

Inefficient SRP Interaction with a Nascent Chain Triggers a mRNA Quality Control Pathway

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SUMMARY

Misfolded proteins are often cytotoxic, unless cellular systems prevent their accumulation. Data presented here uncover a mechanism by which defects in secretory proteins lead to a dramatic reduction in their mRNAs and protein expression. When mutant signal sequences fail to bind to the signal recognition particle (SRP) at the ribosome exit site, the nascent chain instead contacts Argonaute2 (Ago2), and the mutant mRNAs are specifically degraded. Severity of signal sequence mutations correlated with increased proximity of Ago2 to nascent chain and mRNA degradation. Ago2 knockdown inhibited degradation of the mutant mRNA, while overexpression of Ago2 or knockdown of SRP54 promoted degradation of secretory protein mRNA. The results reveal a previously unappreciated general mechanism of translational quality control, in which specific mRNA degradation preemptively regulates aberrant protein production (RAPP).

INTRODUCTION

The cytotoxicity of misfolded and mistargeted proteins is countered by cellular systems that prevent their accumulation (Stefani and Dobson, 2003). Cells have strict quality-control systems in the cytosol and in the endoplasmic reticulum (ER) to distinguish properly folded proteins from misfolded (Buchberger et al.,

2010; Ellgaard and Helenius, 2003). Secretory and membrane proteins that are not transported to their proper cellular destinations are either degraded by the ER associated degradation (ERAD) pathway (Brodsky, 2012; Smith et al., 2011) or form inclusions (Kopito, 2000). When proteins are successfully translocated from the cytosol to the ER, but are not transported further due to misfolding in stress conditions, the unfolded protein response (UPR) is activated. UPR involves activation of stress response genes, general downregulation of protein synthesis, and degradation of misfolded ER proteins (Schröder and Kaufman, 2005; Walter and Ron, 2011). Another mechanism that prevents accumulation of misfolded proteins in the ER during UPR is regulated ire1-dependent decay (RID) (Hollien et al., 2009; Hollien and Weissman, 2006). This mechanism is activated under conditions of general ER stress and reduces the quantity of many mRNAs that encode secretory proteins. Thus, these processes are general responses to stress conditions and have not evolved to specifically triage an individual mutant protein that misfolds. Whether cells can specifically prevent the synthesis of “unwanted,” and potentially hazardous proteins derived from mutated or foreign mRNAs is only poorly understood. If mutant proteins do not fold properly or fail to be delivered to the appropriate cellular compartment, they can be removed by the proteasome system (Ward et al., 1995). Such a posttranslational mechanism, however, does not prevent continued de novo production of the mutant gene product. Currently known systems of mRNA quality control, nonsense mediated decay (NMD), nonstop decay (NSD), and no-go decay (NGD), detect only profound defects, including premature stop codons, lack of a natural stop codon, or stalled ribosomes (Doma and Parker, 2007; Shoemaker and Green, 2012). These

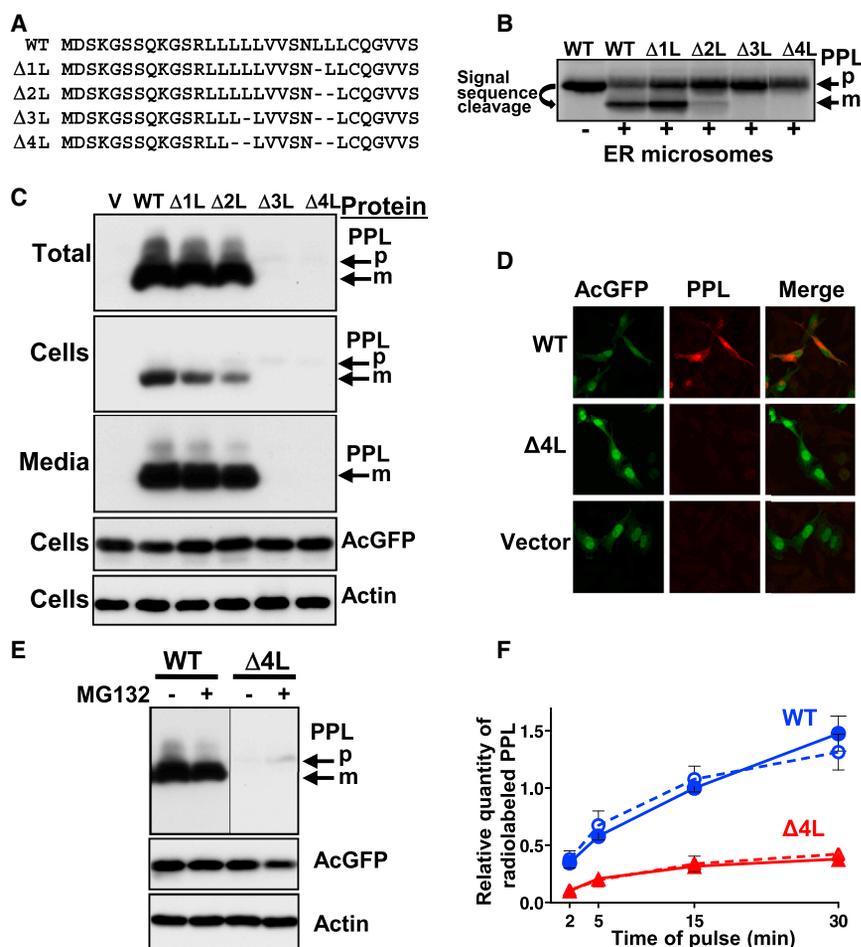


Figure 1. Deletions in the PPL Signal Sequence Lead to Defects in Protein Transport and Expression

(A) Signal sequences of WT and mutated PPLs, deletions indicated by dashes.

(B) WT and mutant PPLs were synthesized in a rabbit reticulocyte translation system *in vitro* in the presence or absence of ER microsomes, analyzed by SDS-PAGE, and detected by autoradiography. Positions of mature PPL (m) and precursor (p) are shown.

(C and D) WT and mutant PPLs were transiently expressed in HeLa Tet-On cells (cells with empty vector [V] were controls) and detected by western blot or by immunofluorescence, respectively. AcGFP, expressed from the same plasmids, and endogenous actin were controls.

(E) Effect of the proteasome inhibitor MG132 on the level of WT and Δ4L PPLs (detection by western blot).

(F) Pulse-labeling analysis of translated WT (blue circles) and Δ4L (red triangles) PPLs in the presence (dashed lines, $n = 4$) and absence (solid lines, $n = 5$) of MG132 (mean \pm SEM). See also Figure S1.

quality control mechanisms are not known to involve sensors that detect misfolded nascent chains or target mRNA templates whose products are not properly folded/targeted because of missense mutations.

