Identification of a novel ADAMTS9/GON-1 function for protein transport from the ER to the Golgi

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ABSTRACT A disintegrin-like and metalloprotease with thrombospondin type I motif (ADAMTS9) is a member of the secreted metalloprotease family that is believed to digest extracellular matrix (ECM) proteins outside of cells. Its Caenorhabditis elegans orthologue, GON-1, is involved in ECM degradation and is required for gonad morphogenesis. ADAMTS9 and GON-1 have similar domain structures, and both have a unique C-terminal domain called the “GON domain,” whose function remains unknown. Here we show that down-regulation of human ADAMTS9 and C. elegans GON-1 results in the inhibition of protein transport from the endoplasmic reticulum (ER) to the Golgi. This phenotype was rescued by the expression of the GON domain localizing in the ER in human cells and C. elegans. We propose a novel function of ADAMTS9 and GON-1 in the ER that promotes protein transport from the ER to the Golgi. This function is GON-domain dependent but protease activity independent.

INTRODUCTION

The A disintegrin-like and metalloprotease with thrombospondin type I motif (ADAMTS) family comprises secreted zinc metalloproteases with a precisely ordered modular organization that includes at least one thrombospondin type I repeat (Kuno \textit{et al.}, 1997; Hurskainen \textit{et al.}, 1999; Clark \textit{et al.}, 2000). ADAMTS9 localizes extracellularly or on the cell surface and cleaves large, aggregating proteoglycans such as aggrecan and versican (Somerville \textit{et al.}, 2003). Similar to most zymogens, the ADAMTS9 protease domain contains an N-terminal propeptide. The propeptide is believed to have a role in maintaining enzyme latency, and excision of the propeptide activates ADAMTS9. The ADAMTS9 propeptide is processed by furin at the cell surface (Somerville \textit{et al.}, 2003) and is necessary for localization to the cell surface (Koo \textit{et al.}, 2007). ADAMTS9 mRNA is widely expressed, especially during embryonic development in both humans and mice (Somerville \textit{et al.}, 2003; Jungers \textit{et al.}, 2005).

In humans, an ADAMTS9 gene variant in the 5′-upstream region (rs4607103) is associated with type 2 diabetes (Grarup \textit{et al.}, 2008; Zeggini \textit{et al.}, 2008), and this allele results in reduced insulin-stimulated glucose uptake (Boesgaard \textit{et al.}, 2009). The molecular mechanisms underlying the effect of ADAMTS9 on the peripheral insulin action and risk of diabetes, however, are unknown. In mice, an ADAMTS9-null allele is early embryonic lethal (Silver \textit{et al.}, 2007), suggesting that ADAMTS9 plays an essential role in organism survival, although the molecular mechanisms of ADAMTS9 involvement during embryogenesis are unknown. GON-1 is the Caenorhabditis elegans orthologue of ADAMTS9 and is required for distal tip cell (DTC) migration during gonad morphogenesis. GON-1 contributes to degradation of the basement membrane or the processing
of extracellular cues required for cell migration by its protease activity (Blelloch et al., 1999). It is of interest that membranes accumulate within the cytoplasm in DTC of gon-1 mutants (Blelloch et al., 1999). This phenotype is not predicted by the known protease functions of GON-1 and ADAMTS-family proteases (Porter et al., 2005). Thus these previous reports suggest that GON-1 and ADAMTS9 have an additional function.

Here we report on the novel function of ADAMTS9 and GON-1, which are required for the protein secretory pathway from the endoplasmic reticulum (ER) to the Golgi in both C. elegans and human cells. It is surprising that this novel function depends on the GON domain without protease activity, and the GON domain plays a role in the ER but not extracellularly. The GON domain exists at the C-terminus of ADAMTS9, ADAMTS20, and GON-1 and in a number of GON-domain proteins in metazoans, suggesting that its role has broad biological significance. This is the first report of the GON-domain function, which is important for protein transport from the ER to the Golgi.

RESULTS
Gonad proliferation defects in a gon-1 mutant are associated with abnormalities in ER structure and function and the ER stress response
A member of the ADAMTS-like metalloproteinase family is encoded by gon-1, which is expressed in DTCs and body wall muscle cells and functions in extracellular matrix (ECM) remodeling in C. elegans (Jones and Riley, 2005). Depletion of GON-1 results in gonad proliferation defects and aberrant membrane morphology in hermaphroditic DTCs (Blelloch et al., 1999). We confirmed this phenotype using RNA interference (RNAi) of gon-1 [gon-1(RNAi)] in wild-type animals and a gon-1 mutant (tm3146) (Figure 1A and Supplemental Figure S1). gon-1(tm3146) harbors a deletion that removes the protease domain, and a termination codon is introduced by a neighboring short insertion (Supplemental Figure S2).

We next evaluated whether protein secretion was impaired in gon-1(RNAi) because the ER structure was abnormal. The protein secretion activity of DTCs was examined based on the distribution of secreted soluble Venus (ssVenus) expressed in DTCs (Fares and Greenwald, 2001). Accumulation of Venus fluorescence was ob-

![Figure 1](image-url)

**Figure 1:** gon-1(tm3146) has an aberrant morphology of the ER and secretion defects. (A) In gon-1 mutants and gon-1(RNAi) animals, gonadal arm extension did not occur. Arrowheads indicate the position of the tip of the gonad. The schematic drawings (right) indicate the morphology of the gonad. Scale bar, 100 μm. (B) ER morphology analyzed by an ER marker, cytochrome b5-Venus. Confocal fluorescence images drastically changed in animals treated with gon-1(RNAi) and gon-1(tm3146) mutants (top). Arrowheads indicate abnormal ER morphology. Bottom, the differential interference contrast (DIC) images. Scale bar, 5 μm. (C) Protein secretion from DTC was blocked in animals with gon-1(RNAi) and gon-1(tm3146) mutants (top). Bottom, the DIC images. Scale bar, 50 μm. (D) Transportation analyzed by a plasma membrane protein marker. Confocal fluorescence images changed in animals treated with gon-1(RNAi) and gon-1(tm3146) mutants (top). Bottom, the DIC images. Scale bar, 5 μm.
We then evaluated whether gon-1 is necessary for the transportation of a plasma membrane protein and a glycosylphosphatidylinositol (GPI)-anchored protein. We generated transgenic worms expressing a lag-2 promoter–driven enhanced GFP (EGFP) fused with the transmembrane (TM) domain of T01F10.1a (TM-EGFP) as a plasma membrane protein and yellow fluorescent protein (YFP)–GPI as a GPI-anchored protein. In wild-type animals, TM-EGFP and YFP-GPI localized to the plasma membrane (Figure 1C, 1D, left, and Supplemental Figure S5A, left). Punctate accumulation of TM-EGFP was observed in DTCs when the transgenic animals were treated with gon-1(RNAi) or in the gon-1(tm3146) background (Figure 1D, middle and right). The YFP-GPI signal accumulated in DTCs when transgenic animals were treated with gon-1(RNAi) (Supplemental Figure S5A, right). ER dysfunction in body wall muscle cells was the gon-1(tm3146) background was examined by evaluating protein secretion in transgenic worms harboring arls37, which expressed and secreted soluble GFP from body wall muscles to the pseudocoelom. The secreted GFP in the pseudocoelom was endocytosed and accumulated in coelomocytes in wild-type animals (Supplemental Figure SSB, left, arrowhead), as previously reported (Fares and Greenwald, 2001). On the other hand, bright fluorescence was observed in the body wall muscle cells of gon-1(tm3146) mutants (Supplemental Figure SSB, right). We also examined the distribution of TM-EGFP expressed in body wall muscle cells. In wild-type animals, TM-EGFP mainly localized to the plasma membrane (Supplemental Figure S5C, left). In contrast, depletion of gon-1 led to the punctate accumulation of TM-EGFP in body wall muscle cells (Supplemental Figure SSC, right), indicating that GON-1 is also required for TM-EGFP transport in body wall muscle cells.

