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

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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.

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).

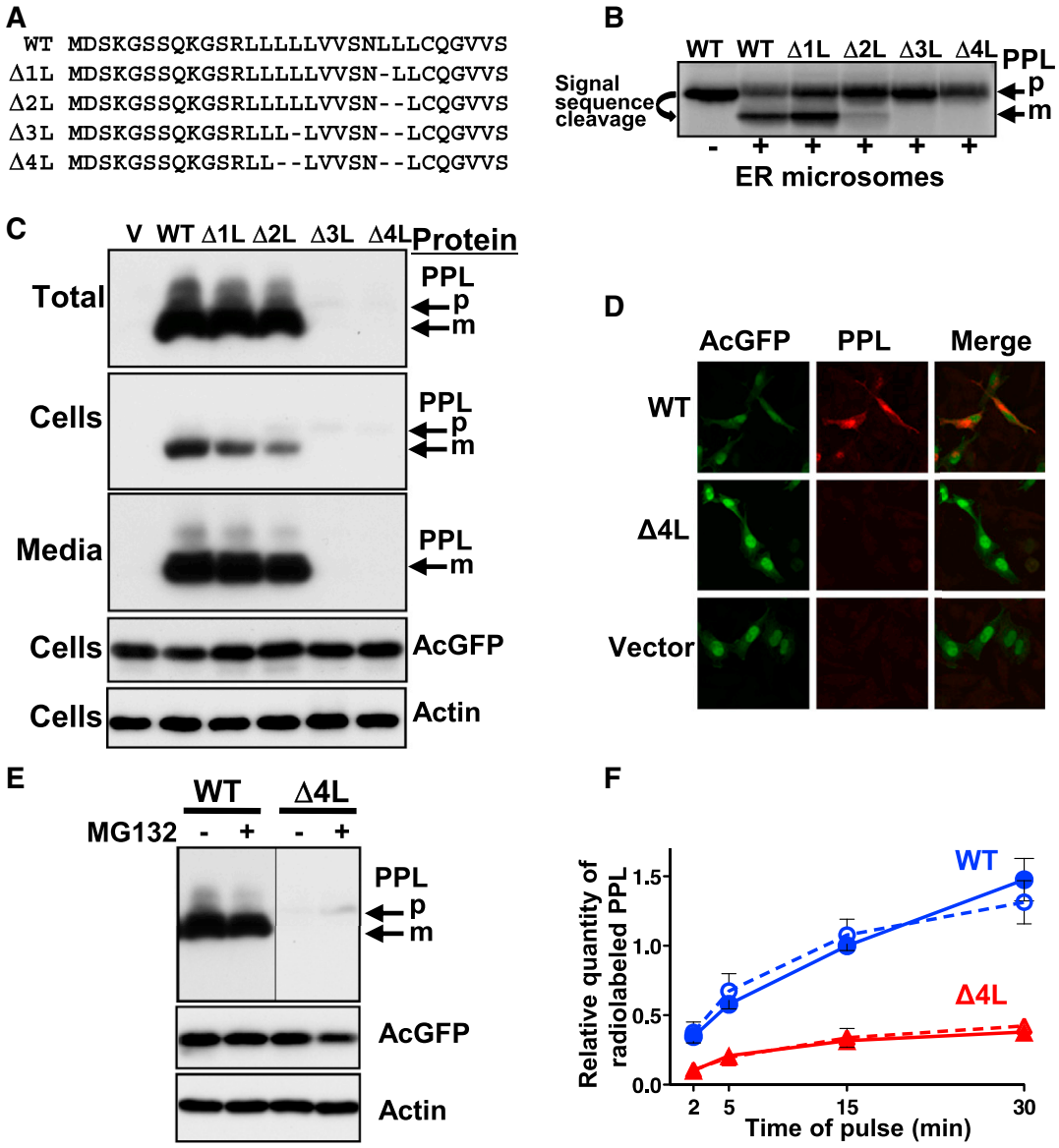


Figure 1. Deletions in the PPL Signal Sequence Lead to Defects in Protein Transport and Expression

(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 ± SEM). See also Figure S1.

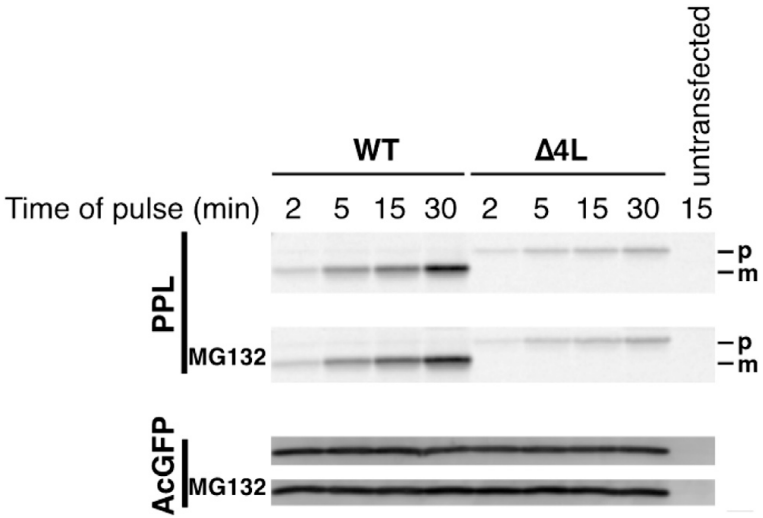


Figure S1. Translation of Mutated Δ4L PPL is Impaired, Related to Figure 1

Analysis of translated WT and Δ4L PPL was done in transiently expressing HeLa Tet-On cells following pulse-labeling with radiolabeled methionine. PPLs were immunoprecipitated at different times after initiation of the pulse, and then analyzed by SDS-PAGE and autoradiography. Cells were treated with MG132 where indicated.

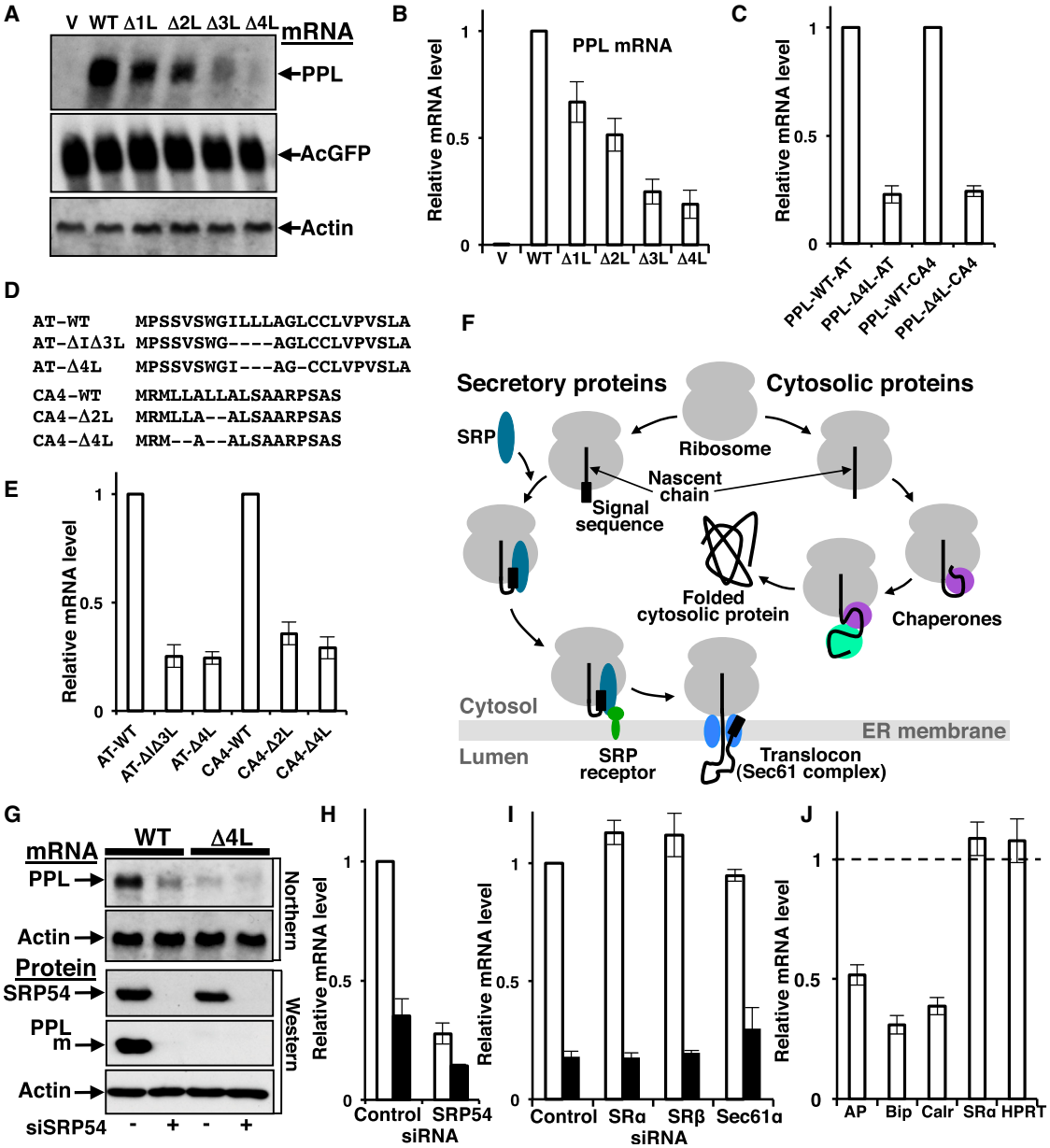

A
V WT Δ1L Δ2L Δ3L Δ4L
mRNA
PPL
AcGFP
Actin
B
PPL mRNA
Relative mRNA level
V WT Δ1L Δ2L Δ3L Δ4L
C
Relative mRNA level
PPL-WT-AT
PPL-Δ4L-AT
PPL-WT-CA4
PPL-Δ4L-CA4
D
AT-WT MPSSVSWGILLLAGLCCLVPVSLA
AT-ΔIΔ3L MPSSVSWG----AGLCCLVPVSLA
AT-Δ4L MPSSVSWGI---AG-CCLVPVSLA
CA4-WT MRMLLALLALSAARPSAS
CA4-Δ2L MRMLLA--ALSAARPSAS
CA4-Δ4L MRM--A--ALSAARPSAS
E
Relative mRNA level
AT-WT
AT-ΔIΔ3L
AT-Δ4L
CA4-WT
CA4-Δ2L
CA4-Δ4L
F
Secretory proteins
Cytosolic proteins
SRP
Ribosome
Nascent chain
Signal sequence
Folded cytosolic protein
Chaperones
Cytosol
ER membrane
Lumen
SRP receptor
Translocon (Sec61 complex)
G
WT
Δ4L
mRNA
PPL
Actin
Northern
Protein
SRP54
PPL m
Actin
Western
siSRP54 - + - +
H
Relative mRNA level
Control SRP54 siRNA
I
Relative mRNA level
Control SRα SRβ Sec61α siRNA
J
Relative mRNA level
AP Bip Calr SRα HPRT

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 ± SD) 42 hr after transfection of HeLa Tet-On with WT and mutated PPL plasmids.