We hypothesized that when an individual protein misfolds or fails to be correctly sorted to the appropriate compartment due to alterations in its protein sequence, processes that specifically reduce its translation could provide an incisive means of reducing potential toxicity caused by the accumulation of misfolded conformers. Here, we demonstrate the existence of such a mechanism for specific mRNA degradation in response to the synthesis of defective proteins.

RESULTS

Mutations in the Signal Sequence of a Secretory Protein Cause a Defect in Protein Transport and Lead to Decreased Production of Mutant Proteins

Secretory proteins contain N-terminal signal sequences (von Heijne, 1983). The signal sequence is cotranslationally recognized by SRP (Walter et al., 1981). The ribosome-nascent chain-SRP complex is targeted to its receptor in the ER membrane where the nascent chain is translocated into ER lumen

PPL was efficiently translocated into ER microsomes as detected by processing of the precursor (signal sequence cleavage) (Figure 1B). Deletion of leucines from the signal sequence reduced translocation, with the number of deleted leucines directly correlating with the degree of the transport defect.

WT PPL expressed in cell culture was efficiently secreted, reflected by the presence of its mature form in the cells and media (Figure 1C). However, the Δ3L and Δ4L mutants did not mature and their protein levels were dramatically decreased. The decrease directly correlated with the number of deleted leucines. In contrast to WT PPL, the Δ4L mutant was not detected by immunocytochemistry (Figure 1D). Notably, levels of AcGFP expressed from an independent promoter on the same plasmid, and of endogenous actin, were not affected (Figures 1C and 1D). Thus, mutations in the signal sequence lead to specific reductions in the quantities of the mutant proteins. The simplest explanation is that mutant proteins are degraded by the proteasome. However, inhibition of the proteasome with MG132 produced only a marginal change (Figure 1E). Hence, reduction in the level of the proteins cannot be explained by proteasomal degradation. Alternatively, the decrease in mutant protein levels could be caused by a reduction in their translation. Pulse-labeling of WT and Δ4L proteins showed that translation of the mutated protein

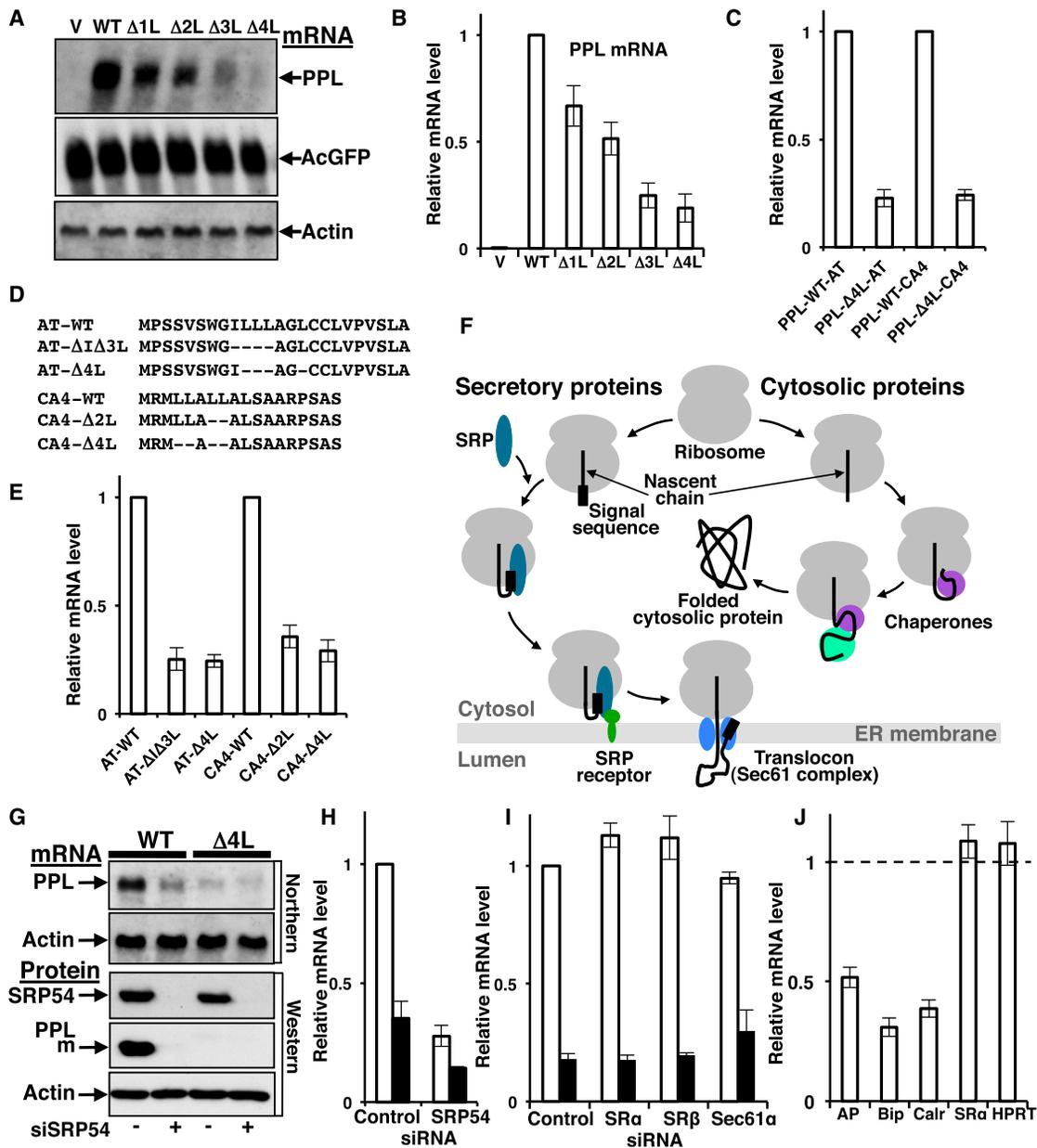


Figure 2. Defects in the Signal Sequence or in a Targeting Factor, SRP, Lead to Decreased Levels of a Secretory Protein mRNA

(A and B) Effect of deletions in signal sequence on PPL mRNA levels. PPL and control mRNAs were analyzed by northern blot (A) or qPCR (B; n = 9, mean \pm SD) 42 hr after transfection of HeLa Tet-On with WT and mutated PPL plasmids.

(C) Presence of a mutated PPL signal sequence (PPL- $\Delta 4L$) in hybrid proteins containing the mature part of $\alpha 1$ -antitrypsin (AT) or carbonic anhydrase IV (CA4) is sufficient to trigger mRNA depletion. mRNA levels measured by qPCR (n = 3, mean \pm SD) are shown relatively to mRNA levels of corresponding hybrid proteins containing WT PPL signal sequence (PPL-WT).

