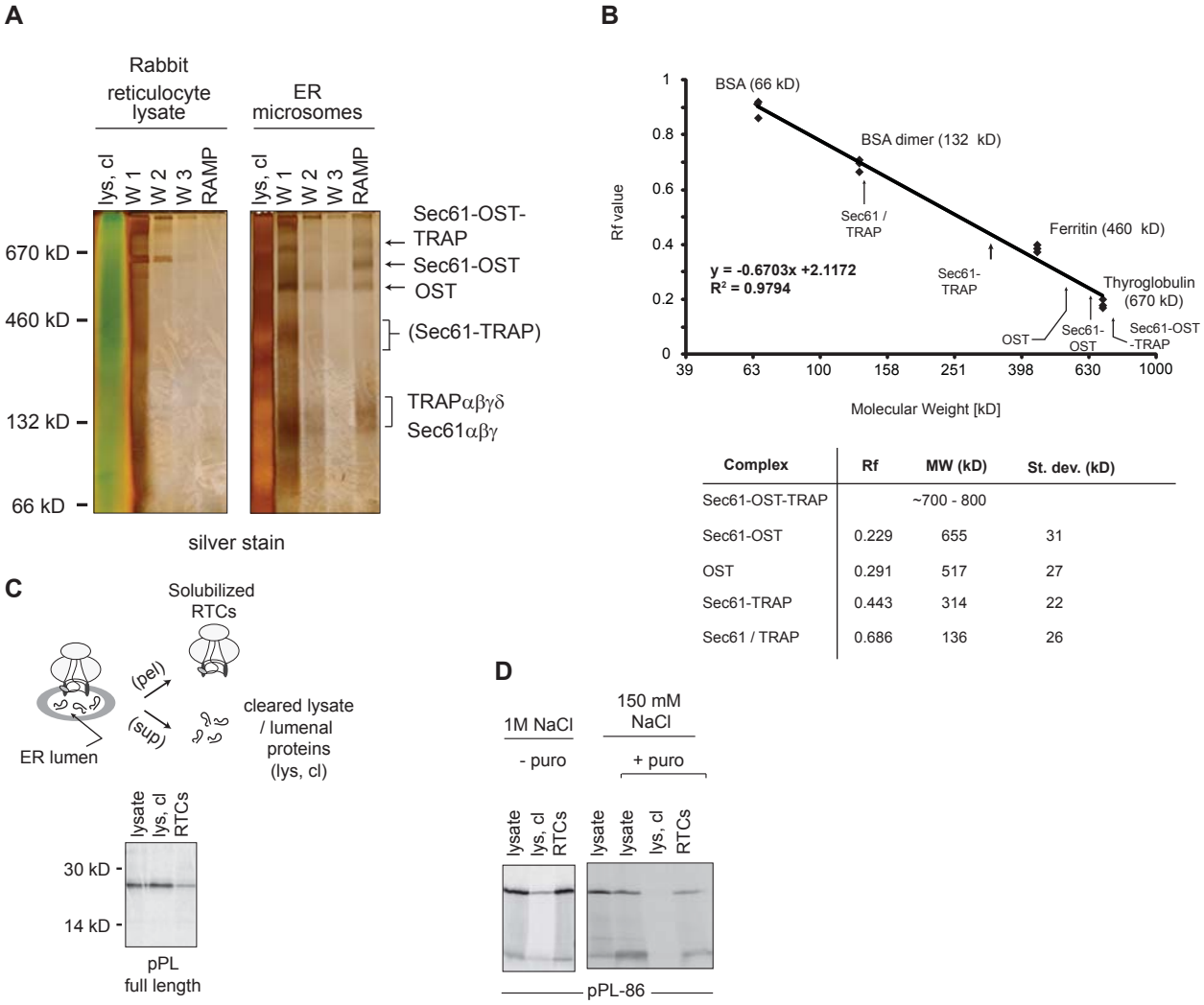
FIgure S1, related to Figure 1



Complex	Rf	MW (kD)	St. dev. (kD)
Sec61-OST-TRAP		~700 - 800	
Sec61-OST	0.229	655	31
OST	0.291	517	27
Sec61-TRAP	0.443	314	22
Sec61 / TRAP	0.686	136	26

Figure S1. RAMP Complex Isolation and Molecular Weight Determination, Related to Figure 1

(A) Rabbit reticulocyte lysate (RRL) was subjected to the same RAMP isolation procedure described in methods (left panel) to confirm specific membrane derivation of observed protein complexes (right panel, identical data from **Figure 1**B). (B) Apparent size of RAMP complexes was determined by plotting the Rf versus the MW of indicated standard molecular markers. Curve fits of three independent experiments are shown along with calculated RAMP complex molecular weight average +/- standard deviation (n=3). Note that the Sec61-OST-TRAP complex Rf value did not fall within the standard curve and the molecular weight is therefore approximated. Molecular weights likely reflect protein masses as well as associated digitonin micelles and therefore are not meant as an absolute size measurement. (C) Full length pPL was translated in the presence of CRMs, and pelleted microsomes were lysed with digitonin. Unlike pPL-86 solubilized RTC intermediates (RTCs), full length pPL remained in the supernatant (sup) fraction after ultracentrifugation. (D) Treatment of pPL-86 RTCs with 1M NaCl or puromycin in 150 mM NaCl did not cleave the peptidyl-tRNA bond nor disassociate nascent chain from the RTC at 4˚C.

