SUPPLEMENTAL MATERIALS

Supplemental Methods:

**Bacterial expression, protein purification and analysis**

Plasmids, bacterial expression and purification of His$_6$-tagged hamster BiP$^{WT}$, BiP$^{44K}$, BiP$^{R197E}$ and BiP$^{E201G}$ were described previously (Awad W., et al. 2008. Proc Natl Acad Sci U S A 105: 1164-9, Gaut J.R. and L.M. Hendershot 1993. J Biol Chem 268: 7248-55). Purified proteins were dialyzed and stored at -80°C in 20 mM Hepes pH7.4, 150 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA, and 10% glycerol.

Mouse P58 (27-504), wildtype or H422Q with or without P58’s J-domain (384-470) fused at its N-terminus were expressed as a His$_6$-Smt3-fusion proteins in E.Coli Rosetta(DE3) strain by inducing with 1 mM IPTG for 6hrs at 18°C, purified on Ni-NTA-agarose under non-denaturing conditions. The Smt3 and His-tag were cleaved with the yeast Ulp1 protease overnight at 4°C and soluble untagged P58(27-504) was purified by gel filtration on Superdex200 and stored in aliquots at -80°C.

P58 J-domain (384-470) wildtype and the H422Q mutant in the pGEX-4T-1 vector (Pharmacia) were expressed in E. Coli BL21 (DE3) as GST-fusion proteins using 1 mM IPTG and 6hrs induction followed by purification using Glutathione Sepharose (Amersham Biosciences), elution with 40 mM glutathione (Sigma), dialysis and storage conditions as above.

**Analysis of BiP’s ATPase activity**

BiP’s ATPase activity and the effects of added J domain on it were measured using a fluorescent polarization assay that detects ADP generated from ATP hydrolysis by displacement
of an AlexaFluor 633-labeled ADP tracer from an anti-ADP antibody (Bell Brook Labs). The assay was performed according to the manufacturer's instructions in a reaction volume of 10µL in K Buffer at 37°C for 1hr using a 10 µM starting concentration of ATP, 0.025 µM BiP and, where indicated 1 µM of purified GST-J and read in Infinite F500 fluorescent plate reader (TECAN). Each was performed in triplicate and included a control of an irrelevant protein (eIF2α 1-185) purified on Ni-NTA-agarose or GST on glutathione-sepharose.

**Generation of a denatured and reduced RNase A affinity matrix and analysis of P58's binding to it:**

Bovine pancreatic Ribonuclease A (RNase A) (Sigma) was chemically coupled to CNBr-activated Sepharose (GE Healthcare) according to the manufacturer’s instructions at ~3 mg (~0.2 µmoles) per mL of bed volume. This resin charged with native RNase A was then stored in 10 mM Tris pH 8.0, 1 mM EDTA, 0.02% sodium azide. Where indicated the RNase A matrix was denatured and reduced by placing 150µL bed volume of charged beads (30 nmoles RNase A) in 1.3 mL of 6 M Guanidine hydrochloride, 0.1 M DTT, 10 mM Tris pH 8.6 at RT overnight. Before use, the denaturant and DTT were washed away and free thiols were blocked with 50mM NEM or the resin was maintained in 1mM DTT to prevent disulfide bonds and folding.
SUPPLEMENTAL Figure legends:

Figure S1:

Translocation of P58 into the ER lumen

A. Alignment of the protein sequence of P58 from mouse, zebrafish and worms showing conservation of a hydrophobic segment at the N-terminus (shaded). The arrow marks the predicted cleavage point of the putative signal peptide. Conserved residues in the portion of the protein displayed here are bolded.

B. Autoradiogram of $^{35}$S-metabolically labeled P58 immunoprecipitated from an in vitro transcription reaction programmed with in vitro transcribed wildtype P58 mRNA (lane 1) or mRNA encoding a protein tagged at its C-terminus (P58-HA, lane 6) or metabolically labeled 293T cells transfected with wildtype P58 (lane 2), P58 tagged with an HA epitope at the N-terminus (lane 4 & 5) or C-terminus (lane 7-8) or of endogenous P58 immunoprecipitated from tunicamycin treated mouse fibroblasts (MEF, lane 3). Anti-P58 or anti-HA tag antibodies were used in the IP as indicated. The two panels are of a shorter and longer exposure of the same gel.

C. Autoradiogram of the indicated fusion proteins between P58 and VCAM1 (a glycoprotein) expressed in metabolically-labeled 293T cells and immunopurified with antiserum to VCAM1. Where indicated the cells had been treated with tunicamcyin before and during the labeling procedure and the position of the glycosylated (G) and non-glycosylated (N) proteins is marked. The domain organization of the various fusion proteins is depicted in cartoon form above the autoradiogram. The hydrophobic segment ($\psi$) and J-domain (J) of P58 are indicated as are the glycosylation sites (G), signal peptide (SP) and transmembrane domain (TM) of VCAM1 (293T cells were transfected by calcium phosphate co-precipitation. pCDNA3
expression plasmids encoding full length mouse P58, deletion mutants and fusions involving human VCAM-1 were constructed by PCR).

**Figure S2:**

Schema of FLAG-P58

Predicted peptide sequence of the FLAG-tagged P58, before (upper-panel) and after (lower panel) cleavage of the pre-pro-trypsinogen signal peptide (PPT-SP).

**Figure S3:**

P58 associates with misfolded insulin.

Autoradiograph of metabolically labeled proteins immunoprecipitated from parental CHO cells and CHO cells stably-expressing FLAG-P58. The cells were transfected with plasmids expressing a fusion of misfolded mutant \( \text{INS2}^{\text{C96Y}} \)-GFP or GFP as a control. Note that a protein identical in size to \( \text{INS2}^{\text{C96Y}} \)-GFP is recovered in the FLAG immunoprecipitate only in cells expressing FLAG-P58 (the stability of the interaction is shown by its resistance to RIPA wash conditions, lane 4).