(D) Deletions (indicated by dashes) in the natural signal sequences of secretory proteins AT or CA4.

(E) Deletions in the hydrophobic core of natural signal sequences of AT or CA4 proteins lead to a decrease in their mRNA levels. Graph shows mRNA levels measured by qPCR (n = 3, mean \pm SD).

(F) Scheme shows differences in biogenesis of secretory and cytosolic proteins. When the signal sequence of a secretory protein emerges from the ribosomal tunnel it is recognized by SRP, and the complex is targeted to SRP receptor and finally to a translocon in ER membrane. Nascent chains of cytosolic proteins do not have signal sequences, however, their nascent chains are recognized by ribosome-associated chaperones and that help them fold in the cytosol.

(G and H) SRP depletion causes a reduction in the level of secretory protein mRNA. Detection of PPL, and actin mRNAs (northern blot), SRP54, PPL and actin proteins (western blot) in HeLa Tet-On cells transfected with siRNA for SRP54 and WT or $\Delta 4L$ PPL plasmids as indicated (G). Quantification of WT PPL (open bars) or $\Delta 4L$ mutant (black bars) mRNAs by qPCR in independent sets of SRP54 knockdown experiments (n = 3, mean \pm SD) (H).

(I) Knockdown of SRP receptor subunits SR α and SR β , or translocon component, Sec61 α , does not affect PPL mRNA level (qPCR, n = 3, mean \pm SD). WT PPL (open bars), $\Delta 4L$ mutant (black bars).

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was dramatically impaired while turnover was not affected (Figures 1F and S1 available online), suggesting that the lower level of mutant translation may be caused by reduction in mutant mRNA.

Deletions in the Signal Sequence of Secretory Proteins Cause a Reduction in Their mRNA Levels

Using two independent techniques, northern blot and quantitative real-time PCR (qPCR), we found that levels of PPL mRNA decreased as the number of deleted leucines in the signal sequence increased (Figures 2A and 2B). This effect was specific for mutant PPLs, as neither AcGFP nor actin mRNAs levels changed (Figure 2A). The same effect was observed in two other cell lines (Figure S2), indicating that the process of mutant mRNA elimination is independent of the cell type. We also constructed hybrid proteins containing WT PPL or mutated PPL Δ 4L signal sequences fused to mature sequences of two other secretory proteins, α 1-antitrypsin (AT) or carbonic anhydrase IV (CA4), and measured their mRNA levels (Figure 2C). The mRNA levels of the hybrid proteins with mutated signal sequences were reduced, indicating that the presence of aberrant signal sequence was sufficient to trigger mRNA depletion regardless of the sequence of the mature secretory protein. These data suggested the existence of a specific mechanism for decreasing mutant mRNA levels.

To establish generality of the mechanism we made deletions in the natural signal sequences of AT and CA4 and found that their mRNA levels were also reduced (Figures 2D–2E), similar to the reductions of mRNA levels of mutated PPL and hybrid proteins (Figures 2B–2C). These results indicate that the effect of mRNA reduction is determined by the defects in the translated signal sequences independently of their amino acid sequences, making the scenario of reduction of mRNA levels due to changes in mRNA secondary structure unlikely. These data demonstrate that the mRNA-depletion mechanism is general and selective.

SRP Depletion Leads to a Decrease in the mRNA Levels of Secretory Proteins

Secretory and cytosolic proteins have different biogenesis pathways. While cytosolic proteins require different ribosome associated chaperones and chaperonins for their proper folding in the cytoplasm, for a secretory protein, the first step in protein transport is recognition of its signal sequence by SRP and formation of the SRP-RNC (ribosome-nascent chain) complex (Figure 2F). Since mRNA levels were reduced by mutations that were predicted to alter signal sequence-SRP interactions, the role of SRP in the mRNA quality control process was independently evaluated by decreasing its intracellular concentration. siRNA was used to efficiently decrease SRP in human cell culture. SRP depletion caused a dramatic reduction in mRNA levels (as detected by northern blot and qPCR) and protein levels of both WT and mutant PPLs (Figures 2G–2H). In the next step of biogenesis the SRP-RNC complex is targeted to the SRP receptor

at the ER membrane, then the RNC is transferred to a translocon and the nascent chain is cotranslationally translocated into the ER lumen, where it is folded with the help of ER chaperones (Figure 2F). Contrary to SRP depletion, no effect on WT or Δ 4L mRNA was found when SRP receptor subunits SR α and SR β or the core component of the translocon, Sec61 α , were depleted (Figure 2I). The data clearly demonstrate that mRNA depletion of a secretory protein occurs when its nascent chain loses interaction with SRP, suggesting that selection of mRNA for degradation takes place at the ribosome during translation, when the N-terminal portion of a mutated nascent chain exits the ribosomal tunnel but is not recognized by SRP.

To address the fate of mRNAs of other potential substrates for SRP, endogenous mRNA levels of secretory and ER resident proteins were measured in SRP depleted cells. mRNA levels of the secretory proteins alkaline phosphatase, as well as ER localized proteins Bip and calreticulin, were dramatically reduced in SRP54 depleted cells (Figure 2J). All of these proteins have signal sequences and are transported from the cytosol. By contrast, mRNA levels of the nonsecreted cytosolic protein hypoxanthine phosphoribosyltransferase (HPRT) and the SRP-independent ER membrane-associated protein SR α (Andrews et al., 1989; Lakkaraju et al., 2007) were not affected (Figure 2J). The data demonstrate that the loss of targeting factor SRP leads to specific elimination of the mRNAs coding for its substrates. Thus, mRNA depletion correlates with an abnormality in the signal sequence recognition process, including either a mutation in the signal sequence or a decrease in the amount of functional SRP.

Decrease in mRNA Level Is Due to mRNA Degradation

Reduction of steady state mRNA levels could be achieved by either reducing transcription or increasing mRNA degradation. To test whether mutant mRNA degradation was increased, mRNA levels were monitored as a function of time after transcriptional inhibition (Figure 3A). WT and mutant PPL were cloned into the pTRE2hyg vector. This vector contains the inducible Tet-response element and a minimal CMV promoter. In HeLa Tet-Off cells cultured in the absence of the antibiotic, the mRNA is transcribed normally, while the addition of doxycycline inhibits transcription. Doxycycline was added 20 hr after transfection to ensure that adequate levels of Δ 4L mRNA still remained at the start of the experiment (Figure 3A, inset). The turnover of WT PPL mRNA (observed by qPCR) as a function of time in doxycycline-treated (versus untreated) cells indicates the normal rate of mRNA degradation (Figure 3A). The Δ 4L PPL mRNA turned over significantly faster than WT PPL mRNA, indicating that the mutant mRNA was preferentially degraded.