FIgure S2, related to Figure 2

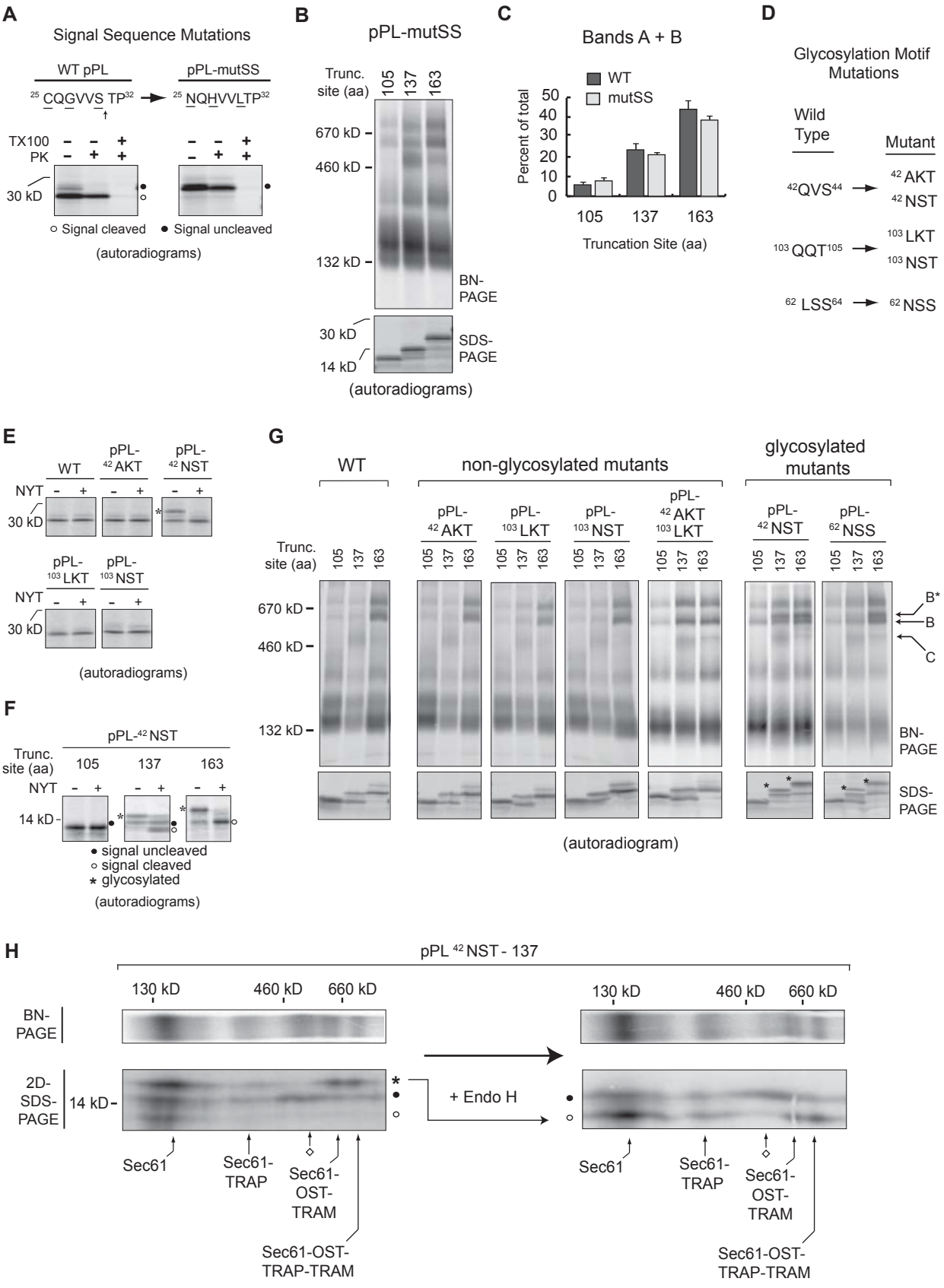


Figure S2. Stabilization of RAMP Complexes A and B Is Not Dependent on Signal Cleavage or Recognition of Glycosylation Motifs, Related to Figure 2

(A) The signal sequence of WT pPL was mutated as depicted (pPL-mutSS) to eliminate signal cleavage while maintaining efficient translocation. SDS-PAGE (autoradiograms) analysis of indicated translation products treated with proteinase K (PK) ± Triton-X 100 (TX-100). (B) pPL-mutSS RAMPs migrate similar to those of WT pPL. Autoradiogram of pPL-mutSS polypeptides truncated as indicated and analyzed by BN-PAGE (top) and SDS-PAGE (bottom). (C) Quantity of truncated WT versus pPL-mutSS recovered in complex A and B as a fraction of total. Results show mean ± SEM (n=6 for WT, n=3 for mutSS). (D) pPL non-cognate glycosylation sites (^{42}QVS and ^{103}QQT) (Nilsson et al., 2003) were eliminated (^{42}AKT and ^{103}LKT) or mutated into consensus sites (^{42}NST and ^{103}NST), whereas pPL ^{62}LSS was mutated into a ^{62}NSS glycosylation site. (E-F) Autoradiograms of full length (E) and truncated (F) pPL mutants translated in the absence and presence of a tripeptide (NYT) glycosylation inhibitor. Asterisk denotes glycosylated polypeptides. tRNA was removed with RNase prior to SDS-PAGE. (G) WT and pPL glycosylation mutants truncated as indicated were analyzed by BN-PAGE (top gels) and SDS-PAGE (bottom gels). pPL ^{42}NST and pPL ^{62}NSS glycosylation resulted in an additional RAMP complex B*. Elimination of non-cognate glycosylation sites did not alter RAMP complex migration on BN-PAGE compared to WT pPL RAMPs. (H) Migration of pPL-^{42}NST-137 on 2D-BN/SDS-PAGE with and without EndoH-mediated glycan removal preceding RAMP release. The symbols "•," "o," and "*" represent signal uncleaved, signal cleaved, and glycosylated peptides respectively.

