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Supplemental Information

Chaperone-Mediated Sec61 Channel Gating during ER Import of Small Precursor Proteins Overcomes Sec61 Inhibitor-Reinforced Energy Barrier

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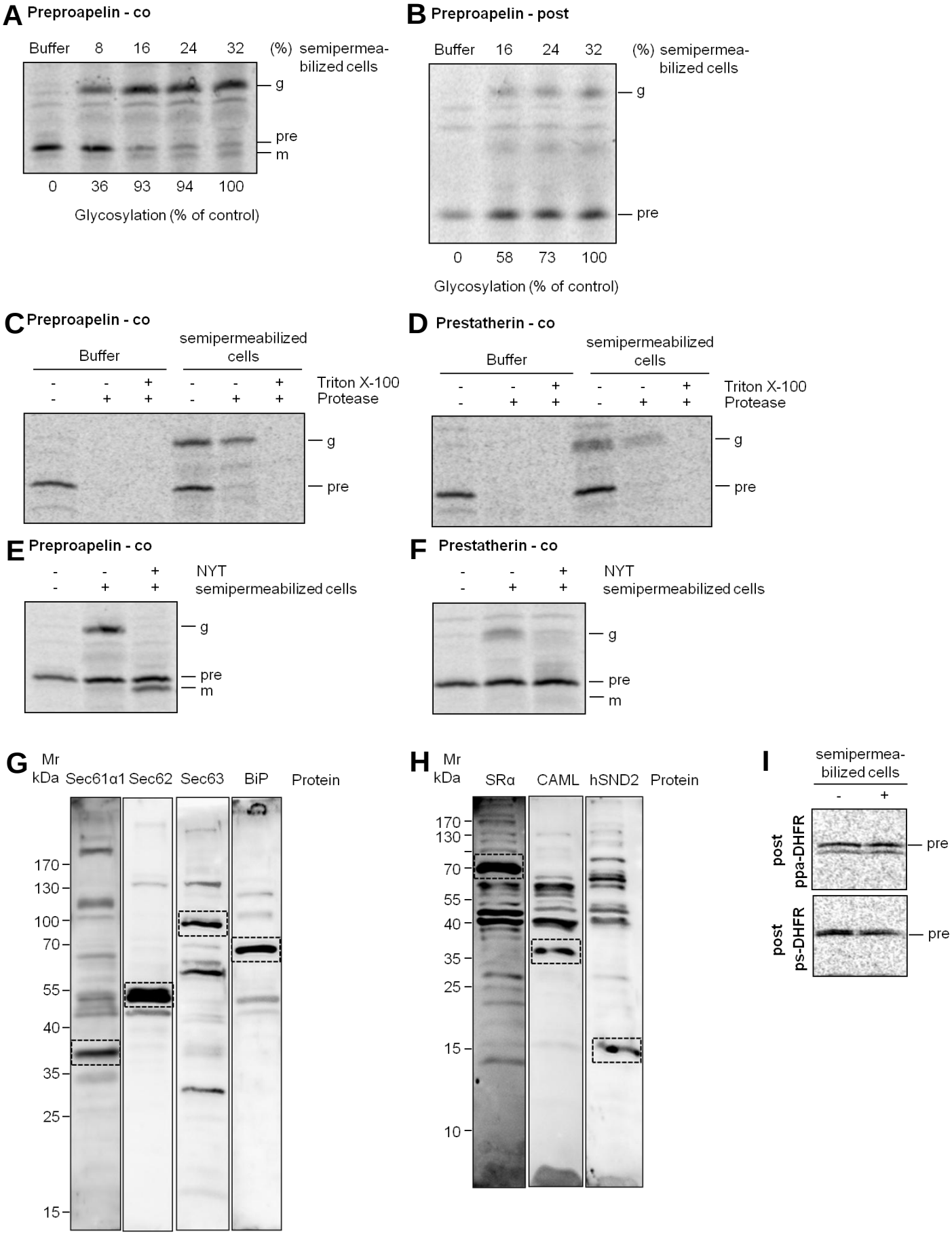
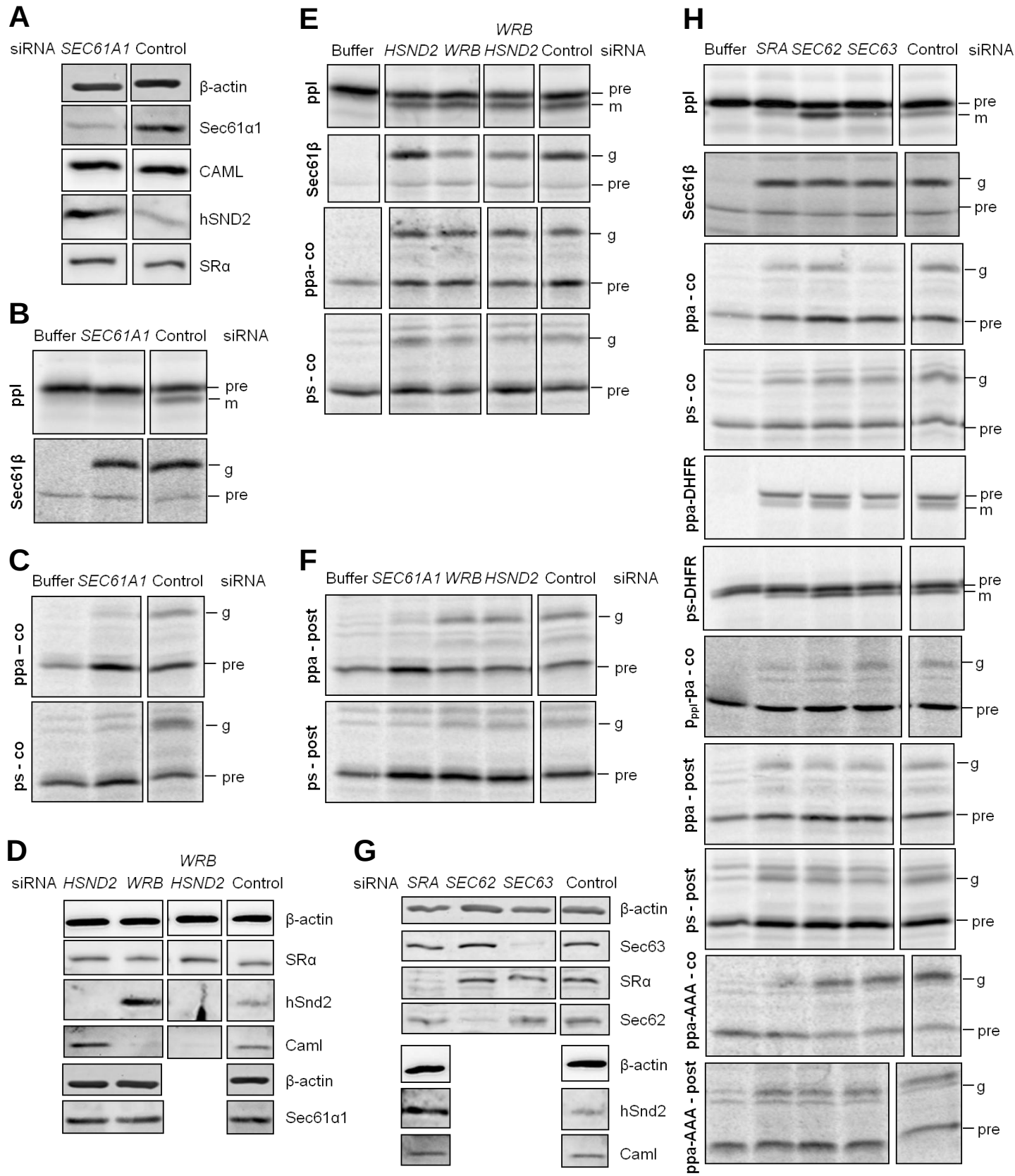