The BIP protein, an ER-stress sensor, is induced when ER functions are impaired in mammalian cells. To determine whether the homologue in the nematode (hsps-4) is also induced in gon-1(RNAi)-treated animals, we used a transgenic allele zcs4 (Calfon et al., 2002) harboring a transcriptional fusion reporter of hsps-4::gfp. In wild-type animals, GFP expression was not observed in DTCs (Supplemental Figure S6A, left). By contrast, gon-1(RNAi) induced GFP expression in DTCs (Supplemental Figure S6A, right).

To more directly evaluate the disturbance of ER functions, we performed reverse transcription-PCR (RT-PCR) analysis of xbp-1. XBP-1 is an important molecule for the unfolded protein response (UPR) in all cells. Under ER stress conditions, xbp-1 mRNA is processed by unconventional splicing, which results in the removal of an intron and a translational frame shift (Schröder and Kaufman, 2005), causing the conversion of the premature unspliced xbp-1 (xbp-1(U)) to the spliced xbp-1 (xbp-1(S)). The amount of xbp-1(S) mRNA was increased in gon-1(RNAi) animals (Supplemental Figure S6B), although the possibility that xbp-1 was induced indirectly in other tissues could not be excluded because xbp-1 might also exist in other cells. We generated transgenic worms that expressed a fusion protein containing a typical ER stress-inducing reagent. Gonad proliferation was also highly disturbed by RNAi knockdown of the T12A2.2 gene in N2 worms, as in gon-1–mutant animals (Supplemental Figure S7). These results suggest that down-regulation of ER function accounts for some of the gonadal defects.

The GON domain localizes to the ER and is important for maintaining functions of the DTC

Other gon-1–mutant alleles—q518 and e1254—retained the pro tease domain but also showed severe gonad morphogenesis defects (Bellocchio and Kimble, 1999) and abnormal ER morphology in almost all DTCs (Supplemental Figure S8). We therefore evaluated which portion of the GON-1 protein is necessary for the structure and function of the ER in DTC. q518 has a stop codon in front of the thrombospondin domain, and e1254 has a stop codon within the thrombospondin domain (Supplemental Figure S2). Transgenic animals that expressed various truncated forms of gon-1 and plag-2::C31E10.7::Venus in the wild-type or the gon-1(tm3146) mutant backgrounds were generated, and ER morphology and gonad proliferation were observed (Figures 2A and 3). We defined ER structure as abnormal when the ER had at least one bright fluorescent circular structure with a diameter of 1 μm or larger (Figure 3A). ER morphology was normal in 97% of wild-type animals, in only 5% of the gon-1(tm3146) mutants, and in only 10% of the tm3146;tmEx[plag-2::gon-1(N-TSP12)] mutants, in which the GON domain was absent. The incidence of normal ER morphology did not differ significantly between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(N-TSP12)] (p > 0.1), and thus plag-2::gon-1(N-TSP12) did not rescue the ER morphology. ER morphology was normal in 81% of the.tm3146;tmEx[plag-2::gon-1(sig_GON)] mutants, which had a signal sequence and the GON domain. The incidence of normal ER morphology differed significantly between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_GON)] animals (p < 0.05), indicating that plag-2::gon-1(sig_GON) rescued the ER morphology. ER morphology in tm3146;tmEx[plag-2::gon-1(sig_GON)::KDEL], which had the signal sequence, the GON domain, and the ER retention signal KDEL, was normal in 92% of the animals. The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_GON)] (p < 0.05), indicating that plag-2::gon-1(sig_GON)::KDEL rescued the ER morphology. ER morphology of tm3146;tmEx[plag-2::gon-1(sig_GON)] had the signal sequence, the Pro domain, and the GON domain, was normal in 92% of the animals. The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_Pro_GON)], which had the signal sequence, the Pro domain, and the GON domain, was normal in 92% of the animals. The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_Pro_GON)] (p < 0.05), indicating that plag-2::gon-1(sig_Pro_GON) rescued the ER morphology. ER morphology in tm3146;tmEx[plag-2::gon-1(sig_GON), pmyo-3::gon-1(FU)], which expressed a fusion protein of the signal sequence and the GON domain in DTCs and expressed the full-length GON-1 protein in muscle, was normal in 98% of the animals. The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_GON), pmyo-3::gon-1(FU)] (p < 0.05), indicating that plag-2::gon-1(sig_GON), pmyo-3::gon-1(FU) rescued the ER morphology. ER morphology in tm3146;tmEx[plag-2::gon-1(E425A)], which had a Glu-425—to-Ala substitution in the active center of the metallopro tease but retained the GON domain, was normal in 77% of the animals. The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(E425A)] (p < 0.05), indicating that plag-2::gon-1(E425A) rescued the ER morphology. ER morphology in tm3146;tmEx[plag-2::gon-1(sig_GON_Venus)], which expressed a fusion protein containing
Venus at the C terminus of sig_GON, was normal in 25% of the animals. plag-2::gon-1(sig_GON) had a weaker rescue activity than did plag-2::gon-1(sig_Venus). The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_GON_Venus)] (p < 0.05). The ER morphology in tm3146;tmEx[plag-2::gon-1(sig_Venus_GON)] rescued the ER morphology. The ER morphology in tm3146;tmEx[plag-2::gon-1(sig_GON_Venus)::KDEL], which expressed a fusion protein in which a KDEL-tag was fused to the C terminus of sig_Venus, was normal in 92% of the animals. The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_Venus_GON);KDEL], which expressed a fusion protein in which a KDEL-tag was fused to the N terminus of the GON domain, was normal in 89% of the animals. The incidence of normal ER morphology was significantly different between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_Venus_GON);KDEL] (p < 0.05), indicating that plag-2::gon-1(sig_Venus_GON)::KDEL rescued the ER morphology. The ER morphology in tm3146;tmEx[plag-2::gon-1(sig_Pro_Venus_GON)], which expressed a fusion protein in which Venus was fused between the Pro domain and the GON domain, was normal in 89% of the animals. The incidence of normal ER morphology did not differ significantly between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_Venus_GON);KDEL] (p < 0.05), indicating that plag-2::gon-1(sig_Pro_Venus_GON) rescued the ER morphology. The ER morphology in tm3146;tmEx[plag-2::gon-1(sig_Pro_Venus_GON)], which expressed GON-1 in muscles but not in the DTCs, was normal in 14% of the animals. The incidence of normal ER morphology did not differ significantly between gon-1(tm3146) and tm3146;tmEx[plag-2::gon-1(sig_Venus_GON);KDEL] (p > 0.1), indicating that pmyo-3::gon-1(FL) did not rescue the ER morphology. These observations were confirmed with two additional alleles. ER morphology in transgenic strains harboring plag-2::gon-1(sig_GON) was rescued, but in transgenic strains harboring plag-2::gon-1(N-TSP12) was not rescued in either the gon-1(q518) or gon-1(e1254) background (Supplemental Figure S8).

