Silencing of Aberrant Secretory Protein Expression by Disease-Associated Mutations

Elena B. Tikhonova[1], Zemfira N. Karamysheva[2], Gunnar von Heijne[3,4] and Andrey L. Karamyshev[1]

*1 - **Department of Cell Biology and Biochemistry,** Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA*
*2 - **Department of Biological Sciences,** Texas Tech University, Lubbock, TX 79409, USA*
*3 - **Department of Biochemistry and Biophysics,** Stockholm University, SE-106 91 Stockholm, Sweden*
*4 - **Sweden and Science for Life Laboratory Stockholm University,** SE-171 21 Solna, Sweden*

***Correspondence to Andrey L. Karamyshev:**Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, 3601 4th Street, Mail Stop 6540, Lubbock, TX 79430, USA. andrey.karamyshev@ttuhsc.edu*
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Abstract

Signal recognition particle (SRP) recognizes signal sequences of secretory proteins and targets them to the endoplasmic reticulum membrane for translocation. Many human diseases are connected with defects in signal sequences. The current dogma states that the molecular basis of the disease-associated mutations in the secretory proteins is connected with defects in their transport. Here, we demonstrate for several secretory proteins with disease-associated mutations that the molecular mechanism is different from the dogma. Positively charged or helix-breaking mutations in the signal sequence hydrophobic core prevent synthesis of the aberrant proteins and lead to degradation of their mRNAs. The degree of mRNA depletion depends on the location and severity of the mutation in the signal sequence and correlates with inhibition of SRP interaction. Thus, SRP protects secretory protein mRNAs from degradation. The data demonstrate that if disease-associated mutations obstruct SRP interaction, they lead to silencing of the mutated protein expression.

Introduction

Secretory and membrane proteins represent more than a third of all proteins in a cell [1]. During their biogenesis, these proteins are transported to different cellular compartments or secreted outside of the cells. Numerous human diseases are associated with protein transport defects [2–4]. Many secretory proteins are synthesized as precursors with N-terminal signal sequences. When a signal sequence of a secretory protein emerges from the ribosome exit tunnel, it is co-translationally recognized by the signal recognition particle (SRP) [5–9]. Formation of a SRP–ribosome–nascent chain complex leads to its targeting to SRP receptor in the endoplasmic reticulum (ER) membrane [10]. Finally, the nascent peptide with its signal sequence is transferred to the Sec61 translocon and a secretory protein is co-translationally translocated into the ER lumen, the signal sequence is cleaved off by the signal peptidase at the luminal side of the ER membrane, and matured proteins are transported further through Golgi outside the cell. These processes are relatively well studied and have been reviewed in detail [11–14].

Signal sequence recognition by SRP is the first and the most important step in targeting of the SRP–ribosome–nascent chain complex to the ER membrane for translocation. Although signal sequences do not have strong amino acid homology, majority of them have similar features and structural organization: a positively charged N-terminal n-region, a hydrophobic core (h-region), and a C-terminal c-region containing cleavage site for a signal peptidase [15,16] (Fig. 1a). Signal sequence integrity is important for protein targeting and translocation through a membrane in prokaryotes and eukaryotes [17–21]. A number of naturally occurring

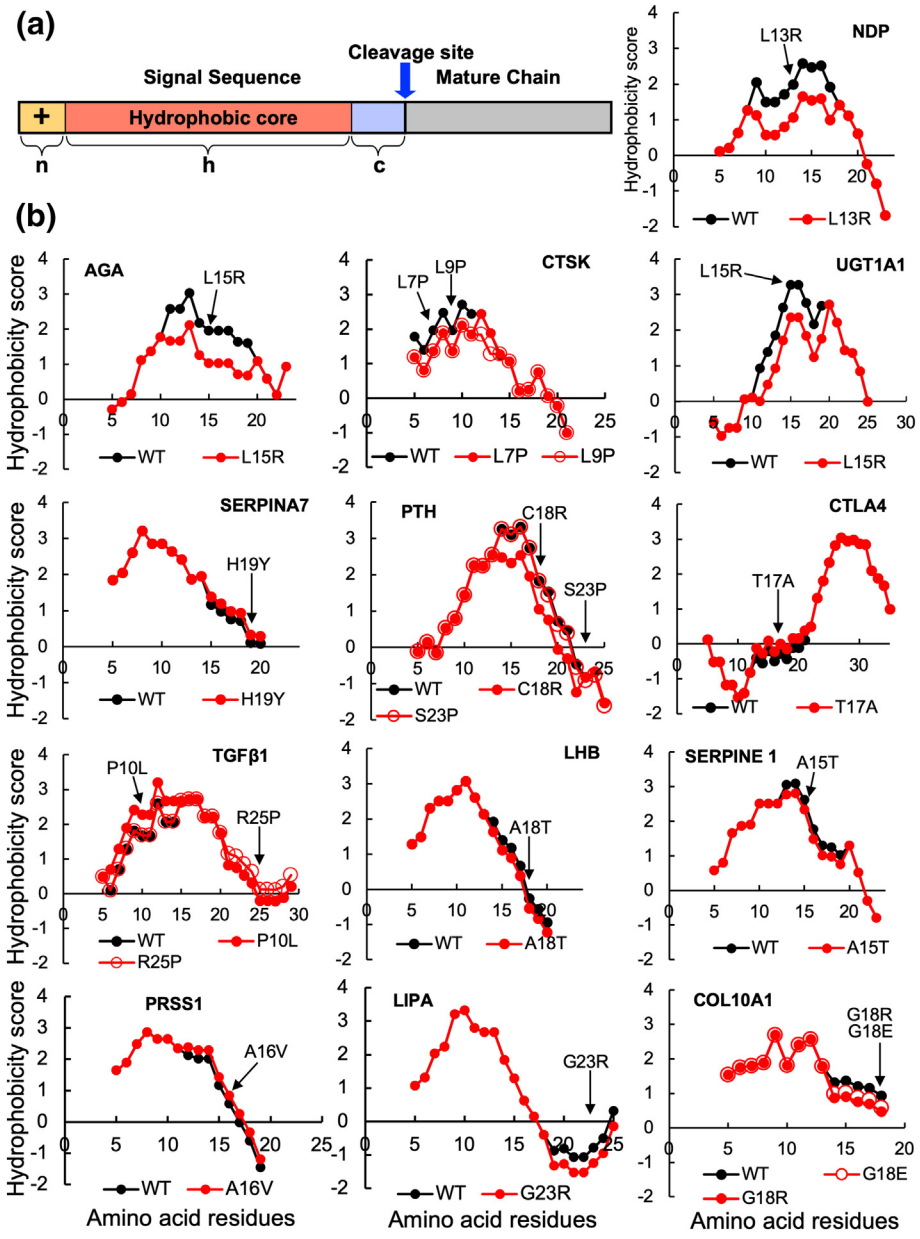

Fig. 1. Signal sequences and aberrations associated with human diseases. (a) Scheme of a signal sequence. Many signal sequences have three regions: N-terminal region, usually contains positively charged amino-acid residues (n-region), stretch of hydrophobic amino acid residues (hydrophobic core, or h-region), and signal sequence cleavage region (c-region). (b) Hydrophobicity plots of the WT and mutated signal sequences of the proteins associated with human diseases (Kyte–Doolittle Scale). Amino acid residue numbers are shown in *X*-axis, and hydrophobicity scores are shown in *Y*-axis. Positions of the disease-associated mutations are marked by arrows. Black symbols indicate WT and red symbols indicate mutants.

mutations in signal sequences have been found, many of them associated with human disease [22–42]. Currently accepted mechanisms for these diseases are connected with defects in the transport or processing of the mutated proteins.