Mammalian expression plasmid encoding pre-pro-insulinC96Y-GFP (Liu M., et al. 2007. Proc Natl Acad Sci U S A 104: 15841-6) was a gift of Peter Arvan.

**Figure S4:**

No discernable affect of P58 on the half-life of misfolded VSV-G.

A. Autoradiograph of VSV-G\(^{6045}\)-GFP immunopurified from parental CHO cells and CHO cells stably-expressing FLAG-P58 following a 30’ labeling pulse and the indicated length of cold chase at the non-permissive temperature of 41˚C.
B. Graphic presentation of the VSV-Gts045-GFP signal from the experiment shown in “A”. Parental CHO cells (○) and CHO cells stably-expressing FLAG-P58 (■).

Figure S5
The effect of BiP concentration on the dissociation of P58 from denatured RNase A in the presence of ATP/Mg$^{2+}$.

Immunoblot of wildtype FLAG-P58 (WT) and a C-terminal truncation mutant FLAG-P58 lacking the J-domain (ΔJ) from cell lysates associated with denatured RNase A. The pre-formed complex was challenged with bacterially expressed wildtype BiP at the indicated concentration in the presence of ADP or ATP. The ratio of P58 retained on the resin is indicated for each experimental pair (by the curved arrow). The lower panels show the amount of BiP retained on the resin by Ponceau-S staining.

Figure S6
A substrate binding mutation compromises BiP’s ability to promote dissociation of P58 from denatured RNase A

A. Immunoblot of FLAG-P58 associated with denatured RNase A in vitro. Where indicated, the pre-formed complex was challenged with 9 μM bacterially expressed wildtype BiP (WT) or a mutant BiP compromised in substrate binding (V461F) in the presence of ADP or ATP. The ratio of P58 retained on the resin was measured and is indicated for each experimental pair (under the curved arrow). The BiP that remained bound to the resin is revealed by Ponceau-S staining of the blot (middle panel) and the input BiP by Coomassie stain (lowest panel).

A plasmid for the bacterial expression and purification of His$_6$-tagged hamster BiP$^{V461F}$ was constructed by PCR in the pQE10, expressed in M15(pREP4) E. coli strain, and purified according to manufacturer’s instructions (Qiagen).
B. ATPase activity of wildtype and BiP$^{V461F}$ in the absence or presence of P58-J, measured by the production of ADP in vitro. Shown is the mean ± SEM of the fluorescence polarization signal (as in figure 6C). The fluorescent polarization signal is inversely-proportional to the ATPase activity.

C. Coomassie-stained SDS-PAGE of BiP$^{WT}$ or BiP$^{V461F}$ mutant retained on glutathione sepharose beads to which GST-J$^{WT}$ had been pre-bound. Where indicated, the binding buffer was supplemented with ADP or ATP (5mM).
A

Mus musculus: MVAPGSVGSRGLAVFPFLVLDQYEGACGVNADVEKHLELGKLLAAGQLADALSQF...504
Danio rerio: MVAVSPVAHKLLSYVPFLVLIDRYEGVNGKGDGIENHLEMGKLLAAGQLADALSHF...504
C. elegans: --------MTIYQHLLILWSSLFASTFAGTAEEVAKHELGSQFLARQFADALTQY...491

B

in vitro: + +
293T: + + + + +
MEF: +
Tun: +
P58: + +
HA-P58: + +
P58-HA: + + +

C

P58(27-393)-VCAM1(34-700): + +
P58(1-393)-VCAM1(34-700): + +
Tun: + +
PPT SP  FLAG tag  linker  p58 (27-504)

MSALLILALVGAAVA   DYKDDDK   LGTELGSM   EGAECG...

Leader peptide peptidase

PPT SP  FLAG tag  linker  p58 (27-504)

MSALLILALVGAAVA   DYKDDDK   LGTELGSM   EGAECG...
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<th>α-Flag</th>
<th>α-GFP</th>
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<tr>
<td>CHO parental</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>GFP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>INS2&lt;sup&gt;C96Y&lt;/sup&gt;-GFP</td>
<td>+ + +</td>
<td>+ + +</td>
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<tr>
<td>RIPPA wash</td>
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![Western Blot Image](image_url)

- **P58**
- **INS2<sup>C96Y</sup>-GFP**
- **GFP**
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<th>-</th>
<th>1.5</th>
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<td>+</td>
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**Ratio (ATP/ADP):**

- **FLAG-P58<sup>WT</sup>**
  - 1.6
  - 1.0
  - 0.5
  - 0.2

- **FLAG-P58<sup>ΔJ</sup>**
  - 0.8
  - 1.1
  - 2.2
  - 1.7

**P58**

**BiP**
A. 

<table>
<thead>
<tr>
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<th>RNaseA&lt;sup&gt;+&lt;/sup&gt;-Seph pulldown</th>
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<tr>
<td>ATP:</td>
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Ratio (ATP/ADP):

1.3 .52 1.2

B. ATPase activity of Bi<sup>WT</sup> and Bi<sup>V461F</sup>

C. GSH-Seph

<table>
<thead>
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<th>GST-J&lt;sup&gt;WT&lt;/sup&gt;</th>
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<tr>
<td>BiP&lt;sup&gt;V461F&lt;/sup&gt;</td>
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</tr>
<tr>
<td>GST-J&lt;sup&gt;WT&lt;/sup&gt;</td>
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BiP inputs:

75 WT V461F

GST-J inputs:

75 50 37 25

BiP inputs:

75 WT V461F