FIgure S3, related to Figure 4

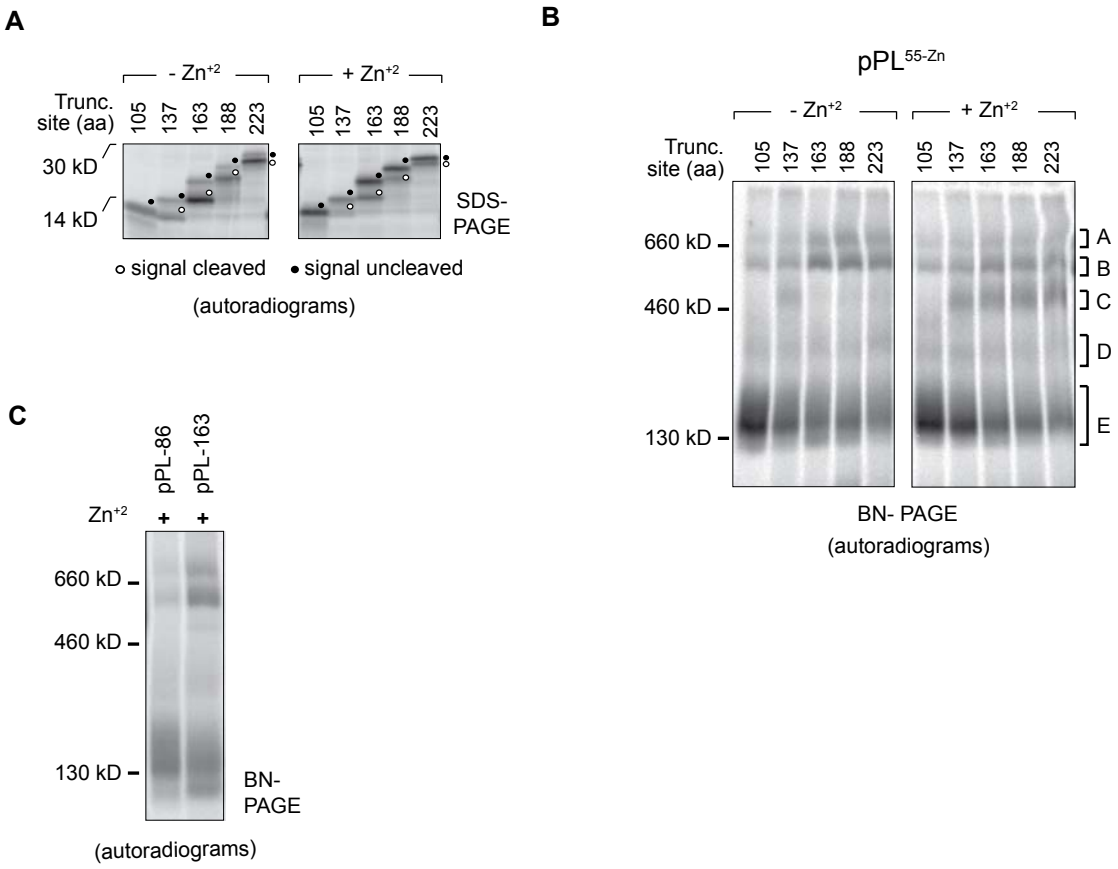


Figure S3. pPL$^{55\text{-}Zn}$ Blocks Translocation in the Presence of Zn^{+2} and Stabilizes RAMP Complex C, Related to Figure 4

(A) Autoradiogram of pPL$^{55\text{-}Zn}$ translated in the presence and absence of Zn^{+2} shows that induced folding of the Zn-finger decreases translocation as monitored by signal sequence cleavage. (B) The presence of Zn^{+2} stabilizes RAMP complex C. Previous studies (Conti et al., 2014) show that the translocation block with pPL$^{55\text{-}Zn}$ is less efficient than pPL$^{45\text{-}Zn}$, which allows observation of both signal cleaved and uncleaved polypeptide on 2D-BN/SDS-PAGE (**Figure 4E**). (C) Translation in the presence of Zn^{+2} did not affect WT pPL migration patterns in the 650 and 750 kD RAMP complexes as nascent chain was extended from 86 to 163 aa.

FIgure S4, related to Figure 5

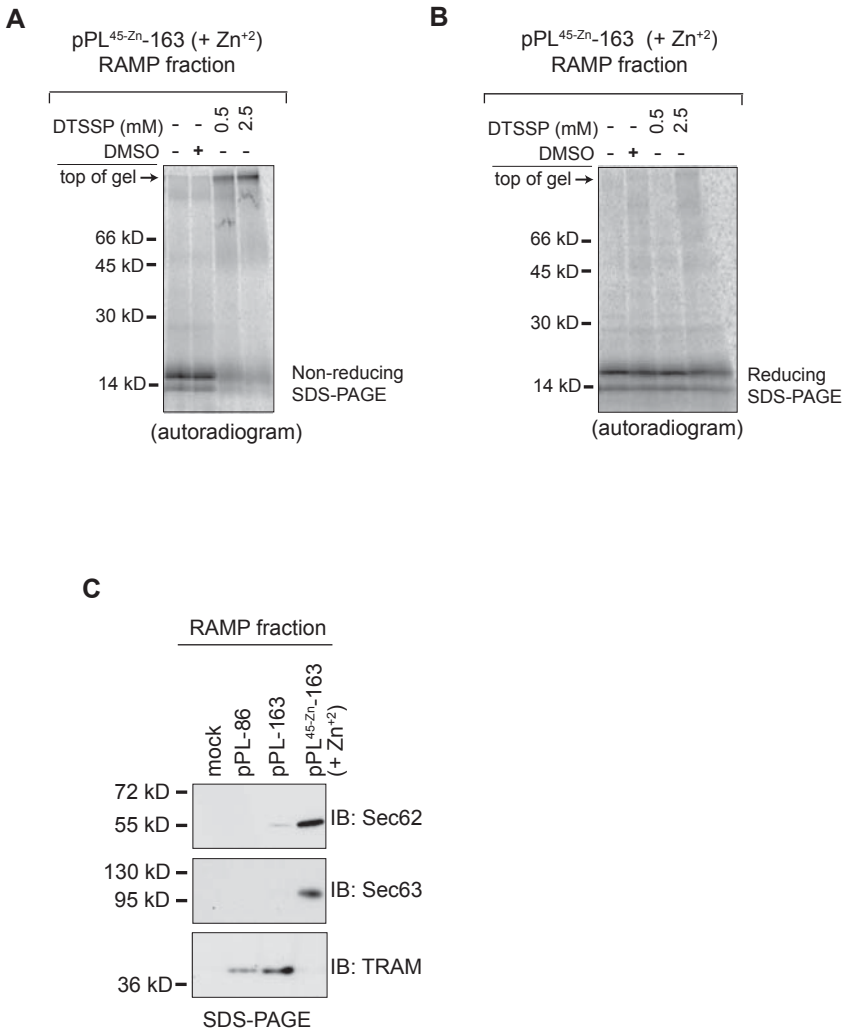


Figure S4. DTSSP Crosslinking and Identification of Complex C Components, Related to Figure 5