Recently, we demonstrated the existence of a novel mechanism for quality control of secretory proteins, named regulation of aberrant protein production (RAPP) [43]. This is a unique quality control pathway among others—it senses interactions of nascent chains during synthesis and degrades mRNAs of proteins that have lost these important interactions [44]. We demonstrated that the pathway degrades mRNAs of model secretory proteins with dramatic deletions in signal sequences when SRP is not able to recognize the altered signal sequences [43]. Although the existence of the pathway is currently established, many details of the mechanism remain unknown. Only

one natural substrate of the RAPP pathway is currently identified—granulin with mutations in signal sequence [45,46]. Here, we reveal that the RAPP pathway is involved in quality control of many secretory proteins with disease-associated mutations. We demonstrate on the example of a number of secretory proteins with disease-associated mutations in their signal sequences that the molecular mechanism is different from the current dogma and connected with stability of the mutated protein mRNAs instead of protein transport defects. We show that many disease-associated mutations lead to degradation of the mutant protein mRNAs, implicating the RAPP pathway as a molecular mechanism of these diseases.

Results

Mutations in the signal sequences of secretory proteins are associated with human diseases

We conducted a literature search for proteins with disease-associated mutations in their signal sequences. Some of these proteins and disease-associated mutations are presented in Table 1. These proteins and the diseases represent a quite diverse group—the proteins have different functions, molecular weight, and signal sequence length. However, many disease-associated mutations have common features: they are located in the hydrophobic core of the signal sequences and led to significant changes in the sequence properties: substitution of a hydrophobic amino acid residue with positively-charged arginine or with helix-breaking proline. Many of these mutations reduce the hydrophobicity of the signal sequence h-regions (Fig. 1), suggesting inhibition of their recognition by SRP. Some mutations were located in the c-regions of the signal sequence and did not disrupt h-region hydrophobicity. The n-region seems to be the one with the fewest disease-related mutations. Only disease-associated form of the Norrie disease protein (NDP) had a deleted n-terminal part of the signal sequence (Δ11 NDP).

Disease-associated mutations in the signal sequences cause the protein secretion and expression defects

We cloned cDNAs for the proteins shown in Table 1 into a vector for expression in cultured human cells and introduced disease-associated mutations by site-directed mutagenesis. First, we tested the expression of the mutated proteins and picked some of them with significant changes in the signal sequences (introducing Arg or Pro into the hydrophobic core) for these experiments. As shown in Fig. 2 for UDP-glucuronosyltransferase (UGT1A1), cathepsin K (CTSK), and aspartylglucosaminidase (AGA), wild-type (WT) proteins were efficiently expressed, CTSK and AGA were also secreted outside of the cells (UGT1A1 is a membrane associated protein and is not released into the media). Disease-associated mutations led to inhibition of these proteins secretion, and they were not detected in the media. However, the mutated proteins were not detected in the cells either, as shown by Western blot and immunofluorescence, demonstrating that their overall expression was impaired (Fig. 2).

Decrease of mutated protein expression cannot be explained by proteasomal degradation alone

Proteasomal degradation is a major way for removal of defective proteins in the cells. To test if decrease in the mutated protein expression was caused by proteasomal degradation, we conducted experiments in the presence of MG-132, a proteasome inhibitor that was added to the cultivated human cells expressing WT and mutated proteins (Fig. 3). The secretory proteins with mutations in the signal sequences were affected little or not at all. Thus, proteasomal degradation of the aberrant mutated secretory proteins is not the primary reason for their decreased expression.

Disease-associated mutations in the signal sequences hydrophobic core cause decrease of their mRNA levels

Using real-time quantitative PCR (RT-qPCR) we found that mRNA levels of the mutated UGT1A1, CTSK, and AGA were significantly reduced relative to the WT, demonstrating that the protein expression defects were caused by the decrease of their mRNA levels (Fig. 4). We also tested the mRNA levels of other secretory proteins with disease-associated mutations (from Table 1). The mutations cause a wide spectrum of effects on mRNA expression, from very strong defects (mutations in the h-region of the signal sequence) to practically no defects in expression (mostly mutations in the c-regions) (Fig. 4). Interestingly, that even charged amino-acid substitutions in the c-region did not lead to mRNA level decrease [Arg in lipase A (LIPA) and Arg or Glu in collagen 10A1 (COL10A1); Table 1 and Fig. 4].

These observations suggest that integrity of the signal sequence hydrophobic core is important for secretory protein mRNA stability, and that low mRNA expression levels of the proteins with disease-associated mutations in this region may contribute to the corresponding human diseases.

mRNA degradation is responsible for decrease in mRNA expression of the proteins with disease-associated mutations

Reduction in mRNA expression levels may be caused by decrease in mRNA synthesis (transcription) or increase in mRNA degradation. To examine if mRNA degradation of the mutants has increased,