A
Preproapelin - co
Buffer 8 16 24 32 (%) semipermeabilized cells
g
pre
m
0 36 93 94 100
Glycosylation (% of control)
B
Preproapelin - post
Buffer 16 24 32 (%) semipermeabilized cells
g
pre
0 58 73 100
Glycosylation (% of control)
C
Preproapelin - co
Buffer
semipermeabilized cells
Triton X-100
Protease
g
pre
D
Prestatherin - co
Buffer
semipermeabilized cells
Triton X-100
Protease
g
pre
E
Preproapelin - co
NYT
semipermeabilized cells
g
pre
m
F
Prestatherin - co
NYT
semipermeabilized cells
g
pre
m
G
Mr kDa
Sec61α1 Sec62 Sec63 BiP Protein
170
130
100
70
55
40
35
25
15
H
Mr kDa
SRα CAML hSND2 Protein
170
130
70
55
40
35
25
15
10
I
semipermeabilized cells
post ppa-DHFR
pre
post ps-DHFR
pre

Figure S1. Transport of preproapelin and prestatherin into the human ER, Related to Figure 1.

Note that under co-translational conditions, precursor polypeptide chains and fully-synthesized precursors were targeted and inserted into Sec61 complexes. Under post-translational conditions, only the targeting and membrane insertion of completed precursor polypeptides occurred. **A-B)** Titration of HeLa cell-derived ER membranes in ppa translocation defined the linear range of the assay. Following semipermeabilization of untreated HeLa cells, ppa was co- (co, **A**) or post-translationally (post, **B**) incubated with the indicated concentrations of ER membranes (8% to 32% v/v of the total translation mix, equivalent to 3,200 to 12,800 cell equivalents/µl). **C-D)** Sequestration assay for demonstration of complete ppa and ps translocation. Following co-translational incubation of ppa **(C)** or ps **(D)** with untreated semipermeabilized HeLa cells (3,200 cell equivalents/µl), membranes were re-isolated in sucrose supplemented with combinations of proteinase K and Triton X-100 as indicated. **E-F)** Transport in the presence of the tripeptide NYT for demonstration of N-glycosylation. Following semipermeabilization of untreated HeLa cells, ppa **(E)** or ps **(F)** were co-translationally incubated with ER membranes (3,200 cell equivalents/µl) in the presence of NYT (0.5 mM). Radioactive samples were subjected to SDS-PAGE and phosphorimaging. Transport efficiencies were calculated as the proportion of N-glycosylation on the total amount of synthesized precursor with the control sample set to 100%. Shown are the relevant parts of representative phosphorimages. Pre: precursor polypeptide; m: mature protein; g: singly or doubly glycosylated protein. **G, H**) HeLa cells were characterized with validated antibodies by Western blot (Lang et al., 2012; Johnson et al., 2013; Haßdenteufel et al., 2017). Shown are representative blot images. The respective protein of interest was identified by its absence in siRNA treated cells (see Figures S2-5) and is boxed with broken lines. **I**) In contrast to Figure 1C, the indicated precursors were post-translationally incubated with the

ER membranes. Notably, there was no signal peptide cleavage in the presence of semi-permeabilized cells under these conditions.