The Mechanism Discriminates Mutant from Wild-Type mRNAs

To determine whether the mutant protein induces a general cellular response that acts on both mRNAs or acts specifically

(J) SRP depletion causes reduction in endogenous mRNA levels of secretory and ER proteins. Measured by qPCR (n = 3, mean \pm SD) mRNA levels in SRP depleted cells are shown relatively those treated with control siRNA (taken as 1). Intestinal alkaline phosphatase (AP) is a secretory protein, Bip and calreticulin (Calr) are ER lumen proteins. mRNA levels of the cytosolic protein HPRT and the SRP-independent ER membrane-associated protein SR α were not altered. See also Figure S2 and Table S1.

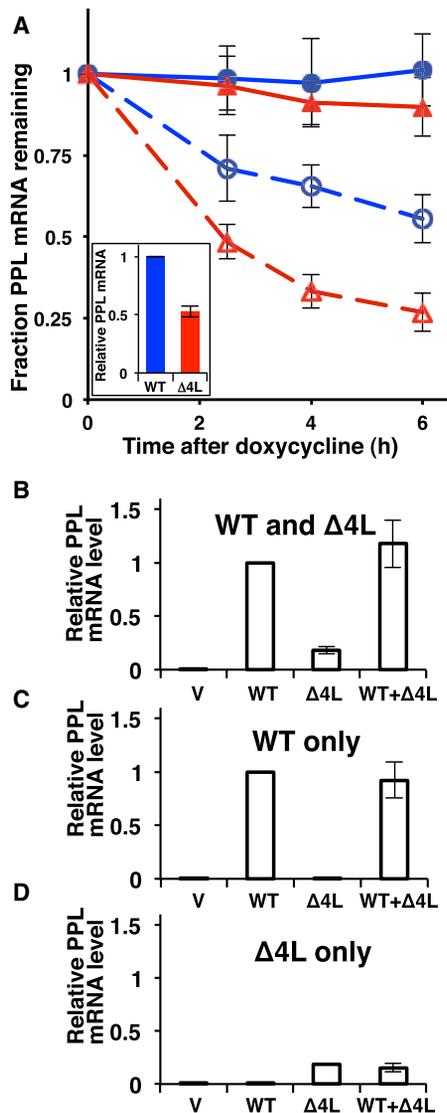


Figure 3. Preferential Degradation of Mutant PPL mRNA

(A) qPCR analysis of WT (blue circles) or Δ4L (red triangles) PPL mRNAs under conditions of transcription inhibition (doxycycline treatment, dashed lines and open symbols) or mock (solid lines and symbols). Doxycycline was added 20 hr after transfection to ensure that adequate levels of Δ4L mRNA still remained at the start of the experiment; the initial WT and Δ4L mutant mRNA content was about 2:1 (inset). The quantity of mRNA at each time point is shown relative to the initial quantity of the respective mRNA species. Data are from three experiments, where each time point contained three repeats in each experiment (mean \pm SD).

(B to D) qPCR analysis ($n = 5$, mean \pm SD) of PPL WT and Δ4L mRNAs using primers amplifying both WT and mutant (B), only WT (C), or only Δ4L (D) in the cultured human cells expressing WT PPL only (WT), Δ4L mutant only (Δ4L), both WT and Δ4L (WT+Δ4L), or transfected with empty vector (V). mRNA levels are shown relative to WT, except in (D), where mRNA levels were scaled to equate the Δ4L level in (D) to that in (B).

See also Figure S3.

on the mutant mRNA, WT and Δ4L PPLs were coexpressed. Protein levels were detected by western blotting (Figure S3) and mRNA levels were determined by qPCR using primers to detect both WT and Δ4L, or specifically the WT or the mutant (Figures 3B–3D). The total amount of PPL message in cells coexpressing mutant and WT was equivalent to the sum of the two mRNAs expressed independently. Thus, the presence of WT protein and mRNA did not prevent degradation of mutant mRNA (Figure 3D), nor did the presence of mutant mRNA lead to increased degradation of WT mRNA (Figure 3C). These data indicate that the cellular machinery discriminates between WT and mutant mRNAs and specifically targets mutant mRNA for degradation.

A Functional Signal Sequence Protects mRNA of a Secretory Protein from Degradation

The results of the SRP knockdown experiment on endogenous transcripts suggest that the mechanism does not involve direct recognition of the abnormal mutant mRNA structures or sequences. Instead, the data suggest a link between the translation of abnormal proteins and the degradation of their mRNA. To directly test this hypothesis, PPL constructs were analyzed that contained a deletion of one, two, or three nucleotides at the beginning of the signal sequence to create either frame-shifts in the open reading frame or the deletion of a codon (Figure 4A). The deletions of one or two nucleotides led to dramatic changes in the protein sequence because of a frame shift in the reading frame and only minor changes (removing one or two nucleotides) in mRNA. By contrast, the deletion of three nucleotides leads to larger changes in mRNA and smaller changes (deletion of one residue) in the protein. As predicted, the mRNA levels of frame-shift mutants were depressed, while the level of mRNA with a deleted codon was not affected (Figures 4B–4C). These results indicate that the translation of the native nascent chain is important. To localize the sequence that is important for protection of mRNA from degradation, additional nucleotide(s) were introduced into frame-shift mutants to restore the PPL reading frame after the signal sequence (Figure 4A). The resultant nascent chains contained dramatic changes in the signal sequence, but no changes in the mature protein. The levels of these mRNAs were low (Figures 4B–4C). These results suggested that the signal sequence protects secretory protein's mRNA from degradation. To test this hypothesis we prepared a PPL construct without a signal sequence, PPL-ΔSS, and found that its mRNA level was decreased (Figure 4D). Similar results were obtained using two other secretory proteins, AT and CA4 lacking signal sequences (Figure 4D). All data together demonstrate that the presence of a functional signal sequence in the nascent chain is important for protection of the mRNA from degradation.