(C) Presence of a mutated PPL signal sequence (PPL-Δ4L) in hybrid proteins containing the mature part of α1-antitrypsin (AT) or carbonic anhydrase IV (CA4) is sufficient to trigger mRNA depletion. mRNA levels measured by qPCR (n = 3, mean ± 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 ± 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 Δ4L PPL plasmids as indicated (G). Quantification of WT PPL (open bars) or Δ4L mutant (black bars) mRNAs by qPCR in independent sets of SRP54 knockdown experiments (n = 3, mean ± SD) (H).

(I) Knockdown of SRP receptor subunits SRα and SRβ, or translocon component, Sec61α, does not affect PPL mRNA level (qPCR, n = 3, mean ± SD). WT PPL (open bars), Δ4L mutant (black bars).

(J) SRP depletion causes reduction in endogenous mRNA levels of secretory and ER proteins. Measured by qPCR (n = 3, mean ± 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 SRα were not altered. See also Figure S2 and Table S1.

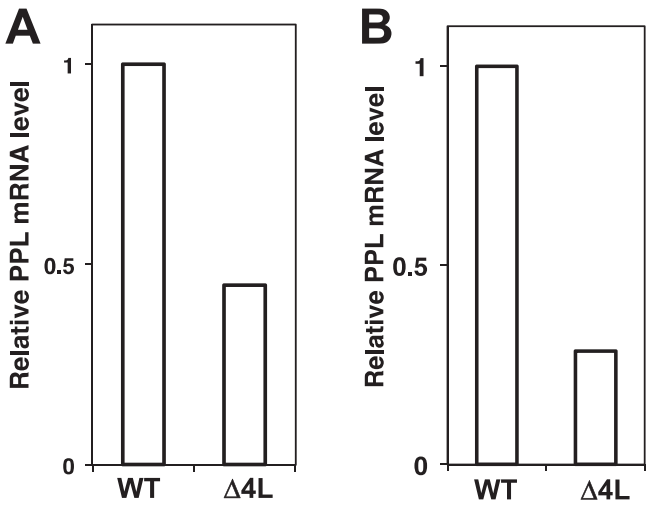


Figure S2. Effect of Δ4L in PPL Signal Sequence on PPL mRNA Levels in Transiently Transfected HEK293 or HeLa, Related to Figure 2

(A and B) PPL mRNAs were analyzed by qPCR for HEK293 (A) or HeLa (B).

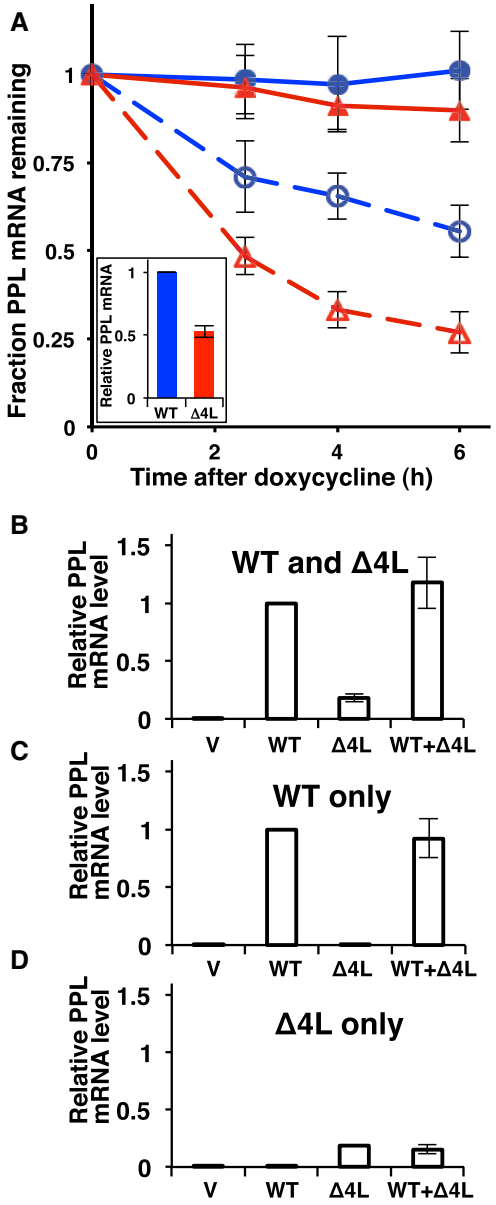


Figure 3. Preferential Degradation of Mutant PPL mRNA

(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 ± SD).

(B to D) qPCR analysis (n = 5, mean ± 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.

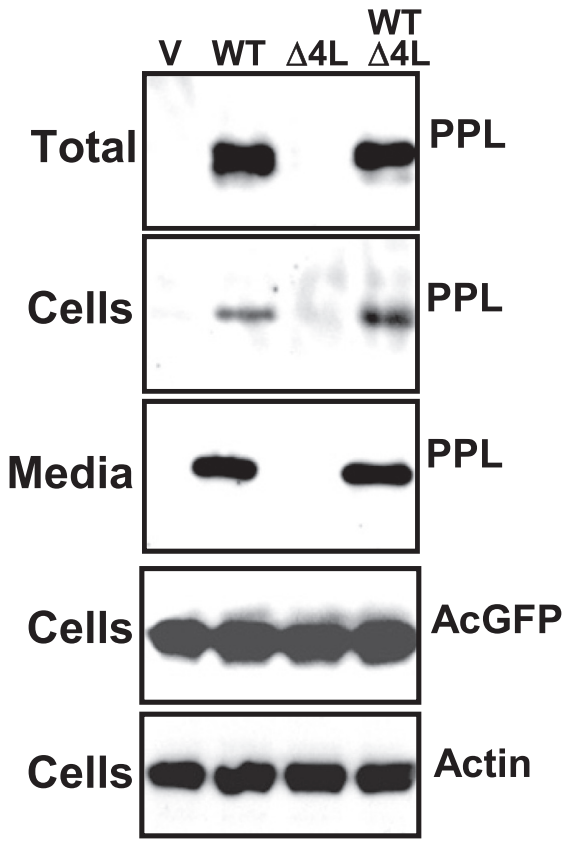


Figure S3. Western Blot Analysis of PPL in HeLa Tet-On Cells Expressing WT PPL, Δ4L Mutant, Both WT and Δ4L, Related to Figure 3

V is an empty vector control and AcGFP and actin were transfection and loading controls.

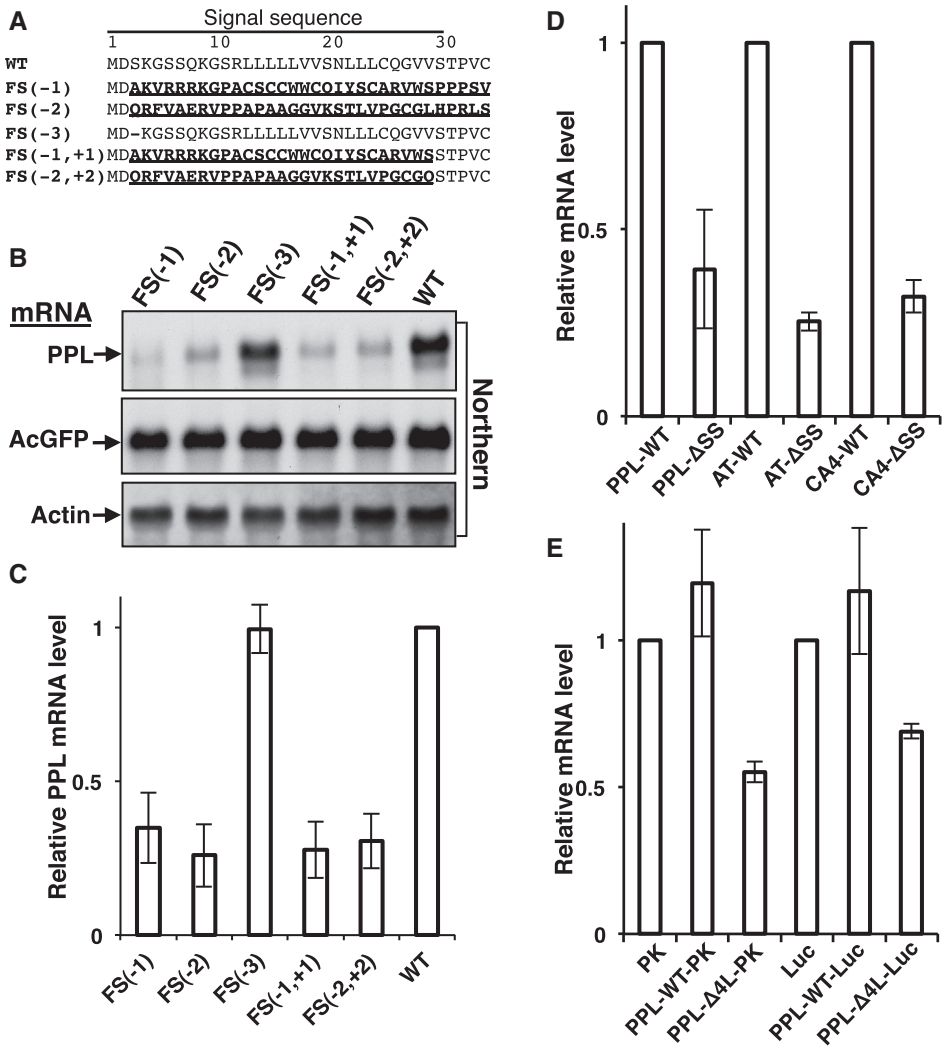


Figure 4. Functional Signal Sequence is Required for Protection of the Secretory Protein mRNA from Degradation

