Supplementary material

Figure Legends

Figure S1. Short secretory protein sequences. The sequences of the proteins used in this study are shown, with signal sequences highlighted in bold and the opsin derived tags underlined. Methionines introduced for radiolabelling are indicated in italics.

Figure S2. Translocation of ppcecA is dependent on nucleotide triphosphates. PpcecAOPG1 was translated in vitro as previously described. The reaction was incubated with 1U apyrase for 10mins at 30°C prior to addition of membranes. Glycosylated forms are indicated and are quantified as a percentage of the total translation products, normalised to the control. Results are displayed as means ± S.E (n=3)

Figure S3. Sucrose gradient centrifugation of human short secretory proteins. In vitro translations of apelin and statherin were subjected to centrifugation through a 5-25% sucrose gradient and collected in fractions. Each fraction was tested for its ability to support ppcecA translocation by incubation with canine pancreatic microsomes. N-glycosylated and unprocessed (pp) forms are indicated.

Figure S4. TRC40 crosslinks to the signal sequence of ppcecA. Translocation competent fractions of ppcecAOPG2 translations were pooled and incubated with BMH crosslinker. Immunoprecipitations were carried out using antibodies against the indicated proteins.

Figure S5. TRC40 binding and WRBcc inhibition using non-nuclease treated reticulocyte lysate. A) In vitro translations were performed with untreated lysate in the presence of recombinant HisTRX (HT) or recombinant TRC40 as indicated. After synthesis was complete the reactions were diluted with buffer and incubated with nickel agarose. The beads were recovered by centrifugation and washed repeatedly before any bound proteins were eluted. Radiolabelled products were analysed by SDS-PAGE and phosphorimaging. Radiolabelled globin binds directly to the resin

(Wahlman et al., 2007). In contrast, the three radiolabelled short secretory proteins: ppcecA, apelin and statherin, are only recovered in the presence of recombinant TRC40 (lanes 2, 4 and 6, see brackets). **B)** PpcecA was synthesised in vitro using untreated lysate and incubated with 10μM MBP or MBP-WRBcc prior to addition of membranes. The membrane fraction was isolated and the products immunoprecipitated using antibodies raised against the opsin tag. Glycosylation is quantified relative to the MBP control (which was set to 100%) and results are expressed as means ± S.E.

Figure S6. Sucrose gradient centrifugation of ppcecA synthesised in lysate depleted of TRC40. Rabbit reticulocyte lysate was immunodepleted of TRC40 or subjected to a mock immunodepletion. Lysates were then used for the in vitro synthesis of ppcecA. Reactions were subjected to sucrose gradient centrifugation and collected in fractions. Each fraction was tested for its ability to support ppcecA translocation by incubation with canine pancreatic microsomes. N-glycosylated and unprocessed (pp) forms are indicated.

Wahlman, J., DeMartino, G. N., Skach, W. R., Bulleid, N. J., Brodsky, J. L. and Johnson, A. E. (2007). Real-time fluorescence detection of ERAD substrate retrotranslocation in a mammalian in vitro system. *Cell* **129**, 943-55.

Figure S1

ppcecAOPG1 (*Hyalophora cecropia*)

MNFSRIFFFVFACLTALAMVNAAPEPKWKLFKKIEKVGQNIRDG*MM*KAGP
AVAVVGQATQIAKGMGPNFYVPFSNKTG

ppcecAOPG2 (*Hyalophora cecropia*)

