PERK EIF2AK3 regulates proinsulin processing by controlling ER chaperones and not through protein synthesis regulation

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Running title: *PERK controls ER chaperone function in β cells*

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1. **SUPPLEMENTARY FIGURES**
2. **SUPPLEMENTARY TABLES**
3. **SUPPLEMENTARY EXPERIMENTAL PROCEDURES**

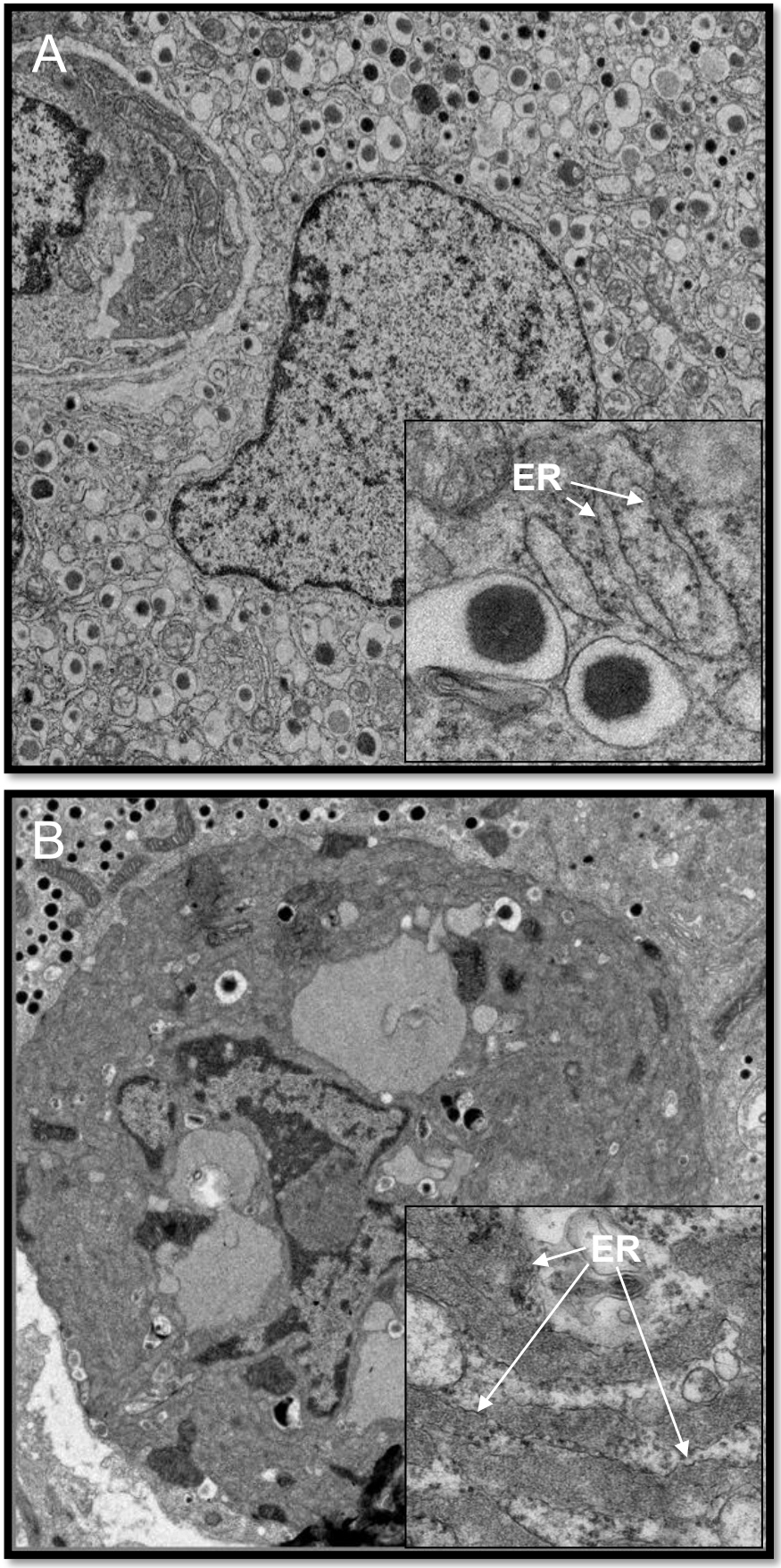


**Figure S1, related to Figure 1.**
A-B. Electron Microscopy images of wild-type mouse pancreatic tissue with its ER exhibiting a thin, ribbon-like structure (A) and PKO mouse pancreatic tissue showing gross expansion of the ER (B).