(A) Non-reducing SDS-PAGE analysis (autoradiogram) of RAMPs derived from pPL$^{45\text{-}Zn}$-163 produced in the presence of Zn^{+2} (denoted by "(+Zn^{+2})") after treatment with DMSO or DTSSP. Nascent chain migration at the top of SDS-PAGE gels upon DTSSP treatment indicated covalent crosslinking to adjacent protein components. (B) Identical samples as in panel (A) were reduced with DTT before SDS-PAGE analysis. DTT treatment reversed covalent crosslinks and allowed nascent chains to migrate similarly to samples treated with DMSO alone. (C) SDS-PAGE western blots confirmed pPL$^{45\text{-}Zn}$-163"(+Zn^{+2}) RAMP fractions contain Sec62 and Sec63.

FIgure S5, related to Figures 5 and 7

LC-MS/MS Summary - Identified Peptides

Translocon Component		Experiment / Samples			
		1a	1b		
Protein ID	Uniprot Accession	mock	$pPL^{45\text{-}Zn}$-163-His	mock	PrP-163-His
Sec61α	J9NXA9	-	FSGNLLVSLLGTWS-DTSSGGPAR[1] YRGQYNTYPIK[1] IIEVGDTPkDR[1,2] FLEVIKPFcVILPEIQkPER[1,2] GQYNTYPIK[2] TWIEVSGSSAKDVAk[2]	-	GQYNTYPIK[1] AFSPTTVNTGR[1] FLEVIKPFcVILPEI-QkPER[2] IIEVGDTPK[1,2] FLEVIKPFcVIL-PEIQKPER[1,2]
Sec62	J9NUW8	-	AkkGEEALFTTR[1] ADLKkDEkSETK[1] AVDcLLDSkWAK[2] ESVVDYcNR[2] GEEALFTTR[2] IQEVGEPSkEEK[1,2]	-	AkkGEEALFTTR[1] GEEALFTTR[1] AVDcLLDSkWAK[2] IQEVGEPSkEEK[2] kENLkDEK[2] IQEVGEPSK[2] ESVVDYcNR[2]
Sec63	Q9UGP8	-	LIMVLAGASEFDPQYNK[1] TkGGWQQkSK[1] EIGSINLkkNEPPLTcPYSLK[1] TIQDLVSLK[1] ITHPVYSLYFPEEK[2] DTEEVELkFPAPGKPG-NYQYTVFLR[2] DATSRPTDNILIPQLIR[2] SkITHPVYSLYFPEEK[2] FPAPGKPGNYQYTVFLR[2] FPAPGkPGNYQYTVFLR[2] QEWWWLYIADR[2] TIQDLVSLkESDR[2] ALLETkSK[2] DDEAEWQELQQSIQR[2] TTQIYTYFVYK[1,2] SQVLDDEDSNNITVG-SLVTVLVK[1,2] IAkAYAALTDEESR[1,2] SPLLQLPHIEEDNLR[1,2] IAkAYAALTDEESRK[1,2] YSGDQILIR[1,2] VLLLSHLAR[1,2] IkTIQDLVSLK[1,2] AYAALTDEESR[1,2]	-	ITHPVYSLYFPEEK[1] EDAVSkEVTR[1] DATSRPTDNILIPQ-LIR[1] QNKDDEAEWQELQ-QSIQR[1] EIGSINLkkNEPPLTc-PYSLK[1] AYAALTDEESRK[1] IAkAYAALTDEESR[1] IkTIQDLVSLK[1] DDEAEWQELQQ-SIQR[1] VLLLSHLAR[1] TIQDLVSLK[1] SkITHPVYSLY-FPEEK[2] FPAPGKPGNYQY-TVFLR[2] YSGDQILIR[1,2] AYAALTDEESR[1,2] SPLLQLPHIEED-NLR[1,2]
Rpn I (Ribo-phorin I)	E2RQ08	-	ELVLkSAVEAER[2] NVESYTkLGNPTR[2]	-	VTAEVVLAHPGG-GSTAR[1] ALTSEIASLQSR[1] kSLETEHkALTSEI-ASLQSR[1] NVESYTkLGNPTR[2] HFDETINR[2] GEDEEENNLEVR[1,2]
OST48	Q05052	-	-	-	YSQTGNYELAVA-LSR[2] ELGSEcGIEFDEEK[2]
Rpn II	F1PBH2/ F1PCT7	-	-	-	SIVEEIEDLVAR
HSPA5 (BiP)	F1PIC7	-	DNHLLGTFDLTGIPPAPR[2]	-	
TRAPγ	J9P906	-	EDAVSkEVTR[1]	-	EDAVSkEVTR[1]
Sec61β	P60467	-	FYTEDSPGLK[1] TTSAGTGGMWR[2]	-	QQSEEDLLLQD-FSR[1] SPSkAVAAR[2] FYTEDSPGLK[2]
Sec61γ	P60058	-	LIHIPINNIIVGG[2]	-	-

k = DTSSP-modified lysine
c = iodoacetamide -modified cysteine

[1] = detected in N_1
[2] = detected in N_2
[1,2] = detected in both repeated experiments, N_1 and N_2

Figure S5. Identified LC-MS/MS Peptide Sequences, Related to Figures 5 and 7
Unique peptide sequences designated in **Figure 5**E and **7**E are listed according to experiment /sample identities and the translocon component from which they are derived. Lower case "c" indicates a cysteine residue modified by iodoacetamide. Lower case "k" indicates a lysine modified by a DTSSP adduct. Peptides detected in either the first (N_1), second (N_2), or both experimental repeats are denoted by "[1]", "[2]", or "[1, 2]", respectively. The general criterion for positive protein detection in experimental samples was two or more high probability peptide matches in both experimental repeats, N_1 and N_2, compared to none in corresponding mock controls. Also listed are peptides corresponding to translocon proteins that failed to meet the above criteria.

FIgure S6, related to Figure 5

LC-MS/MS Protein Indentifications and Translocon Component Peptide Matches from Experiment 2b and 2c.