Fig. S2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β-actin
Sec61α1
CAML
hSND2
SRα
B
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ppl
pre
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g
pre
C
Buffer SEC61A1 Control siRNA
ppa – co
g
pre
ps - co
g
pre
D
WRB
siRNA HSND2 WRB HSND2 Control
β-actin
SRα
hSnd2
Caml
β-actin
Sec61α1
E
WRB
Buffer HSND2 WRB HSND2 Control siRNA
ppl
pre
m
Sec61β
g
pre
ppa- co
g
pre
ps - co
g
pre
F
Buffer SEC61A1 WRB HSND2 Control siRNA
ppa - post
g
pre
ps - post
g
pre
G
siRNA SRA SEC62 SEC63 Control
β-actin
Sec63
SRα
Sec62
β-actin
hSnd2
Caml
H
Buffer SRA SEC62 SEC63 Control siRNA
ppl
pre
m
Sec61β
g
pre
ppa - co
g
pre
ps - co
g
pre
ppa-DHFR
pre
m
ps-DHFR
pre
m
pPpl-pa - co
g
pre
ppa - post
g
pre
ps - post
g
pre
ppa-AAA - co
g
pre
ppa-AAA - post
g
pre

Figure S2. Effects of Sec61α1, Sec62, Sec63, SRα, hSnd2 and Wrb depletion on transport of short presecretory proteins, Related to Figures 2 and 3.

A, D, G) Representative Western blots validating protein content. **B-C, E, F, H)** Phosphorimages of representative SDS-PAGE gels. Prior to preparation of semipermeabilized cells, HeLa cells were treated with the indicated siRNA(s). Precursors were co- (co) or post-translationally (post) incubated with the indicated ER membranes. Pre: precursor polypeptide; m: mature protein; g: glycosylated protein.

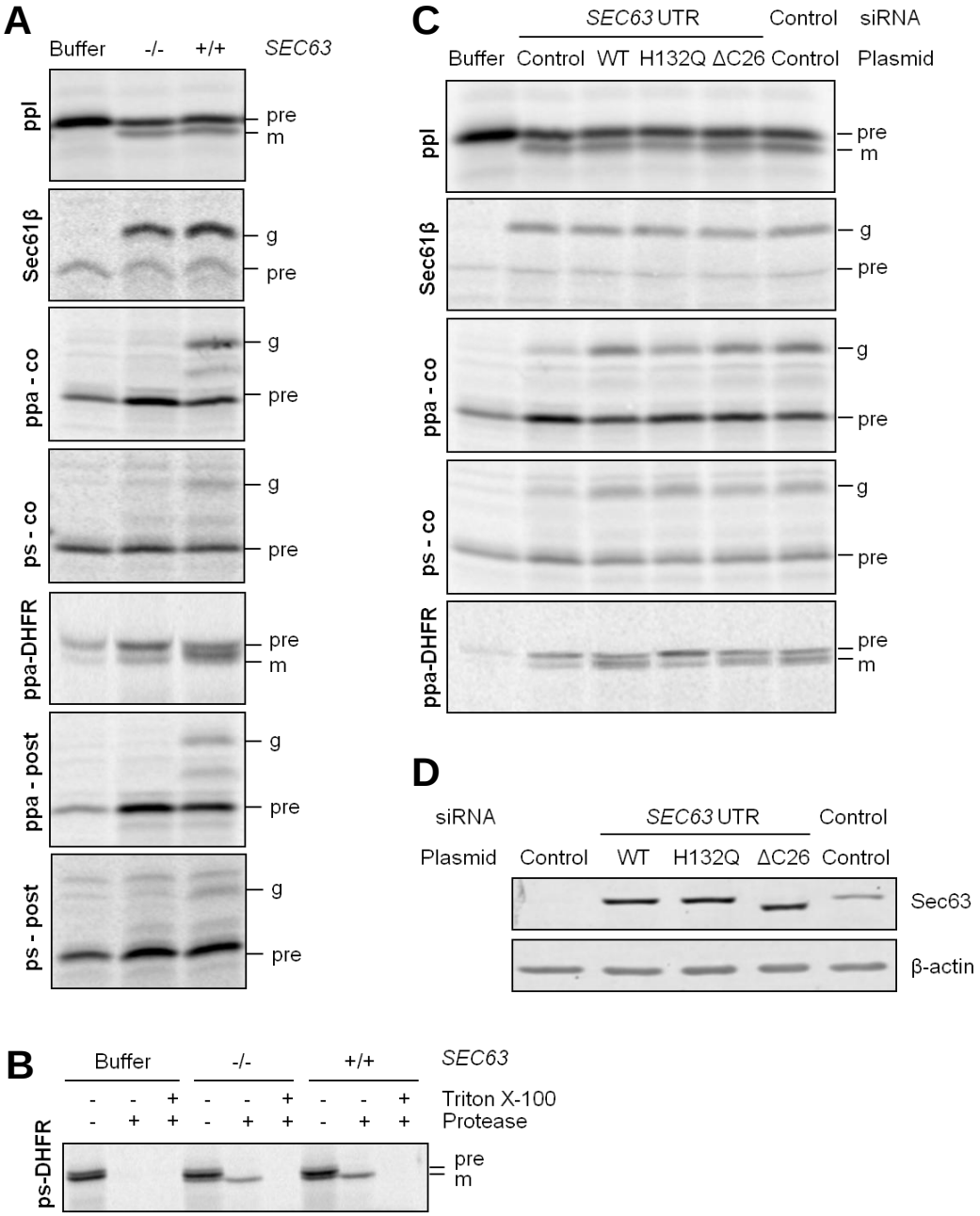
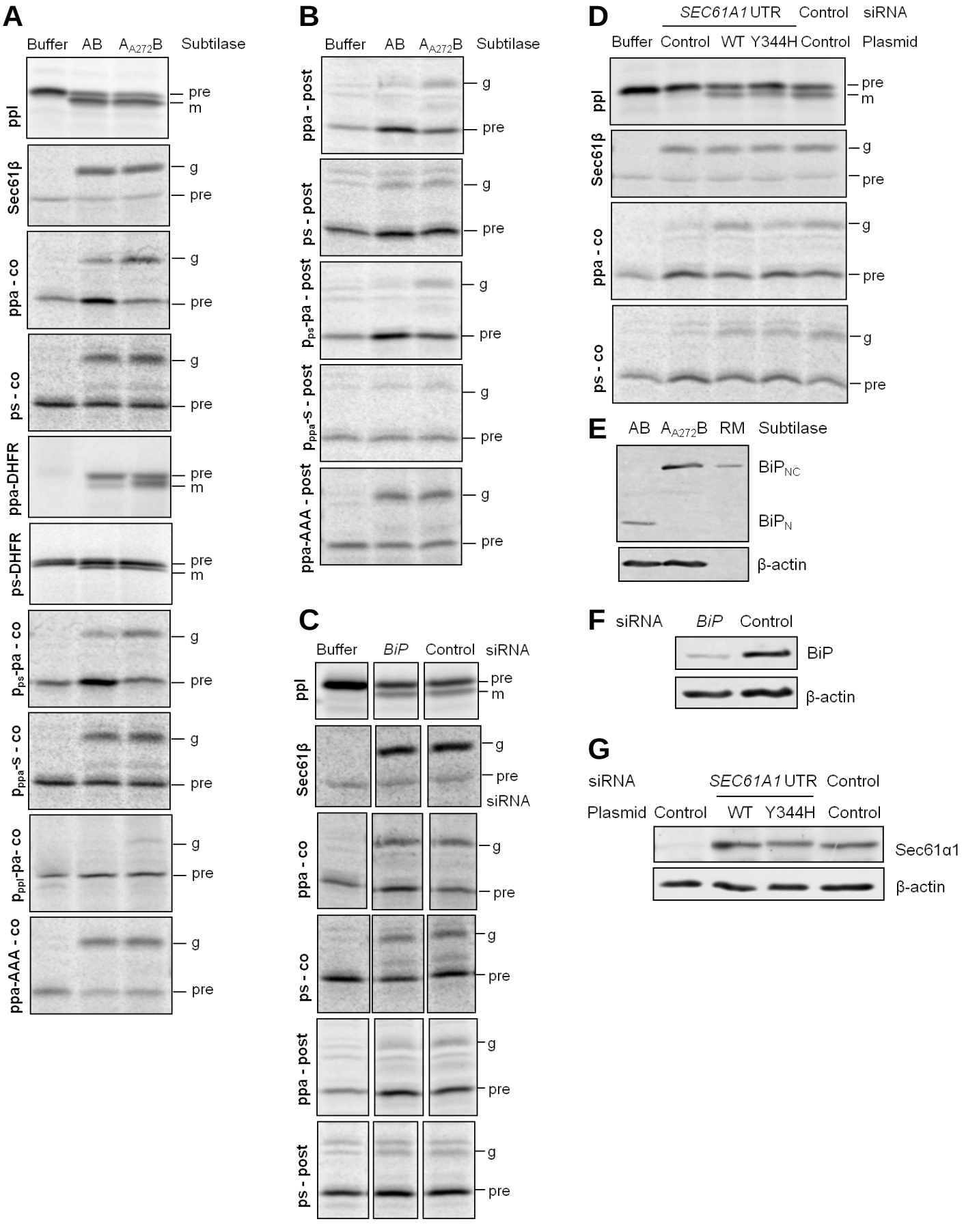