(A) N-terminal sequences of PPL mutants with amino acid substitutions in bold and underlined, and deletions as dashes. FS(−1), FS(−2), FS(−3) contain deletions of one, two, three nucleotides, correspondingly, in the third codon. FS(−1, +1), FS(−2,+2) contain additional insertion of one or two nucleotides in the codon 29 to restore original reading frame. mRNAs were analyzed by northern blots (B) or by qPCR (C; n = 3, mean ± SD).

(D) Deletions of natural signal sequences from PPL, AT, and CA4 lead to decrease in their mRNA levels (qPCR, n = 3, mean ± SD).

(E) Presence of a mutated signal sequence in hybrid cytosolic proteins leads to their mRNA depletion (qPCR, n = 3, mean ± SD).

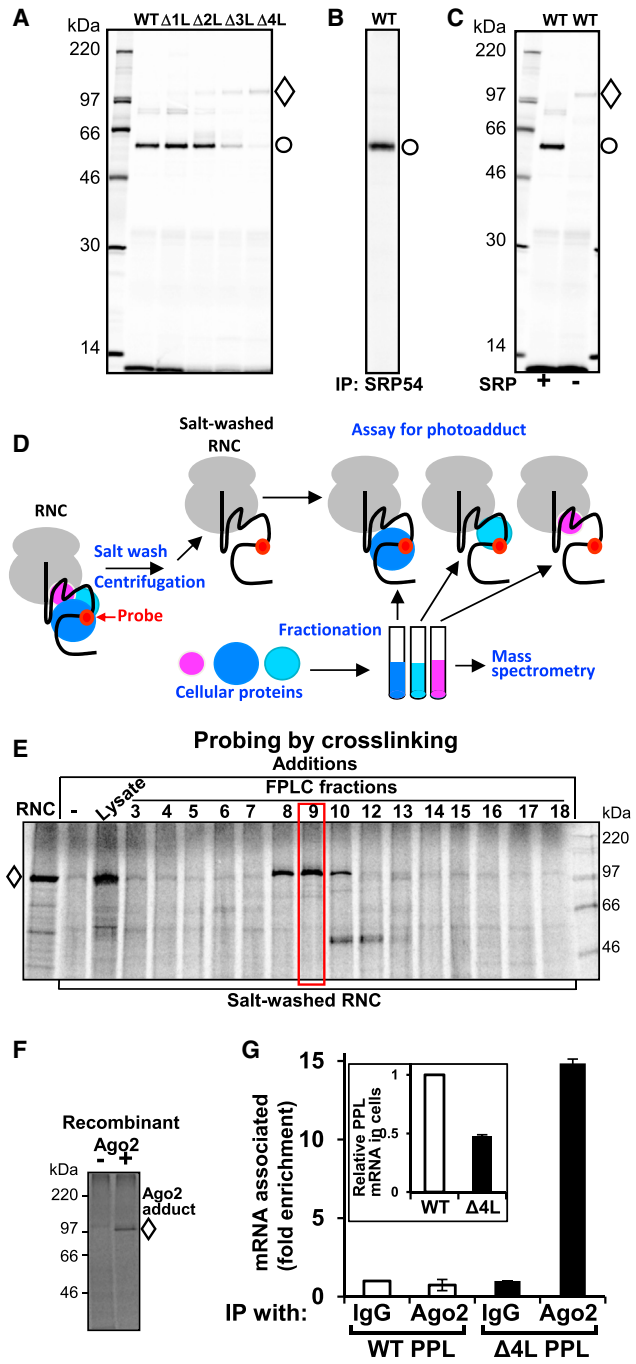


Figure 5. Ago2 is in Close Proximity to Mutant Nascent Chains and Associated with Mutant mRNA

(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.

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

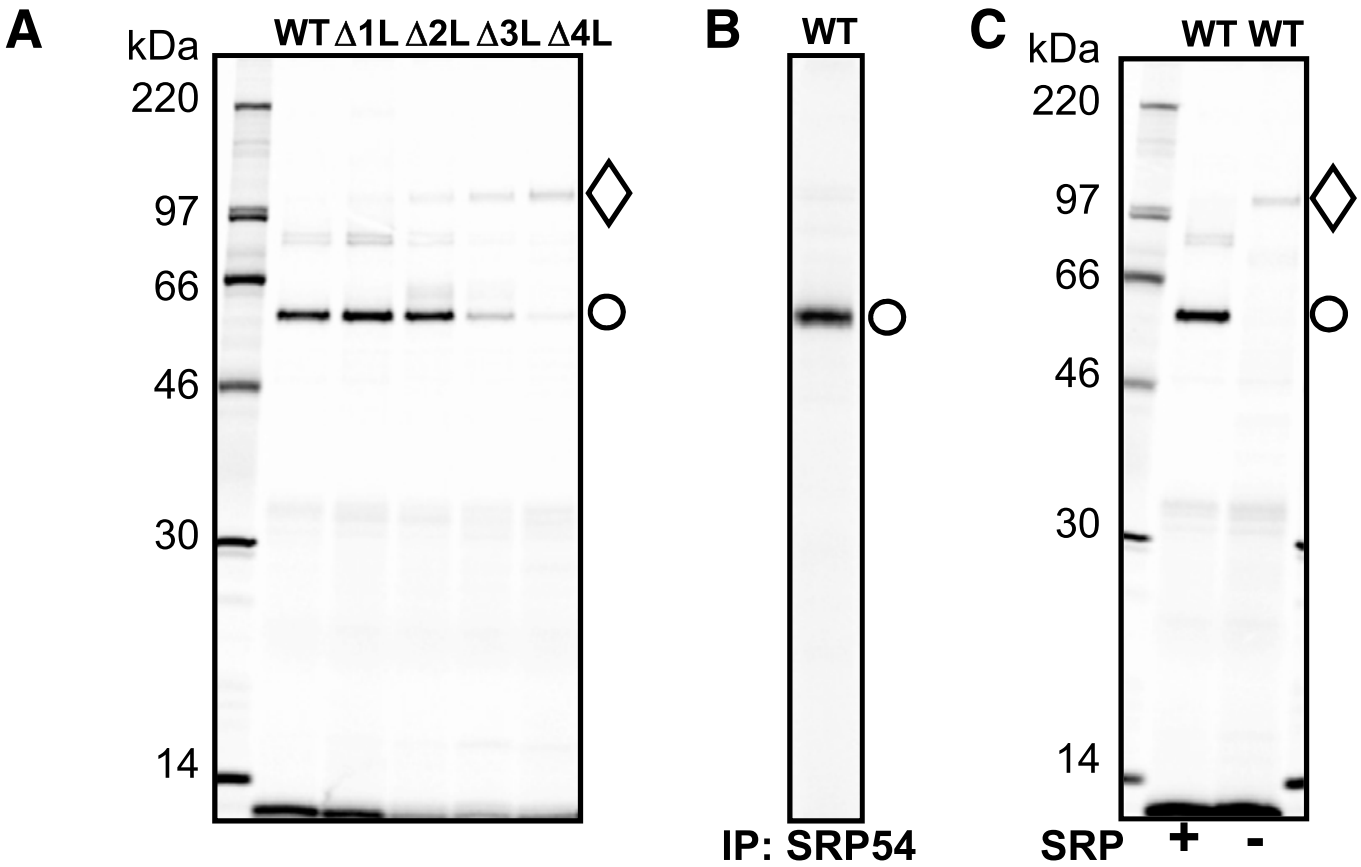
(D) Scheme of the method for identification of proteins interacting with nascent chains (iPINCH).

(E) Identification of fractions that contain the ~100 kDa protein. Δ4L 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 Δ4L PPL mutant with photocrosslinking probe. Samples were photocrosslinked and analyzed as above.

(G) mRNA of Δ4L 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 Δ4L mRNA still remained at the start of the experiment: the WT and Δ4L mutant mRNA content in cell lysates 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 ± SD.

See also Figure S4 and Table S2.