Table 1. Mutations in signal sequences and human diseases

Gene (protein)	Human disease	Mutation	Signal sequence + 2 amino acid residues[a]	Reference
AGA (aspartylglucosaminidase)	Aspartylglucosaminuria	L15R	MARKSNLPVLLVPF**L**LCQALVRCSS	[22]
CTSK (cathepsin K)	Pycnodysostosis	L7P	MWGLKV**L**LLPVVSFALY	[23,24]
		L9P	MWGLKVLL**L**PVVSFALY	
UGT1A1 (UDP-glucuronosyltransferase)	Crigler–Najjar disease	L15R	MAVESQGGRPLVLG**L**LLCVLGPVVSHA	[25]
SERPINA7 (serpin peptidase inhibitor A7)	Thyroxine-binding globulin deficiency	H19Y	MSPFLYLVLLVLGLHATI**H**CAS	[26]
NDP (Norrie disease protein)	Norrie disease	L13R	MRKHVLAASFSM**L**SLLVIMGDTDSK	[27]
		Δ11	MLSLLVIMGDTDSK	
PTH (parathyroid hormone)	Hypoparathyroidism	C18R	MIPAKDMAKVMIVMLAI**C**FLTKSDGKS	[28,29]
		S23P	MIPAKDMAKVMIVMLAICFLTK**S**DGKS	
TGFB1 (TGFβ1, transforming growth factor beta 1)	Renal function decline, osteoporosis, proliferative diabetic retinopathy	P10L	MPPSGLRLL**P**LLLPLLWLLVLTPGRPAAGLS	[37–40]
		R25P	MPPSGLRLLPLLLPLLWLLVLTPG**R**PAAGLS	
CTLA4 (cytotoxic T-lymphocyte associated protein 4)	Autoimmune disease	T17A	MACLGFQRHKAQLNLA**T**RTWPCTLLFFLLFIPVFCKA	[30]
LHB (luteinizing hormone beta polypeptide)	Hypogonadotropic hypogonadism	A18T	MEMLQGLLLLLLLSMGG**A**WASR	[31]
SERPINE1 (serpin peptidase inhibitor E1)	Fibrinolytic bleeding disorder	A15T	MQMSPALTCLVLGL**A**LVFGEGSAVH	[32]
PRSS1 (serine protease 1)	Chronic pancreatitis	A16V	MNPLLILTFVAAALA**A**PF	[33,41]
COL10A1 (collagen type X alpha 1)	Schmid metaphyseal chondrodysplasia	G18R	MLPQIPFLLLVSLNLVH**G**VF	[34,35]
		G18E		
LIPA (lipase A)	Wolman disease	G23R	MKMRFLGLVVCLVLWPLHSEGS**G**GKL	[36]

[a] Signal sequence cleavage sites are underlined, and positions of mutations are in bold font.

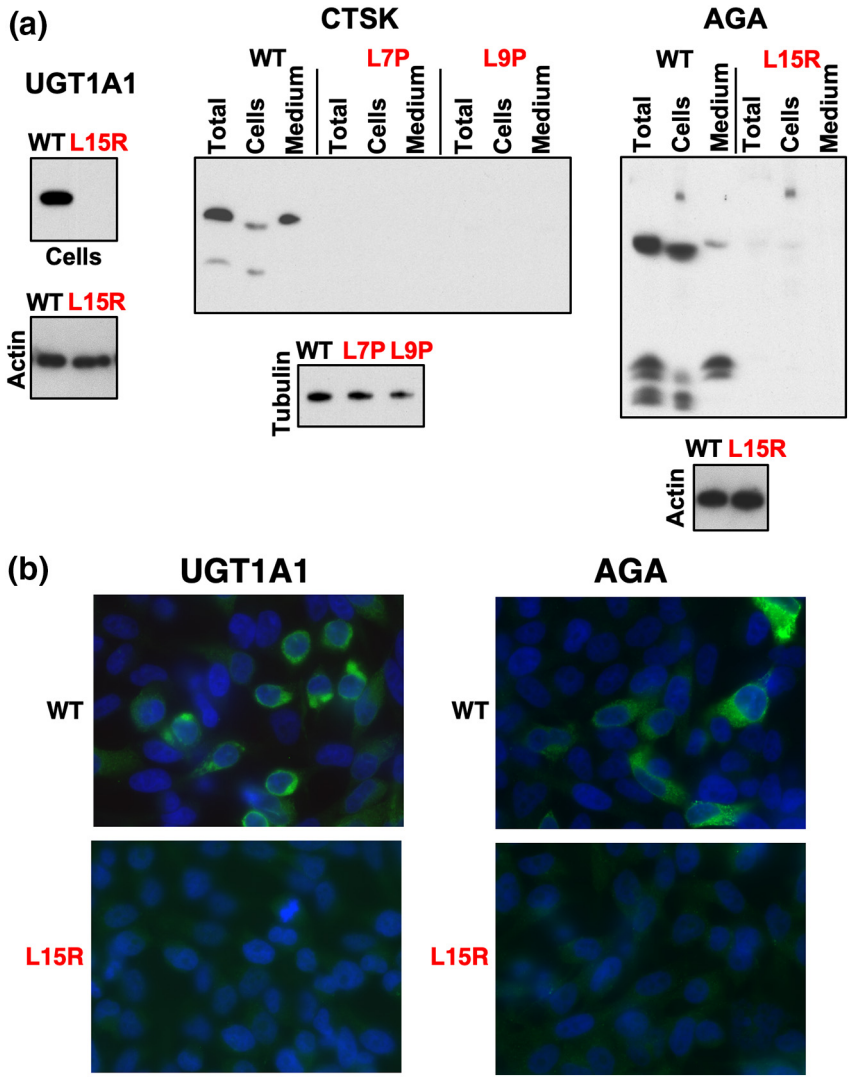

Fig. 2. Clinical mutations in the signal sequences of secretory proteins inhibit protein expression. (a) Western blot analysis of the WT and mutated UGT1A1, CTSK, and AGA, transiently expressed in HeLa Tet-On cells. Expression of the secreted proteins AGA and CTSK was evaluated in the cell lysates, secreted fractions (media), and total samples. Although UGT1A1 is translocated into ER, it is not released into the medium and remained bound through a membrane anchor to a membrane; thus, UGT1A1 was analyzed in the cell lysates only. AGA is observed in several forms on the Western blots because it is cleaved during maturation. Actin and tubulin proteins from the cell lysate samples were used as loading controls. (b) Expression of the WT and mutated UGT1A1 and AGA proteins in HeLa Tet-On cells was analyzed by immunofluorescence (green). Blue is a DAPI staining.

we conducted experiments under conditions when transcription was inhibited by the antibiotic actinomycin D. WT and mutated forms of the secretory proteins were transiently expressed in cultured human cells in the presence of the antibiotic. As it is shown in the Fig. 5a and b, mRNA turnover of the tested mutant proteins with alterations in the h-region was much faster than for the WT forms. However, the mRNA turnover of the LIPA mutant with mutation in the c-region, which did not change mRNA expression level (Fig. 4), was similar to the WT one (Fig. 5c). These data demonstrate that the preferential degradation of the mRNAs of the proteins with mutations in the h-region is implicated in the reduced mRNA expression.