A

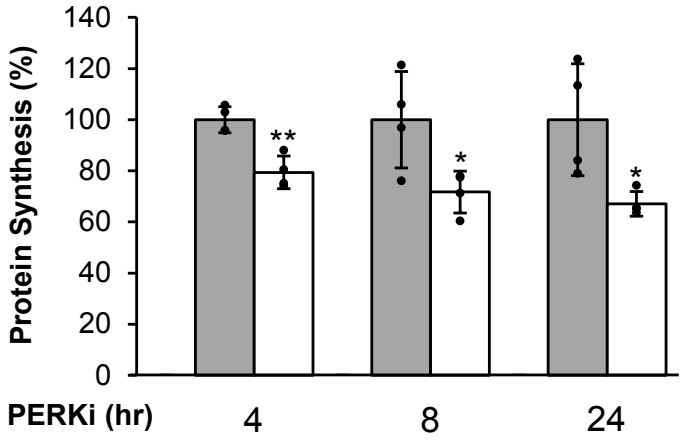


**Figure S2, related to Figure 2.**
A. MIN6 cells treated for 4, 8, or 24 hrs. with 1μM PERKi (Pi). . The methionine analog HPG (50 μM) was added 90 minutes before harvest. Newly synthesized proteins were quantified by fluorescence detection of the incorporated derivatized methionine using ImageJ software as described in Experimental Procedures. Quantification represents n=4 per treatment. Statistical significance was calculated relative to the non-impacted control; *$p \leq 0.05$, **$p < 0.01$.

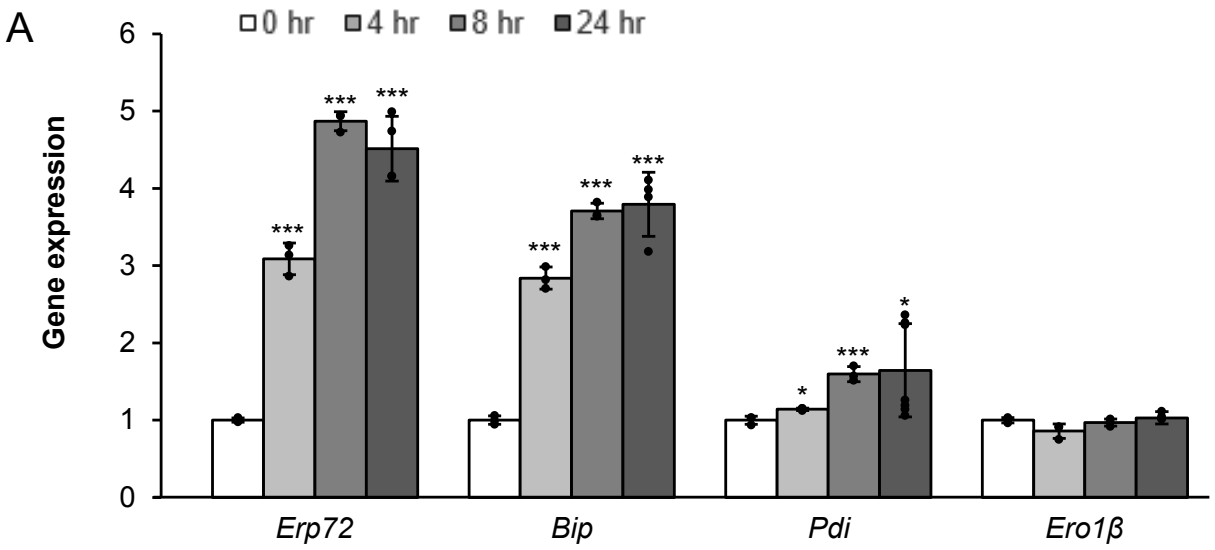


**Figure S3, related to Figure 4.**
A. mRNA expression levels quantified in MIN6 cells treated with 1μM PERKi. Quantification represents n=4 per treatment. Statistical significance is calculated relative to 0 hr. control; *p≤0.05, ***p<0.001.