Because muscle expression of GON-1 results in the growth and expansion of gonadal tissues but not elongated gonads in the gon-1(q518) mutant (Blelloch and Kimble, 1999), we classified gonad proliferation as either “enlarged gonad” or “elongated gonad” (Figure 3B) according to the criteria for gonad proliferation described previously (Blelloch and Kimble, 1999). The gonads of tm3146;tmEx[plag-2::gon-1(N-TSP12)] did not rescue gonad proliferation (p > 0.1). On the other hand, transgenic animals harboring a lag-2 promoter-driven transgene that contained the GON domain, except for tm3146;tmEx[plag-2::gon-1(sig_Venus_GON)], rescued gonad proliferation (p < 0.05; Figures 2 and 3B). The gonads of tm3146;tmEx[plag-2::gon-1(sig_GON)] were enlarged in 20% of the animals. The gonads of tm3146;tmEx[plag-2::gon-1(sig_Venus_GON);KDEL] were enlarged in 58% of the animals. This result indicates that plag-2::gon-1(sig_GON);::KDEL had a higher rescue activity than plag-2::gon-1(sig_GON). The gonads of tm3146;tmEx[plag-2::gon-1(sig_Venus_GON)] were enlarged and elongated in 33 and 4% of the animals,
FIGURE 3: The GON domain is important to operate in the ER for maintaining the ER morphology and gonadal proliferation in *C. elegans*. (A) Rescue activities of the ER morphology by various gon-1 transgenes are shown. The transgenes used are the same as those in Figure 2. Genetic backgrounds (N2 or tm3146) are shown at the bottom. The graph represents the combined results of five independent experiments (n > 40 for each experiment). Bars represent mean ± SE. (B) Rescue activities of gonadal proliferation by various gon-1 transgenes are shown. Transgenes used are the same as those in A and in Figure 2. Populations of enlarged gonads and elongated gonads are shown with the white boxes and shaded boxes, respectively. Genetic backgrounds (N2 or tm3146) are shown at the bottom. The graph represents the combined results of five independent experiments (n > 40 for each experiment). Bars represent mean ± SE.
respectively. The gonads of tm3146;tmEx[plag-2::gon-1(sig_Venus_GON)::KDEL] were enlarged and elongated in 69 and 4% of the animals, respectively, indicating that plag-2::gon-1(sig_Venus_GON)::KDEL had a higher rescue activity than plag-2::gon-1(sig_Venus_GON).

To investigate the underlying mechanisms of such different rescue activities among tm3146;tmEx[plag-2::gon-1(sig_Venus_GON)], tm3146;tmEx[plag-2::gon-1(sig_Pro_Venus_GON)], and tm3146;tmEx[plag-2::gon-1(sig_GON_Venus)], we compared the localization of plag-2::gon-1(sig_Venus_GON) and tm3146;tmEx[plag-2::gon-1(sig_Pro_Venus_GON)], which had strong rescue activities, and plag-2::gon-1(sig_GON_Venus), which had a weak rescue activity. When gon-1(sig_Venus_GON) was expressed in DTCs, a mesh-like structure was observed. On the other hand, gon-1(sig_GON_Venus) localized diffusely to the cytoplasm of DTCs (Figure 2B). When gon-1(sig_Pro_Venus_GON) was expressed in DTCs, a mesh-like structure was faintly observed. To further analyze the localization of gon-1(sig_Venus_GON) in DTCs, we immunostained dissected DTCs expressing plag-2::gon-1(sig_Venus_GON) with an anti-HDEL antibody that stains the ER in C. elegans (Pourkarimi et al. 2012). gon-1(sig_Venus_GON) colocalized with the anti-HDEL immunoreactivity (Figure 2C).

These findings suggest that gon-1 functions both inside and outside of the cell. On the basis of these data, we propose that localization of the GON domain in the ER is important for intracellular function and that localization of gon-1, both inside and outside of the cell, is important for gonad proliferation.

Depletion of ADAMTS9 in a mammalian cell line inhibits transport from the ER to the Golgi

To determine whether the gon-1–like protein function is conserved in mammalian cells, we performed small interfering RNA (siRNA)–mediated knockdown of ADAMTS9, a human homologue of GON-1, in HEK293 cells. Staining with an ER-Tracker showed that ADAMTS9 depletion induced deformation of the ER morphology (Figure 5A). ER-Tracker–positive globular structures were observed in ADAMTS9 siRNA–treated cells but rarely in the control siRNA–treated cells. To confirm the abnormal ER structures in the ADAMTS9 siRNA–treated cells, we examined the ER structure with electron microscopy. We observed tightly stacked whirling membrane patterns in ADAMTS9-depleted cells but not in the control siRNA–treated cells (Supplemental Figure S10).