MNFSRIFFFVFACLTALAMVNAAPEPKWKLFKKIEKVGQNIRDG*MM*KAGP
AVAVVGQATQIAKGMNGTEGPNFYVPFSNKTG

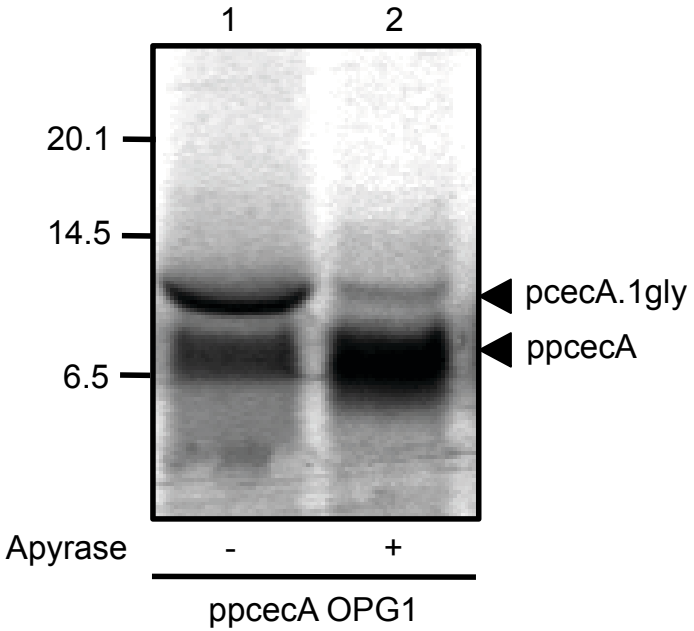
ApelinOPG2 (human)

MNLRLCVQALLLLWLSLTAVCGGSLMPLPDGNGLEDGNVRHLVQPRGSR
NGPGPWQGGRR
KFRRQRPRLSHKGPMPFMNGTEGPNFYVPFSNKTG

StatherinOPG2 (human)

