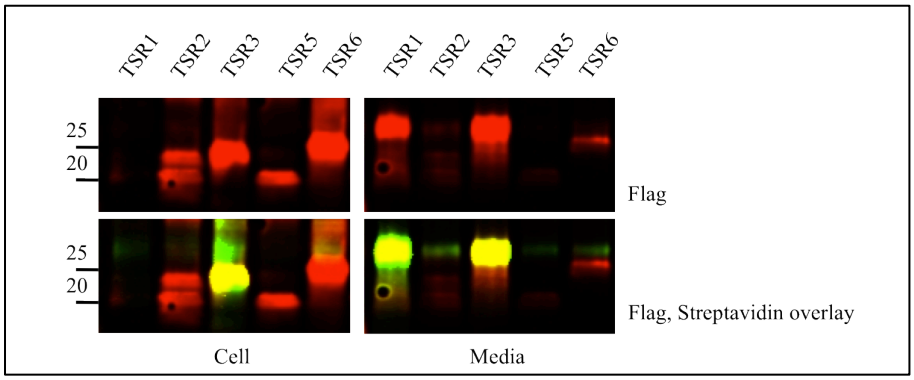

Fig. D2. 6AF labeling of single TSRs from ADAMTS13: TSRs 1 and 3 from ADAMTS13 label with 6AF, indicating that they are fucosylated. TSRs 2 and 5 are not expressed efficiently. TSR6 is expressed, albeit poorly, but does not label with 6AF.

Appendix E. Pulse-chase studies

The pulse-chase experiments in section 4.3 demonstrate that TSR-containing proteins take a long time to be folded and secreted. Since *O*-fucosylation occurs only on properly folded TSRs, this suggests that *O*-fucosylation could also happen post-translationally since full-length proteins are seen in the early time points. To more directly test this using a metabolic labeling system, pulse-chase studies (similar to those in section 4.3) were performed in the presence of 6-alkynyl fucose (6AF) to be able to follow fucosylation on [^{35}S]-Methionine labeled protein. CHO cells grown in the presence of 6AF were transfected with ADAMTSL1-MycHis$_6$ and pulsed with [^{35}S]-Methionine for 10 minutes. As a control, cells transfected with the heavy chain of human IgG, were used. The cells were then chased in the presence of unlabeled methionine and 6AF for several time points. The cells were then lysed, reacted with azido-biotin, and ADAMTSL1 was immunoprecipitated with anti-Myc antibody (for ADAMTSL1 transfected cells) or with Protein G agarose (for IgG transfected cells). A part of the eluate (20%) from the immunoprecipitation was run directly on a gel and autoradiographed as a measure of total ADAMTSL1. The remainder was enriched a second time with streptavidin-agarose to isolate the fucosylated ADAMTSL1 population. All samples were run on a gel and autoradiographed (Fig. E1).

As expected, the anti-Myc-IP samples showed similar signals in increasing time points indicating that the pulse efficiently labeled the protein. Interestingly, some of the 6AF-labeled ADAMTSL1 was detected at the earliest chase time (0 minutes) of the double enrichment (Myc/Strep double), indicating that fucose is added to the protein during the earliest stages of its biosynthesis, probably co-translationally as suggested by the data in Figures 4.3 and 4.4. The double enrichment samples also showed an increase in signal from 0 to 40 minutes of chase after

which the signal drops off at 120 minutes (Fig. E1), suggesting that additional fucose is added to the protein after translation is complete. Similar results were seen for ADAMTS13, although sufficient amounts of the protein could not be purified from media for this analysis (Fig. E2). These results supports the data from section 4.3 that folding of the protein takes a significant amount of time and that fucose is gradually added to the protein during this time of folding.

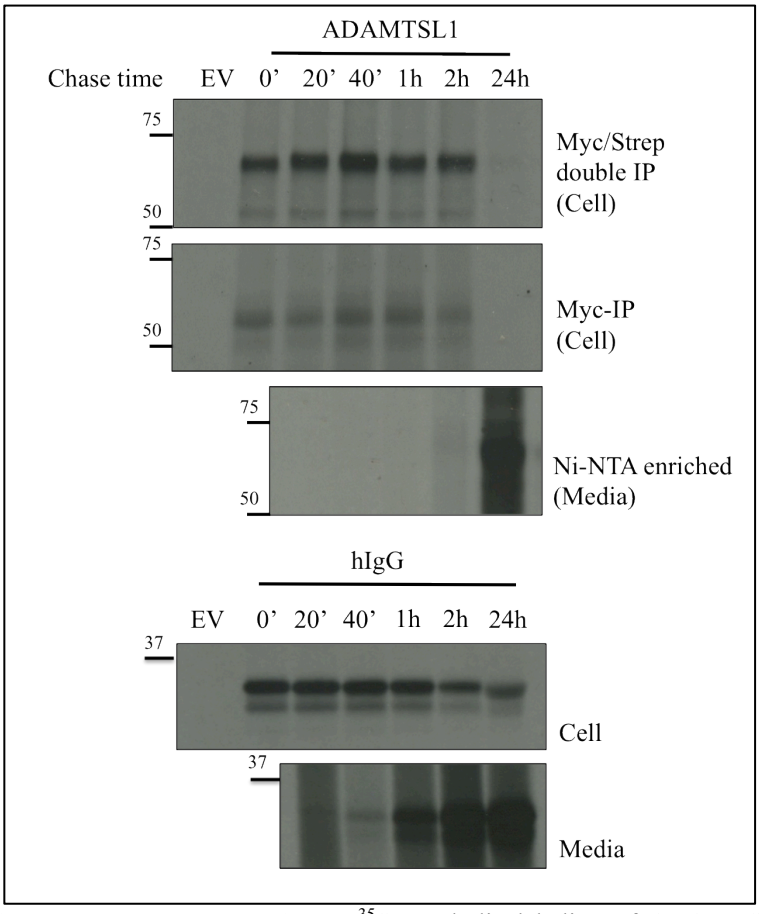

Fig. E1. *O*-Fucosylation can be post-translational: ^{35}S-metabolic labeling of ADAMTSL1-myc in Pro5 cells grown in 6AF. After pulse-chase, cell fractions were subjected to either myc-IP (panel 2) or a myc/streptavidin double enrichment (panel 1). Media samples were NiNTA enriched (panel 3). Similar studies on human IgG (panel 4, 5), an unrelated protein with no *O*-fucosylation sites, showed the protein to be much more rapidly secreted.

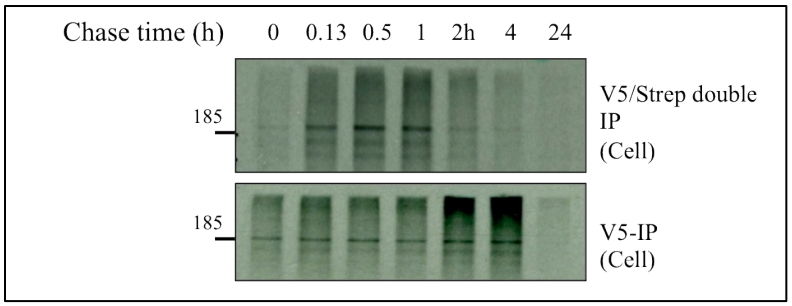

Fig. E2. *O*-Fucosylation on larger proteins may occur post-translationally: ^{35}S-Metabolic labeling of ADAMTS13 similar to ADAMTSL1 in Fig. E1 shows an increase in fucosylation in ADAMTS13 from 0 to 1 h (top panel) with no increase in total ADAMTS13 synthesized.