Disease-associated mutations in the signal sequence hydrophobic core inhibit interaction with SRP

SRP recognizes signal sequences when they are exposed from the polypeptide tunnel on the ribosome. The h-region is the most important part of the signal sequence for this interaction [17]. Many disease-associated mutations in the h-region lead to significant changes in the hydrophobicity profiles of the signal sequences (Fig. 1a). This observation suggests that these disease-associated mutations may interfere with SRP interaction. To test this hypothesis, we applied site-specific photo-crosslinking [17,21,47,48] to examine SRP interactions with mutated signal sequences. This technique makes possible to incorporate a photo-crosslinking probe in a specific position in a polypeptide nascent chain during *in vitro* translation (Fig. 6a). In these experiments, the photo-crosslinking probe is incorporated into the nascent chain by a modified εANB-Lys-tRNA^{amb} in response to an amber-stop codon in the mRNA. An amber-stop codon (UAG) was introduced in the positions corresponding to the middle of the signal sequences of the WT and the mutated secretory proteins. Translational intermediates of the WT and mutated forms of the secretory proteins were obtained by translating *in vitro*

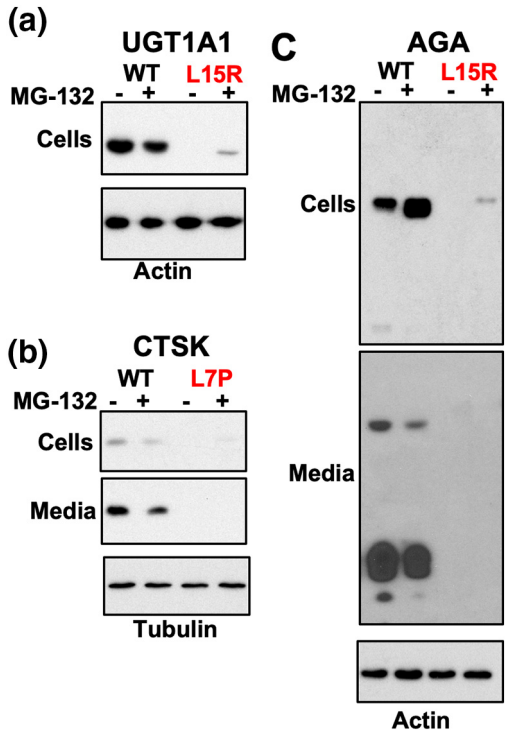

Fig. 3. Inhibition of proteasome does not restore expression of the secretory proteins with disease-associated mutations in the signal sequence. Effect of proteasome inhibitor, MG-132, on WT and mutant proteins expression was studied. HeLa Tet-On cells were transfected with the plasmids expressing WT and mutated CTSK, AGA, and UGT1A1 proteins. Cells were grown for 20 h after plasmid DNA transfection and then treated with 10 μM MG-132 (+) or DMSO (–) for 8 h before samples collection. Expression of UGT1A1 (a) was examined in the cell lysates only (it is not released into the medium), while CTSK (b) and AGA (c) were tested in the cells and media (secreted proteins) by Western blot. Actin and tubulin proteins from the cell lysates were used as loading controls.

truncated mRNAs with an amber-stop codon in the presence of modified εANB-Lys-tRNA^{amb} and [^{35}S] methionine. The mRNAs were truncated to make translational intermediates of the desired length (86-amino-acid residues in these experiments). When the ribosome reaches the end of the truncated mRNA, it does not dissociate but remains bound to the mRNA and the nascent chain, forming a ribosome-mRNA-nascent chain complex. After UV-irradiation, the photo-crosslinking probe form covalent bonds with nearby molecules, and the photo-adducts are analyzed by gel electrophoresis and autography. All tested WT secretory proteins formed photo-adducts with a protein corresponding in size to SRP54, a subunit of SRP (Fig. 6b and c). The quantity of these photo-adducts were increased after addition of purified canine SRP, confirming that the photo-adducts are indeed to the subunit of SRP, as observed by us earlier [17,43]. However, nascent chains with

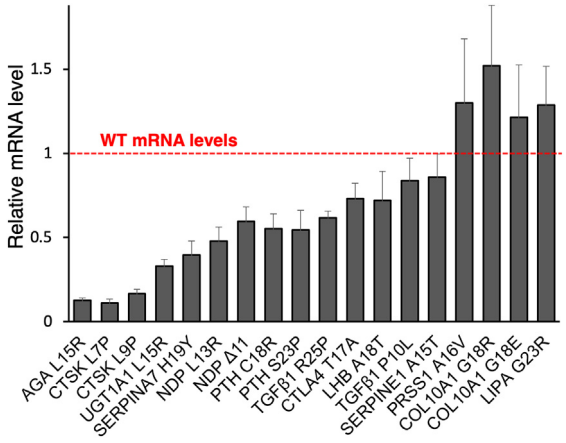

Fig. 4. Mutations in signal sequences of secretory proteins inhibit their mRNA expression. HeLa Tet-On cells were transfected with the plasmids expressing WT and mutated proteins with disease-associated mutations in the signal sequences. mRNA levels were measured by RT-qPCR and shown relatively to corresponding WT mRNA levels (marked by red dash line). The data are presented as mean values with standard errors (n = 3–7).

Arg or Pro in the hydrophobic core of the signal sequences of AGA, CTSK, and UGT1A1 did not form photo-adducts with SRP54 (Fig. 6b). Notably, charged amino acids in the c-region did not prevent SRP54 interaction (Fig. 6c). The efficiency of SRP interactions correlates well with the mRNA expression and stability (Figs. 4 and 5), suggesting that SRP protects mRNAs of secretory proteins from degradation.

Defective SRP leads to mRNA degradation of secretory proteins associated with human diseases

As shown in Fig. 4, the disease-associated mutations lead to the full spectrum of mRNA expression defects—from very strong effects (mRNA degradation) to no change in mRNA levels. The degree of mRNA degradation correlates with the reduced ability of the mutated signal sequence to interact with SRP. These observations suggest pathological activation of RAPP, a protein quality control pathway, as a mechanism for mRNA degradation of the mutated proteins. RAPP is the unique pathway that senses interactions of the nascent chains with SRP at the ribosome during translation and directs mRNAs for degradation if these interactions are disrupted [43,44]. The effects of the mutations inhibiting nascent chain interactions with SRP and inducing mRNA degradation are consistent with this scenario.

What about the other mutant proteins for which mRNA expression was not changed? Are these proteins subjects for the RAPP protein quality control? As we demonstrated earlier, the RAPP pathway may be activated by changes in the substrate for SRP due to mutations in the hydrophobic core of the signal sequence, and by the defects in the binding partner,