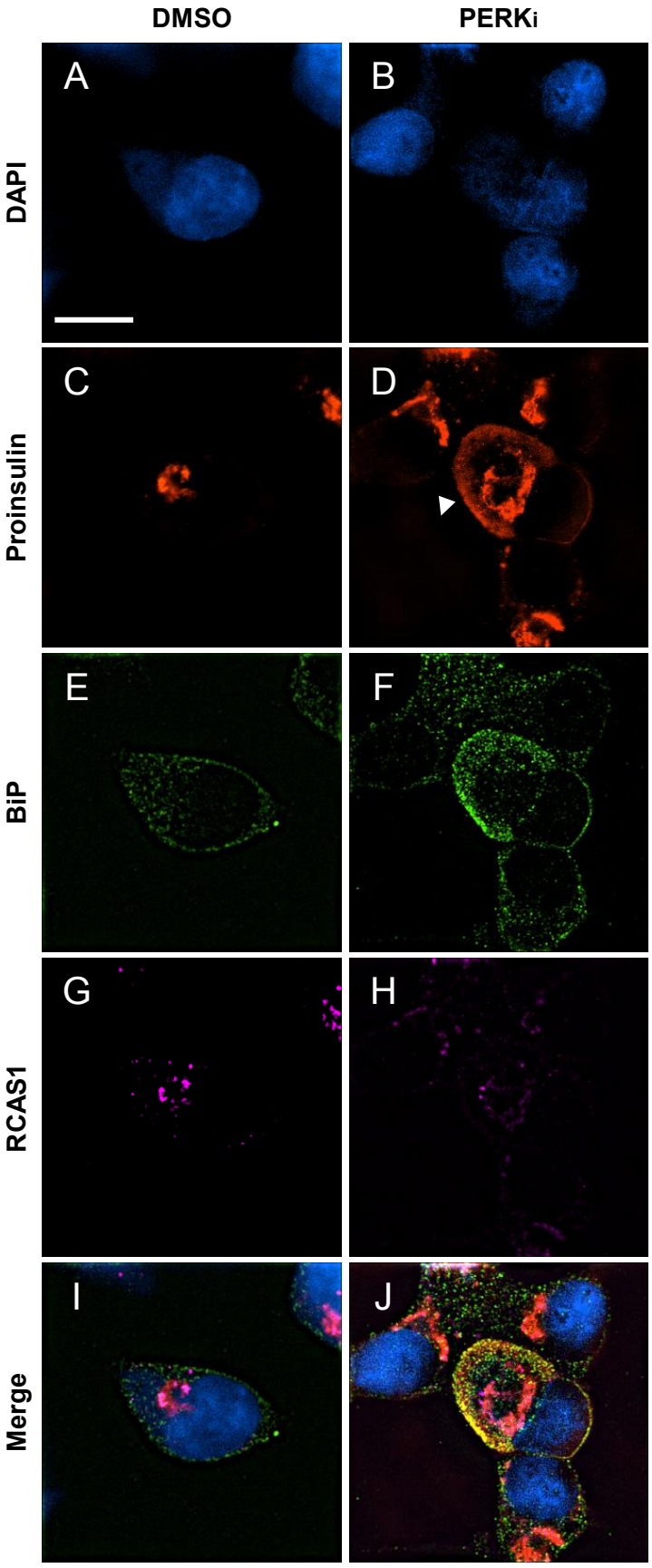


**Figure S4, related to Figure 5.**
A-J. Immuno-detection of DAPI (blue), proinsulin (red), BiP (green), and RCAS1(a Golgi-specific marker; magenta) images in *INS1*-832/13 cells treated for 24 hours with 1μM PERKi or equal volume of DMSO. Cells were imaged on a high-resolution microscope to show increased co-localization of proinsulin and BiP staining as the ER becomes distended and the cell becomes impacted under PERKi conditions. White arrowhead indicated impacted cell. Scale bar = 10μm.

