Supplemental Material for:

The role of p58^{IPK} in protecting the stressed endoplasmic reticulum

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Supplemental Figures

Rutkowski et al., Fig. S1

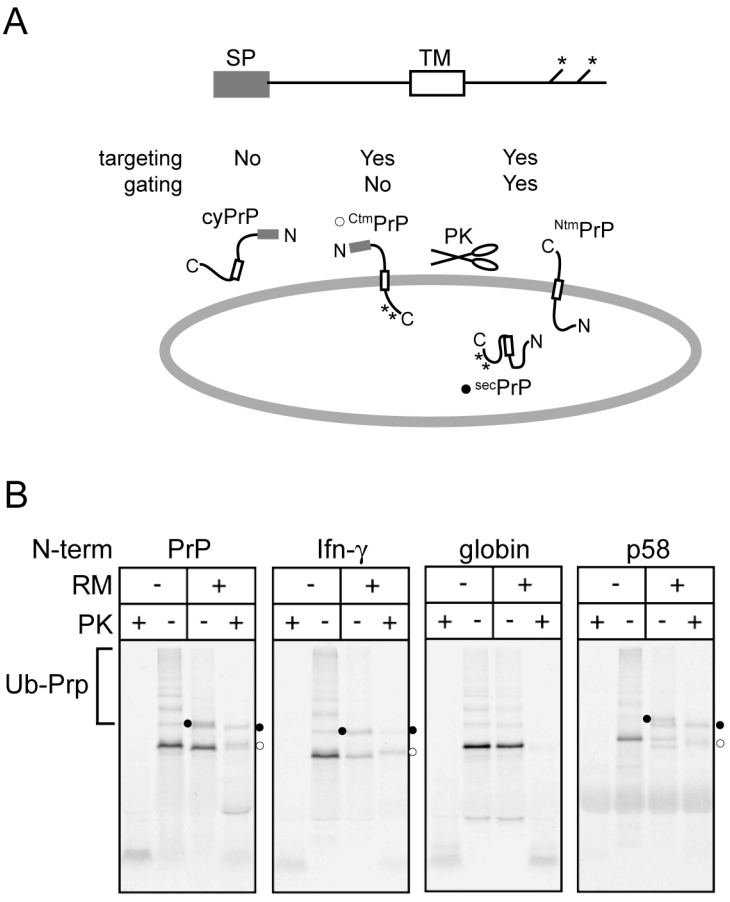

Figure S1. The putative p58^{IPK} signal sequence functions in a heterologous context (A) Experimental design. A previously described assay system (Kim et al., 2002) was used to quantify the targeting and translocation functionality of the putative p58 signal sequence. In this assay, the test sequence of interest is fused to the N-terminus of a reporter protein derived from the prion protein (PrP). The PrP reporter contains both a potential transmembrane (TM) domain and two glycosylation sites near the C-terminus (see line diagram). Successful ER targeting mediated by the test sequence results in translocation and/or membrane insertion of the reporter protein, resulting in glycosylation and/or protease protection of the translocated domains (see diagram). The relative 'strength' of the test sequence in its interaction with the ER translocon to initiate translocation (an event termed translocon gating) can be inferred from the distribution of topological forms generated (see Kim et al., 2002, for details). The schematic diagram illustrates the various topological forms of PrP, their corresponding interpretations, and how they are discriminated on the basis of protease protection and glycosylation (stars). (B) The N-termini of the indicated proteins were fused to the PrP reporter and tested for translocation. The constructs were translated in vitro in the presence or absence of ER-derived rough microsomes (RM), followed by digestion with proteinase K (PK). In the absence of RM, all of the constructs are ubiquitinated (resulting in the ladder of bands indicated by the bracket) and no protease protection is observed. The construct containing the globin test sequence is not translocated in the presence of RM as evidenced by its continued ubiquitination and lack of either glycosylation or protease protection. By contrast, each of the other constructs is targeted to and translocated/inserted into the RM, resulting in the absence of ubiquitination and generation of glycosylated PrP (indicated by the closed circle). Upon PK digestion, glycosylated PrP is either completely protected (indicative of successful targeting and gating) or partially digested to generate a C-terminal fragment (open circle, indicative of targeting but no gating). Based on the relative ratios of these forms, the p58 signal sequence appears to be more efficient than the Ifnγ signal and comparable to the PrP signal. The double bands observed in the presence of RM for p58 is due to heterogeneity of signal sequence cleavage.