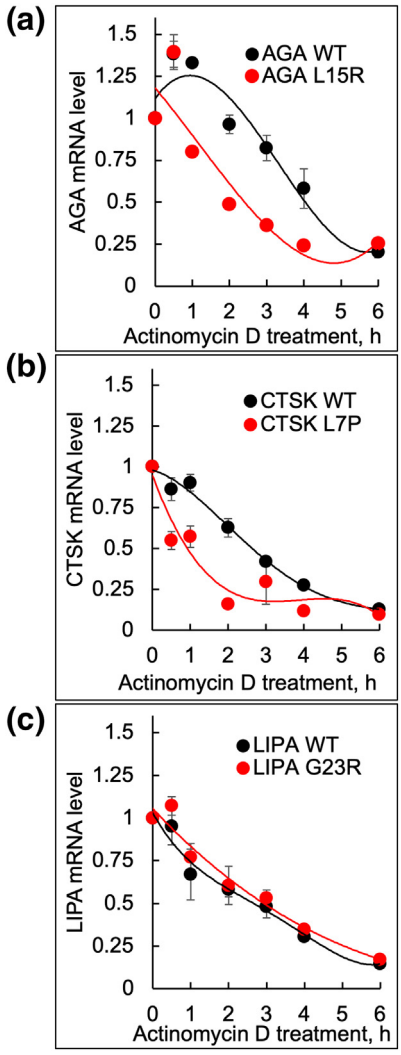

Fig. 5. Decrease of the mRNA expression of the secretory proteins with mutations in the hydrophobic core of the signal sequences is caused by mRNA degradation. Experiments were conducted with AGA (a) and CTSK (b) proteins with mutations in the hydrophobic core (h-region), and LIPA (c) with the mutation in the signal sequence c-region. HeLa Tet-On cells were transfected with plasmids expressing WT and mutated proteins and cultivated for 20 h to ensure that sufficient levels of the mutant mRNAs still remained at the beginning of the experiment (at point "0"). Then actinomycin D, an inhibitor of transcription, was added, and incubation was continued for indicated periods of time. mRNA levels were measured by RT-qPCR at each point and presented relatively to the mRNA level in the point zero for each construct. Error bars are standard errors ($n = 3$). Trendline was used to present dynamic of the mRNA level changes during actinomycin D treatment. Black symbols indicate WT, and red symbols indicate mutants.

SRP, and lead to mRNA degradation [43]. To test the hypothesis that the disease-associated proteins are controlled by the RAPP pathway, we conducted experiments with knockdown of the SRP54 subunit of SRP by the use of siRNA. In the control cells, the secretory proteins were efficiently expressed and secreted into the media (Fig. 7b and c). SRP54 was efficiently depleted by specific siRNA as SRP54 mRNA and the protein were not detected by RT-qPCR, and by Western blot and immunofluorescence, respectively (Fig. 7a, inset; Fig. 7b and c). The mRNAs of the WT forms of all tested secretory proteins were substantially depleted in the cells with SRP54 knockdown (Fig. 7a). The secreted proteins were not detected by Western blot in the media, and the overall expression of the mutated proteins in the cells was dramatically impaired as demonstrated by Western blot and immunofluorescence (Fig. 7b and c). These data strongly indicate that all tested disease-associated secretory proteins are surveyed by the RAPP quality control during translation and are subjected to mRNA elimination in the absence of SRP. However, RAPP does not act on defective proteins with mutations that do not interfere with recognition of the signal sequence by SRP.

In conclusion, our data demonstrate that if disease-associated mutations in secretory proteins obstruct SRP interaction, they lead to silencing of the mutated protein expression through mRNA degradation (Fig. 8).

Discussion

SRP-dependent protein targeting initiates the major pathway for transport of secretory and membrane proteins. The process involves co-translational recognition of signal sequences by SRP. A number of secretory proteins with disease-associated mutations in the signal sequences were examined in this study (Table 1). Defects in the transport of mutant proteins through the secretory pathway are often assumed to underlie the diseases. Here we demonstrate that the molecular mechanism behind the diseases is different from the current dogma for some of the secretory proteins with disease-associated mutations and is connected with mRNA instability instead of protein transport defects. This process is initiated when SRP is not able to interact efficiently with mutated signal sequences at the ribosome. Effects on the SRP interaction play a key role for triggering mRNA degradation of the mutated proteins. Dramatic mutations introducing helix-breaking proline or interfering with hydrophobicity of the h-region (introduction of charged amino acids such as arginine) lead to significant mRNA degradation (Figs. 4 and 5). These mutations inhibit interaction with SRP as we demonstrated by the use of site-specific photo-crosslinking technique (Fig. 6b). However, charged amino acid residues outside of the hydrophobic core of the signal sequence did not have a visible effect on mRNA expression and stability (Figs. 4 and 5) and did not interfere with SRP interaction (Fig. 6c). Analysis of a

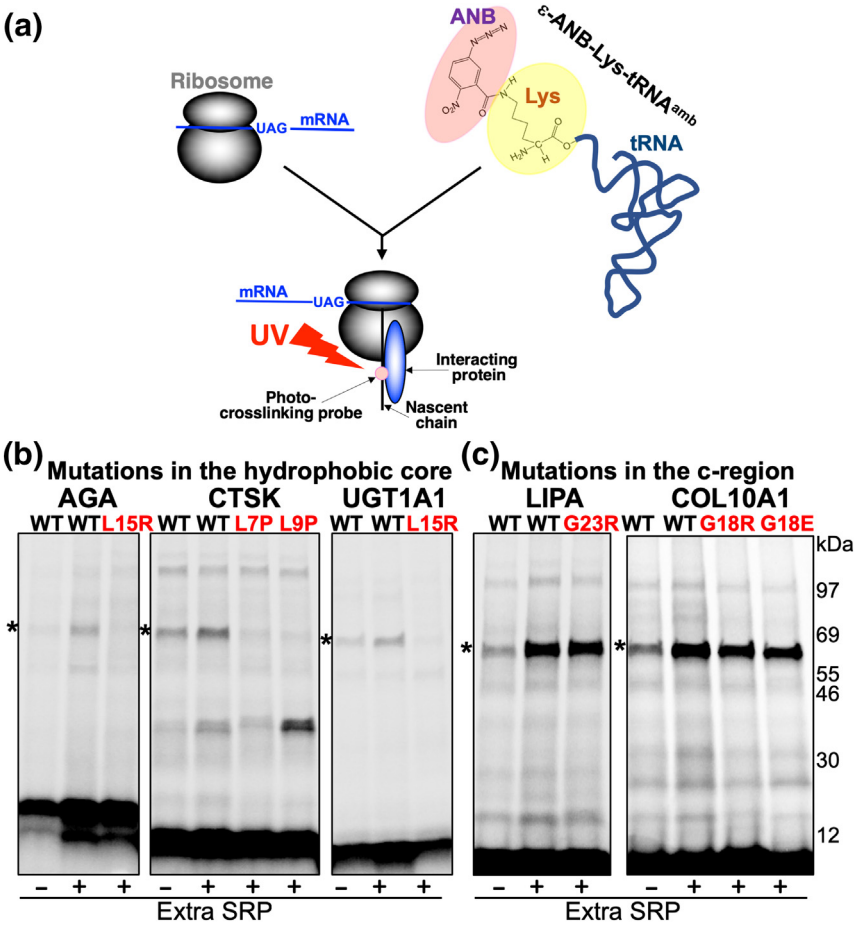

Fig. 6. Mutations in the signal sequence hydrophobic core disrupt interaction with SRP while mutations in the c-region do not. (a) Scheme of the site-specific photo-crosslinking. The technique is based on the tRNA-mediated incorporation of a photo-crosslinking probe into the nascent chains during translation *in vitro*. Modified amber-suppressor εANB-Lys-tRNA^{amb} was used in our experiments. This tRNA incorporates εANB-Lys into position of the nascent chain corresponding to an amber-stop codon in the mRNA. Ribosome–nascent chains are formed by translation of the truncated mRNA *in vitro* in the presence of εANB-Lys-tRNA^{amb}. The length of the nascent chains depends on the length of the truncated mRNAs. When the nascent chains are exposed from the polypeptide tunnel at the ribosome, they interact with their natural partners. Photo-crosslinking probes form covalent bonds to these interacting factors upon UV irradiation. Photo-adducts are detected by the shift in electrophoretic mobility of the nascent chains during electrophoresis and autoradiography. (b, c) Photo-crosslinking of secretory proteins with mutations in the hydrophobic core (b) and in the c-region of the signal sequences (c). Ribosome–nascent chain complexes containing N-terminal 86 residues of the WT and mutant proteins were produced *in vitro* in the presence or absence of extra added purified SRP as indicated. After UV irradiation, the samples were analyzed by electrophoresis and autoradiography. SRP54-photo-adducts are marked by asterisks. Positions of the molecular weight markers are shown.