Presence of a Mutant Signal Sequence in Hybrid Cytosolic Proteins Leads to Their mRNA Depletion

While the lack of a functional signal sequence in secretory proteins leads to significant decrease of their mRNA levels, the mRNAs of cytosolic proteins are stable despite the fact that they do not have signal sequences. The biogenesis of cytosolic proteins is significantly different from that of secretory proteins (Figure 2F). During de novo synthesis of cytosolic proteins,

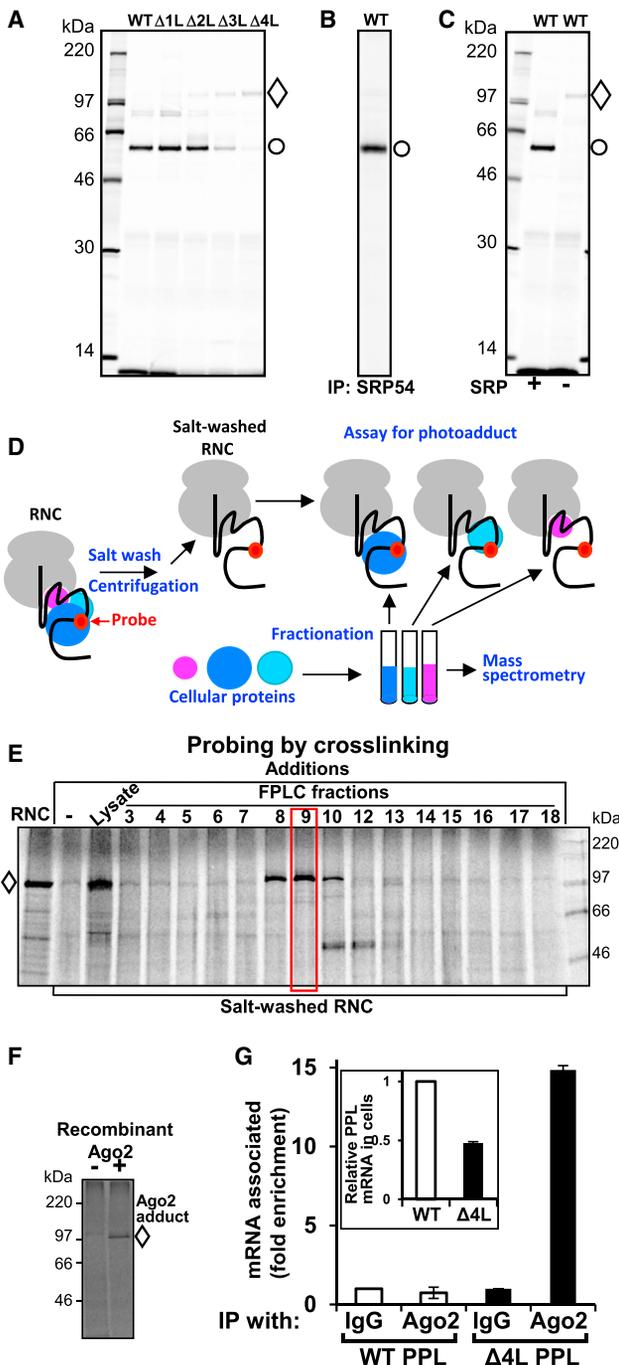


Figure 5. Ago2 is in Close Proximity to Mutant Nascent Chains and Associated with Mutant mRNA

(A) Deletions in the PPL signal sequence lead to reduced interaction with SRP54 and increased proximity to a ~100 kDa protein. Photocrosslinking patterns of WT and mutated PPLs are shown. RNCs containing the first 86 residues of WT and mutant PPLs with a photoreactive probe in the signal sequence were produced *in vitro* in the presence of SRP. Following UV irradiation, samples were analyzed by electrophoresis and autoradiography. The positions of photoadducts containing ~50 kDa (circle) or ~100 kDa (diamond) proteins are shown.

(B) Immunoprecipitation (IP) using SRP54 specific antibody demonstrates that the ~50 kDa protein is SRP54.

with WT PPL when the reaction was not supplemented with SRP (Figure 5C). This behavior implicated the ~100 kDa protein in the initial step of the process that leads to the specific degradation of mutant PPL mRNA or WT PPL mRNA when SRP is limiting.

Argonaute2 is in Close Proximity to Mutant Nascent Chains during Translation and Associates with Mutant mRNA

To identify proteins interacting with nascent chains we combined site-specific photocrosslinking with protein fractionation and mass spectrometry. This allowed identification of proteins involved in short-lived, transient interactions during translation. The approach includes: *in vitro* preparation of salt-washed RNC complexes with an incorporated photocrosslinking probe, by the use of modified amber-suppressor tRNA (salt-washing removes ribosome-associated proteins); fractionation of cellular proteins, and selection of fractions containing the protein of interest using the photocrosslinking assay; and identification of proteins in the selected fractions by mass spectrometry (Figure 5D). This method for identification of proteins interacting with nascent chains (iPINCH) is a valuable tool for identification of partners in transient protein-protein interactions during translation.

RNCs of $\Delta 4L$ PPL containing the photocrosslinking probe were prepared in an *in vitro* translation system. As expected the nascent chain of the mutated protein photocrosslinked the ~100K protein (Figure 5E, lane RNC). Washing the RNCs with high-salt buffer removed most of the ~100 kDa protein, but upon adding lysate back to salt-washed RNCs, photocrosslinking to the ~100 kDa protein was reconstituted (Figure 5E, lane “Lysate”). A crude reticulocyte preparation of ribosome-associated proteins, which restored photoadduct formation, was fractionated and fractions containing the protein of interest were identified (Figure 5E, fractions 8–10). Analysis by mass spectroscopy revealed that the protein was Argonaute2 (Ago2) (Table S2). Ago2, a member of the argonaute protein

(C) WT PPL crosslinks to the ~100 kDa protein are maximized when lysate is not supplemented with SRP.

(D) Scheme of the method for identification of proteins interacting with nascent chains (iPINCH).

(E) Identification of fractions that contain the ~100 kDa protein. $\Delta 4L$ RNCs were prepared as above and treated with a high-salt buffer. The latter were combined with lysate or aliquots of fractionated rabbit reticulocyte ribosome-associated proteins, incubated, UV irradiated and analyzed as in A. The photocrosslinking pattern of the fraction examined by mass spectrometry is boxed.

(F) Recombinant Ago2 is in close proximity to mutant nascent chain. Purified recombinant human Ago2 or buffer was added to salt-washed RNCs of $\Delta 4L$ PPL mutant with photocrosslinking probe. Samples were photocrosslinked and analyzed as above.

(G) mRNA of $\Delta 4L$ PPL is preferentially enriched in Ago2 immunoprecipitates. A short incubation time (20–24 hr after plasmid transfection) was used to ensure that significant levels of $\Delta 4L$ mRNA still remained at the start of the experiment: the WT and $\Delta 4L$ mutant mRNA content in cell lysate was about 2:1 before IP (inset). Mouse monoclonal Ago2 antibody was used for IP. Data are from two qPCRs, reactions were in triplicates, mean \pm SD.

See also Figure S4 and Table S2.