Vesicular transport was observed using the temperature-sensitive vesicular stomatitis virus glycoprotein (VSVG; ts045) fused to GFP. In this experimental system, VSVG-GFP first accumulates in the ER when cultured at 40°C and when the temperature is shifted to 32°C, the molecules are translocated from the ER to the plasma membrane through the Golgi. Localization of VSVG-GFP was evaluated by immunostaining with an anti-GM130 antibody or an anti-KDEL antibody. As expected, in the cells treated with control siRNA or ADAMTS9 siRNA, when cultured at 40°C, VSVG-GFP fluorescence localized to the ER, as shown by colocalization of anti-KDEL antibody staining (Supplemental Figure S11), but not to the Golgi, as shown by colocalization of anti-GM130 antibody staining (Figure 5B). After 20 min at the permissive temperature, in the cells treated with control siRNA, VSVG-GFP fluorescence localized to the Golgi in 76% of the cells, based on the colocalization of anti-GM130 antibody staining (Figure 5, B and C), but not in the ER, based on the colocalization of anti-KDEL antibody staining (Supplemental Figure S11). VSVG-GFP fluorescence localized to both the Golgi and the ER in 22% of the cells. At later time points, the VSVG-GFP was eventually translocated to the plasma membrane (unpublished data). In contrast, in ADAMTS9 siRNA–treated cells, 20 min after transfer to the permissive temperature, VSVG-GFP fluorescence localized to the Golgi in 18% of the cells and to both the Golgi and the ER in 27% of the cells. VSVG-GFP fluorescence localized to the ER in 55% of the cells, as shown by colocalization of anti-KDEL antibody staining (Figure 5C and Supplemental Figure S11). VSVG-GFP fluorescence did not colocalize with anti-GM130 antibody immunoreactivity in 55% of the cells (Figure 5B). These results indicate that ADAMTS9 knockdown caused the accumulation of VSVG-GFP within the ER 20 min after shifting the temperature to 32°C in HEK293 cells (Figure 5, B and C, and Supplemental Figure S11).

To investigate other phenotypes in mammalian cells, we examined whether ADAMTS9 is required for the secretory function in HEK293 cells. Knockdown of ADAMTS9 caused the accumulation of GFP fused with the prolactin (PRL) secretory signal within the cells (Supplemental Figure S12). We also examined whether frame-switch splicing of XBP1 is observed in ADAMTS9 siRNA–treated mammalian cells. This was indeed the case in HEK293 cells (Supplemental Figure S13A). Although the cell morphology changed a little,
To investigate whether the GON domain alone could rescue the ER-to-Golgi transport phenotype in HEK293 cells, as observed in *C. elegans*, we transfected VSVG-GFP and gon-1(sig_GON) into HEK293 cells treated with ADAMTS9 siRNA or control siRNA. In this experiment, the cells became rounder (Figure 5A), and too strong ER stress may cause apoptosis, we examined cell viability of ADAMTS9 siRNA–treated HEK293 cells. The cell viability was unchanged between control and ADAMTS9 siRNA–treated cells (Supplemental Figure S13B).

**FIGURE 4:** Time course of alteration of ER structure and protein secretion from DTC after KillerRed-mediated, light-induced inactivation of the GON domain. (A) A schematic diagram of the fusion protein used in B–E and Supplemental Figure S8. Domains are indicated as described in Figure 2A. (B) ER morphology of gon-1 mutants that expressed plag-2::gon-1(sig_KillerRed_GON) and plag-2::C31E10.7::Venus was observed at the indicated time points by confocal fluorescence microscopy after illumination with a green light (photoablation) or not (control). ER morphology changed dramatically between 10 and 24 h after irradiation, as shown by arrowheads. Scale bar, 5 μm. (C) Quantification of the results in B. The graph represents the combined results of six independent experiments (n > 100 for each experiment). Bars represent mean ± SE. (D) Accumulation of ssVenus within DTC in gon-1 mutants that expressed plag-2::gon-1(sig_KillerRed_GON) and plag-2::ssVenus was observed at the indicated time points by confocal fluorescence microscopy after illumination with a green light (photoablation) or not (control). ssVenus accumulation in DTC was observed after 2 h of irradiation. (E) Quantification of the results in D. The graph represents the combined results of five independent experiments (n > 90 for each experiment). Bars represent mean ± SE.
The role of ADAMTS9 in protein transport was evaluated. At 40°C, VSVG-GFP accumulated in the ER, as shown by colocalization of anti-KDEL antibody staining in 100% of the gon-1(sig_GON)-introduced and control siRNA-treated cells and in 100% of the gon-1(sig_GON)-introduced ADAMTS9 siRNA-treated cells (Figure 6, A and B, and Supplemental Figure S14). After 20 min at the permissive temperature, VSVG-GFP localized to the Golgi, as shown by colocalization of anti-GM130 antibody staining in 85% of the gon-1(sig_GON)-introduced and control siRNA-treated cells. After 20 min at the permissive temperature, VSVG-GFP localized to the Golgi in 83% of the gon-1(sig_GON)-introduced and ADAMTS9 siRNA-treated cells (Figure 6, A and B). VSVG-GFP did not localize to the ER, as shown by colocalization of anti-KDEL antibody staining in these cells (Supplemental Figure S14). These observations support the idea that the GON domain, but not the protease activity, is required for cargo transport from the ER to the Golgi in HEK293 cells.

We next analyzed the subcellular localization of various GON-domain proteins in mammalian cells to determine protein structure–function relationship. To do this, we transfected gon-1(sig_GON_Venus), gon-1(sig_Venus_GON), ADAMTS9(sig_GON_Venus), or ADAMTS9(sig_Venus_GON) into HEK293 cells and observed Venus fluorescence localization. When HEK293 cells were transfected with plasmids expressing gon-1(sig_Venus_GON) and ADAMTS9(sig_Venus_GON), fluorescence was observed in the ER, as shown by colocalization with the calnexin immunoreactivity. In contrast, gon-1(sig_GON_Venus) and ADAMTS9(sig_GON_Venus) localized diffusely throughout the cytoplasm (Supplemental Figure S15). These findings are similar to the localization of the GON domain in the nematode. Taken together, these observations of HEK293 cells were similar to the phenotypes found in the nematode.

Because cargo molecules accumulated in the ER, and ER stress was induced under the ADAMTS9-depleted condition, we evaluated whether secretory proteins are normally O-linked glycosylated in the Golgi and degraded normally by the proteasome system. We expressed α1-antitrypsin (AAT) to monitor secretion in HEK293 cells treated with or without ADAMTS9 siRNA and found that ADAMTS9 knockdown caused the accumulation of AAT within the cells and a reduction of AAT in the medium (Supplemental Figure S16, A–C), similar to the accumulation of the prolactin-GFP fusion protein described earlier. We used the enzyme endoglycosidase H (Endo H), which removes N-linked glycosylation, but not O-linked glycosylation, from proteins in the siRNA-plasmid transfection experiment, because the GON-domain nucleotide sequences are very different between human and C. elegans, the transfected siRNA should not knock down the GON domain mRNA from the rescue plasmid. These cells were stained with an anti-GM130 antibody or an anti-KDEL antibody, and localization of VSVG-GFP was evaluated. At 40°C, VSVG-GFP accumulated in the ER, as shown by colocalization of anti-KDEL antibody staining in 100% of the gon-1(sig_GON)-introduced and control siRNA–treated cells and in 100% of the gon-1(sig_GON)-introduced ADAMTS9 siRNA–treated cells (Figure 6, A and B, and Supplemental Figure S14). After 20 min at the permissive temperature, VSVG-GFP localized to the Golgi, as shown by colocalization of anti-GM130 antibody staining in 85% of the gon-1(sig_GON)-introduced and control siRNA–treated cells. After 20 min at the permissive temperature, VSVG-GFP localized to the Golgi in 83% of the gon-1(sig_GON)-introduced and ADAMTS9 siRNA–treated cells (Figure 6, A and B). VSVG-GFP did not localize to the ER, as shown by colocalization of anti-KDEL antibody staining in these cells (Supplemental Figure S14). These observations support the idea that the GON domain, but not the protease activity, is required for cargo transport from the ER to the Golgi in HEK293 cells.