MKFLVFAFILALMVSMIGADSSEEKFLRRIGRFGYGYGPYQPVPEQPLYPQ
PYQPQYQQYTF*MM*MNGTEGPNFYVPFSNKTG

Figure S2


1
2
20.1
14.5
6.5
pcecA.1gly
ppcecA
Apyrase
-
+
ppcecA OPG1

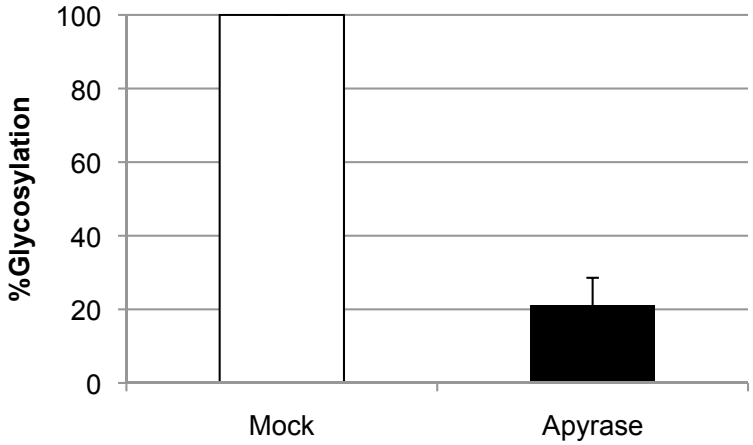

%Glycosylation
100
80
60
40
20
0
Mock
Apyrase

Figure S3

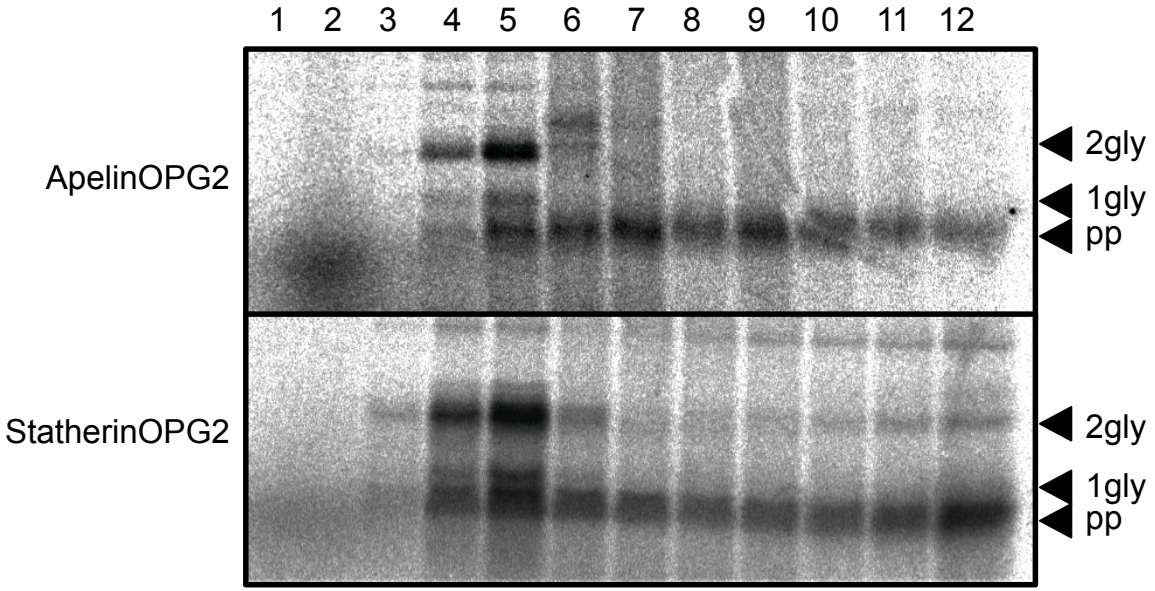

1 2 3 4 5 6 7 8 9 10 11 12
ApelinOPG2
2gly
1gly
pp
StatherinOPG2