			Experiment / Sample		
			2a	2b	2c
Protein Category	Uniprot Accession	Protein Description	mock	pPL$^{45\text{-}Zn}$-163 -His	pPL-163 -His
Translocon Components					
	Q9UGP8	human Sec63		17	1
	J9NUW8	Sec62		3	
	F1PIC7	BiP		6	7
	J9NXA9	Sec61α		5	5
	E2RQ08	Ribophorin I		5	6
	Q05052	OST 48		1	2
	E2RAB2	gp180 / Ribosome Binding Protein		1	3
	E2QY54	TRAPδ		1	2
	F1PBH2	Ribophorin II			4
	P61010	SRP54		1	
	P60467	Sec61β		1	1
	J9P906	TRAPγ		1	1
	J9NTJ9	SRP receptor α		1	
	F1PJP5	STT3A		1	1
	E2RGF1	RAMP4		1	1
	E2R4X3	DAD1		1	1
	P60058	Sec61γ			1
	P16967	TRAPα			1
Unknown Significance					
	F1PLR0	Uncharacterized protein GN=GNB2L1		4	1
	P25291	Pancreatic secretory granule membrane major glycoprotein GP2		3	
	E2RQJ8	Uncharacterized protein (Fragment) GN=SLC30A7]		2	
	F1PRS5	Uncharacterized protein GN=ATP13A1 PE=3		2	2
	F1PQL8	Uncharacterized protein GN=ACTG1		2	1
	E2RJW8	Uncharacterized protein GN=PGRMC1		2	1
	P60524	Hemoglobin subunit beta GN=HBB			2
	J9NVT1	Uncharacterized protein GN=UBC			2
Ribosomal Proteins					
	E2R8R8	Uncharacterized protein GN=RPS9		3	4
	E2QXF3	60S ribosomal protein L15 GN=RPL15		3	
	F2Z4Q5	40S ribosomal protein S3a GN=RPS3A		2	1
	F1PKR6	Uncharacterized protein (Fragment) GN=RPS16		2	
	E2RIA8	Uncharacterized protein GN=RPL8		2	
	F1Q0Z2	Uncharacterized protein GN=RPL7			2

# of Unique Identified Peptides

Figure S6. Additional Proteins Detected in Experimental Sample 2b and 2c by LC-MS/MS, Related to Figure 5

All proteins identified in experimental samples 2b (pPL$^{45\text{-}Zn}$-163-His) and 2c (pPL-163-His) are shown. The general criterion for protein identification in experimental samples was two or more high probability peptide matches compared to none in their corresponding mock control (2a). Proteins identified were parsed into three categories: translocon components, “proteins of unknown significance,” and ribosomal proteins. In addition, translocon proteins with a single peptide identified are also listed.

FIgure S7, related to Figure 7

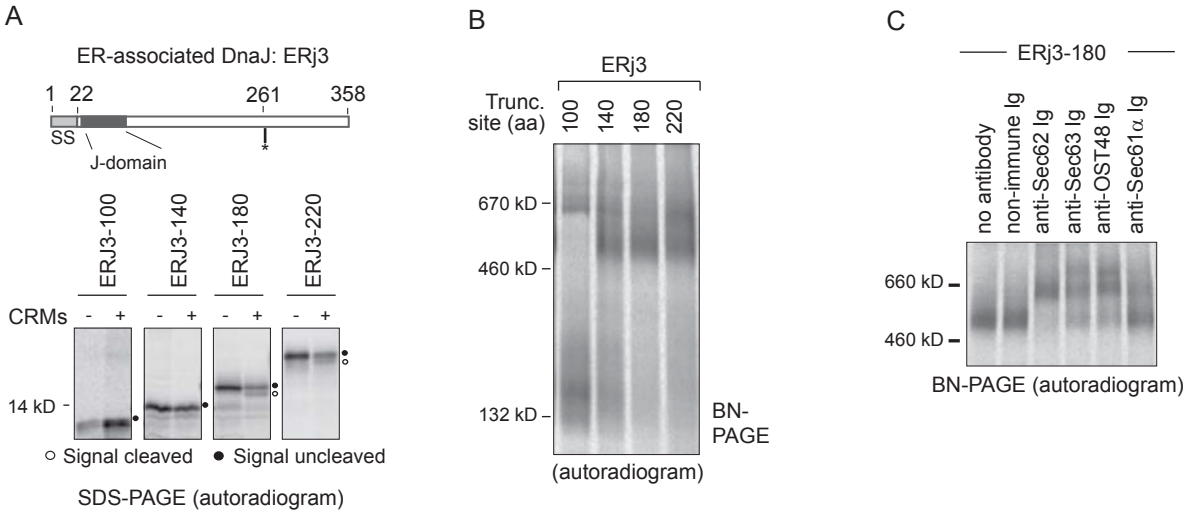


Figure S7. ERj3 Stabilizes Sec62/63-Containing RAMP Complex C, Related to Figure 7

(A) Schematic of ERj3 depicts the presence of a helical J-domain immediately C-terminal to the signal sequence (SS). Truncated nascent chain translated in the presence and absence of CRMs treated with RNase prior to SDS-PAGE. Partial signal cleavage is observed at chain lengths of 180 and 220. (B) RAMP fractions prepared from indicated ERj3 truncation intermediates were analyzed by BN-PAGE and form a prominent 500 kD complex at truncation 140 and longer. (C) Gel-shifts of ERj3-180 RAMP complexes on BN-PAGE show the 500 kD complex contains Sec62, Sec63, OST48, and Sec61.

Supplemental Experimental Procedures

Reagents

All chemicals were purchased from Sigma (St. Louis, MO,USA) unless otherwise noted.