number of secretory proteins with multiple disease-associated mutations in the signal sequences allowed us to reveal the full spectrum of their effects on the mRNA degradation—from very severe to no effect (Fig. 4). These data suggest the existence of diverse molecular mechanisms for diseases, or even mixture of several mechanisms, where mRNA degradation plays a certain role when targeting by SRP is disrupted by a mutation. We propose that the position of the disease-associated mutations in the signal sequence may lead to different consequences and trigger mRNA degradation if they are located in the signal sequence h-region, or inhibit cleavage of the signal sequence (processing) if they are located in the c-region (Fig. 8).

There are several pathways that monitor mRNA and protein quality at the different levels in response to a number of defects. mRNAs with premature stop codons are removed by nonsense-mediated decay (NMD), translationally stalled and truncated mRNAs are degraded by no-go decay (NGD), mRNAs lacking natural stop codons are detected by non-stop decay (NSD), truncated polypeptides are degraded by the ribosome quality control complex (RQC), and misfolded proteins are removed by several systems including ER-associated

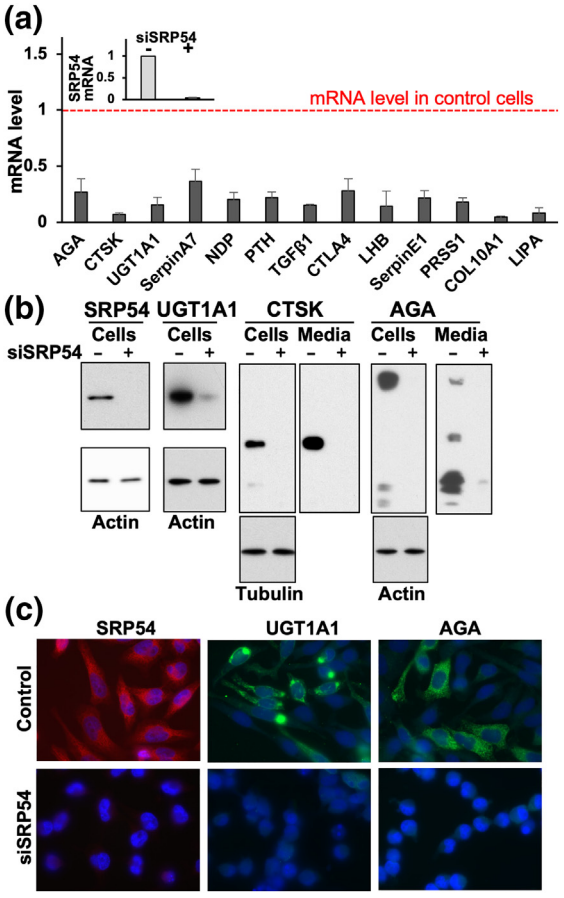

Fig. 7. SRP is required for protection of mRNAs of secretory proteins associated with human diseases from degradation. Effects of SRP54 knockdown on expression of secretory proteins are shown. HeLa Tet-On cells were transfected with siRNA for SRP54 to knockdown SRP54 prior to the second transfection with the plasmids expressing secretory proteins. (a) mRNA levels of the secretory proteins in SRP54 knockdown cells as measured by RT-qPCR and shown as mean values relatively to those in control cells (n = 2–4). Corresponding mRNA level in control cells is marked by red dash line. SRP54 mRNA level in SRP54 knockdown and control cells is shown in inset (n = 14). Errors bars are standard errors. (b) Protein expression of secretory proteins in SRP54 knockdown cells. CTSK and AGA expression was analyzed in the cell lysates and media, and UGT1A1 was tested in the cell lysates only (it is associated with plasma membrane) by Western blot. Actin and tubulin proteins were used as loading controls. SRP54 depletion was confirmed by Western blot. (c) Expression of SRP54 (red), UGT1A1 (green), and AGA (green) in HeLa Tet-On cells was detected by immunofluorescence. Blue is a DAPI staining.

degradation (ERAD), unfolded protein response (UPR), and ubiquitin/proteasome (for review, see Ref. [44]). Recently, we also reported existence of a novel mechanism for protein quality control, RAPP [43], that surveys nascent chain interactions during translation and directs mutated protein mRNAs for degradation. This pathway is the first mechanism that senses mutated proteins and transfers information about aberrant proteins to the mRNA degradation machinery [43,44]. Detailed analysis of the consequences of the disease-associated mutations led us to a conclusion that the mRNA degradation observed is associated with activation of the RAPP pathway. Indeed, there is a strong correlation between the loss of SRP interaction with altered signal sequences and the level of mRNA degradation. This is a major characteristic of RAPP [43,44]. The alternative interpretation by the involvement of other types of mRNA and protein quality controls is unlikely because RAPP is the only known mechanism that examines the status of co-translational interactions of secretory proteins and degrades mRNAs of aberrant proteins with mutations in signal sequences. Thus, RAPP detects the defective proteins and eliminates their mRNAs. Moreover, our data clearly demonstrate that the RAPP pathway is involved in quality control of all tested secretory proteins, as it is demonstrated by the SRP54 depletion experiment (Fig. 7). These observations suggest that RAPP is a general mechanism of protein quality control involved in survey of secretory SRP-dependent proteins, and possibly membrane proteins that use SRP as a targeting factor. However, our results revealed that only mutations in the hydrophobic core of the signal sequence that interfere with SRP interaction triggered the RAPP response. These data demonstrate that in addition to a targeting function, SRP has a certain function in protection of secretory protein mRNAs from the RNA degradation machinery. Our results also demonstrate that the molecular mechanisms of some cases of aspartylglucosaminuria, pycnodystosis, Crigler–Najjar syndrome, thyroxine-binding globulin deficiency, Norrie disease, and probably many other human diseases are associated with pathological RAPP pathway activation.