A
Buffer
-/-
+/+
SEC63
ppl
pre
m
Sec61β
g
pre
ppa - co
ps - co
ppa-DHFR
ppa - post
ps - post
C
SEC63 UTR
Control
siRNA
Buffer
Control
WT
H132Q
ΔC26
Control
Plasmid
D
siRNA
SEC63 UTR
Control
Plasmid
Control
WT
H132Q
ΔC26
Control
Sec63
β-actin
B
Buffer
-/-
+/+
SEC63
Triton X-100
Protease
ps-DHFR
pre
m

Figure S3. Effects of murine *SEC63* knock-out on transport of short presecretory proteins and complementation of *SEC63* siRNA effects, **Related to Figure 3.**

A-C) Phosphorimages of representative SDS-PAGE gels. **D)** Representative Western blot validating protein content. Semipermeabilized cells were prepared from murine *SEC63*-null cells **(A, B)** or HeLa cells **(C, D)**. Prior to preparation, HeLa cells were treated with *SEC63*-UTR siRNA and transfected with the indicated plasmids. Precursors were co- (co) or post-translationally (post) incubated with the indicated ER membranes. Pre: precursor polypeptide; m: mature protein; g: glycosylated protein.

Fig. S4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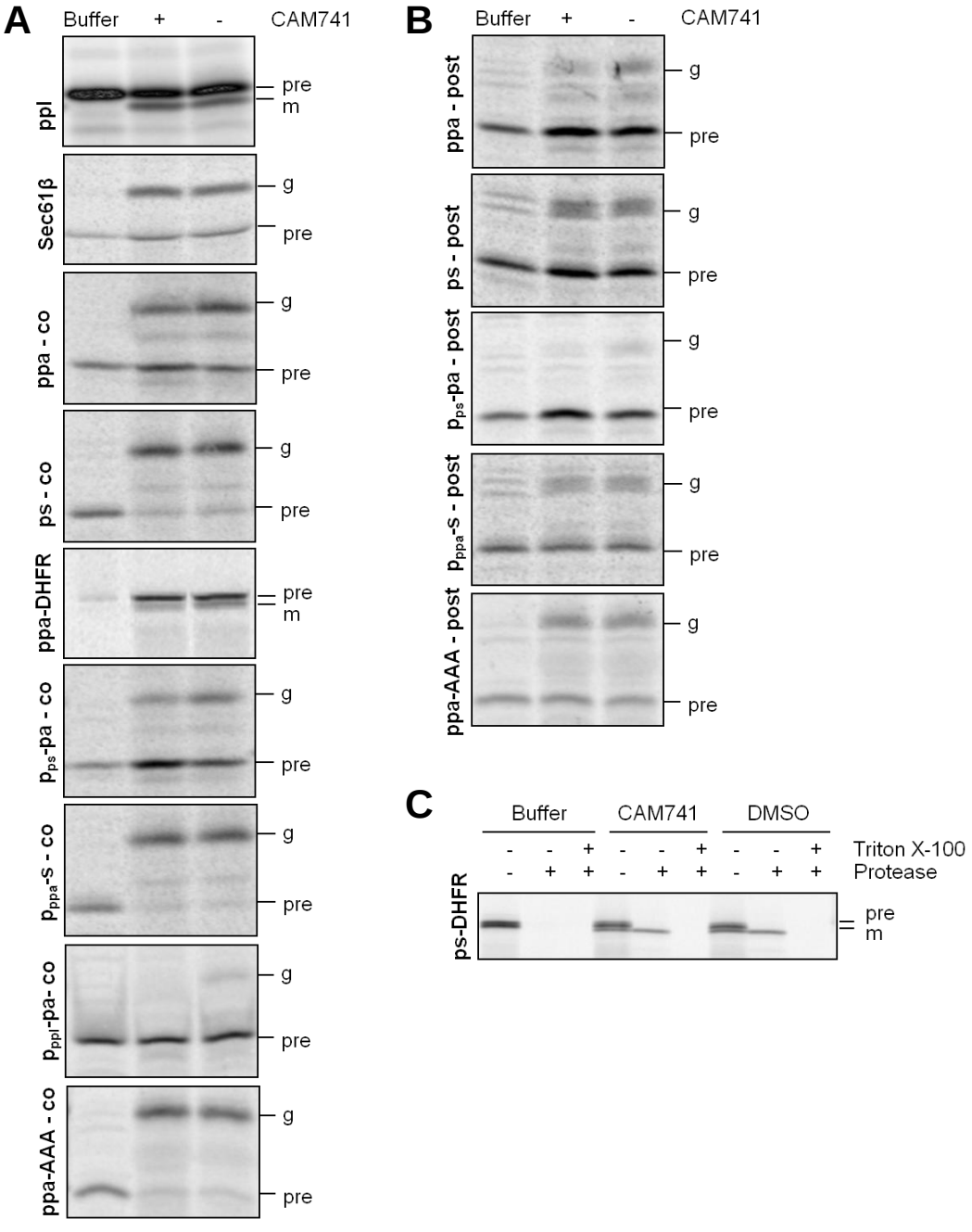
Buffer
AB
$A_{A272}B$
Subtilase
ppl
Sec61β
ppa - co
ps - co
ppa-DHFR
ps-DHFR
p_{ps}-pa - co
p_{ppa}-s - co
p_{ppl}-pa- co
ppa-AAA - co
pre
m
g
B
ppa - post
ps - post
p_{ps}-pa - post
p_{ppa}-s - post
ppa-AAA - post
C
Buffer
BiP
Control
siRNA
D
SEC61A1 UTR
Control
siRNA
Buffer
Control
WT
Y344H
Control
Plasmid
E
AB
$A_{A272}B$
RM
Subtilase
BiP_{NC}
BiP_N
β-actin
F
siRNA
BiP
Control
BiP
β-actin
G
siRNA
SEC61A1 UTR
Control
Plasmid
Control
WT
Y344H
Control
Sec61α1
β-actin

Figure S4. Effects of of subtilase toxin and *BIP* siRNA on transport of short presecretory proteins and complementation of *SEC61A1* siRNA effects, Related to Figure 4.