We next analyzed the subcellular localization of various GON-domain proteins in mammalian cells to determine protein structure–function relationship. To do this, we transfected gon-1(sig_GON_Venus), gon-1(sig_Venus_GON), ADAMTS9(sig_GON_Venus), or ADAMTS9(sig_Venus_GON) into HEK293 cells and observed Venus fluorescence localization. When HEK293 cells were transfected with plasmids expressing gon-1(sig_Venus_GON) and ADAMTS9(sig_Venus_GON), fluorescence was observed in the ER, as shown by colocalization with the calnexin immunoreactivity. In contrast, gon-1(sig_GON_Venus) and ADAMTS9(sig_GON_Venus) localized diffusely throughout the cytoplasm (Supplemental Figure S15). These findings are similar to the localization of the GON domain in the nematode. Taken together, these observations of HEK293 cells were similar to the phenotypes found in the nematode.

Because cargo molecules accumulated in the ER, and ER stress was induced under the ADAMTS9-depleted condition, we evaluated whether secretory proteins are normally O-linked glycosylated in the Golgi and degraded normally by the proteasome system. We expressed α1-antitrypsin (AAT) to monitor secretion in HEK293 cells treated with or without ADAMTS9 siRNA and found that ADAMTS9 knockdown caused the accumulation of AAT within the cells and a reduction of AAT in the medium (Supplemental Figure S16, A–C), similar to the accumulation of the prolactin-GFP fusion protein described earlier. We used the enzyme endoglycosidase H (Endo H), which removes N-linked glycosylation, but not O-linked glycosylation, from proteins in the siRNA-plasmid transfection experiment, because the GON-domain nucleotide sequences are very different between human and C. elegans, the transfected siRNA should not knock down the GON domain mRNA from the rescue plasmid. These cells were stained with an anti-GM130 antibody or an anti-KDEL antibody, and local-
secretory pathway. Endo H cleaved all the carbohydrate chains of AAT that remained within the cells in both control and ADAMTS9 siRNA-treated cells (Supplemental Figure S16C, Cell). On the other hand, irrespective of siRNA treatment, secreted AAT was Endo H resistant due to the oligosaccharide modification that occurred in the Golgi apparatus (Supplemental Figure S16C, Medium). Thus glycosylation was not affected by ADAMTS9 siRNA treatment.

To examine whether ADAMTS9 is required for ER-associated degradation (ERAD) in the ER, we transfected an ATT variant, null Hong Kong (NHK), which fails to be transported to the Golgi and is degraded by ERAD (Hosokawa et al., 2003), in HEK293 cells. We examined the time course of the degradation of the NHK variant in a cycloheximide-chase experiment with or without ADAMTS9 siRNA. Degradation of the NHK variant did not differ between cells treated with ADAMTS9 siRNA and control siRNA (Supplemental Figure S16D). This finding suggested that degradation of AAT(NHK) by ERAD is not dependent on ADAMTS9.

**DISCUSSION**

Distal tip cells and body wall muscle cells express gon-1, which encodes a ADAMTS-like metalloprotease that functions in ECM remodeling (Jones and Riley, 2005). The gon-1 gene has an essential role in gonad development in *C. elegans* (Blelloch and Kimble, 1999). The present study revealed that *C. elegans* GON-1, an ADAMTS9 homologue, and human ADAMTS9 also have another function. GON-1 and ADAMTS9 are involved in the mechanisms of protein transport from the ER to the Golgi. In addition, the GON domain at the C-terminus of GON-1 and ADAMTS9 localizes to the ER, and the GON domain is important for ER functions.

In *C. elegans*, down-regulation of gon-1 led to ER deformation and gonad proliferation defects, and these phenotypes were rescued by GON-domain expression without the protease domain in the ER of DTCs. To investigate whether the role of the GON domain in the ER of DTCs is sufficient for gonad proliferation, we compared transgenic animals harboring two...
transgenes—gon-1(sig_GON) in DTCs and gon-1(FL) in muscle—with transgenic animals harboring only the gon-1(sig_GON) in DTCs or with transgenic animals harboring gon-1(FL) in muscle. Gonad proliferation recovered to a greater extent in transgenic animals harboring both transgenes—gon-1(sig_GON) in DTCs and gon-1(FL) in muscle—than in transgenic animals harboring only the gon-1(sig_GON) in DTCs or only the gon-1(FL) in muscle. Our findings strongly suggest that the GON-1 protein functions both inside and outside DTCs. The GON domain functions in the ER for protein transport from the ER to the Golgi, and at the same time, the protease domain of GON-1 functions outside DTCs to digest ECM proteins. gon-1(sig_GON)::KDEL, which has an ER-retention signal, rescued DTC function better than did gon-1(sig_GON) in the gon-1 mutant (Figures 2 and 3). These data support the conclusion that the GON domain acts in the ER.

DTCs are specialized for the stimulation and navigation of gonad proliferation and generate some of the secretory or membrane proteins that are required for gonad navigation, such as UNC-5 (Su et al., 2000), and gonad proliferation, such as LAG-2 (Gao and Kimble, 1995). GPI-anchored proteins are also suggested to be involved in gonad proliferation (Murata et al., 2012). Our data imply that depletion of GON-1 impairs the transport of LAG-2, other germ cell growth factors, and DTC navigation factors to the plasma membrane. For this reason, the gonad of the gon-1 mutant fails to proliferate and undergo morphogenesis. Future studies to examine the candidate proteins transported by the GON domain function to control gonad proliferation will provide important data.

To elucidate the role of the GON domain, we performed chromatophore-assisted light inactivation using the phototoxic fluorescent protein KillerRed and inactivated the GON domain. We observed the time course of the changes in ER morphology and protein secretion inhibition. In the experiments, secretory proteins accumulated in the ER within the early hours after GON-domain depletion. Cells expressing higher levels of membrane proteins contain a number of nonbranching structures, including whorls and open loops (Snapp et al., 2003). Furthermore, the accumulation of VSVG-GFP was observed in both the spherically aggregated, abnormal ER and the mesh-like, normal ER structures in the ADAMTS9 siRNA-treated cells after 20 min at the permissive temperature. These results suggest that the abnormal ER structure might be induced secondarily by the accumulation of proteins in the ER.