Among six protein subunits of SRP, SRP54 is the only protein directly involved in the signal sequence recognition. SRP54 has three domains: N-terminal (N), GTP-binding (G), and signal sequence binding (M) domain [9]. Recently, several mutations were identified in SRP54 G domain of the patients with inherited neutropenia and Shwachman–Diamond-like syndrome, indicating that these disease-causing mutations may interfere with its GTPase function [49,50]. Our data suggest that defects in SRP54 may also lead to weakening of the signal sequence binding and potentially lead to human diseases. The mutations should be localized in the SRP54 M domain in that case. Although the disease-causing mutations were not identified in the SRP54 M domain yet, we predict that they will action through the pathological RAPP pathway activation.

Thus, our findings demonstrate that interactions of the polypeptide nascent chains with their natural partners (SRP in this study) during translation are an important step in the protein biogenesis. Disruptions

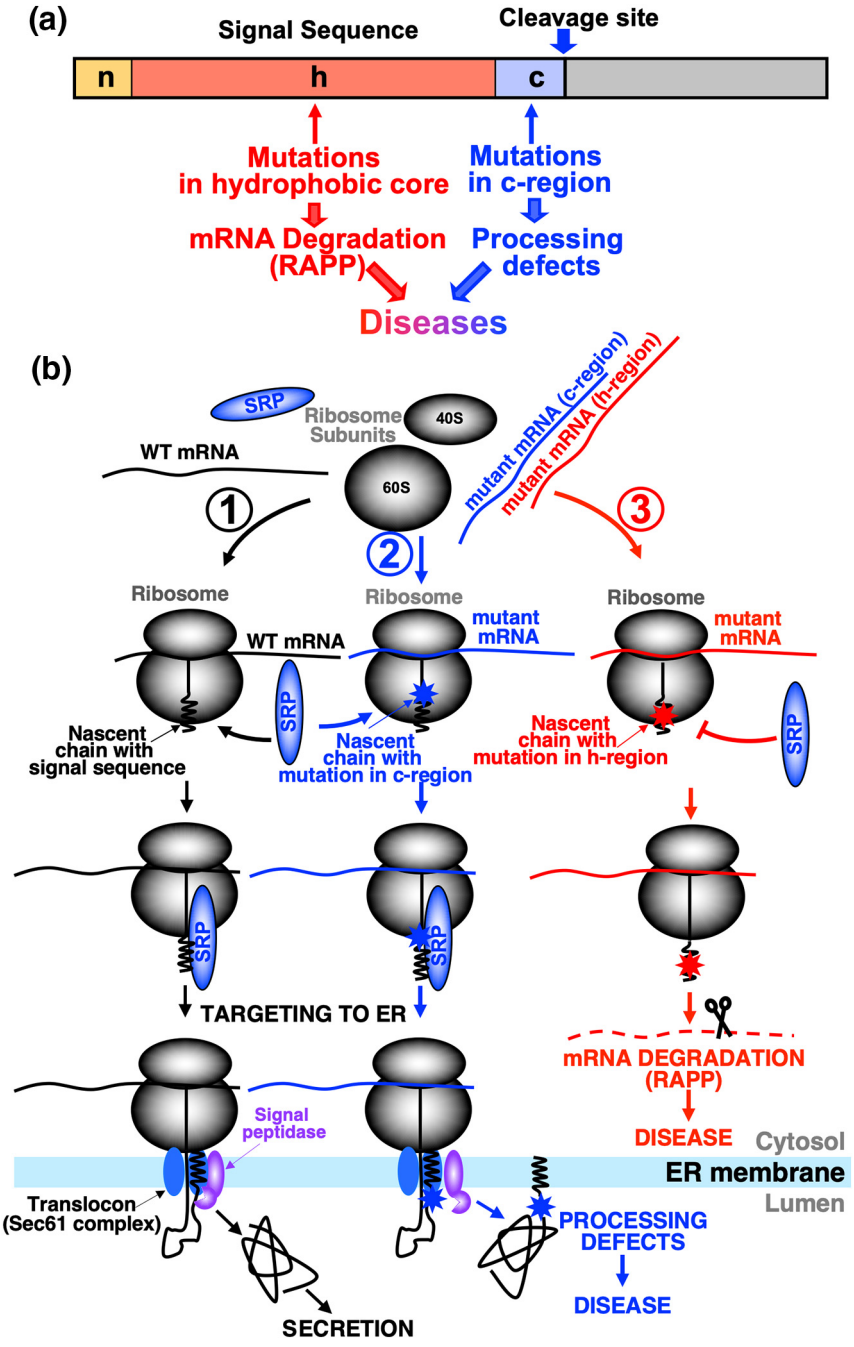

Fig. 8. Signal sequence mutations and possible molecular mechanisms of the associated diseases. (a) Scheme of the signal sequence and location of the disease-associated mutations and their potential effects. (b) Scheme of possible scenarios of biogenesis of the WT (1) and mutated proteins with defects in the c-region (2) and in the hydrophobic core (h-region) (3) of the signal sequences. (1) Normally, secretory proteins start their synthesis at the cytoplasmic ribosomes. SRP recognizes signal sequences when they emerge from the ribosome tunnel during translation. This interaction leads to a normal targeting of the ribosome–nascent chain complex to the ER membrane and finally secretion outside of the cells. (2) When mutations are located in the signal sequence c-region, they usually do not inhibit SRP interaction, and the mutated proteins are targeted to the ER membrane and successfully translocated into the ER lumen. However, often mutations in that region prevent cleavage of the signal sequence by the signal peptidase. If it happens, the mutated protein remains bound to the ER membrane and is not able to be transported further. The molecular basis of these disease-associated mutations is most likely linked to defects in processing. (3) When a mutation in the signal sequence (mostly in h-region) prevents interaction with SRP, ribosome–nascent chain complex is unable to be targeted to ER, and the mRNA of the mutated protein is degraded in the RAPP pathway. Thus, the molecular mechanism of the diseases in that scenario is connected with pathological activation of the RAPP pathway.

of these interactions may pathologically activate the RAPP pathway leading to silencing of the defective protein expression and as a result to a number of human diseases.

Materials and Methods

Cloning, mutagenesis, DNA, and RNA techniques

The clones containing ORFs of the genes *LIPA* (BC012287.1), *COL10A1* (NM_000493.3), *PRSS1* (NM_002769.4), *SERPINE1* (NM_000602.4), *LHB* (NM_000894.2), *TGFB1* (NM_000660.4), *PTH* (NM_000315.2), *NDP* (NM_000266.3), *SERPINA7* (NM_000354.5), *UGT1A1* (NM_000463.2), and *CTSK* (NM_000396.3) were obtained from Life Technologies, and *CTLA4* (NM_005214.4) and *AGA* (BC012392.1) were obtained from Sino Biologicals. All cDNAs were cloned into pCS2 vector under control of the CMV promoter. Site-directed mutagenesis was performed by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) or with Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

All clones and mutations were confirmed by DNA sequencing. Isolation of total RNA from cultured human cells was done using the NucleoSpin RNA kit (Clontech). cDNA samples for real-time quantitative PCR (RT-qPCR) reactions were prepared using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCRs were done on Quant Studio 12 K Flex Real-Time PCR System with using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Comparative $\Delta\Delta C_T$ method was used to quantify the qPCR results [51]. To prepare constructs for *in vitro* transcription, DNA fragments were synthesized using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). PCR products were cleaned using NucleoSpin Gel and PCR Clean-up kit (Clontech). These PCR products were used for *in vitro* transcription by SP6 RNA polymerase. Resulting mRNA products were purified using RNeasy Mini kit (Qiagen) and used for *in vitro* translation and crosslinking experiments. All primers used in this study were synthesized by Sigma-Aldrich, and the sequences are available upon request.