A-D) Phosphorimages of representative SDS-PAGE gels. **E-G)** Representative Western blots validating protein content. Prior to preparation of semipermeabilized cells, HeLa cells were treated with active or inactive subtilase toxin (**A, B, E**), with *BIP* **(C, F)** or *SEC61A1*-UTR siRNA and transfected with the indicated plasmids (**D, G)**. Precursors were co- (co) or post-translationally (post) incubated with the indicated ER membranes. Pre: precursor polypeptide; m: mature protein; g: glycosylated protein.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Buffer
+
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CAM741
ppl
pre
m
Sec61β
g
pre
ppa - co
g
pre
ps - co
g
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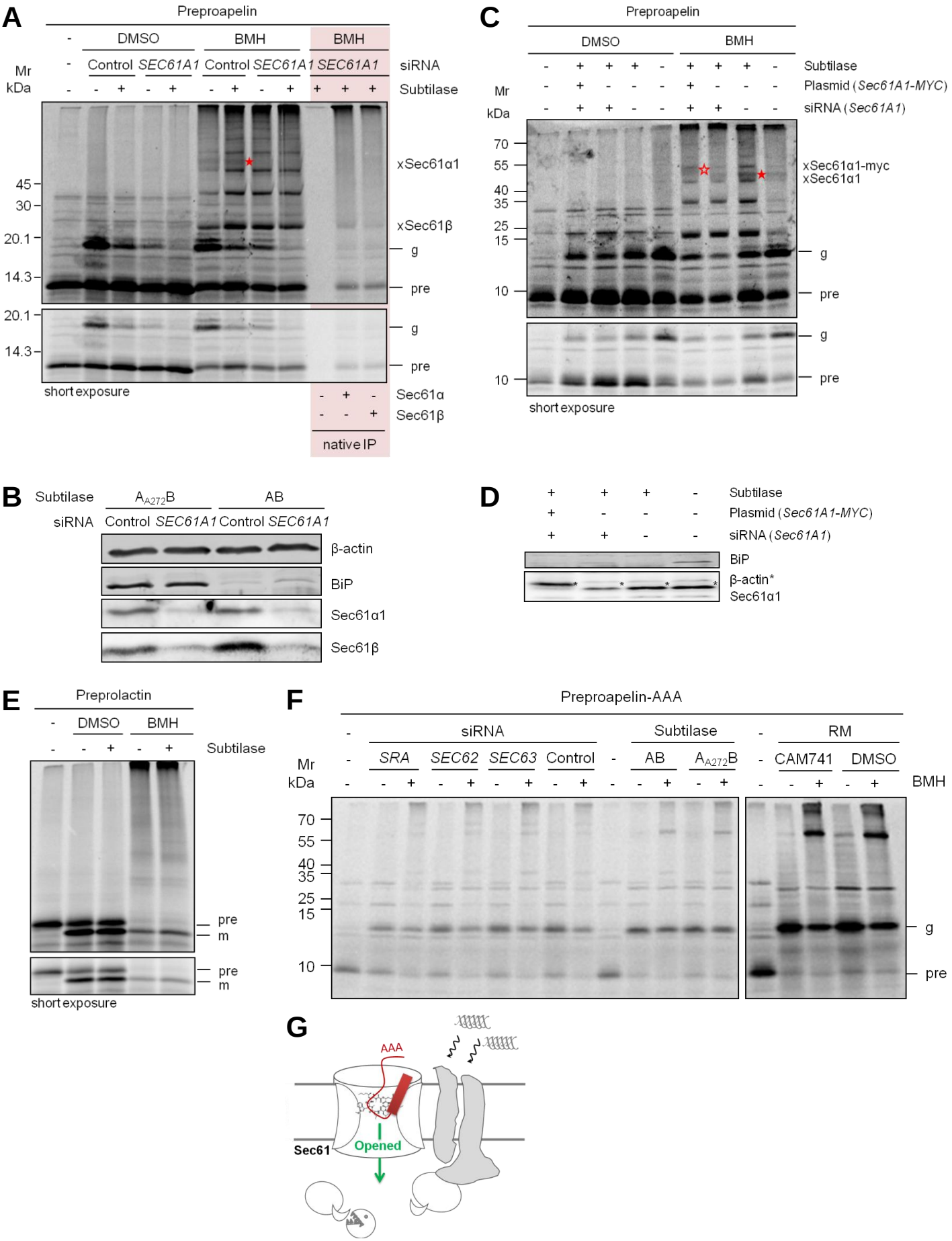
pre
m
p_{ps}-pa - co
g
pre
p_{ppa}-s - co
g
pre
p_{ppl}-pa- co
g
pre
ppa-AAA - co
g
pre
B
Buffer
+
-
CAM741
ppa - post
g
pre
ps - post
g
pre
p_{ps}-pa - post
g
pre
p_{ppa}-s - post
g
pre
ppa-AAA - post
g
pre
C
Buffer
CAM741
DMSO
Triton X-100
Protease
ps-DHFR
pre
m

Figure S5. Effects of CAM741 on transport of short presecretory proteins, Related to Figure 5.

A-C) Phosphorimages of representative SDS-PAGE gels. Precursors were co- (co) or post-translationally (post) incubated with canine pancreatic rough microsomes in the presence of solvent or CAM741. Pre: precursor polypeptide; m: mature protein; g: glycosylated protein.

Fig. S6


A
Preproapelin
DMSO
BMH
BMH
Mr
kDa
Control
SEC61A1
Control
SEC61A1
SEC61A1
siRNA
Subtilase
45
30
20.1
14.3
xSec61α1
xSec61β
g
pre
20.1
14.3
g
pre
short exposure
Sec61α
Sec61β
native IP
C
Preproapelin
DMSO
BMH
Subtilase
Plasmid (Sec61A1-MYC)
siRNA (Sec61A1)
Mr
kDa
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55
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35
25
15
10
xSec61α1-myc
xSec61α1
g
pre
10
g
pre
short exposure
B
Subtilase
A272B
AB
siRNA
Control
SEC61A1
Control
SEC61A1
β-actin
BiP
Sec61α1
Sec61β
D
Subtilase
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siRNA (Sec61A1)
BiP
β-actin*
Sec61α1
E
Preprolactin
DMSO
BMH
Subtilase
pre
m
pre
m
short exposure
F
Preproapelin-AAA
siRNA
Subtilase
RM
Mr
kDa
SRA
SEC62
SEC63
Control
AB
A272B
CAM741
DMSO
BMH
70
55
40
35
25
15
10
g
pre
G
AAA
Sec61
Opened

Figure S6. Identification of Sec61α1 and Sec61β as crosslinking partners of preproapelin upon co-translational translocation in the absence of BiP or Sec61 complex and crosslinking of ppl and preproapelin-AAA, Related to Figure 6.

A-D) Identification of crosslinking partners of ppa in the absence of Sec61 complex (**A, B**) or BiP (**A-D**) and in the presence of tagged Sec61α, respectively (**C, D**). HeLa cells were treated with *SEC61A1*-UTR siRNA or control siRNA and transfected with *SEC61A1*-Myc/6His or control plasmid, and then treated with subtilase AB or inactive subtilase variant $A_{A272}B$. Following the preparation of semipermeabilized cells used for co-translational translocation of ppa, membranes were obtained by sedimentation, re-suspended in XL-buffer, and supplemented with BMH or DMSO for crosslinking. Radioactive samples were subjected to SDS-PAGE and phosphorimaging. Where indicated, immunoprecipitation with validated antibodies was carried out after crosslinking and membrane solubilisation under non-denaturing conditions (0.65% CHAPS and 0.4 M KCl; Tyedmers et al., 2000; **A**). Relevant crosslinking products of ppa to Sec61α1 (filled red asterisk), Sec61α1-Myc/6His (open red asterisk) or Sec61β (blue asterisk) are indicated. Pre: precursor polypeptide; g, glycosylated protein. **B, D)** Protein content was validated by Western blot. We note that the tagged Sec61α comigrated with ß-actin (**D**). **E, F)** Crosslinking of fully-synthesized ppl (**E**) and ppa-AAA (**F**). Prior to preparation of semipermeabilized cells, HeLa cells were treated with the indicated siRNA (Table 1) or subtilase toxin. Alternatively, canine pancreatic rough microsomes were treated with solvent or CAM741. The presecretory proteins ppl and ppa-AAA were co-translationally incubated with the indicated ER membranes, which were sedimented and re-suspended in XL-buffer before crosslinking with BMH. Radioactively labelled samples were subjected to SDS-PAGE and phosphorimaging. pre: precursor polypeptide; g: glycosylated protein; m, mature protein. **G)** Schematic representation of