2gly
1gly
pp

Figure S4

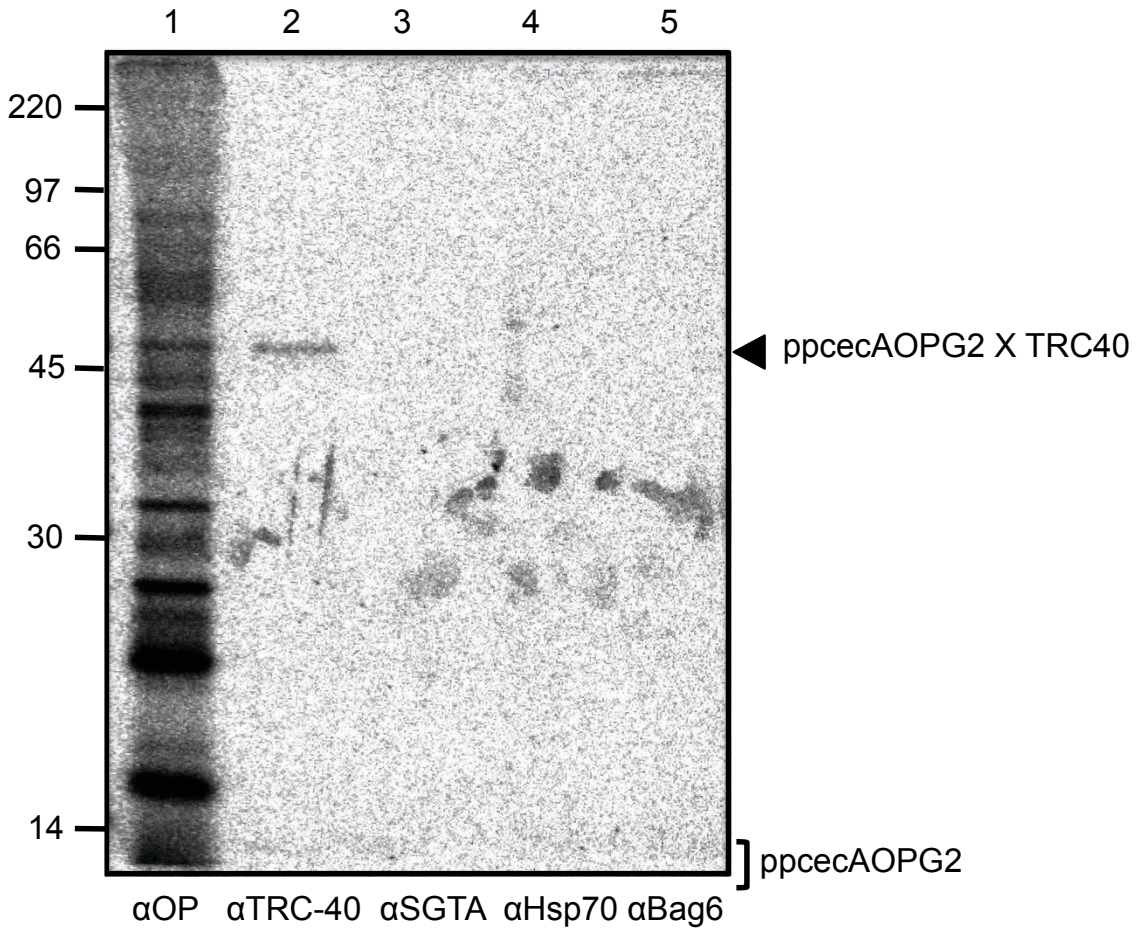

1
2
3
4
5
220
97
66
45
30
14
ppcecAOPG2 X TRC40
ppcecAOPG2
αOP
αTRC-40
αSGTA
αHsp70
αBag6

Figure S5

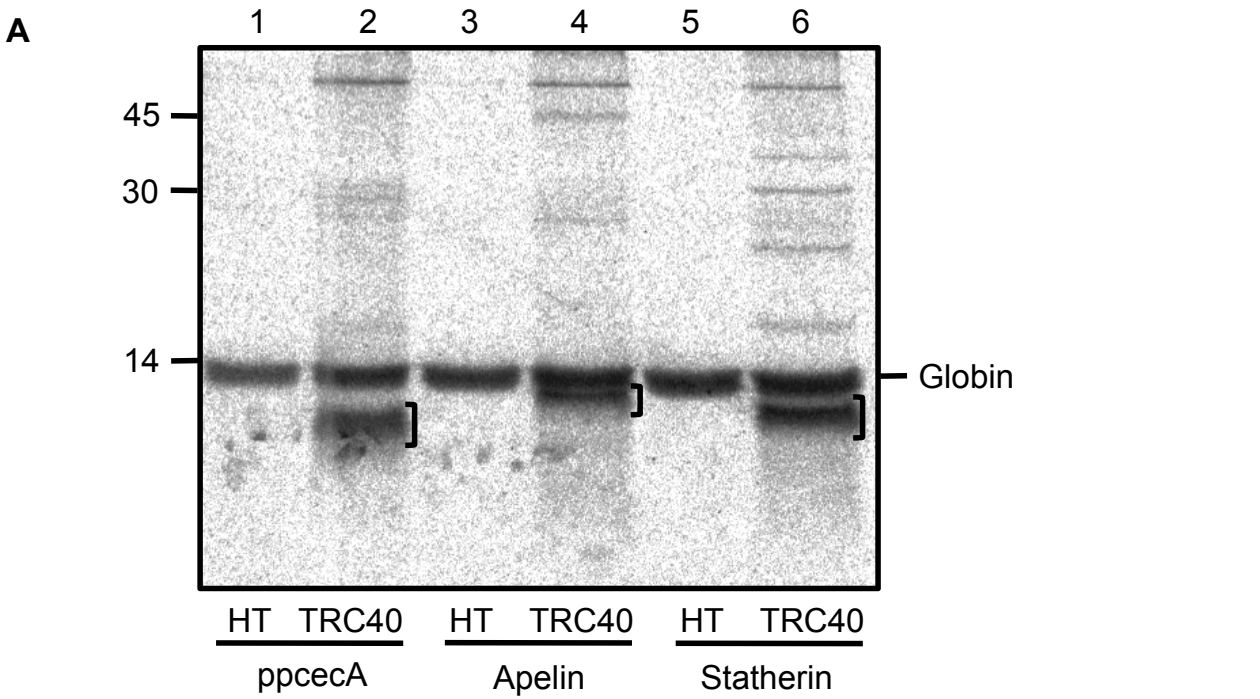

A
1
2
3
4
5
6
45
30
14
Globin
HT
TRC40
HT
TRC40
HT
TRC40
ppcecA
Apelin
Statherin
B
1
2
MBP
WRBcc
100
80
60
40
20
0
% Glycosylation
MBP
WRBcc

Figure S6