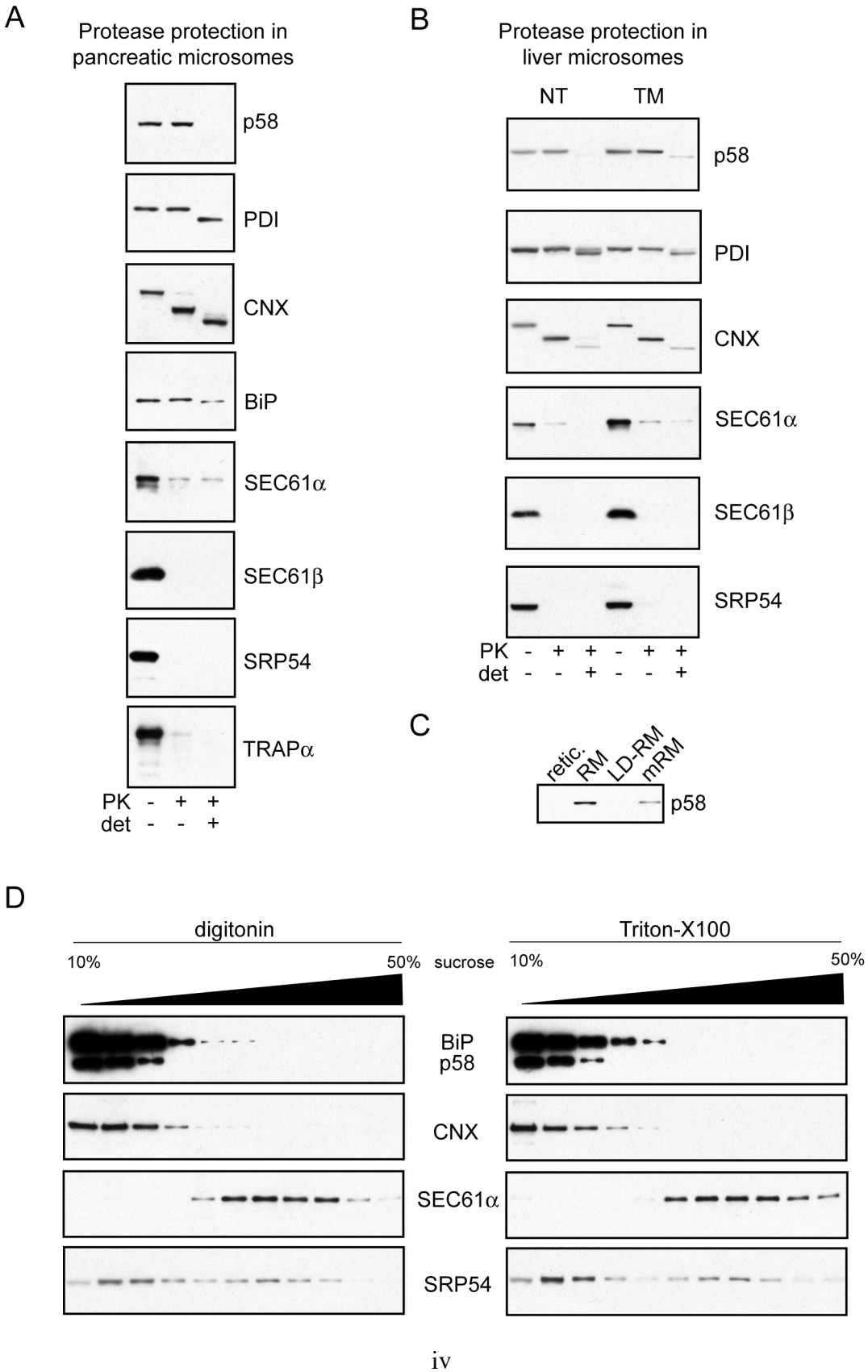
A
Protease protection in pancreatic microsomes
p58
PDI
CNX
BiP
SEC61α
SEC61β
SRP54
TRAPα
PK - + +
det - - +
B
Protease protection in liver microsomes
NT
TM
p58
PDI
CNX
SEC61α
SEC61β
SRP54
PK - + + - + +
det - - + - - +
C
retic.
RM
LD-RM
mRM
p58
D
digitonin
Triton-X100
10%
50%
sucrose
10%
50%
BiP
p58
CNX
SEC61α
SRP54

Figure S2. Analysis of p58^{IPK} topology in liver and pancreas microsomes
(A) ER-derived canine pancreatic microsomes were digested with PK in the presence or absence of detergent and probed by immunoblot against the indicated antigens. The partial protection of some proteins (e.g., BiP, PDI, Calnexin) even in the presence of detergent is due to tightly folded domains resistant to complete protease digestion. The small degree of protease protection observed for SEC61α and TRAPα is due to steric shielding by tightly-bound ribosomes.
(B) Liver microsomes from nontreated or TM-injected mice were digested with PK as above, and probed by immunoblot as indicated.
(C) p58^{IPK} levels were assessed by immunoblot in reticulocyte lysate, canine pancreatic microsomes (RM), microsomes depleted of lumenal contents (LD-RM), and mock-depleted microsomes (mRM).
(D) Pancreatic microsomes were solubilized in either 1% digitonin or 1% Triton-X100 under physiologic salt conditions and fractionated by velocity sedimentation as in Figure 3E. The p58/BiP immunoblot is intentionally overexposed to illustrate the complete lack of any p58^{IPK} co-fractionating with SEC61α.