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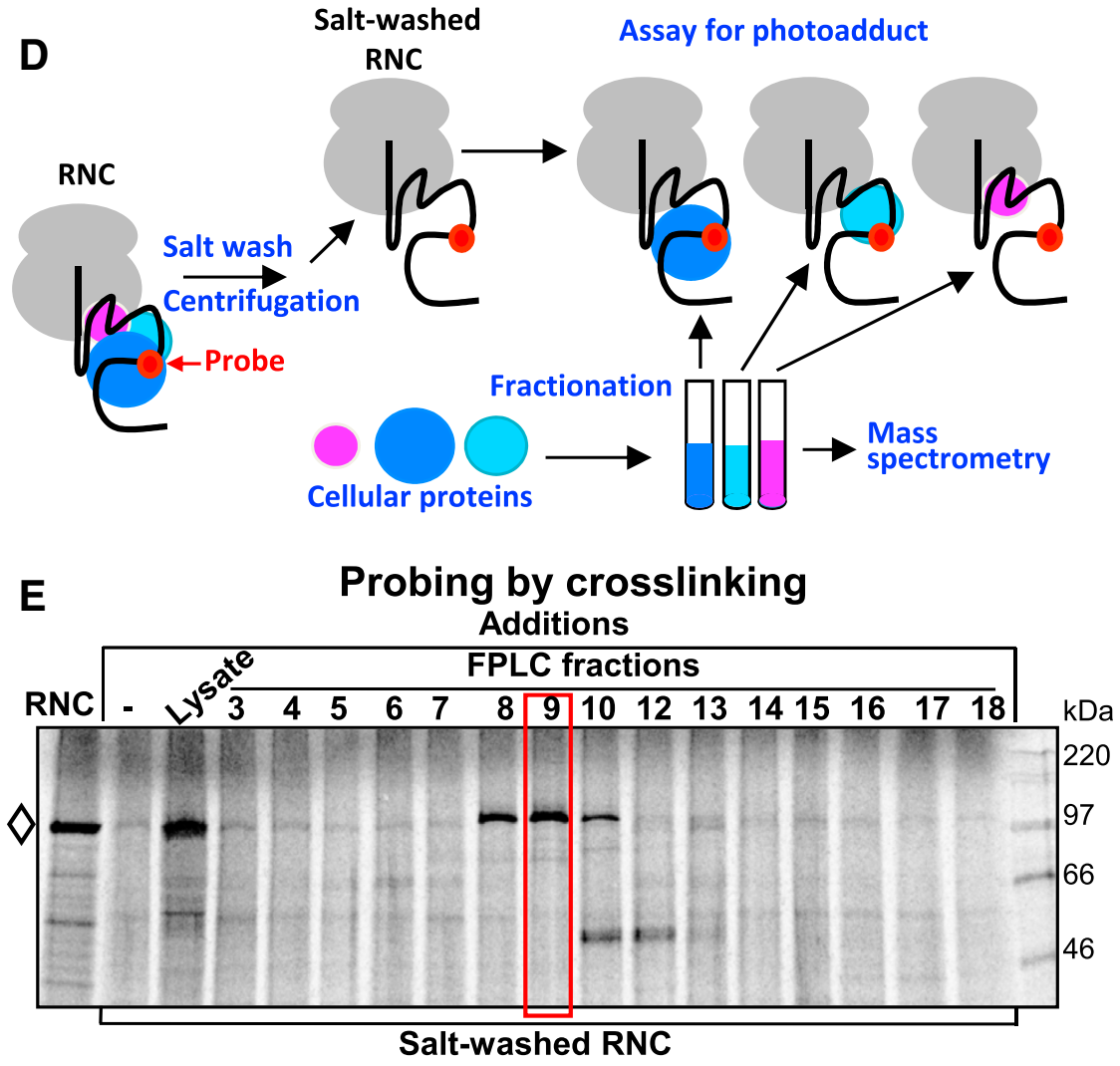

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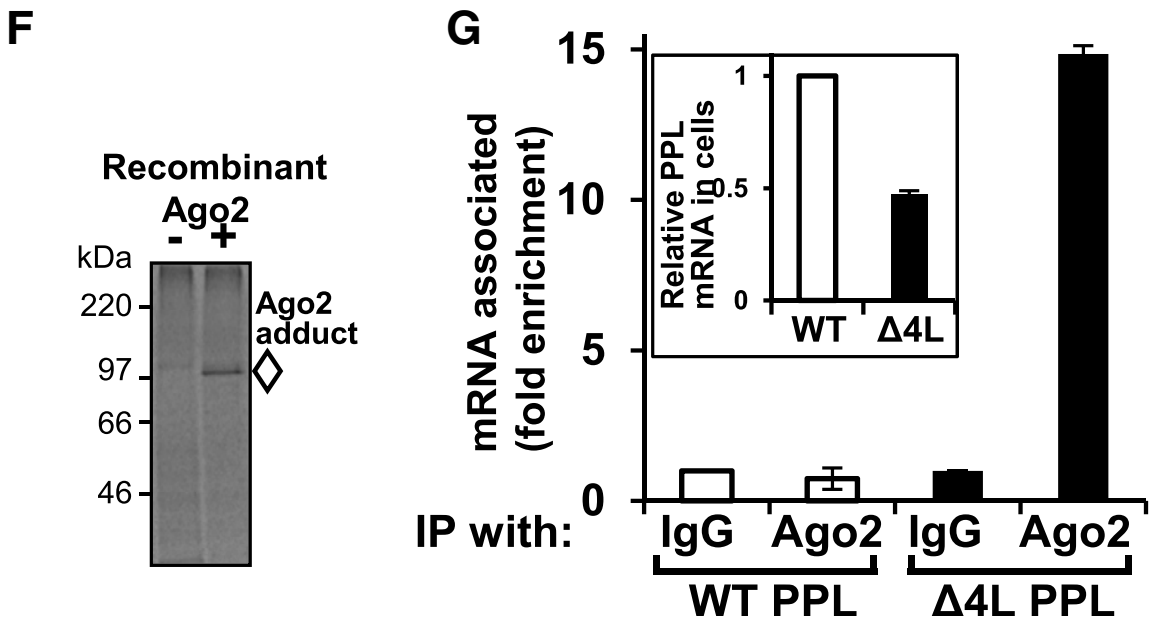

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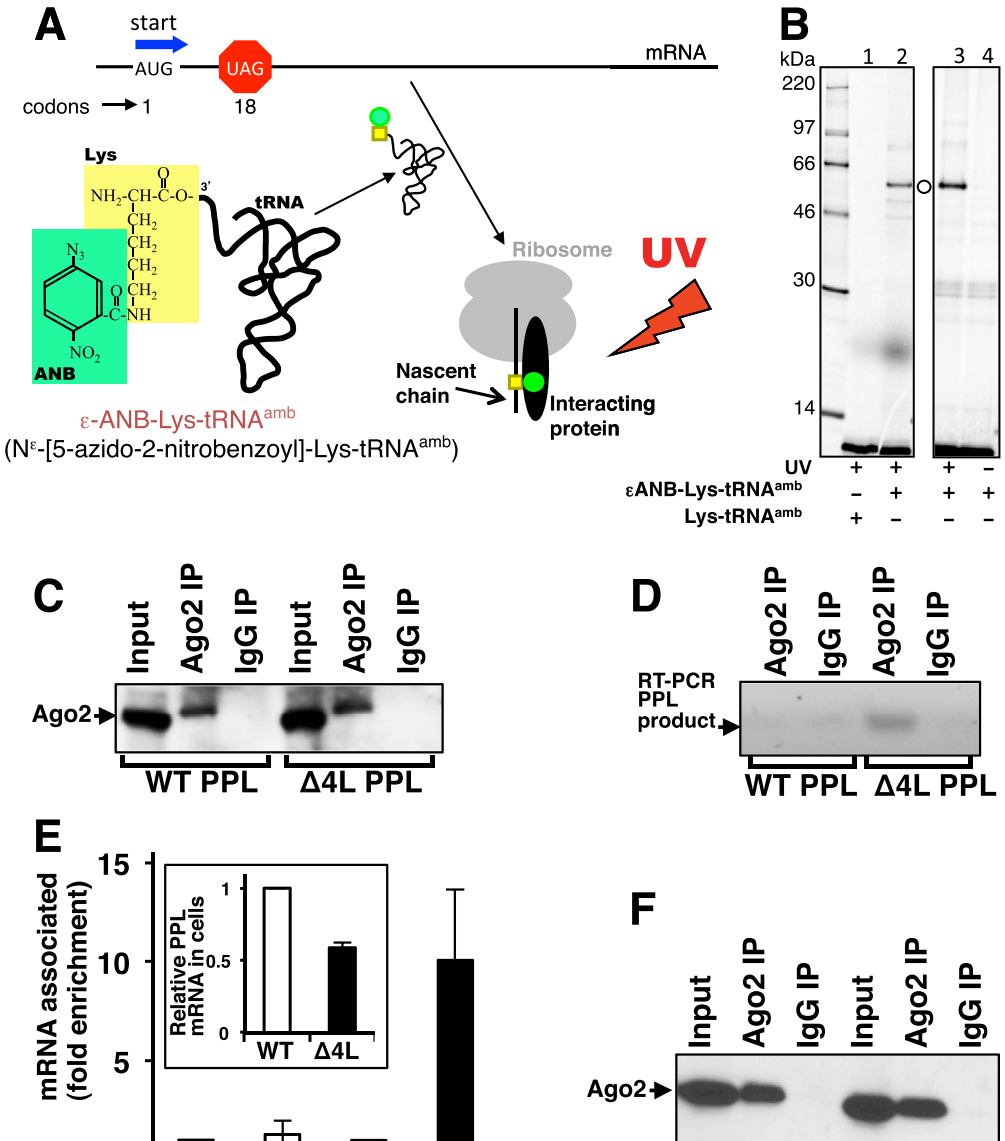
A
start
AUG
UAG
mRNA
codons
1
18
Lys
tRNA
Ribosome
UV
Nascent chain
Interacting protein
ANB
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(Nε-[5-azido-2-nitrobenzoyl]-Lys-tRNAamb)
B
kDa
1 2 3 4
220
97
66
46
30
14
UV
εANB-Lys-tRNAamb
Lys-tRNAamb
C
Input
Ago2 IP
IgG IP
Ago2
WT PPL
Δ4L PPL
D
RT-PCR PPL product
E
mRNA associated (fold enrichment)
Relative PPL mRNA in cells
WT
Δ4L
IP with:
IgG
Ago2
F