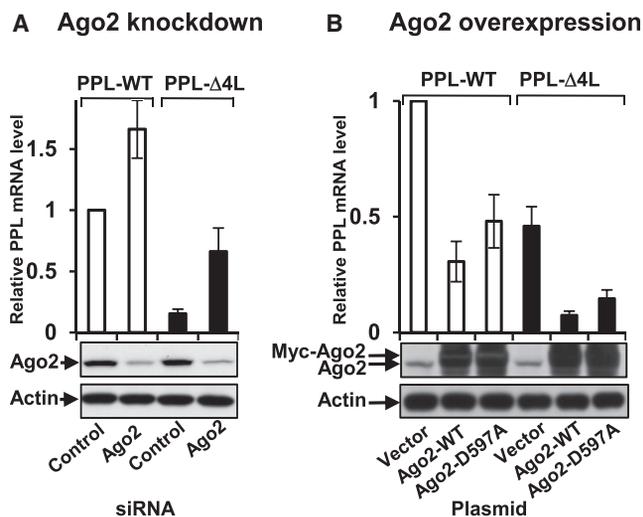


Figure 6. Process of mRNA Degradation Involves Ago2

(A and B) Ago2 knockdown inhibited reduction of PPL mRNA (A), while overproduction of recombinant WT or mutant D597A Myc-Ago2 reduced the level of PPL mRNA (B). Bars are PPL mRNA levels from northern blot ($n = 3$ [but $n = 2$ for D597A], mean \pm SEM). WT PPL mRNA levels are open bars, $\Delta 4L$ mutants are black bars. Depletion or overexpression of Ago2 was confirmed by western blot, actin was a control. See also Figure S5.

family, is involved in gene silencing (Hammond et al., 2001; Hutvagner and Simard, 2008; Martinez et al., 2002; Tolia and Joshua-Tor, 2007). The identification of Ago2 was validated independently by reconstituting the photoadduct using recombinant human Ago2 purified from insect cells (Figure 5F), confirming its close proximity to mutated PPL-nascent chain during translation.

We tested whether Ago2 preferentially interacts with the mutant mRNA. RNA immunoprecipitation (RNA-IP) of endogenous Ago2 from human cells expressing WT or mutant PPLs revealed that mutant $\Delta 4L$ PPL mRNA was enriched in Ago2 immunoprecipitates (Figures 5G and S4C–S4F).

To test functional involvement of Ago2 in the specific reduction of mutant mRNA levels we measured PPL mRNA levels in the cultured cells in which Ago2 was depleted or overexpressed. The reduction of mutant PPL mRNA was significantly inhibited in cells when Ago2 was knocked-down ($\Delta 4L$ mRNA level increased ~ 4.4 -fold, Figure 6A), while WT mRNA level was elevated in Ago2 knockdown cells to a lesser degree (~ 1.7 -fold). The results suggest that Ago2 is involved in the process of specific mRNA degradation. For Ago2 overexpression experiments, Ago2-containing plasmids were transfected a day before transfection with PPL plasmids. A short incubation time (~ 24 hr after PPL plasmids' transfections) was used to ensure that significant level of $\Delta 4L$ mRNA (about 45% of WT PPL mRNA level) still remained in the control (Figure 6B). When Ago2 was overexpressed, the level of $\Delta 4L$ mRNA was dramatically reduced (~ 6.2 -fold), while WT PPL mRNA level was decreased to a lesser degree (~ 3.3 -fold) (Figure 6B).

Although Ago2 possesses ribonuclease H activity (slicer activity) and amino acid residues in the active site are known

(Liu et al., 2004; Song et al., 2004), not all Ago2-dependent processes require its nuclease activity. To test if Ago2 slicer activity is required for mutant PPL mRNA degradation we conducted experiments with a slicer defective Ago2, containing mutation D597A (Liu et al., 2004; Rivas et al., 2005). Overproduction of Ago2-D597A leads to significant decrease in the level of $\Delta 4L$ mRNA, and to lesser degree, WT PPL mRNA (Figures 6B). Although the effect of Ago2-D597A was slightly less than that of Ago2-WT, the results demonstrate that overproduction of slicer mutant Ago2 leads to decreased levels of PPL mRNAs. Overproduction of Ago2-WT or mutant Ago2-D597A under conditions when endogenous Ago2 was knocked-down independently confirmed this observation (Figure S5A) and showed that the effects were specific (Figure S5B). These results indicate that Ago2 is involved in the process, but its slicer activity is not required. Interestingly, knock down of other proteins of the argonaute family, Ago1, Ago3, and Ago4, and proteins involved in small RNA biogenesis, Dicer and Drosha, as well as proteins involved in miRNA-dependent association with Ago2, TNRC6A, B, C, were without measurable effect on $\Delta 4L$ PPL mutant mRNA levels (Figures S5C–S5H). Modest increases in mRNA levels of WT PPL mRNA in some cases was observed, reflecting involvement of the above proteins in normal PPL mRNA biogenesis, however, the knockdowns did not significantly affect the process of mutant mRNA elimination.

DISCUSSION

Misfolded/mistargeted proteins, as well as their aggregates can be cytotoxic, leading to many diseases (Stefani and Dobson, 2003). However, the fact that protein aggregates do not accumulate in unstressed cells despite their potential continued production indicates the existence of efficient quality control machinery (Kopito, 2000). Cells have developed multiple mechanisms for protection from these “unwanted” products at different levels. One of the protection levels is labeling with ubiquitin and degradation of already synthesized aberrant proteins by the proteasome (Buchberger et al., 2010; Smith et al., 2011). Energy-dependent degradation of mutant proteins and their futile translation are expensive processes and cells evolved mechanisms to prevent synthesis of mutant cytotoxic proteins. NMD directing mRNAs with premature stop codons for degradation, NSD acting on mRNAs without a natural stop codon, and NGD acting on mRNAs on stalled ribosomes, are examples of quality control on the template mRNAs with dramatic aberrations (Doma and Parker, 2007; Shoemaker and Green, 2012). However, none of these systems are known to act on mutant mRNA templates that code for proteins with small aberrations in their primary structure. The mechanism reported here fills this gap in quality control by specifically acting on mutant mRNA templates encoding proteins that have lost interactions with their normal binding partners at the ribosome exit site.