Plasmid DNA

Bovine preprolactin (pPL) or Hamster Major Prion Protein (PrP) cDNA was inserted into pSP64 (Promega, Madison, WI, USA) as previously described to generate plasmid pSP-BPI or pSP-PrP (Hay et al., 1987; Skach et al., 1993; Skach and Lingappa, 1993). ERj3 cDNA was obtained from transOMIC technologies (Huntsville, AL, USA). All mutations, deletions, and stop codons were introduced into full length preprolactin or prion protein DNA fragments by PCR overlap extension (Ho et al., 1989) and subcloned into pSP64 via HindIII and EcoRI restriction sites. All cloned regions of cDNA generated by PCR were verified by DNA sequencing. $pPL^{45\text{-}Zn}$ and $pPL^{55\text{-}Zn}$ replaced 29 pPL amino acids starting at the designated residue with the *S. Cerevisiae* ADR1a zinc finger sequence (Conti et al., 2014; Denzer et al., 1995; Parraga et al., 1988).

Antibodies and Immunogenic Peptides

Polyclonal anti-Sec61α serum (ProteinTech Group, Chicago, IL, USA) was generated in rabbits against the synthetic peptide CEIFVKEQSEVGSMGALL. Fab fragments were prepared by Ig digestion with papain exactly as described (Andrew and Titus, 2001a). All components were exchanged into PBS using 10,000 Da cut-off centrifugal filter units (EMD Millipore Chemicals, Darmstadt, Germany). Anti-OST48 and anti-KCP2 were gifts

from Dr. Stephen High (University of Manchester, Manchester, UK). Anti-Sec62 serum was a kind gift from Dr. Richard Zimmermann (Saarland University, Homburg, Germany). Anti-Sec63 was purchased from ProteinTech Group, Inc (Chicago, IL, USA). Purified anti-TRAPα and anti-TRAM were a kind gift from Dr. Kent Matlack and were generated against the synthetic peptides, CLPRKRAQKRSVGSDE and CADSPRNRKEKSS. Competing immunogenic and control peptide sequences (Genescript, Piscataway, NJ, USA) are as follows: TRAM, CADSPRNRKEKSS, and control peptide (pPL residues 86-110), SGSNSCHTSSLPTPEDKEQAQQTHHEVL. Antibody Ig fractions were affinity purified on protein A-agarose beads for gel-shifts (Bio-Rad, Hercules, CA, USA) (Andrew and Titus, 2001b).

In Vitro Translation

Full length or truncated RNA transcripts were synthesized using SP6 polymerase from PCR-amplified cDNA templates in reactions containing 40 mM Tris-Acetate (pH 7.5), 6 mM MgAcetate, 2 mM spermidine, 10 mM DTT (Roche Diagnostics, Indianapolis, IN, USA), G(5')pppG RNA CAP analog (New England Biolabs, Ipswich, MA, USA) and ribonucleic acids for 60 min at 40ºC as described (Carlson et al., 2005). Truncation sites correspond to the last designated 3' codon of pPL or PrP included in the cDNA template, which was replaced with a GTG codon (valine) to stabilize the peptidyl-tRNA during SDS-PAGE analysis. Where indicated, the last ten residues were replaced with histidine. Translations (10 μL) were carried out at 24ºC for 60 min with 4 μL S7-nucleased rabbit reticulocyte lysate (RRL) in 10 mM Tris-Acetate, 100 mM KAcetate, 2 mM MgAcetate, 2 mM DTT supplemented with creatine kinase (Roche Diagnostics,

Indianapolis, IN, USA), RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA), 1mM of each of 19 amino acids minus Met, 20 mM GTP, 20 mM ATP, 0.05 μCi/μL EasyLabel ^{35}S-Methionine (PerkinElmer, Waltham, MA, USA), and 20% (v/v) RNA transcript (Carlson et al., 2005; Matsumura et al., 2011). Unless otherwise designated, *in vitro* translation reactions contained CRMs (final OD_{280} absorbance of 5.0). RRL and CRMs were prepared according to previously published procedures (Carlson et al., 2005; Matsumura et al., 2011). Acetylated NYT peptide (50 μM) was added to translations where indicated to inhibit N-linked glycosylation.

Isolation of RAMP Proteins

ER microsomes were collected through a 0.6 M sucrose cushion in buffer A (50 mM HEPES-KOH (pH 7.5), 100 mM KCL, 5 mM MgAcetate, 1 mM DTT) by ultracentrifugation at 187,000 x g. After resuspension in buffer A, microsomes were lysed in 8 volumes of buffer B (1.2% digitonin, 100 mM HEPES-OH (pH 7.6), 15 mM MgAcetate, 15% glycerol, and 5 mM DTT) containing 150 mM NaCl. Additional bulk CRMs were added to samples when performing 2D-BN/SDS-PAGE to aid in the visualization of translocon components by silver stain. Digitonin was purchased either from EMD Millipore Chemicals (Darmstadt, Germany) or Sigma (St. Louis, MO, USA). Solubilized RTCs were washed by sequential rounds of pelleting at 350,000 x g for 60 min and resuspension in buffer B with 400 mM NaCl (or 150 mM NaCl where specified). RAMP proteins were eluted by treating resuspended RTCs in buffer B with 1mM puromycin (EMD Millipore Chemicals, Darmstadt, Germany), 1 mM GTP, and 1M NaCl at 4ºC for 60 min. Efficient PrP release from ribosomes required increasing the NaCl

concentration to 2M. Ribosomes were removed by diluting the solution to 500 mM NaCl in buffer B and pelleting at 350,000 x g for 60 min. The resultant supernatant comprised the RAMP fraction. Where indicated, RAMP fractions were incubated for 1 hour at 24ºC.