In human cells, siRNA-mediated knockdown of ADAMTS9 inhibited transport of the membrane protein VSVG-GFP from the ER to the Golgi and secretion of the secretory proteins PRL-GFP and AAT. Bright oval structures were observed in the ER under the same conditions. Inhibition of protein transport from the ER to the Golgi was recovered by GON-domain expression in the ER. These findings suggest that the GON domain has a common intracellular function: the export of a broader variety of cargo proteins from the ER to the Golgi in human and C. elegans.

We searched the Pfam database for proteins from other species that have the GON domain and investigated the size of those proteins, the position of the GON domain, the existence of the signal sequence, TM regions, and the metalloprotease domain. Various species have proteins with the GON domain. The size of those proteins varies from 219 to 2289 residues. Most of them have a signal sequence or a TM region, consistent with the notion that the GON domain is involved in ER-to-Golgi transport and is associated with proteins in the secretory pathway. Those proteins, however, do not necessarily have a metalloprotease or a thrombospondin domain. The metalloprotease domain is not required for GON-domain function, based on the phylogenic origins described earlier, as well as our own observations. The GON domain is present in the most-C-terminal region in these proteins (Supplemental Table S1). These results are consistent with our finding that gon-1(sig_Venus_GON) had a higher rescue activity than gon-1(sig_GON_Venus). In addition, the presence of Venus in the C-terminus disrupted the localization of the GON domain in both C. elegans and mammalian cells. Thus the C-terminus of the GON domain most likely has an important role in its localization and eventually its function.

Newly synthesized secretory proteins are transported from the ER to the Golgi by COPII-coated vesicles. A large number of proteins, including coat proteins that help vesicle budding, proteins that regulate cargo export, and receptors that bind to secretory cargoes, are required for the biogenesis of COPII-coated vesicles. COPII-coated vesicles comprise the sec23/24 and sec13/31 complexes (Gurkan et al., 2006). Export of cargo proteins from the ER is mediated by sar-1, sec16, and sec12 (Gurkan et al., 2006). TFG-1 regulates cargo export from the ER (Witte et al., 2011). ERGIC-53, Emp24p, surf4, and TANGO1 function as cargo receptors (Nakamura et al., 1998; Muniz et al., 2000; Mitrovic et al., 2008; Nyfeler et al., 2008; Saito et al., 2009). These cargo receptors are believed to mediate cargo loading into COPII vesicles, and the cargo proteins are then exported from the ER to the Golgi. ERGIC-53 has a role in the transport of coagulation factors V and VIII, cathepsin C and Z, and AAT. Emp24p is involved in the transport of Gas1p, a GPI-anchored protein. Surf4 has a role in the transport of soluble proteins. TANGO1 has a role in the transport of collagen VII. These factors are all considered to be involved in transportation of limited types of cargoes. On the other hand, depletion of gon-1 led to accumulation of TM-EGFP, a TM protein, and a GPI-anchored protein, as well as secretory proteins such as sVenus, suggesting that the GON domain acts on a variety of cargoes in the secretory pathway. Thus the function of the GON domain appears to differ from those of other transport factors. The amounts and types of cargoes to be transported differ among cell types, but mechanisms of substrate specificity and cell specificity involved in cargo export are not fully elucidated. Of interest, not all cells express GON-1 and ADAMTS9 in the nematode and human, but all cells have some mechanisms of transport from the ER to the Golgi. The dissimilarities among cell types that require the GON domain are not clear. Future studies are needed to elucidate the molecular mechanisms of the action of the GON domain in the ER.

MATERIALS AND METHODS

Nematode strains

The deletion mutant strain of gon-1(tm3146) was obtained as described (Gengyo-Ando and Mitani, 2000). The deletion allele was identified by PCR amplification with the primers spanning the deleted region as described in Supplemental Table S2 and Supplemental Figure S2. gon-1(tm3146) was derived from the wild-type Bristol strain N2. Genetic crosses were carried out by the standard genetic protocols (Brenner, 1974).

Generation of transgenic strains

The transgenic strains used in this work are listed in Supplemental Table S3. The sequences of the primers for construction of plasmids used for microinjection are listed in Supplemental Table S4. For expression of target proteins in DTC, the promoter region of lag-2 was amplified by PCR from the C. elegans genomic DNA using the primers of plag-2-PW and plag-2-RV and inserted into the pPD95.79 vector plasmid (a gift from A. Fire, Stanford University), giving rise to pPD95.79_plag-2. The GFP cDNA that was originally inserted in pPD95.79 was replaced with Venus at KpnI and EcoRI sites (pPD95.79_plag-2::Venus).
To construct pmyo-3::gon-1, the cDNA of gon-1 was prepared by RT-PCR using the primers of gon-1-FW and gon-1-RV and inserted into pPD114.95 (a gift from A. Fire) using the BamHI site.

To construct plag-2::Venus used in Figure 1, a fragment coding for SEL-1-derived signal peptide (ss) was amplified by PCR using the primers of SEL-1-ss-FW and SEL-1-ss-RV. The PCR fragment was inserted into plag-2::Venus using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA).

To construct plag-2::C31E10.7::Venus used in Figure 1 and the plag-2::Imp-1::Venus and plag-2::aman-2::Venus used in Supplemental Figure S4, the Venus fragment was inserted into the pFX_VT vector plasmid using the NotI and BglII sites, giving rise to pFX_VT_Venus (Gengyo-Ando et al., 2006). The cDNAs of C31E10.7, Imp-1, and aman-2 were amplified by RT-PCR using the primers of C31E10.7-FW and C31E10.7-RV, Imp-1-FW and Imp-1-RV, and aman-2-FW and aman-2-RV, respectively. C31E10.7 and Imp-1 cDNA fragments lacking the termination codon were digested and inserted into the pFX_VT_Venus vector using the TthI site.

To construct plag-2::Venus::lgg-1 and plag-2::Venus::rab-5 used in Supplemental Figure S4, the cDNAs of lgg-1 and rab-5 were amplified by RT-PCR using the primers of lgg-1-FW and lgg-1-RV, and rab-5-FW and rab-5-RV, respectively. Each cDNA fragment was digested and inserted into the pFX_VT_Venus vector using the NotI site.

To construct plag-2::Venus::rlgg-1 and plag-2::Venus::rab-5 used in Supplemental Figures S6 and S8, the cDNA of the unspliced form of lgg-1 was amplified by PCR using the primers of lgg-1-FW and lgg-1-RV, and inserted into the plasmid pPD95.79_plag-2::Venus using the In-Fusion PCR Cloning Kit. The fragment was inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lmp-1 and plag-2::Venus::aman-2 used in Supplemental Figure S4, the lmp-1 and aman-2 cDNAs were amplified by RT-PCR using the primers of lmp-1-FW and lmp-1-RV, and aman-2-FW and aman-2-RV, respectively. The cDNAs were inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 lacking the nucleotides from 4 to 243 was amplified by RT-PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into pFX_VT using the In-Fusion PCR Cloning Kit. The fragment was inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lsg-1 and plag-2::Venus::lsg-2 used in Supplemental Figures S5 and S8, the cDNA of lsg-1 was amplified by PCR using the primers of lsg-1-FW and lsg-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lexbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lexbp-1 was amplified by PCR using the primers of lexbp-1-FW and lexbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

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To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.