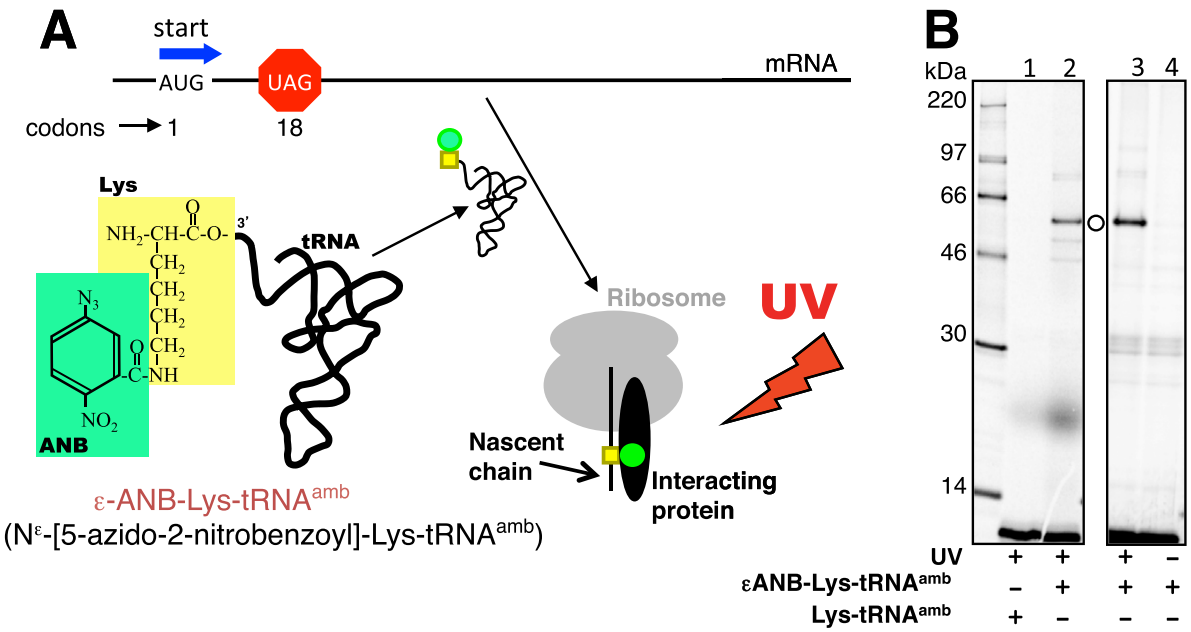

Figure S4. Cotranslational Site-Specific Photocrosslinking and RNA IP of Endogenous Ago2, Related to Figure 5

(A) Scheme of the site-specific incorporation of photocrosslinking probe into a protein. When an mRNA is mutated to contain an amber stop codon in position 18 of PPL, protein synthesis is prematurely terminated at this location in the absence of an aminoacylated amber suppressor tRNA (aa-tRNA^{amb}). However, when a translation incubation is supplemented with an aa-tRNA^{amb} that has a photoreactive moiety covalently attached to the amino acid side chain (εANB-Lys-tRNA^{amb}), the photoreactive and uncharged εANB-Lys is incorporated into the nascent chain at position 18. A homogeneous population of RNCs with photoreactive nascent chains is obtained by translating truncated mRNAs that lack a stop codon (other than the amber codon at position 18 that is translated by the amber suppressor) because normal termination does not occur and the nascent chain remains attached to the ribosome-bound peptidyl-tRNA. The length of the nascent chain is then dictated by the length of the truncated mRNA.

(B) Formation of crosslinking products is photo-probe dependent. Nascent 86-residue preprolactin was synthesized in vitro in a wheat germ translation system containing canine SRP in the presence of unmodified Lys-tRNA^{amb} lacking a photoprobe (lane 1) or of εANB-Lys-tRNA^{amb} (lanes 2–4). Samples were irradiated with UV light (lanes 1–3) or not irradiated (lane 4). Samples were analyzed by 10%–15% gradient SDS-PAGE and autoradiography. The positions of photoadducts to SRP54 (open circle) is shown.

(C–F) mRNA of Δ4L PPL is Preferentially Enriched in Ago2 Immunoprecipitates. (C-D) Additional panels for Figure 5G. IP was done with mouse monoclonal Ago2 antibody or control IgG. Ago2 was detected in immunoprecipitates by western blot using Ago2 rabbit antibodies (C). RT-PCR analysis of WT and the mutant PPL mRNAs in immunoprecipitates (D). (E and F) Experiment similar to those shown in Figures 5G and S4C, but with usage of independent Ago2 antibody (rabbit) for IP. WT and Δ4L PPL mRNAs were quantified by qPCR (E). The WT and Δ4L mutant mRNA content in cell lysates was about 2:1 before IP (inset). Data are from two qPCRs, reactions were in triplicates, mean ± SD. Ago2 was detected in immunoprecipitates by western blot using Ago2 mouse monoclonal antibodies (F).

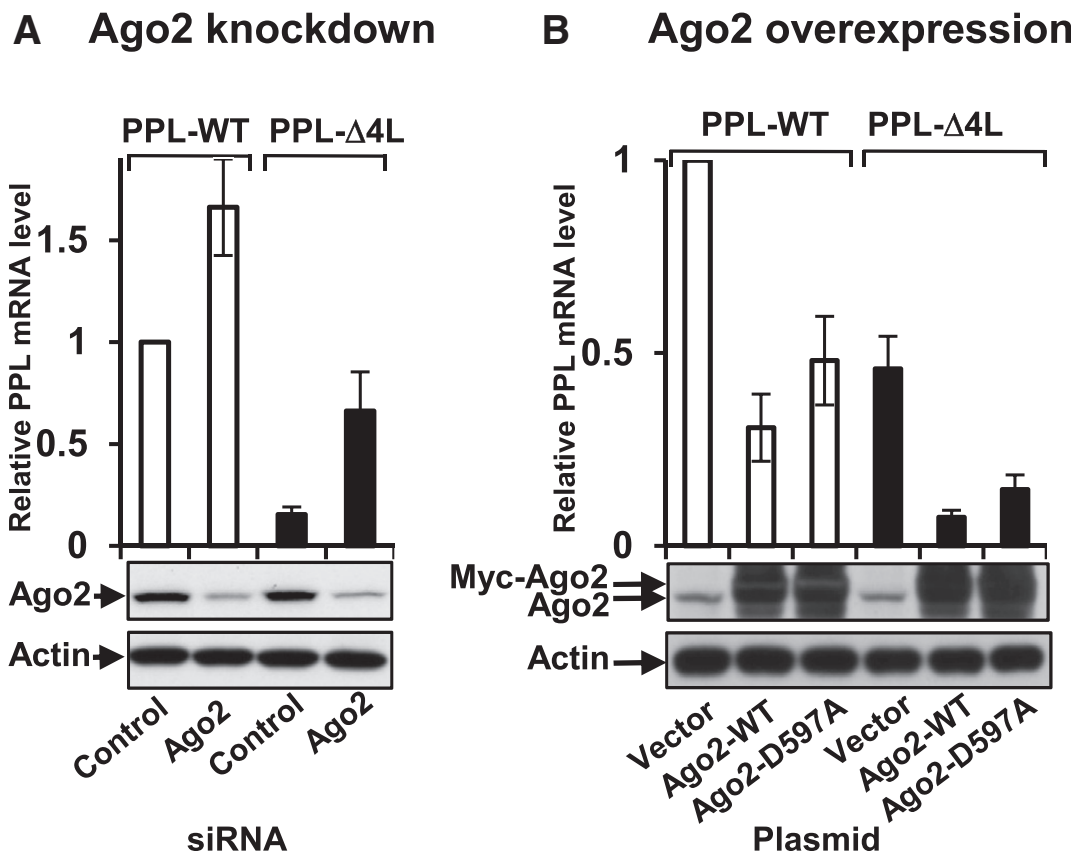

Figure 6. Process of mRNA Degradation Involves Ago2

(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 ± SEM). WT PPL mRNA levels are open bars, Δ4L mutants are black bars. Depletion or overexpression of Ago2 was confirmed by western blot, actin was a control. See also Figure S5.