It is worth noting that SRP54 is an especially important control in these experiments because it represents a well-studied cytosolic protein that is transiently recruited to the cytosolic face of the translocon [analogous to the topology and previously proposed model for p58^{IPK} (Oyadomari et al., 2006)]. We find that SRP54 (which co-fractionates with microsomes from both pancreas and liver) is stripped by high salt (Fig. 3C), is accessible to cytosolic protease (Sup. Fig. S2A, B), and can be found to at least partially co-fractionate with the translocon on velocity gradients of detergent solubilized microsomes (Sup. Fig. S2D). None of these properties is shared by p58^{IPK} in the same experiments, arguing strongly against its localization at the cytosolic face of the translocon.

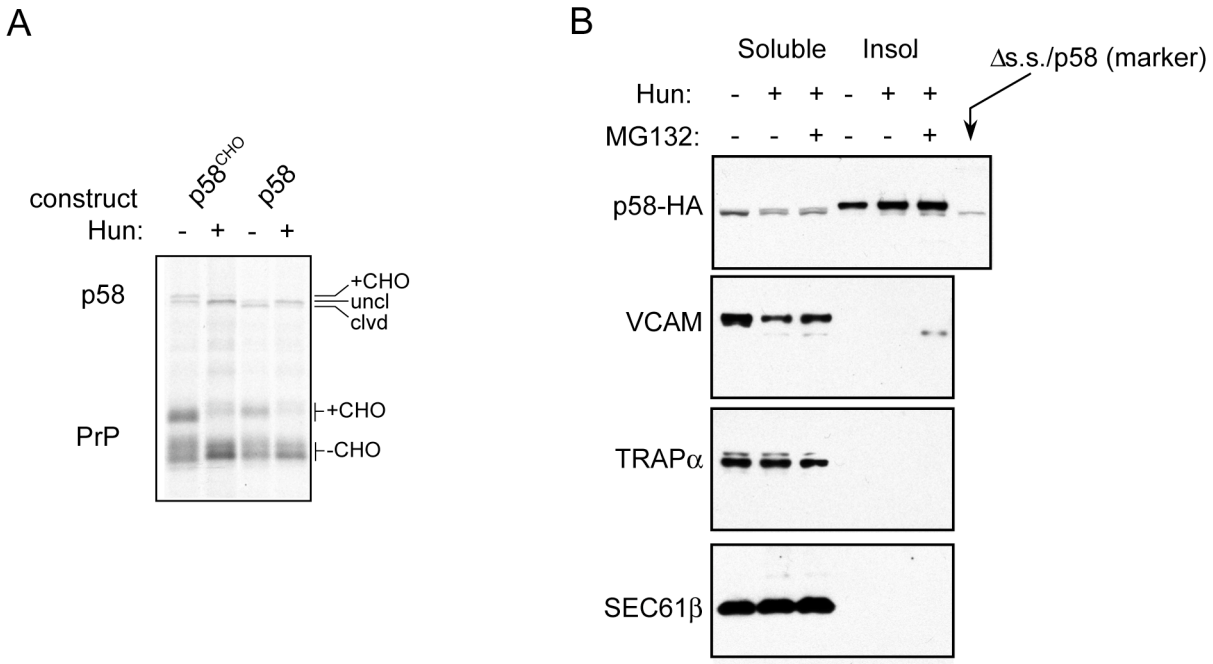

Figure S3. Hun inhibits p58 translocation and promotes p58 aggregation
(A) HeLa cells were cotransfected with PrP and either $p58^{CHO}$ or p58, followed by treatment with or without 100 nM Hun-7293 for thirty minutes, then by a 10 minute pulse labeling and immunoprecipitation (using PrP- and HA-specific antibodies). Inhibition of PrP translocation is seen by inhibition of production of the glycosylated form of PrP (+CHO). For both $p58^{CHO}$ and p58, Hun-7293 treatment leads to production of signal sequence-uncleaved (and for $p58^{CHO}$, unglycosylated) p58.
(B) HeLa cells cotransfected with p58 and VCAM1 were treated for 6 h with 100 nM Hun-7293 in the presence or absence of 10 μM MG132. Cells were lysed in 1% Triton X-100, 50 mM Hepes, pH 7.4, 100 mM NaCl, separated into soluble and insoluble fractions by centrifugation for 10 min in a microcentrifuge, and probed by immunoblot with antibodies against p58 (HA), VCAM1, TRAPα, or SEC61β. The effect of Hun-7293 and MG132 treatment on both VCAM1 and p58 can be seen as an increased amount of non-translocated insoluble protein and some reduction of soluble, correctly processed protein. Translocation for p58 is accompanied by signal sequence cleavage, while VCAM1 translocation results in its glycosylation. TRAPα and SEC61β serve as internal controls for efficient detergent extraction in all three conditions, and illustrate that not all proteins are found in aggregates in the presence of Hun-7293 and MG132.

Note that although translocation of p58 is reasonably efficient in Hela cells as judged by pulse-labeling and immunoprecipitation (panel A), the non-translocated material

appears to be particularly prone to aggregation and accumulation even in the absence of Hun-7293 or MG132 (panel B). This is probably a combination of its high overexpression in Hela cells and the presence of an unprocessed hydrophobic signal sequence. That notwithstanding, VCAM1 and p58 co-fractionate into insoluble aggregates only in the presence of both Hun-7293 and MG132, the same conditions where their interaction by crosslinking was previously observed (Oyadomari et al., 2006).