Chemical Crossinking

RAMP complexes in a reduced concentration of 50 μM DTT were treated with 2 mM 3,3´-Dithiobis[sulfosuccinimidylpropionate] (DTSSP) (ThermoFisher Scientific Inc., Waltham, MA, USA) that was diluted from freshly prepared 50x stock solution in DMSO. Reactions were carried out for 10 minutes at 4°C and quenched with 20 mM Tris-Cl pH 7.5 followed by SDS-PAGE, BN-PAGE, or Ni-NTA purification.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

BN-PAGE was carried out on 4.5 to 12% acrylamide gradient gels and a 4% acrylamide stack in 50 mM BisTris-HCl (pH 7.0) and 60 mM aminocaproic acid as described previously (Schagger and von Jagow, 1991; Shibatani et al., 2005; Wittig et al., 2006). RAMP samples were mixed with 1/40th volume of loading buffer (5% Coomassie Brilliant Blue G250 in 500 mM aminocaproic acid (pH 7.0)) and gels were run at 4ºC at 75V for 14 hours with a cathode buffer of 0.02% Coomassie Brilliant Blue G-250, 15 mM Bis-Tris, 50 mM Tricine at pH 7.0, and an anode buffer of 50 mM Bis-Tris (pH 7.0). The voltage was then increased to 500V until the dye front reached the gel edge. For western blots, the cathode buffer was replaced before increasing the voltage with that of an identical formulation, except Coomassie Brilliant Blue G-250 concentration was

reduced to 0.002%. Standard calibration proteins (GE HealthCare, Little Chalfont, United Kingdom) (thyroglobulin [670 kDa], ferritin [460 kDa], and bovine serum albumin [65 kDa monomer, 130 kDa dimer]) were run on every gel. Protein complexes were visualized with modified Blum silver stain methodology (Blum et al., 1987). Briefly, gels were fixed for 60 min in 40% ethanol and 10% acetic acid, washed three times with 30% ethanol for 20 minutes, and washed once in ddH_2O for 20 minutes. After a 5 minute sensitization step in 0.02% sodium thiosulfate, gels were washed three times in ddH_2O for 30s and infused with a 0.1% silver nitrate solution for 20 minutes at 4ºC. Gels were developed in 3% sodium carbonate and 0.05% formalin after three rinses in ddH_2O.

Phosphorimage analysis was used to detect and quantify ^{35}S-containing nascent chain bands with a Personal Fx Phosphoimager and Quantity One software (Bio-Rad, Hercules, CA, USA). For antibody gel-shifts, 100 ng of purified Igs or Fab fragments were incubated with 10 μL RAMP samples for 1 hour at 4ºC with or without ~25x molar concentration of specified peptide as indicated, followed by BN-PAGE analysis as described above.

1st and 2nd Dimension SDS-PAGE

SDS-PAGE analysis was performed using 8-18% gradient running gels and 4% stacking gel (Gallagher, 2001; Laemmli, 1970). For 2D-BN/SDS-PAGE, the first dimension BN-PAGE strips were excised, incubated in 62.5 mM Tris-HCL (pH 6.8), 10% glycerol, 2% SDS and 1% DTT for 10 minutes, and then incubated in 2% iodoacetamide for 10 min at 24ºC (Shibatani et al., 2005). Strips were placed directly onto the top of the

SDS stacking gel and were subjected to electrophoresis. Proteins were visualized by silver-staining or autoradiography as above.

Photocrosslinking

N^{ε}-5-azido-2-nitrobenzoyl-lysine (ANB-Lys) was incorporated into nascent chains by supplementing translation reactions with either ANB-Lys-$tRNA^{UAG}$ or ANB-Lys-$tRNA^{lys}$ (1μM final concentration). ANB-Lys−tRNAs were synthesized as previously described (Johnson and Cantor, 1980; Johnson et al., 1978; Krieg et al., 1986; McCormick et al., 2003; Sadlish et al., 2005) either using FPLC-purified Yeast tRNA or using *in vitro*-transcribed (with T7 RNA polymerase) *E. coli* $tRNA^{lys}$ that was mutated to contain an engineered CUA anticodon (i.e. $tRNA^{UAG}$). tRNAs were aminoacylated with ^{14}C-lysine (GE HealthCare, Little Chalfont, United Kingdom) and then reacted with N-5-azido-2-nitrobenzoyloxysuccinimide ester (Thermo Fisher Scientific, Waltham, MA, USA). Photocrosslinking was performed by irradiating translation samples on ice for 10 min with collimated 300-500 nm UV light from a 500W mercury arc lamp (Oriel, Stratford, CT, USA). Membranes were collected through sucrose cushions and crosslinked products were analyzed by SDS-PAGE after digestion with RNase A (Roche Diagnostics, Indianapolis, IN, USA) to remove attached peptidyl-tRNA.

Immunoprecipitation

Translation samples were first denatured in Phosphate-buffered saline (PBS) containing 1% SDS at 40ºC for 5 minutes and then diluted to 0.05% SDS with 20 volumes of 1% Triton X-100 in PBS. Samples were then incubated overnight at 4ºC with 10 μL of

Protein A-agarose beads (Bio-Rad, Hercules, CA, USA) and 1 μL Sec61α antiserum or immunoaffinity (peptide) purified TRAM Ig (~300 ng/μL). Beads were washed six times with PBS containing 1% TX-100, and immunoprecipitates were eluted with standard loading buffer for subsequent SDS-PAGE analysis as above.

Pegylation Assays

CRMs containing specified *in vitro* translated products were pelleted by ultracentrifugation (187,000 x g for 10 min) and resuspended as above, except that the DTT concentration was reduced to 50 μM. Samples were split, treated with 0.5 M NaCl or 1% digitonin, and incubated with polyethyleneglycol maleimide-5,000 Da (PEG-mal) (Sigma, St. Louis, MO,USA) or not at 4ºC for 1 hour. Reactions were quenched with 200 mM DTT and analyzed by SDS-PAGE followed by phosphorimaging.

Western Blotting

SDS-PAGE gels were transferred in 25 mM Tris, 192 mM glycine, 20% (w/v) methanol (pH 8.3) to PVDF membrane (EMD Millipore Chemicals, Darmstadt, Germany) at 50V and 350 mA for 1 hour. For BN-PAGE, gels were agitated in transfer buffer for 1 hour before transfer to reduce Coomassie concentrations within the gel. After transfer, membranes were blocked with 5% (w/v) non-fat dry milk in TTBS (0.05% Tween in Tris-buffered saline, pH 7.4) for 1 hr, incubated with indicated primary antibody at ~ 25 ng/mL, and then incubated with 5 ng/mL anti-rabbit Ig HRP (Santa Cruz Biotechnolgy Inc, Dallas, TX, USA) in the same buffer. Before and after secondary antibody incubation, membranes were washed with TTBS six times for 5 minutes. After two

additional wash steps in Tris-buffered saline pH7.5, HRP signal was detected using Supersignal West Femto Substrate (ThermoFisher Scientific Inc., Waltham, MA, USA) and film exposure (Light Labs, Dallas, TX, USA) for 1 second to 30 minutes. Western blot signals were aligned with autoradiographic signals, which were obtained by inactivating HRP with 0.02% NaN_3 or standard harsh stripping procedures and exposing membrane to film for prolonged durations. No autoradiographic signal was detected on film in the time scale used for Western Blots (30 min maximum).