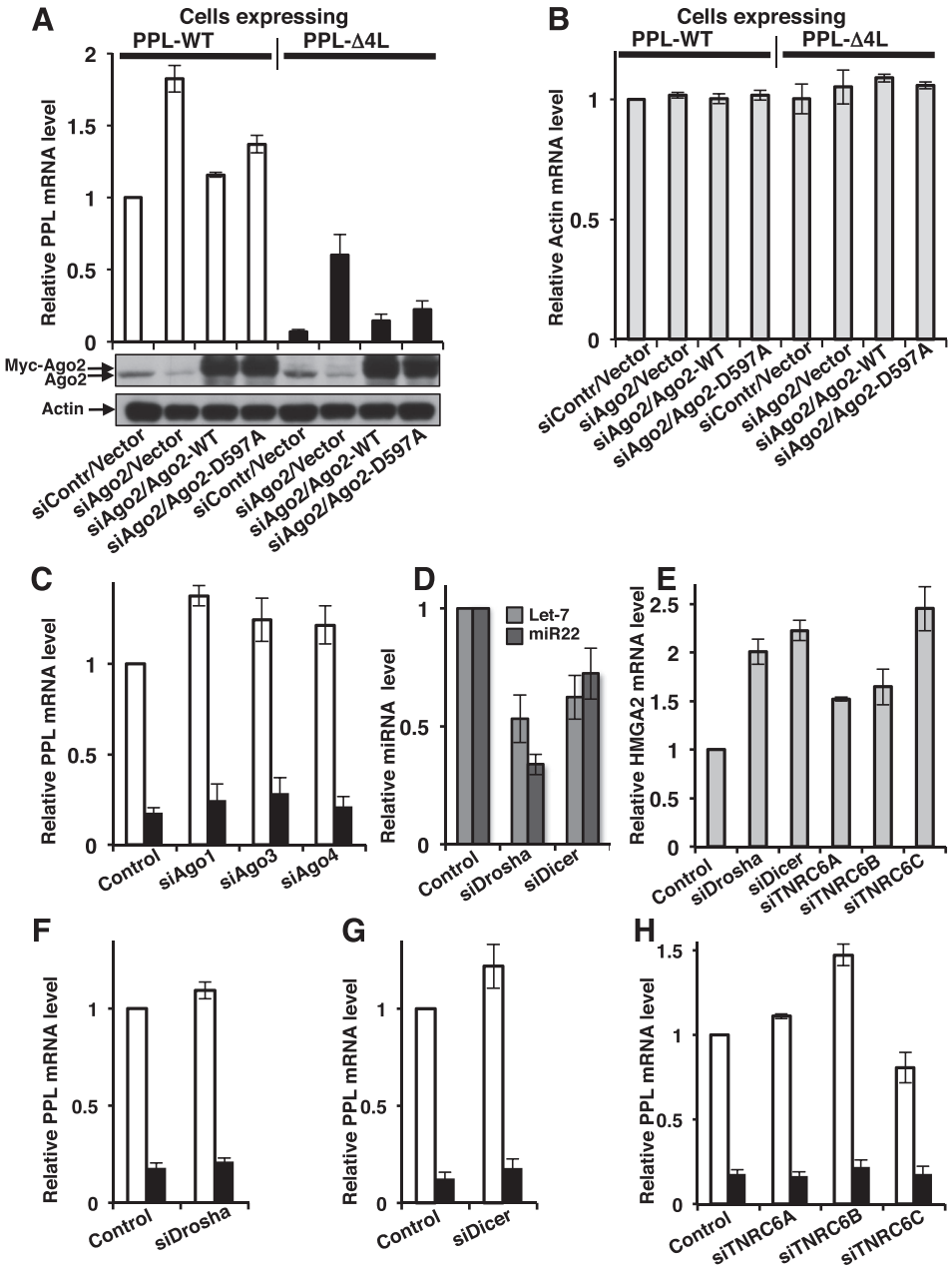
A
Cells expressing
PPL-WT
PPL-Δ4L
Relative PPL mRNA level
Myc-Ago2
Ago2
Actin
siContr/Vector
siAgo2/Vector
siAgo2/Ago2-WT
siAgo2/Ago2-D597A
B
Relative Actin mRNA level
C
Control
siAgo1
siAgo3
siAgo4
D
Let-7
miR22
Relative miRNA level
siDrosha
siDicer
E
Relative HMGA2 mRNA level
siTNRC6A
siTNRC6B
siTNRC6C
F
G
H

Figure S5. Ago2 Slicer Activity, as well as Other Argonautes, Human Paralogues of GW182, and Proteins Involved in Small RNA Biogenesis are Not Required for Specific Degradation of the Mutant Δ4L PPL mRNA, Related to Figure 6

(A) Effect of overproduction of Ago2-WT or mutant Ago2-D597A on WT PPL (open bars) or Δ4L mutant (black bars) mRNA levels under conditions of endogenous Ago2 knockdown. The cells were first treated with endogenous Ago2 specific siRNAs (target 3′-UTR of endogenous Ago2, the sequence is absent in the recombinant Ago2) or control siRNA (siContr), Ago2 expression plasmids were transfected the next day, and PPL containing plasmids were transfected a day later, and cells were incubated for 42 hr after PPL transfections. Bars are PPL mRNA levels from northern blot (n = 3, mean ± SEM). Depletion or overexpression of Ago2 was confirmed by western blot, actin was a control. (B) Actin mRNA levels in samples of the experiment presented in A (northern blot, n = 3, mean ± SEM).

(C) Knockdowns of Ago1, Ago3, and Ago4 do not suppress PPL Δ4L mRNA reduction.

(D–H) Drosha and Dicer knockdowns lead to decreased level of mature miRNAs (Kumar et al., 2007), as it is shown on the examples of Let-7 and miR22 (D) and consequently to increase in mRNA levels of miRNA regulated targets, as it is shown for a known Let-7 target (Lee and Dutta, 2007), HMGA2 (E). However, Drosha and Dicer knockdowns do not suppress PPL Δ4L mRNA reduction (F and G). Consistent with miRNA-dependent regulation of HMGA2 expression, knockdowns of TNRC6A, B, C lead to increase its mRNA level (E), however, do not suppress PPL Δ4L mRNA reduction (H).

mRNA and miRNA levels were measured by qPCR (n = 3, but controls n = 9 for C, F, H; n = 3 for D and E; n = 4 for G; mean ± SD). WT PPLs are open bars, Δ4L mutants are black bars (A, C, F–H), Let-7 and miR22 levels are light gray and dark gray bars, correspondingly (D). See also Table S1.

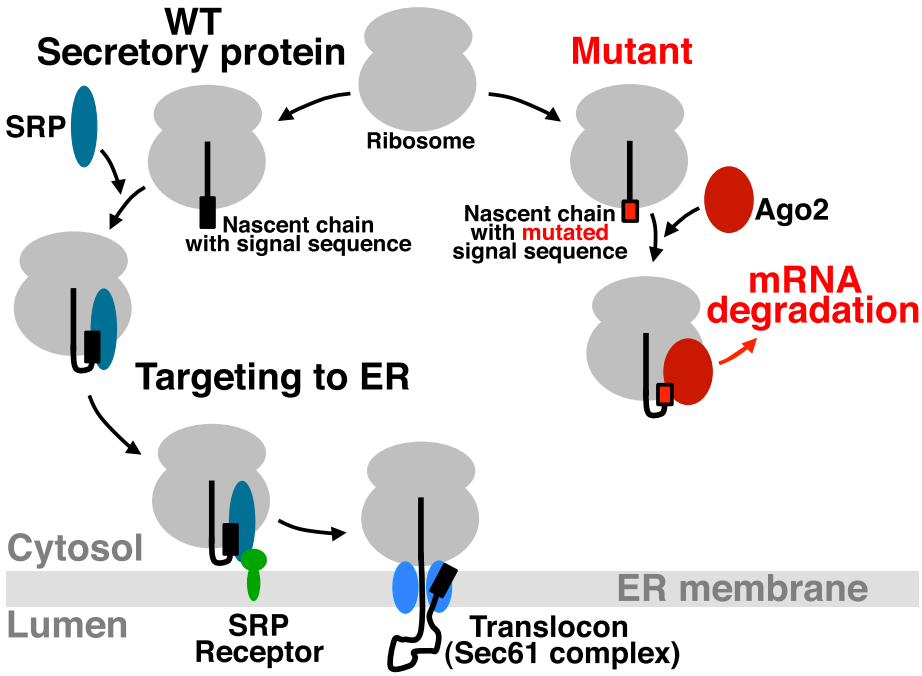

Figure 7. Model for Regulation of Aberrant Protein Production

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.

EXTENDED EXPERIMENTAL PROCEDURES

Plasmids, DNA Techniques, mRNA, and tRNA

pSPBP4 (Krieg et al., 1986) was used as a source of bovine PPL gene. Plasmids for expression of PPL in human cell cultures were made with pBI-CMV2 (Clontech) and pCS2 vectors. For the mRNA stability/degradation experiments, WT and mutant PPLs were cloned under control of the Tet-response element into vector pTRE2hyg (Clontech). For Ago2 expression in human cell culture, human Ago2 was cloned into a modified pCS2-Myc vector containing FseI and AscI sites. For this purpose, the Ago2 gene was amplified from human fetal thymus cDNA (Clontech) by two consecutive PCR reactions with the primers 1823-1825 and the primers 1826-1827 (Table S3). The resulting construct contains the human Ago2 with six Myc repeats at the N terminus under control of the CMV promoter. The construct and protocol employed for expression and purification of human recombinant Ago2 from insect cells was described previously (Ye et al., 2011). Carbonic anhydrase IV (CA4), and α1-antitrypsin (AT), were cloned into pBI-CMV2 vector and deletions in signal sequences were done by site-directed mutagenesis. CA4 and AT lacking signal sequences were made by PCR, hybrids containing the mature part of AT (Pro27–Lys418), or CA4 (Ala19–Arg312) and the WT PPL signal sequence (Met1-Thr31) or the corresponding PPL Δ4L signal sequence were prepared by overlapping PCR. Pyruvate kinase (PK) (plasmid kindly provided by Yuh Min Chook) (Zhang et al., 2011), and firefly luciferase (Luc) (pGL4.17, Promega) were cloned into pCS2 vector and the hybrids containing WT PPL or Δ4L signal sequence (Met1-Thr31) and PK (Ser2-Pro531) or Luc (Glu2-Val550) sequences were prepared by overlapping PCR. Site-directed mutagenesis was performed by a PCR technique using Pfu turbo or Pfu Ultra DNA polymerases (Stratagene). All mutations were confirmed by DNA sequencing. DNA fragments for in vitro transcription were generated by standard PCR techniques. For in vitro translation and photocrosslinking experiments mRNAs were transcribed in vitro using SP6 RNA polymerase and PCR-produced DNA fragments of the desired length as before (Flanagan et al., 2003) and purified using the RNeasy Mini kit (QIAGEN). N^{ε}-(5-azido-2-nitrobenzoyl)-Lys-$tRNA^{amb}$ (εANB-Lys-$tRNA^{amb}$) was prepared as described earlier (Flanagan et al., 2003; McCormick et al., 2003). This tRNA contains an anticodon that recognizes an amber stop codon in mRNA. Briefly, $tRNA^{amb}$ was synthesized in vitro by T7 RNA polymerase, purified by chromatography on MonoQ column, aminoacylated with [^{14}C]Lys in vitro, purified again on the same column, and chemically modified with ANB. This εANB-Lys-$tRNA^{amb}$ was used for photocrosslinking experiments.