To construct plag-2::Venus::lxbp-1(D81) used in Supplemental Figures S5 and S6, the cDNA of lxbp-1 was amplified by PCR using the primers of lxbp-1-FW and lxbp-1-RV, and inserted into the plasmid pPD95.79_plag-2 using the BamHI site.
Microinjection of the described DNA constructs was performed as previously reported (Mello et al., 1991) with appropriate selection markers. Images were taken with a BX-51 microscope (Olympus, Center Valley, PA) or a confocal laser-scanning microscope LS5M Pascal (Carl Zeiss, Jena, Germany).

RNA interference in C. elegans
The feeding RNA interference was performed as previously described (Timmons et al., 2001). Briefly, the E. coli strains of HT115(DE3) harboring double-strand RNA expression plasmids were plated on NGM medium containing 1 mM isopropyl-ß-D-thiogalactoside and 100 µM ampicillin. At 12 h after platting the bacteria, L1 or L4 larvae were placed on the plates and cultured at 20°C, except for the experiment shown in Supplemental Figure S6C. In Supplemental Figure S6C, the animals were cultured at 12°C to reduce the constitutive activation of xbp-1.

RT-PCR analysis
Total RNAs from the worms and HEK293 cells were extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (5 mg) was used for reverse transcription with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using the oligo-dT primer and amplified with the ExTag polymerase (Takara, Otsu, Japan) using the primers of sp-xbp-1-FW and sp-xbp-1-RV for the nematode xbp-1, sp-XBP1-FW and sp-XBP1-RV for the human XBP1, GAPDH-FW and GAPDH-RV for the human glyceraldehyde-3-phosphate dehydrogenase, and ama-1-FW and ama-1-RV for the nematode ama-1 (Supplemental Table S5). PCR products were separated on 10% polyacrylamide gels, stained with 5 µg/ml ethidium bromide, and photographed.

Transfection of plasmids and siRNA into HEK293 cells
HEK293 cells were cultured in DMEM media supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. Analyses of siRNA-mediated knockdown were performed with ON-TARGETplus SMARTpool l-005779-00 or ON-TARGETplus siCONTROL Non-targeting siRNA (Dharmacon RNAi Technologies, Lafayette, CO) for knockdown of the ADAMTS9 gene or the nontargeting control, respectively. The cells were then transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen) and analyzed at 72 h after the transfection.

Plasmids pCMV_gon-1(sig_GON), pCMV_gon-1(sig_Venus_GON), pCMV_ADAMTS9(sig_Venus_GON), and pCMV_ADAMTS9(sig_GON_Venus) were generated by inserting the cDNAs of gon-1(sig_GON), gon-1(sig_Venus_GON), gon-1(sig_GON_Venus), ADAMTS9(sig_Venus_GON), and ADAMTS9(sig_GON_Venus), respectively, into pEGFP-N3. The cDNAs of ADAMTS9(sig) and ADAMTS9(GON) were prepared by RT-PCR using the primers of ADAMTS9(sig)-FW and ADAMTS9(sig)-RV, and ADAMTS9(GON)-FW and ADAMTS9(GON)-RV, respectively.

To analyze the localization of GON domain fused with Venus, 1.6-µg plasmids of pCMV_gon-1(sig_Venus_GON), pCMV_gon-1(sig_GON_Venus), pCMV_ADAMTS9(sig_Venus_GON), or pCMV_ADAMTS9(sig_GON_Venus) were transfected into cells cultured in two-well chamber slides (Lab-Tek; Nalge Nunc, Rochester, NY) with Lipofectamine 2000.

pCMV_AAT and pCMV_NHK were generated by insertion of the cDNA of AAT or NHK into a plasmid pAcGFP from which AcGFP had been removed (Clontech). AAT and NHK were gifts from Randal J. Kaufman (Sanford-Burnham Medical Research Institute). To construct pCMV_AAT, the AAT cDNA was amplified using the primers of AAT-FW and AAT-RV. To construct pCMV_NHK, the AAT cDNA was amplified using NHK-FW and NHK-RV as primers and pCMV_AAT as a template and fused with the In-Fusion PCR Cloning Kit. Aliquots of 200 ng of plasmid were transfected into the cultured cells in 35-mm dishes with Lipofectamine 2000 and analyzed at 24 h after transfection.

pCMV_AAT-Flag was generated by inserting the cDNA of AAT into pCMV_Flag, which was generated by replacing the AcGFP tag of pAcGFP with the Flag tag. To construct pCMV_AAT-Flag, the AAT cDNA was amplified using AAT-Flag-FW and AAT-Flag-RV as primers. Seventy-two hours after the ADAMTS9 or control siRNA transfection, the cells were transfected with pCMV_AAT. Aliquots of 200 ng of plasmid were transfected into cultured cells in 35-mm dishes with Lipofectamine 2000 and analyzed at 24 h after transfection.

pCMV_PRL-GFP was generated by inserting the signal sequence of PRL into pAcGFP (Voigt et al., 1996). Aliquots of 200 ng of plasmid were transfected into cultured cells in 35-mm dishes with Lipofectamine 2000 and analyzed at 24 h after transfection.

Visualizing the endoplasmic reticulum
To stain the HEK293 cells with an ER-Tracker, the cells were cultured with 100 nM ER-Tracker Blue-White DPX (Invitrogen) in the culture media for 30 min at 37°C with 5% CO2. After washing with phosphate-buffered saline (PBS), the cells were observed by a microscope with a 40x water-immersion objective. Images were taken with a BX-51 microscope (Olympus) with an epiluminescence light source.

VSVG trafficking
To analyze the protein transport from the ER to the Golgi, with or without the GON-domain function, 1.6 µg of VSVG-GFP (a gift from J. Lippincott-Schwartz, National Institutes of Health) and 0.1 µg of pCMV_gon-1(sig_GON) were transfected into the cells cultured in two-well chamber slides (Lab-Tek) with Lipofectamine 2000. Cells were transfected with VSVG-GFP ts045 (a temperature-sensitive mutant of the VSVG protein) and incubated at 40°C for 16 h. To follow VSVG transport through the secretory pathway, the cells were transferred to 32°C, and after 20 min they were fixed with 4% paraformaldehyde and imaged using fluorescence microscopy.