For quantitative western blots in **Figure 6** exclusively, Immobilon-FL membranes were used (EMD Millipore Chemicals, Darmstadt, Germany), Aquablock WB reagent was used for membrane blocking (East Coast Bio, Inc., North Berwick, ME, USA), IRdye800 -conjugated anti-rabbit secondary antibodies were used (Rockland Immunochemicals, Inc., Limerick, PA, USA) and signal was detected and quantitated with Odyssey Infrared Imaging Systems and software (LI-COR Biosciences, Inc, Lincoln, NE, USA). Percentage of input that each sample represented on immunoblots was calculated using its signal and the equation of linear curve fits of standard input dilutions run on every gel.

Ni-NTA Purification

DTSSP-crosslinked RAMPs containing 50 μM DTT were denatured in 1% (w/v) SDS and diluted 20-fold in Buffer P [1% (v/v) Triton X-100 in Phosphate-buffer saline (PBS) supplemented with 500 mM NaCl and 30 mM imidazole]. RAMPs were incubated for 10 hr at 4°C with 2.5 μL Ni-NTA-agarose (Qiagen, Valencia, CA, USA) in a total volume of 1 mL, then pelleted, and washed six times. Samples from experiment 1(**Figure 5**) and

those corresponding to **Figure 7** were eluted with 250 mM imidazole in buffer P and analyzed by BN-PAGE. For experiment / samples 2a-c (**Figure 5**), beads were washed with PBS alone for the last three washes. These purified RAMP complexes were then either denatured and trypsinized on Ni-NTA beads for mass spectrometry or eluted with 8M Urea, 1% SDS, 200 mM DTT, and 0.01% Bromophenol Blue in 10 mM Tris-CL pH 7.5 at 40°C for 30 min followed by SDS-PAGE analysis.

LC – MS/MS

RAMPs bound on Ni-NTA beads (**Figure 5**, experiment / samples 2a-c) were processed by the following serial treatments: 1) 8 M electrophoresis grade urea, 1.0 M Tris (pH 8.5), 8 mM CaCl2, and 0.2 M methylamine in 20 mM DTT for 30 minutes at 50°C, 2) 50 mM iodoacetamide for 30 minutes at room temperature in the dark, 3) 100 mM DTT for 15 minutes at room temperature, 4) Dilution to final 2M urea concentration with ddH_2O, 5) Addition of 250 ng proteomics grade trypsin (Sigma, St. Louis, MO,USA) overnight at 37°C, and 6) adding $1/10^{th}$ volume of neat (88%) formic acid. Peptide digests were solid phase extracted using C18 Tips (Thermo Scientific, San Jose, CA).

For experiment / samples 1a-b (**Figure 5**) or those corresponding to **Figure 7**, Ni-NTA eluates were split evenly and analyzed on separate halves of the same BN-PAGE gel. Complex C was visualized by silver stain on one half of the gel (**Figure 5**C), which was aligned with the second gel half using MW standards to locate the parallel unstained gel portion. These unstained gel portions at ~500 kD for mock, $pPL^{45\text{-}Zn}$-163, and/or PrP-167-His samples were removed for in-gel trypsin proteolysis exactly as described previously (Shevchenko et al., 2006).

Trypsin digested peptides were either 1) separated using a NanoAcquity UPLC system, 75 µm x 25 cm BEH 130 UPLC column containing 1.7 µm C18 resin (Waters, Milford, MA, USA), 300 nl/min flow rate, and 7.5 - 30% acetonitrile gradient over 60 min in a mobile phase containing 0.1% formic acid and then analyzed with a LTQ Velos dual pressure linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA) using electrospray ionization and Captive Spray Source, or 2) separated using an UltiMate 3000 nano UPLC system, 75 µm x 25 cm PepMap RSLC UPLC EasySpray column containing 2 µm C18 resin (Thermo Scientific, San Jose, CA), and chromatography conditions as described above, and analyzed with an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA). Both analyses used the instrument's data-dependent scanning feature and dynamic exclusion to collect MS/MS spectra using CID and HCD fragmentation in the LTQ Velos and Orbitrap Fusion, respectively.

MS/MS results from each sample were analyzed with the Proteome Discoverer 1.4 software package using the Sequest HT and Percolator modules. LC-MS/MS spectra were compared to a database of all canine protein sequences available from the Uniprot Consortium (www.uniprot.org) (The UniProt, 2014). Only a fragment of canine Sec63 existed in the Uniprot repository (accession P82008), therefore the database was supplemented with the highly homologous human Sec63 sequence (accession Q9UGP8) (Muller et al., 2010). *In silico*, each database protein was reduced, alkylated with iodoacetamide (adding 57.021 atom mass units to each Cys), and trypsinized to generate a list of peptides. Incomplete trypsin digestion at Arg and Lys was accounted for by considering peptides with up to two missed cleavages. In

addition, every lysine side chain was considered both in native form or modified with a reduced and alkylated DTSSP adduct (adding 145.02 atomic mass units). 2 Da precursor and 1 Da MS/MS monoisotopic mass tolerances were used to match database-derived theoretical spectra to experimental LTQ-generated spectra. Respectively, 20 ppm precursor and 0.6 Da monoisotopic tolerances were used to match spectra generated by the higher resolution Orbitrap mass spectrometer.

The final Percolator module compared real and decoy database spectra matches to assign each identified peptide with a q-value to indicate false discovery rates. Q-values of 0.01 or less were considered high confidence peptide matches. The general criterion for positive protein detection was two or more high confidence peptide matches in experimental samples compared to none in corresponding mock control. For samples in which two separate identical experiments were analyzed, protein detection in both repetitions (N_1 and N_2) was required to be considered a genuine protein identification.

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