ppa-AAA translocation upon depletion of Sec62, Sec63 or BiP by siRNA (waved arrows) or toxin (pacman) treatment, or in the presence of CAM741. Without the help by accessory translocon components, insertion into the Sec61-channel efficiently occurs without trapping of ppa-AAA precursor polypeptides.

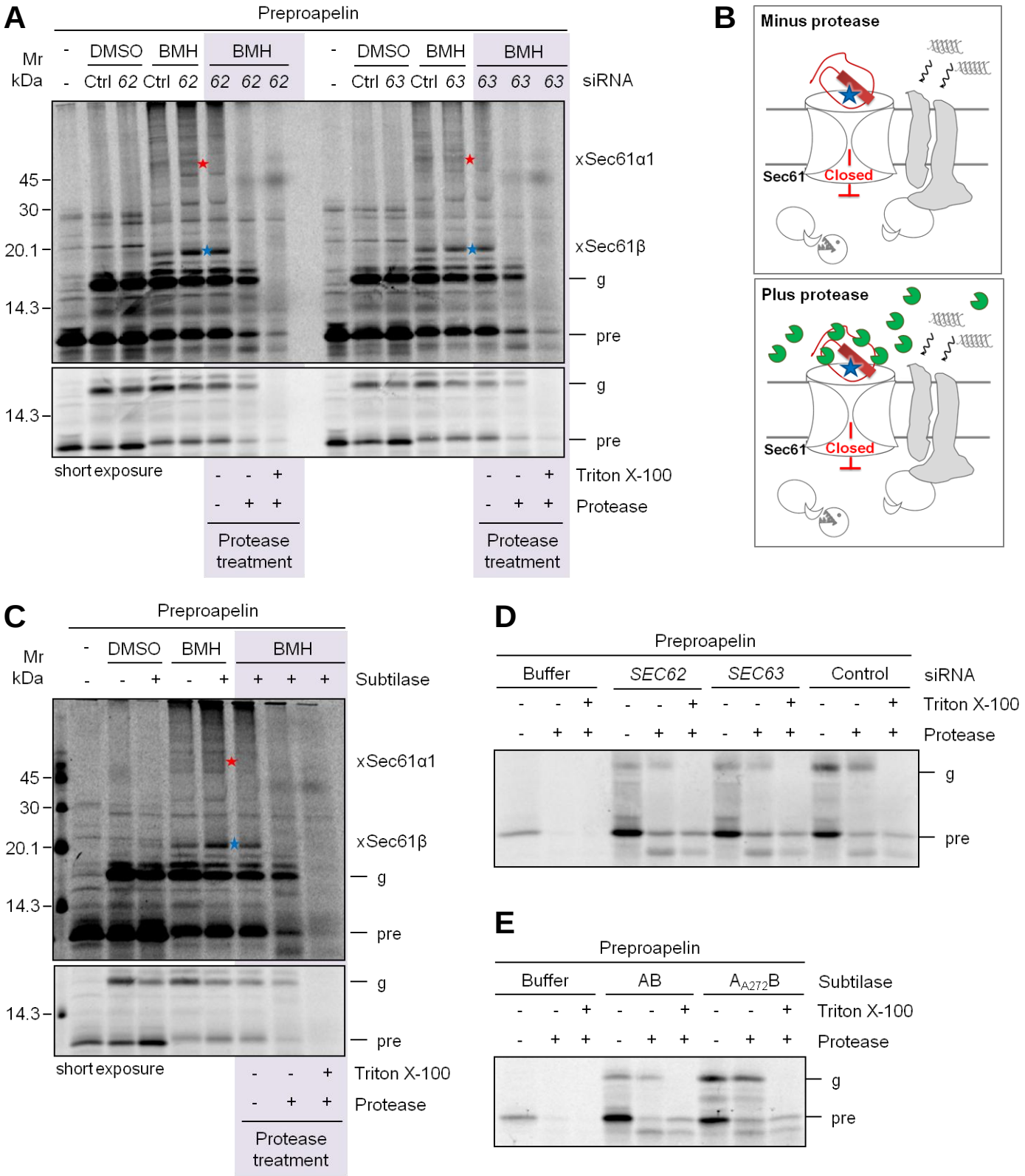
A
Preproapelin
Mr
kDa
- DMSO BMH BMH
- Ctrl 62 Ctrl 62 62 62 62
- DMSO BMH BMH
- Ctrl 63 Ctrl 63 63 63 63
siRNA
45
30
20.1
14.3
xSec61α1
xSec61β
g
pre
14.3
g
pre
short exposure
- - + Triton X-100
- + + Protease
Protease treatment
B
Minus protease
Sec61
Closed
Plus protease
Sec61
Closed
C
Preproapelin
Mr
kDa
- DMSO BMH BMH
- - + - + + + + Subtilase
45
30
20.1
14.3
xSec61α1
xSec61β
g
pre
14.3
g
pre
short exposure
- - + Triton X-100
- + + Protease
Protease treatment
D
Preproapelin
Buffer SEC62 SEC63 Control siRNA
- - + - - + - - + - - + Triton X-100
- + + - + + - + + - + + Protease
g
pre
E
Preproapelin
Buffer AB A272B Subtilase
- - + - - + - - + Triton X-100
- + + - + + - + + Protease
g
pre

Figure S7. Localisation of preproapelin and its crosslinking products after co-translational translocation in the absence of Sec62, Sec63, or BiP, Related to Figure 6.