In this study we report the discovery of a previously unappreciated mechanism of translational quality control, in which translation of a defective protein leads to specific degradation of its mRNA. This mechanism monitors the absence of normal interactions of nascent chains early during translation and transfers the

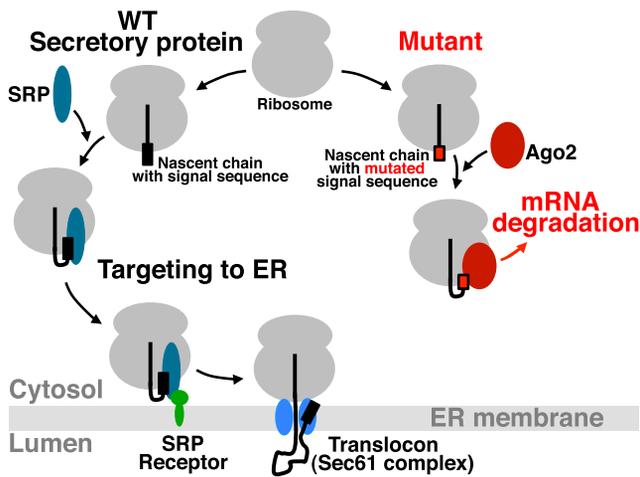


Figure 7. Model for Regulation of Aberrant Protein Production

When the nascent chain of a WT secretory protein containing a signal sequence emerges from the ribosome polypeptide exit tunnel during translation it is recognized by SRP. This interaction leads to targeting of the complex to SRP receptor in the ER membrane and finally to the translocon. The nascent chain is cotranslationally translocated into the ER lumen, where it is folded with the help of ER chaperones. However, when the nascent chain of a secretory protein contains critical mutations preventing SRP recognition, the unbound nascent chain is exposed outside the polypeptide tunnel. Despite the absence of its interaction with SRP, a defective secretory protein does not typically behave as a cytosolic protein because of the absence of appropriate sequences/conformations/signals required for normal cytosolic nascent chains interactions. Thus, the space normally occupied by SRP at the ribosome remains unoccupied. Ago2 occupies the space and directs mRNA for degradation initiating the RAPP process.

signal to mRNA degradation machinery that allows cells to prevent synthesis of these “unwanted” aberrant proteins. This process is distinct from known quality control mechanisms. It involves surveillance of newly synthesized nascent chain interactions and triggers mRNA degradation in response to a loss of these normal interactions during translation. This mechanism can discriminate between normal and mutated mRNAs even when they are coexpressed (Figures 3B–3D). This specificity is graded, as demonstrated by the strong correlation between decreased efficiency of SRP–nascent chain interaction and mRNA degradation (Figures 2A–2B and 5A). Therefore, the system balances the quantity of synthesized protein with decreased ability for targeting and secretion. Notably, this process is not a generalized stress response but rather is specifically directed to reduce the burden of nontransportable proteins as well as potentially other types of newly synthesized misfolded proteins. Furthermore, as demonstrated by two different approaches, introduction of deletions in signal sequences of several secretory proteins and assessment of mRNA levels of secretory and ER lumen proteins under conditions when SRP was depleted, the mechanism is general (Figures 2).

Based on our data, we propose that the loss of cotranslational interactions between a nascent chain and factors required for transport and/or folding (SRP in this study, but possibly ribosome-associated chaperones for cytosolic proteins (Hartl et al., 2011; Pechmann et al., 2013; Raue et al., 2007), and other

targeting factors for nuclear, mitochondrial, or peroxisomal proteins) can trigger a cellular response that leads to specific elimination of the corresponding mRNA (Figure 7). According to this hypothesis, Ago2 is associated with the ribosome within the reach of the nascent chains that are either defective or unable to bind their normal binding partners after emerging from the translating ribosome and the encoding mRNA. This association correlates with mRNA elimination. Although it is still unknown if Ago2 is a direct sensor, the data demonstrate that Ago2 is functionally involved in the process. This mechanism of regulation of aberrant protein production (RAPP) would provide high flexibility for regulating the synthesis of aberrant proteins regardless of their sequence and structure, without negative effects on the production of undamaged proteins in the same cell.

The following observations support this model: (1) Ago2 cotranslationally crosslinks to mutant nascent chains that have lost the ability to bind SRP (Figure 5A); (2) in the absence of SRP, nascent WT PPL photocrosslinks instead to Ago2 (Figure 5C), indicating that Ago2 occupies the space when it is not occupied by SRP; (3) Ago2 was found preferentially associated (directly or nondirectly) with the mutant mRNA (Figures 5G and S4C–S4F); (4) overexpression of Ago2 or depletion of SRP in human cells decreases the levels of PPL mRNA, while depletion of Ago2 stabilizes the level of mutant mRNA (Figure 6); (5) increase in Ago2 crosslinking efficiency directly correlates with the number of leucines deleted from the PPL signal sequence, a decrease in efficiency of RNC interaction with SRP, and a decrease in the level of mutant mRNA (Figures 2A, 2B, and 5A); (6) mRNAs of secretory proteins with defective signal sequences were efficiently degraded despite the absence of homology in signal sequences (Figure 2E); (7) SRP knockdown leads to mRNA degradation of even WT protein, while defects in other downstream components of the protein translocation machinery were without effect (Figures 2G–2I).

Several different scenarios were examined to determine which signals are used for selection for degradation of mRNAs coding aberrant proteins. The above model is the simplest model that explains all the data. Indeed, direct surveillance of mRNA sequence by a quality control mechanism is unlikely (see discussion to Figure 4). A model in which the decreased stability is due to mislocalization of ER-targeted mRNAs, or a mechanism involving surveillance of the overall change in the structure of synthesized aberrant secretory protein as a result of folding in the cytosol, an inappropriate environment, is inconsistent with the observations that knock down of SRP receptor or translocon protein Sec61 α did not effect the mRNA level of secretory proteins, by contrast to SRP knockdown (Figure 2). All these defects in components of the secretory machinery abolish protein translocation, but only SRP knock down leads to mRNA elimination. Recognition of a specific sequence or features of an aberrant protein (e.g., a mutated signal sequence) is inconsistent with data on secretory proteins lacking their signal sequences or with scrambled N termini (Figure 4). This scenario also cannot explain secretory protein mRNA depletion when SRP was knocked-down (Figures 2G, 2H, and 2J). Therefore the simplest model is that the quality control system does not recognize specific sequences of a mutant protein or overall changes in its

structure, but rather, the absence of normal interactions of nascent chains early in translation.

How does this mechanism discriminate aberrant secretory proteins with defects in signal sequences from cytosolic proteins that are not natural substrates for SRP and therefore do not interact with SRP? In this regard, it is important to note that the presence of signal sequence is not the only feature that distinguishes secretory proteins from cytosolic proteins. The removal of a signal sequence from a secretory protein will not typically make it truly cytosolic (having failed translocation, it will be localized to the cytosol, but it will lose its natural binding partners, will not likely be correctly folded, and will be directed for degradation), just as adding a signal sequence to a cytosolic protein will not typically make it truly secretory (although exceptions are known).

Other factors interact with cytosolic proteins during their translation. A special network of chaperones linked to protein synthesis, CLIPS (Albanèse et al., 2006; Pechmann et al., 2013), and ribosome-associated protein biogenesis factors, RPBs (Raue et al., 2007), represent the very complex machinery at the ribosome peptide exit tunnel. Examples include ribosome associated chaperones Hsp70 SSB (Willmund et al., 2013), MPP11, and Hsp70L1 (Otto et al., 2005), chaperonin TRiC as well as numerous others (see for details reviews by Hartl et al., 2011; Pechmann et al., 2013). Thus, in the case of cytosolic proteins, while their nascent chains do not bind SRP, they interact with other factors. These interactions may prevent Ago2 association and thus would not trigger RAPP.