Cell culture and transfection techniques

HeLa Tet-On cell line (Clontech) was used in all experiments. Cells were grown in Dulbecco's modified Eagle's medium–high glucose (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Sigma Aldrich) and penicillin (100 units/mL) and streptomycin (100 μg/mL) mixture (Sigma-Aldrich) at 37 °C with 5% CO_2. siRNAs transfections were done using Lipofectamine RNAiMAX (Invitrogen) as described [43] after cells were cultivated for 16–18 h with starting cell count of 0.5×10^5 cells/mL. The plasmid DNA transfections were done next day after siRNA transfections (where applicable) or after 20–24 h of cell growth with starting cell count of 2×10^5 cells/mL using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. When indicated, plasmid-transfected cells were treated with 10 μM of MG-132 or DMSO (control) for 8 h at 37 °C with 5% CO_2. In experiments with inhibition of transcription, cells were treated with 8 μM of actinomycin D (Sigma-Aldrich) for different periods of time as indicated. Artificial OmpA mRNA [52] was added to the samples of the actinomycin D experiment before total RNA purification to use it for normalization in RT-qPCR.

Western blotting

Expression levels of secretory proteins were tested in cells, media, and total samples.

Cytoplasmic or membrane-associated proteins were analyzed in cells only. Proteins were separated on SDS-PAGE, transferred to PVDF membrane, and detected by standard Western blotting techniques. The antibodies used in this study were as follows: anti-SRP54 (mouse monoclonal, catalog number 610940) was from BD Bioscience; β-actin (mouse monoclonal, catalog number 66009-1-Ig), anti-AGA (rabbit polyclonal, catalog number 17299-1-AP), anti-CTSK (rabbit polyclonal, catalog number 11239-1-AP), and anti-UGT1A1 (rabbit polyclonal, catalog number 23495-1-AP) were from Proteintech Group Inc.; peroxidase-conjugated goat anti-mouse were from Jackson ImmunoResearch Laboratories); and ECL anti-rabbit IgG and horseradish peroxidase-linked whole antibody from donkey were from GE Healthcare (catalog number NA934V). Anti-tubulin rabbit polyclonal antibody was a gift from Dan Webster (TTUHSC).

Immunostaining and microscopy

HeLa Tet-On cells were grown on glass cover slips, transfected with siSRP54 RNA (where needed) and then with plasmid DNA carrying WT or mutant cDNAs. The cells were grown for 40–46 h, then fixed in 4% paraformaldehyde solution, washed in Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich) and treated with permeabilization buffer containing 0.2% Triton X-100, 3% BSA, and DPBS for 20 min at 4 °C. Permeabilized cells were incubated with the primary antibody in the same buffer for 1 h at room temperature and washed once in 0.2% Triton X-100, then three times with DPBS for 5 min. Incubation with the secondary antibody was done in 0.2% Triton X-100, 3% BSA, and DPBS at room temperature for 30 min in the dark. After wash, cells were mounted on the slide with ProLong Gold antifade reagent with DAPI (Life Technologies) overnight. Alexa Fluor 488 Goat anti-Rabbit IgG (Invitrogen, catalog number A11008) or Alexa Fluor 555 Goat anti-mouse IgG (Life Technologies, catalog number A21422) were used as a secondary antibody for AGA and UGT1A1 or SRP54 correspondingly. Zeiss Axiovert 200 M Microscope (TTUHSC Imaging Center) was used to collect the images.

In vitro translation and site-specific photocrosslinking

Site-specific photocrosslinking technique used in the study was based on tRNA-mediated incorporation of photo-reactive probes into the polypeptide nascent chains during *in vitro* translation [21,47,48]. Modified amber-suppressor εANB-Lys-$tRNA^{amb}$ was used for incorporation of the photo-probe into polypeptide position corresponding to amber-stop mutation in mRNA (Fig. 6a). Amber mutations were introduced by site-directed mutagenesis into positions corresponding to A19 in AGA; S13 in CTSK, P10 in UGT1A1; H18 in LIPA and N14 in COL10A1 (see amino acid sequences of the signal sequences in Table 1).

Truncated mRNAs were prepared from PCR fragments by SP6 RNA polymerase and purified as described above. The mRNAs were truncated in the ORF and did not contain natural stop-codons, which allowed to prepare ribosome–nascent chain complexes (or translational intermediates) with desired length of the polypeptide nascent-chains. Translational intermediates (86 amino acid residues long in this study) were synthesized in 15 μL of reaction containing rabbit reticulocyte lysate (Green Hectares, LLC), 2.6 mM magnesium acetate, 70 mM potassium acetate, 0.8 U/μL RNasin (Promega), 0.8 μCi/μL [35S] methionine, and 1 μg of mRNA and 15 pmol of εANB-Lys-tRNA^{amb} (tRNA Probes, LLC). Purified canine SRP (40 nM; tRNA Probes, LLC) was added where indicated. Samples were incubated for 40 min at 26 °C in the dark and then subjected to UV irradiation for 15 min on ice (Newport Oriel, 500-W mercury arc lamp). Ribosome–nascent chain complexes were pelleted by centrifugation, treated with RNase A (Sigma-Aldrich), and analyzed as described [43]. Typhoon FLA 9000 Biomolecular Imager was used for phosphorimaging of the gels. [Methyl-^{14}C] methylated protein molecular weight markers (PerkinElmer) were used to estimate molecular weight of the photo-adducts.

Hydrophobicity of the signal sequences

Hydrophobicity plots were calculated by the Kyte and Doolittle method [53] using the ExPASy server [54] (https://web.expasy.org/protscale/).

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Author contributions: A.L.K. and Z.N.K. developed principal concept, and conducted literature review and experimental design. E.B.T. designed and performed the experiments. G.v.H. provided expertise on signal sequences, role of mutations in targeting, and mechanism of protein transport. A.L.K. planned and coordinated the study. A.L.K. and E.B.T. wrote the manuscript. All authors participated in the data analysis, discussed, and edited the manuscript.

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Abbreviations used:
SRP, signal recognition particle; ER, endoplasmic reticulum; RAPP, regulation of aberrant protein production; WT, wild-type; εANB-Lys-tRNA^{amb}, N^{ε}-(5-azido-2 nitrobenzoyl)-Lys-tRNA^{amb}.

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