A-E) Prior to preparation of semipermeabilized cells, HeLa cells were treated with the indicated siRNA or subtilase cytotoxin. ppa was co-translationally incubated with the indicated ER membranes, which were sedimented and resuspended in XL-buffer before sequestration analysis (i.e., treatment with proteinase K (green pacman in **B**) in the absence or presence of Triton X-100) and/or crosslinking with BMH plus subsequent sequestration analysis. Samples were subjected to SDS-PAGE and phosphorimaging. Relevant crosslinking products of ppa to Sec61α1 or Sec61β are indicated by red and blue asterisks, resepctively. Pre: ppa; g: glycosylated pa. **B**) Schematic representation of ppa translocation upon depletion of Sec62, Sec63 or BiP by siRNA (waved arrows) or toxin (pacman) treatment.

Table S1. Amino acid sequences of the model proteins, Related to Figure 1.

Underlined: SP or TMD; italics: linker dipeptide; red: mutagenized positively charged cluster; blue: putative translational pausing element.

Name	Sequence
ppa OPG2	MNLRLCVQALLLLWLSLTAVCG GSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGPMPF MNGTEGPNFYVPFSNKTG
p_{ppl}-pa OPG2	MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS GSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGPMPF MNGTEGPNFYVPFSNKTG
p_{ps}-pa OPG2	MKFLVFAFILALMVSMIGA GSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGPMPF MNGTEGPNFYVPFSNKTG
ppa-AAA OPG2	MNLRLCVQALLLLWLSLTAVCG GSLMPLPDGNGLEDGNVRHLVQPRGSRNGPGPWCGGAAAFRRQRPRLSHKGPMPF MNGTEGPNFYVPFSNKTG
ps OPG2	MKFLVFAFILALMVSMIGA DSSEEKFLRRIGRFGYGYGPYQPVPEQPLYPQPYQPQYQQYTF*MM* MNGTEGPNFYVPFSNKTG
p_{ppa}-s OPG2	MNLRLCVQALLLLWLSLTA DSSEEKFLRRIGRFGYGYGPYQPVPEQPLYPQPYQPQYQQYTF*MM* MNGTEGPNFYVPFSNKTG
ppl	MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS TPVCPNGPGNCQVSLRDLFDRAVMVSHYIHDLSSEMFNEFDKRYAQGKGFITMALN SCHTSSLPTPEDKEQAQQTHHEVLMSLILGLLRSWNDPLYHLVTEVRGMKGAPDAIL SRAIEIEEENKRLLEGMEMIFGQVIPGAKETEPYPVWSGLPSLQTKDEDARYSAFYNL LHCLRRDSSKIDTYLKLLNCRIIYNNNC
Sec61β OPG1	MPAPASSTSVGSGSRSPSKLSAPRSAGSGGGSTLKQRKTTTSTTAARSRAPGGAGTGG MW RFYTDDSPGIKVGPVPVLVMSLLFIASVFMLHIWGKYNRS GPNFYVPFSNKTG
DHFR	MVRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLVIMGRK TWFSIPEKNRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLIEQPELASKVDMVWI VGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEIDLGKYKLLPEYPGVLSEVQEE KGIKYKFEVYEKKD

Table S2. Characteristics of the model precursor proteins, Related to Figure 1.

SP: signal peptide; TMD: transmembrane domain; total: precursor excluding OPG1/2 tag; tagged: precursor including OPG1/2 tag; ΔG^{pred}: ΔG prediction; $N\text{-}in^{pred}$: N-in prediction.

Precursor variant	**Length (amino acids)** (SP/total/tagged)	**Charges** (SP)	**ΔG^{pred}** (SP or TMD)	**$N\text{-}in^{pred}$** (total/tagged)
ppa	22/77/95	1+	-0.19	0.94/0.88
ppa-DHFR	22/266/-	1+	-0.19	0.70/-
p_{ps}-pa	19/74/92	1+	-0.91	0.96/0.92
p_{ppl}-pa	30/85/103	3+1-	5.36	0.66/0.98
ppa-AAA	22/77/95	1+	-0.19	0.93/0.88
ps	19/62/82	1+	-0.91	0.36/0.33
ps-DHFR	19/251/-	1+	-0.91	0.31/-
p_{ppa}-s	22/65/85	1+	-0.19	0.32/0.26
ppl	30/229/-	3+1-	5.36	0.66/-
Sec61β	-/96/109	-	0.51	0.06/0.16

Table S3. Sequences of siRNAs used in this study, Related to Experimental Procedures.

Name	Target sequence	Source	Concentration (nM)	Time (h)
BIP siRNA	AAGCGGCTGTTTACTGCTTTT	Qiagen	30	48
SEC61A1-UTR siRNA	AACACTGAAATGTCTACGTTT	Applied	20	96
SEC61B-UTR siRNA	ACCCAACATTTCTTGGACCAA	Qiagen	20	96
SEC62 siRNA	AAGGCTGTGGCCAAGTATCTT	Applied	20	96
SEC63-UTR siRNA	AAGGGAGGTGTAGTTTTTTTA	Applied	20	96
HSND2 siRNA #2	CACCTTAAGGATGTGATCCTA	Qiagen	20	96
SRA-UTR siRNA	CACCAGAGCTTTGCTAATAAT	Qiagen	15	96
WRB siRNA	TGACACGTATGTACTAGTGAA	Qiagen	20	96