The crosslinking and functional studies described here implicate Ago2 in the process of specific degradation of mRNAs in response to synthesis of mutated nascent chains not capable of efficiently binding SRP. Ago2 is a member of the argonaute protein family. These proteins are highly conserved and divided into three subfamilies: Ago-like, PIWI-like, and group 3 (Hutvagner and Simard, 2008; Tolia and Joshua-Tor, 2007). Mammals contain four Ago-like subfamily members, and Ago2 is the only member known to possess RNA cleavage activity (Liu et al., 2004; Meister et al., 2004). Historically, and most commonly known, Ago2 function is associated with noncoding RNAs (miRNAs or siRNAs) and its participation in formation of the RNA-induced silencing complex (RISC) responsible for gene silencing through mRNA cleavage (Hammond et al., 2001; Martinez et al., 2002). Ago2 has also been reported to play a key role in translational repression, but by incompletely understood mechanisms (Djuranovic et al., 2011; Filipowicz et al., 2008). Recently, Ago2 has been associated with multiple additional functions in the cell (Meister, 2013). Ago2 is involved in RNA Interference (RNAi)-mediated regulation of gene expression, miRNA biogenesis, protection from viruses, transcriptional regulation, and even alternative splicing (Ameyar-Zazoua et al., 2012; Cernilogar et al., 2011; Czech and Hannon, 2011; Djuranovic et al., 2011; Fabian et al., 2010; Filipowicz et al., 2008; Hutvagner and Simard, 2008). Although many Ago2 functions are most often associated with its ability to bind small RNAs (Fabian et al., 2010), recent examples indicate that small RNAs are not involved in all Ago2 functions. A PUF-Ago-eEF1A complex responsible for attenuation of translational elongation does not depend on small RNAs (Friend et al., 2012). Ago2

recruitment to chromatin is also RNAi independent (Moshkovich et al., 2011). Small RNA-independent recruitment to mRNA was also recently demonstrated for one of two argonautes in *Drosophila* (Pinder and Smibert, 2013). The wide variability in Ago2 reliance on small RNAs and RNase activity may reflect multiple parallel functions in the cell. Experiments with Ago2 knock down and its overproduction demonstrated that it is functionally involved in the RAPP process (Figure 6). Ago2 was found in proximity to mutated nascent chains during their synthesis, with direct correlation between the severity of a mutation in signal sequence and more efficient crosslinking of the nascent chain to Ago2 (Figure 5A). Moreover, we have found that mutant protein mRNA was specifically enriched in Ago2 IP experiments (Figures 5G, and S4C–S4F). Although questions remain about whether Ago2 binds mutant mRNA directly, during translation or at later stages, the data suggest Ago2 is in close proximity to the mutant nascent chain and mRNA at the same time at the ribosome. Association of Ago2 with mutated nascent chains during translation is consistent with earlier observations of Ago2 association with translating ribosomes (Landthaler et al., 2008; Nelson et al., 2004). It was also shown that Ago2 forms a complex containing L5, L11 ribosomal proteins, along with 5S RNA (Ishizuka et al., 2002). Experiments with slicer inactive Ago2, as well as experiments on Dicer and Drosha-knockdowns (Figures 6B and S5) suggest that Ago2's function in RAPP is small RNA- and slicer-independent.

Although precise role for Ago2, its ability to act as a sensor in the process, as well as other details of the mechanism remain obscure, the data presented here indicate that the stability of mRNA is dictated by the balance of nascent chain interactions with specific cellular factors at the ribosome exit site, thereby providing a means to selectively reduce the load of defective proteins at an early point in translation by specifically degrading their mRNAs. RAPP provides a mechanism by which defects in the signal sequence of a secretory protein, or in the secretory machinery, lead to a dramatic reduction in substrate mRNA and thus expression of the protein.

EXPERIMENTAL PROCEDURES

Protein translocation was studied using an in vitro translation/translocation system in the presence of purified canine microsomes or by maturation of the proteins in human cell culture. Protein visualization in the cells was conducted by immunocytochemistry and confocal microscopy. Cellular mRNA levels were analyzed by northern blot or qPCR, and proteins by western blot. For the mRNA stability/degradation experiments, WT and mutant PPLs cloned under control of the Tet-response element into pTRE2hyg were expressed in HeLa Tet-Off cells (Clontech) in the presence/absence of doxycycline. SRP54, Ago2, and other protein depletions in human cell culture were achieved by siRNA technique. Site-specific photocrosslinking using tRNA-mediated incorporation of a photoreactive probe into a nascent chain during in vitro translation was conducted as before (Krieg et al., 1986; McCormick et al., 2003). Method for identification of proteins interacting with nascent chains employs: (1) in vitro preparation of salt-washed RNCs containing the photocrosslinking probe in specific position of the nascent chain; (2) fractionation of the active lysates and assay of fractions containing the protein of interest using photocrosslinking with salt-washed RNCs; and (3) identification of proteins in the selected, purified fractions by mass spectrometry. SRP and microsomes for in vitro experiments were purified from canine pancreas. Recombinant human Ago2 was expressed in and purified from cultured insect cells (Ye et al., 2011).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.12.017>.

AUTHOR CONTRIBUTION

A.E.P. and Z.N.K. contributed equally to this work. A.L.K. and P.J.T. wrote the paper; A.E.J., G.v.H., S.R., Z.N.K., A.E.P., and D.S.G. edited the paper. A.L.K. conducted majority of the experiments; Z.N.K. conducted mutant mRNA degradation specificity experiments (coexpression of WT and mutant PPLs experiments), Ago2 RNA IP experiments, and provided ideas and design for some experiments; A.E.P. conducted pulse-labeling experiment; D.S.G. conducted transcription inhibition experiment; H.H. conducted Dicer depletion experiment; S.T.-K.-S. conducted confocal microscopy and provided technical support; I.M.N. participated in the initial stage of the project, conducted preliminary experiments on translation/translocation of mutant PPLs in vitro, prepared some mutant PPL constructs; H.O. conducted some fractionations of reticulocyte proteins for mass spectrometry; Q.L. provided purified recombinant Ago2 and presented comments; S.R., A.E.J., G.v.H. participated in the initial stages of the project; S.R. directed and designed experiments for the first identification of Ago2, A.E.J. supervised the photocrosslinking experiments, G.v.H. provided ideas for SRP nascent chain interaction experiments. A.L.K. and P.J.T. are cocorresponding authors.

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