Antibodies

The following antibodies were used in the current work: mouse monoclonal prolactin antibody (Thermo Fisher), mouse monoclonal antibody specific to human SRP54 (BD Bioscience), affinity-purified mouse monoclonal antibody JL-8 (Clontech) for AcGFP, mouse monoclonal anti-human Ago2 antibody (Wako), rabbit Ago2 antibody from Millipore and Cell Signaling, and mouse monoclonal anti-actin antibody (Millipore), normal mouse and rabbit Igg (Millipore). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used as secondary antibody for western blots. The antibodies used for immunocytochemistry were prolactin antibody (Thermo Fisher) as the primary, and Rhodamine (TRITC)-conjugated AffiniPure goat anti-mouse IgG as the secondary antibody (Jackson ImmunoResearch Laboratories).

Cell Culture, Transfections, Western Blot Analysis, mRNA Stability/Degradation Experiments

Expression of WT and mutated PPLs was conducted in cultures of HeLa Tet-On (Clontech), HeLa (American Type Culture Collection, ATCC), or HEK293 (ATCC) human cells. Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% Fetal Calf Serum (Gemini Bio-Products), 50 U of penicillin and 50 μg of streptomycin (Invitrogen) at 37°C with 5% CO2. Plasmid transfections were conducted with Lipofectamine 2000 (Invitrogen). When indicated, the cells were treated with 10 μM MG132 or DMSO for 8.5 hr. In the case of recombinant Myc-Ago2 expression in human cells, the corresponding plasmid or the pCS2-Myc vector (control) were transfected into HeLa Tet-On cells, and WT or Δ4L PPL plasmids were transfected the next day. For western blotting proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF Immobilon membranes (Millipore) and incubated with the appropriate antibody. The ECL Plus kit (GE Healthcare) was used for detection.

HeLa Tet-Off (Clontech) cells were used in mRNA stability/degradation experiments. To that end, WT or Δ4L PPLs were cloned into the pTRE2hyg vector under the inducible Tet-response element and were cotransfected with the pBI-CMV2 plasmid encoding for AcGFP (for normalization) into HeLa Tet-Off cells. In HeLa Tet-Off cells cultured in the absence of the antibiotic, mRNA is normally transcribed. However, the addition of doxycycline specifically inhibits mRNA synthesis from the gene controlled by the Tet-response element. Cells were grown for 20 hr after transfection and then treated with doxycycline for 2.5, 4, and 6 hr (untreated cells were controls). Total RNA was purified using the NucleoSpin RNA II kit (Clontech). WT or mutant PPL mRNA levels were determined by qPCR.

Confocal Microscopy and Immunocytochemistry

HeLa Tet-On cells were grown on glass coverslips, transfected with WT and Δ4L PPLs expressing plasmids, and grown for 48 hr. Cells were fixed with 4% (v/v) paraformaldehyde and permeabilized with 0.2% (v/v) Triton X-100 for 10 min. After a 1 hr treatment at room temperature with blocking buffer containing 5% goat serum, 1.5% BSA, 0.1% gelatin, 0.01% sodium azide in PBS (phosphate-buffered saline: 1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4, pH7.4), the cells were incubated at 4°C overnight with prolactin antibody, washed with PBS, then incubated with secondary antibodies at room temperature for 1 hr, and washed with PBS. Confocal images were taken with the Bio-Rad MCR-1000 Laser Scanning Confocal Imaging System.

Pulse-Labeling Experiments

HeLa Tet-On cells were transfected with WT or Δ4L PPL plasmids as above. Twenty hours after transfection, cells were starved for 40 min in DMEM media without methionine/cysteine (Invitrogen) supplemented with 1 mM glutamine in the presence or absence of 20 μM MG132. Efficient inhibition of the proteasome by MG132 was confirmed by monitoring the fluorogenic peptide (Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin, Bachem) (Kaneko et al., 2009) and by testing inhibition of proteasome in vivo by using a known proteasome substrate SOD1 fusion protein expressed in human cultured cells (Somalinga et al., 2011). Cells were labeled with EasyTag EXPRESS ^{35}S Protein Labeling Mix (100 μCi/ml, Perkin Elmer). At the 0, 2, 5, 15, and 30 min of labeling, media and cells were collected. Cells were lysed in RIPA buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) IGEPAL, 0.5% (w/v) deoxycholic acid, complete protease inhibitor cocktail [Roche]) on ice for one hour, and lysates were cleared by centrifugation (13,000 g, 30 min, 4°C). Separate aliquots of the samples were used either for immunoprecipitation of PPL or for SDS-PAGE analysis followed by western blotting to detect AcGFP. For immunoprecipitation, the cell lysates were incubated with prolactin antibody overnight at 4°C, then 40 μl of protein G-Sepharose (Calbiochem) were added, and incubation was continued for 4 hr. Beads were collected by centrifugation, washed 3 times with RIPA buffer, and samples were analyzed by SDS-PAGE. Gels were visualized on a Typhoon 9410 Variable Mode Imager (GE Healthcare). The presence of labeled PPL in the media was also examined by immunoprecipitation, and was found to be below the detection limit at the time points of the experiment. Quantification was performed using the Quantity One Software (Bio-Rad). Each preprolactin sample was normalized to its AcGFP content. The amount of radiolabeled PPL was calculated relative to WT PPL after 15 min of labeling without MG132.

RNA Interference Experiments

siRNAs were synthesized by Dharmacon. HeLa Tet-On cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen), followed by transfection with WT or mutant PPL plasmids the next or second day after siRNA transfection. In the experiment with endogenous Ago2 knockdown and expression of recombinant WT Ago2 or D597A mutant, the cells were first treated with endogenous Ago2 specific siRNAs (target 3′-UTR of endogenous Ago2, a sequence absent in the recombinant Ago2), Ago2 expression plasmids were transfected the next day, and PPL expression plasmids were transfected a day later. Cells were incubated for 42 hr after PPL transfections. The following siRNAs were used for knock down of SRP54 (sense: GAAAUGAACAGGAGUCAAUdTdT) (Ren et al., 2004), Ago1 (GCACAGUAUUUCAAGCAGA, CAACGAACGGGUCGACUUU), Ago3 (UCAUGUAACUGGACGCGUA, AGAGAACAGUAGCGCAGUA), Ago4 (GUGGACAGAUGAAACGAAA, CAAUAUGGAGGCCGGAAUA), TNRC6A (GCCUAAAUAUUGGUGAUUA, CCGGUUCAGUGCAGAAUAA), TNRC6B (GCACUGCCCUGAUCCGAUA, GGAGUGCCAUGGAAAGGUA), TNRC6C (CUAUUAACCUCGCCAAUUA, GAAUUCUUCUAGACAGAUA), Dicer (UAAAGUAGCUGGAAUGAUG, GAAUAUGGUUGUUUGAAGA), Drosha (GGCCAACUGUUAUAGAAUA, GGCCAACUGUUAUAGAAUA), SRα (GCGAGGAGUUCAUUCAGAA, GAGCUUGAGUCGUGAAGAC), SRβ (UUAGAGCGGUUUAAGUCUU, CAUGAGAGUUUGAGGCUUC), Sec61α (ACUUUGAGAUCUUCGUUAA, GUGUCAUCCUGCCGGAAAU), for Ago2 knockdown Ago2-specific siRNAs were as published (Chu et al., 2010). Silencer Negative Control #1 siRNA (Ambion) or control siRNA (ACUACAUCGUGAUUCAAACUU) were used as controls.

cDNAs, Quantitative Real Time-PCR

Total RNA was purified using the NucleoSpin RNA II kit (Clontech) or Trizol (Ambion). cDNAs were synthesized using a High-Capacity cDNA reverse transcription kit (Applied Biosystems). qPCR was conducted using Power SYBR Green and the 7900HT Fast Real-Time PCR System (Applied Biosystems). PPLs, both WT and mutants, were detected by 1857-1858 or 1852-1877 primer sets (Table S3). The PPL WT-specific primer set was 1932-1934, and the PPL Δ4L mutant-specific primer set was 1933-1934. The following primers were used to detect expression of SRP54 (1880-1881), AcGFP (1913-1914), actin (1896-1897), CA4 (4426; RTPrimerDB ID 4426), AT (2157-2158), alkaline phosphatase (1981-1982), Bip (1987-1988), calreticulin (1989-1990), SRα (2121-2122), SRβ (2238-2239), HPRT (1894-1895), PK (2281-2282, primers specific to cloned PK only), Luc (2148-2149), Ago1 (2226-2227), Ago3 (2228-2229), Ago4 (2230-2231), TNRC6A (2046-2047), TNRC6B (2300-2301), TNRC6C (2234-2235), Dicer (Dicer F- Dicer R), Drosha (2236-2237), Sec61α (2240-2241), HMGA2 (2312-2313). let-7b, miR-22 and U6 snRNA were analyzed by TaqMan MicroRNA Assays (Applied Biosystems), qPCR data were analyzed by the comparative C_T method (Schmittgen and Livak, 2008).

Northern Blot

RNA probes for northern blots were generated by in vitro transcription using SP6 RNA polymerase (New England Biolabs) from PCR templates. The SP6 promoter sequence was introduced into a primer, and the primer sets were designed to generate antisense RNA probes. The 2001-2002 set was used to generate the PPL-specific probe, while the 2003-2004 set generated the AcGFP-specific probe. A plasmid carrying PPL and the pBI-CMV2 plasmid carrying AcGFP were used as PCR templates to make probes. To produce an actin control probe, total RNA was purified from HeLa Tet-On cells, and actin cDNA was produced using SuperScript II Reverse Transcriptase (Invitrogen). The cDNA was amplified by PCR with 2009-2010 primers. The product of the first PCR reaction served as a template for a second PCR reaction with primers 2011-2012. Finally, an antisense actin RNA probe was synthesized using SP6 RNA polymerase. RNA probes for hybridization were purified via the NucleoSpin RNA II kit (Clontech), and labeled using the BrightStar Psoralen-Biotin Nonisotopic Labeling Kit (Ambion). Total RNAs were purified from cultured human cells and separated by agarose gel electrophoresis in the presence of formaldehyde before being transferred to BrightStar-Plus positively charged nylon membranes (Ambion) using Model 785 Vacuum Blotter (Bio-Rad). The RNA on the membranes were detected by hybridization with the above probes in ULTRAhyb buffer and detected by BrightStar BioDetect Kit (Ambion) according to the manufacturer's instructions. Northern blots were quantified by ImageJ software (NIH); PPL mRNA levels were calculated relatively to PPL WT with a control transfection.