Transmission electron microscopy
Transmission electron microscopy (TEM) analyses of C. elegans were performed as previously described (Maruyama et al., 2005). In brief, the worms were anesthetized in M9 buffer with 8% ethanol for 5 min and then cut into two or three pieces in the primary fixative solution (2% glutaraldehyde, 1% paraformaldehyde, 50 mM sodium cacodylate, 200 mM sucrose, 1 mM MgCl2, pH 7.4). After primary fixation for 2 h at room temperature, the pieces were postfixed in the primary fixative solution with 1% osmium tetroxide and incubated on ice for 2 h. They were then stained en bloc with 2% uranyl acetate for 2 h and dehydrated in a graded ethanol series. After replacement of ethanol with methyl methacrylate, samples were embedded in Rigolac mixture (Tamura and Kushida, 1960). Thin sections were stained with uranyl acetate and lead citrate and subjected to electron microscopic observations using one-slot copper grids precoated with Formvar. The prepared samples were observed with an H-7000 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 keV. TEM analyses of HEK293 cells were performed at the Hanaichi Ultrastructure Research Institute (Okazaki, Japan). Control siRNA–treated cells or ADAMTS9 siRNA–treated cells were fixed with 2% glutaraldehyde in 100 mM phosphate buffer, postfixed in osmium tetroxide at 4°C, dehydrated, and embedded in EPON812.
Ultrathin sections were analyzed using a transmission electron microscope (JEM-1200EX; JOEL, Peabody, MA) at 80 keV.

**Immunostaining**

HEK293 cells were plated on chamber slides ~24 h prior to transfection. Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 2 min. The cells were then blocked with PBS containing 5% bovine serum albumin (BSA). The cells were incubated with primary antibodies for 1 h, washed, incubated with the secondary antibodies conjugated with Alexa 488 or 594 for 1 h, washed, and observed. Images were taken with a BX-51 microscope (Olympus) and a confocal laser-scanning microscope LMS5 Pascal (Carl Zeiss). Antibodies were purchased from the following companies: anti-GM130 (BD Biosciences, San Diego, CA), anti-calnexin (BD Biosciences), anti-KDEL (Enzo Life Sciences, Plymouth, PA), and anti-AAT (Dako, Glostrup, Denmark). Secondary antibodies for immunostaining were obtained from Invitrogen.

Immunostaining of DTC was performed as previously described (Maruyama et al., 2005). In brief, transgenic animals that expressed plag-2::gon-1(sig_Venus_GON) were placed on a subbed slide in 5.0 ml of M9 containing 1 mM levamisole. Heads or tails were cut off using a 25-gauge needle, extruding the gonad and intestine. Cut animals were fixed with 1.0% paraformaldehyde in PBS for 10 min and permeabilized with PBS containing 0.1% Triton X-100 for 3 min. Samples were blocked in PBS containing 2.5% BSA for at least 30 min. Fixed worms were incubated with an anti-HDEL antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Samples were washed three times with PBS containing 0.5% BSA, incubated with a secondary antibody conjugated with Alexa 594 for 1 h, washed, and observed. Images were taken with a confocal laser-scanning microscope LMS5 Pascal (Carl Zeiss).

**Western blotting**

Cell lysates were separated by 10% SDS–PAGE. Proteins were blotted onto a polyvinylidene fluoride membrane in blotting buffer (Bio-Rad, Hercules, CA). After blocking in 1% Blocking Reagent (Amersham-Pharmacia Biotech, GE Healthcare Bio-Sciences, Piscataway, NJ), specific proteins recognized by each antibody were detected using ECL reagents (Pierce, Thermo Fisher Scientific, Rockford, IL) and LAS-3000 mini (FujiFilm, Tokyo, Japan). Tubulin was detected using ECL reagents (Pierce, Thermo Fisher Scientific, Rockford, IL) and LAS-3000 mini (FujiFilm, Tokyo, Japan). AAT(NHK) was detected by an anti-AAT antibody (Dako). Secondary antibodies recognized by each antibody were detected using ECL reagents (Pierce, Thermo Fisher Scientific, Rockford, IL) and LAS-3000 mini (FujiFilm, Tokyo, Japan). Mouse anti-gon-1 antibodies were detected using an anti-mouse secondary antibody (Pierce, Thermo Fisher Scientific, Rockford, IL) and LAS-3000 mini (FujiFilm, Tokyo, Japan).

**Cycloheximide chase experiment**

For analysis of AAT(NHK) degradation, HEK293 cells were transfected with pCMV-NHK. After 24 h, transfected HEK293 cells were treated with 10 μg/ml cycloheximide for 0, 1, and 2 h. Protein degradation was examined by Western blotting with an anti-AAT antibody described earlier. The amounts of the NHK protein were normalized by the α-tubulin expression as an internal control.

**Immunoprecipitation**

Immunoprecipitation was performed by adding protein A Sepharose beads (GE Healthcare, Piscataway, NJ) to the cell extracts, incubating for 1 h in a cold room, and washing with Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) buffer. After precipitation of protein A Sepharose beads, the supernatants were collected in other tubes. Anti–flag M2-agarose beads (Sigma-Aldrich) were then added to the supernatants and incubated for 4 h in a cold room, washed with TBS buffer, and resuspended in the sample buffer for SDS–PAGE.

**Endo H treatment**

Cell extracts were denatured with 1X Glycoprotein Denaturing Buffer at 100°C for 10 min. After adding 1X G5 Reaction Buffer, 500 U of Endo H (New England Biolabs, Ipswich, MA) was added, and the reaction mixture was incubated for 1 h at 37°C. Separation of reaction products was visualized by SDS–PAGE and Western blotting as described.

**Chromophore-assisted light inactivation**

For photoactivation experiments, gon-1(tm3146) mutants expressing plag-2::gon-1(sig_KillerRed_GON) and plag-2::C31E10.7::Venus, plag-2::ssVenus or plag-2::xbp-1(D81)::Venus were exposed to a light-emitting diode array (572 nm) for 0, 2, 5, 10, and 24 h after the L4 stage. Transgenic animals were observed on the slide at the indicated time points, and then they were recovered from the slide to NGM plates at 20°C. The time course of the morphological changes of the ER was observed using the transgenic animals that expressed plag-2::C31E10.7::Venus. We defined an abnormal ER morphology as one that was observed as a bright fluorescent circular structure with a diameter of 1 μm or larger (Figure 4, B and C). Time courses of the accumulation of secretory proteins were observed using the transgenic animals that expressed plag-2::ssVenus. We used an oil-immersion objective with a 100x magnification and recorded the images with a DP30 charge-coupled device camera (Olympus) using a 500-ms exposure time in all of the experiments. We measured the fluorescence intensity using the ImageJ (National Institutes of Health, Bethesda, MA; Figure 4, D and E). The time course of the xbp-1 splicing was observed using the transgenic animals that expressed plag-2::xbp-1(D81)::Venus. We used an oil-immersion objective with a 100x magnification and recorded images using a DP30 camera with a 500-ms exposure time. We measured the fluorescence intensity using ImageJ (Supplemental Figure S9).

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