RNA Immunoprecipitation

HeLa Tet-On cells transfected either with WT PPL or Δ4L expression plasmids were grown for 24 hr after transfection and lysed in buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, RNAsin (Promega) and protease inhibitors (Roche). Endogenous Ago2 was immunoprecipated from cell lysates with an Ago2 mouse monoclonal antibody (Wako) and Protein G-Agarose (Roche) according manufacturer's recommendations. Beads incubated with normal mouse IgG (Millipore) served as a negative control. After incubation for 4 hr, beads were washed several times using cell lysis buffer. RNA/protein complexes were eluted from the beads using buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS and divided into two aliquots used either for western blotting or RNA extraction. RNA was extracted using Trizol LS reagent (Invitrogen), treated with DNase and used for cDNA production and subsequent semiquantitative RT–PCR (Karamysheva et al., 2003) or qPCR reactions. Ago2 was detected in immunoprecipitates by western blot using Ago2 rabbit antibodies from Cell Signaling. A similar RNA immunoprecipitation experiment was performed using rabbit polyclonal Ago2 antibody (Millipore) and normal rabbit IgG (Millipore) as a negative control. Ago2 was detected in immunoprecipitates by western blot using Ago2 mouse monoclonal antibodies (Wako). Data analyzed by the fold enrichment method (Ciccone et al., 2004).

Canine SRP, ER Microsomes, Ago2 Purification

Canine SRP was purified as described previously (Walter and Blobel, 1983b), except that the sucrose density sedimentation was omitted. Canine endoplasmic reticulum rough microsomes (ER microsomes or RMs) were prepared as before (Walter and Blobel, 1983a). Expression and purification of recombinant human Ago2 was described previously (Ye et al., 2011).

In Vitro Translation and Photocrosslinking

Translations (30 min, 30°C) of full-length mRNA were performed in a total volume of 10 μl containing nuclease-treated reticulocyte lysate, 0.8 μl EGS-M [15 mM ATP, 15 mM GTP, 120 mM phosphocreatine, 0.12 U/μl creatine phosphokinase, 0.375 mM of each amino acid except methionine in 90 mM HEPES (pH 7.5)], 50 μg/ml bovine tRNA, 2.5 mM magnesium acetate (MgAcetate), 70 mM potassium acetate, 0.8 U/μl RNasin, 0.35 μCi/μl [^{35}S]methionine, 0.8 equivalents of ER microsomes (RMs). Translated proteins were separated by 12% SDS-PAGE and visualized by phosphoimager.

For photocrosslinking experiments, in vitro translations (25 μl total volume; 26°C, 30-40 min in the dark) contained wheat germ cell-free extract (Erickson and Blobel, 1983), 25 μCi of [^{35}S]methionine, 25-35 pmole of εANB-Lys-tRNA^{amb} or unmodified Lys-tRNA^{amb}, the desired truncated mRNA, 60 nM canine SRP (Walter and Blobel, 1983b) where indicated, and other components as described previously (Do et al., 1996; McCormick et al., 2003). After photolysis (Do et al., 1996), ribosomes were pelleted, resuspended and treated with RNase A and EDTA (Etchells et al., 2005), and analyzed by 10%–15% gradient SDS-PAGE. Photoadducts were detected by phosphorimager. The quantity of each photoadduct was normalized to the total amount of the corresponding nascent chain, and then compared to the WT PPL crosslink normalized in the same manner.

For immunoprecipitation of nascent chain photoadducts to SRP54, ribosome pellets from photolyzed 50-μl translations were resuspended in 55 μl of 0.25% (w/v) SDS, 100 mM Tris-HCl (pH 7.5) and placed at 55°C for 30 min. After 5 μl were removed for direct analysis by SDS-PAGE, the remaining 50 μl were supplemented with 500 μl immunoprecipitation buffer [140 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2% (v/v) Triton X-100], followed by 2.5 μl SRP54-specific antibody. After overnight shaking at 4°C, 40 μl of protein G-Sepharose (Calbiochem) were added and incubated for 2 hr. Beads were recovered by centrifugation, washed twice with immunoprecipitation buffer and one time with immunoprecipitation buffer lacking Triton X-100. Samples were analyzed by SDS-PAGE and visualized as above.

Photocrosslinking Assay for the Identification of Proteins Interacting with Mutant Nascent PPL

Fractions of cell lysates were tested for the presence of proteins forming a photoadduct with mutant PPL. For this purpose salt-washed ribosome nascent chain complexes (salt-washed RNCs) of mutated Δ4L PPL-86 truncated mRNA were prepared. Translations were conducted in the dark for 40 min as above, except that reactions were conducted in a total volume of 225–500 μl. At the end of translation, KAcetate was added to 300–400 mM and samples were incubated on ice for 10–15 min. Salt-washed RNCs were collected by centrifugation through sucrose cushions as above, except that the sucrose cushions contained 300 mM KAcetate. Pellets were resuspended in buffer (50 mM HEPES [pH 7.5], 40 mM KAcetate, 5 mM $MgCl_2$), aliquoted, and combined with fractionated proteins or purified Ago2. After incubation on ice for 15 min, samples were photolyzed and analyzed by SDS-PAGE as above.

Purification of the ~100 kDa Protein that Is Photocrosslinked to a Mutant Nascent PPL

Two independent approaches for protein purification and mass spectrometry conducted at two different facilities resulted in the identification of Ago2 as a crosslink partner of nascent mutant PPL (Table S2). The first approach was based mainly on an earlier published method (Otto et al., 2005). Ribosomes were isolated from 200 ml of rabbit reticulocyte lysate (Green Hectares, Oregon, WI) by centrifugation at 200,000 g for 1.5 hr. Each of the ribosomal pellets was washed once with 10 ml of buffer S1 (20 mM HEPES (pH 7.4), 5 mM MgAcetate, 1 mM dithiothreitol, 1 mM PMSF) containing 50 mM KAcetate. Ribosome-associated proteins were released by resuspending the ribosomal pellets in S1 containing 400 mM KAcetate. High-salt washed ribosomes were separated from released proteins by centrifugation at 200,000 g for 1.5 hr. Supernatant was diluted with 4 volumes 20 mM HEPES (pH 7.4) containing 1 mM PMSF. The material was applied to a MonoS HR5/5 column (Amersham Pharmacia) in two identical successive runs. Bound proteins were eluted with a 50–1,200 mM, 25-ml linear KAcetate gradient in 40 mM HEPES (pH 7.4) and 1 ml fractions were collected. Fractions 8–10 from both MonoS runs were pooled and diluted with 3 volumes of 40 mM HEPES (pH 7.4). The material was loaded onto a MonoQ HR5/5 column (Amersham Pharmacia). Bound proteins were eluted with a 50–1,000 mM, 18-ml linear KAcetate gradient in 40 mM HEPES (pH 7.4). One ml fractions were collected and 5 μl of fraction 3 −18 were tested in the crosslinking assay described above. A strong nascent chain photocrosslink to a protein of ~100 kDa, was observed in three fractions (8–10), with most in fraction 9 (Figure 5E). To identify the crosslink partner, 400 μl of fraction 9 was concentrated to a volume of 100 μl and the sample was applied to 6%–16.5% BN-PAGE followed by a second dimension separation under denaturing conditions as described (Otto et al., 2005). Spots from the second-dimension gel were excised and analyzed via mass spectrometry (Table S2, Experimental Approach 1).

In the second approach, ribosomes were isolated from 20 ml of rabbit reticulocyte lysate (Green Hectares, Oregon, WI) by centrifugation at 540,000 g (Beckman TLA100.3 rotor) for 1 hr. Ribosome-associated proteins were released by resuspending of the ribosomal pellet in buffer containing 45 mM HEPES (pH 7.5), 4.5 mM MgAcetate, 440 mM KAcetate, 2 mM glutathione, and then separated from salt-washed ribosomes by centrifugation as above. The supernatant was dialyzed 3 times against 25 mM HEPES (pH 7.5), 40 mM KAcetate, 2 mM glutathione, and proteins were separated on a FPLC using a 40–1,200 mM KAcetate linear gradient in 25 mM HEPES (pH 7.5), 2 mM glutathione with a MonoQ 5/50GL column (GE Healthcare). Aliquots (2.5 μl) of each fraction were tested using the crosslinking assay as above. Two fractions that showed a strong crosslink to a ~100 kDa protein were dialyzed as above and used for second chromatography on a 1-ml HiTrap Heparin HP column (GE Healthcare) with a 40–1200 mM KAcetate linear gradient in 25 mM HEPES (pH 7.5), 2 mM glutathione. The resulting fractions were tested for crosslinking, and one fraction with a strong crosslink to a ~100 kDa protein was selected. Its proteins were separated by SDS-PAGE and the ~100 kDa band was excised from the gel. Mass spectrometry was conducted by the Protein Chemistry Core Facility, UT Southwestern Medical Center. After in-gel digestion with trypsin, the peptide mixture was subjected to nano LC/MS/MS analysis. Nano-HPLC/electrospray mass spectrometry was performed on a ThermoFinnigan LTQ instrument, coupled with an Agilent 1100 Series HPLC system. The separation was achieved with a 75 micron i.d. C18 reverse-phase chromatography column. The resulting files were searched against NCBI-nr protein sequence databases through the Mascot search engine (Matrix Science). The results are shown in Table S2 (Experimental Approach 2). Both applied protein identifications independently revealed Ago2 protein in the selected fractions (Table S2).