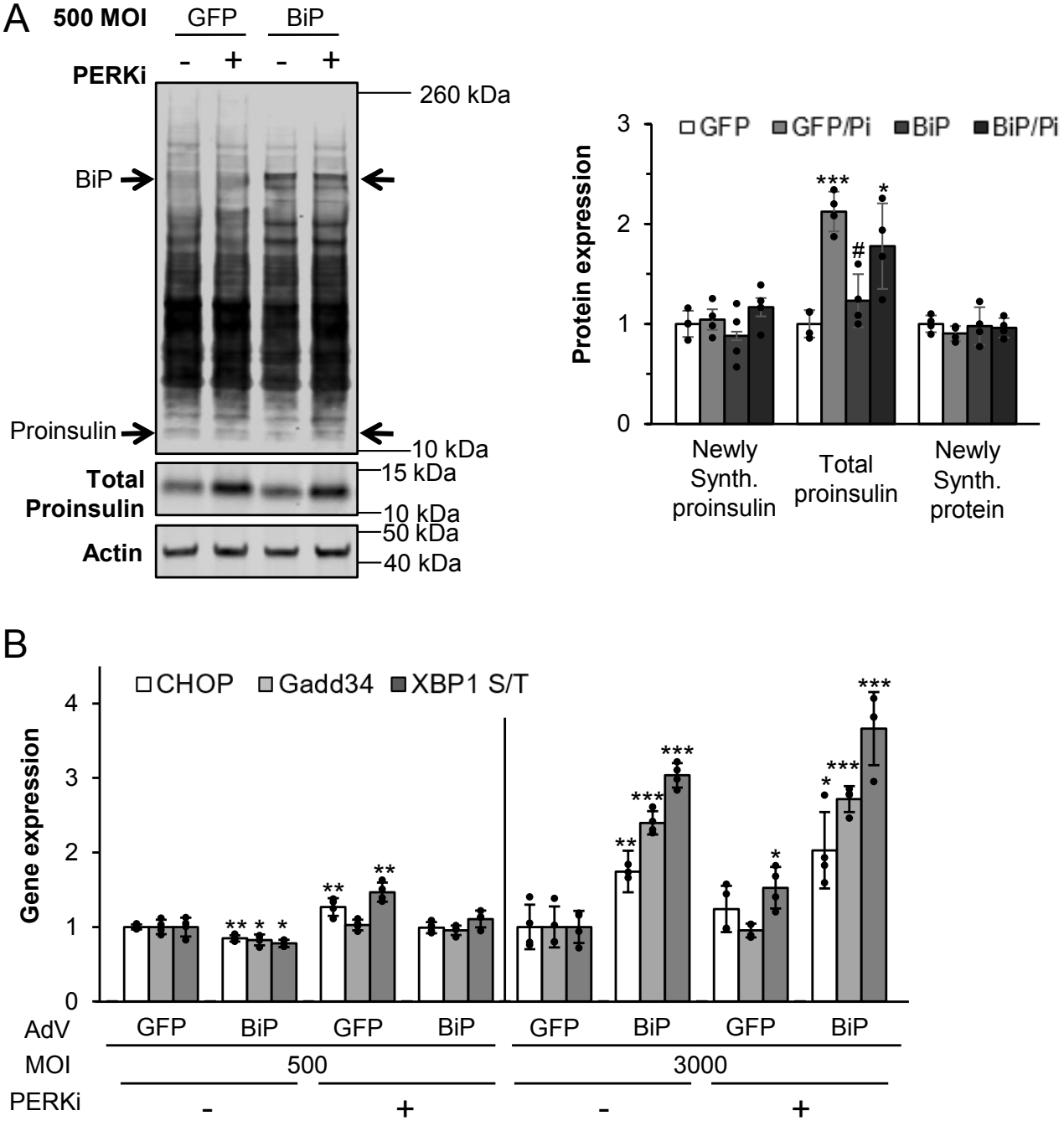


**Figure S5, related to Figure 6.**

A. *INS1* 832/13 cells treated for 24 hrs. with 500nM PERKi (Pi). Puromycin (10µg/mL) was added 15 minutes prior to harvest. Incorporation of puromycin into newly synthesized total protein was detected first by blotting with anti-puromycin. Puromycin-incorporated newly synthesized proinsulin was visualized by reprobing the same blots with anti-C-peptide. A representative blot is shown with quantification representing n=4 per treatment. Statistical significance is calculated relative to the AdV-GFP/DMSO control; #p=0.07, *p≤0.05, and ***p≤0.001.

B. mRNA expression levels in *INS1* 832/13 cells relative to control cells. Cells were infected with 500 MOI or 3000 MOI of adenovirus for 8 and 2 hrs, respectively, and media was replaced with fresh media. Cells were then treated for 24 hrs. with 500nM PERKi. Quantification represents n=4 per treatment. Statistical significance is calculated relative to the AdV-GFP/DMSO control for each MOI; *p≤.05, **p≤0.01, and ***p≤0.001.

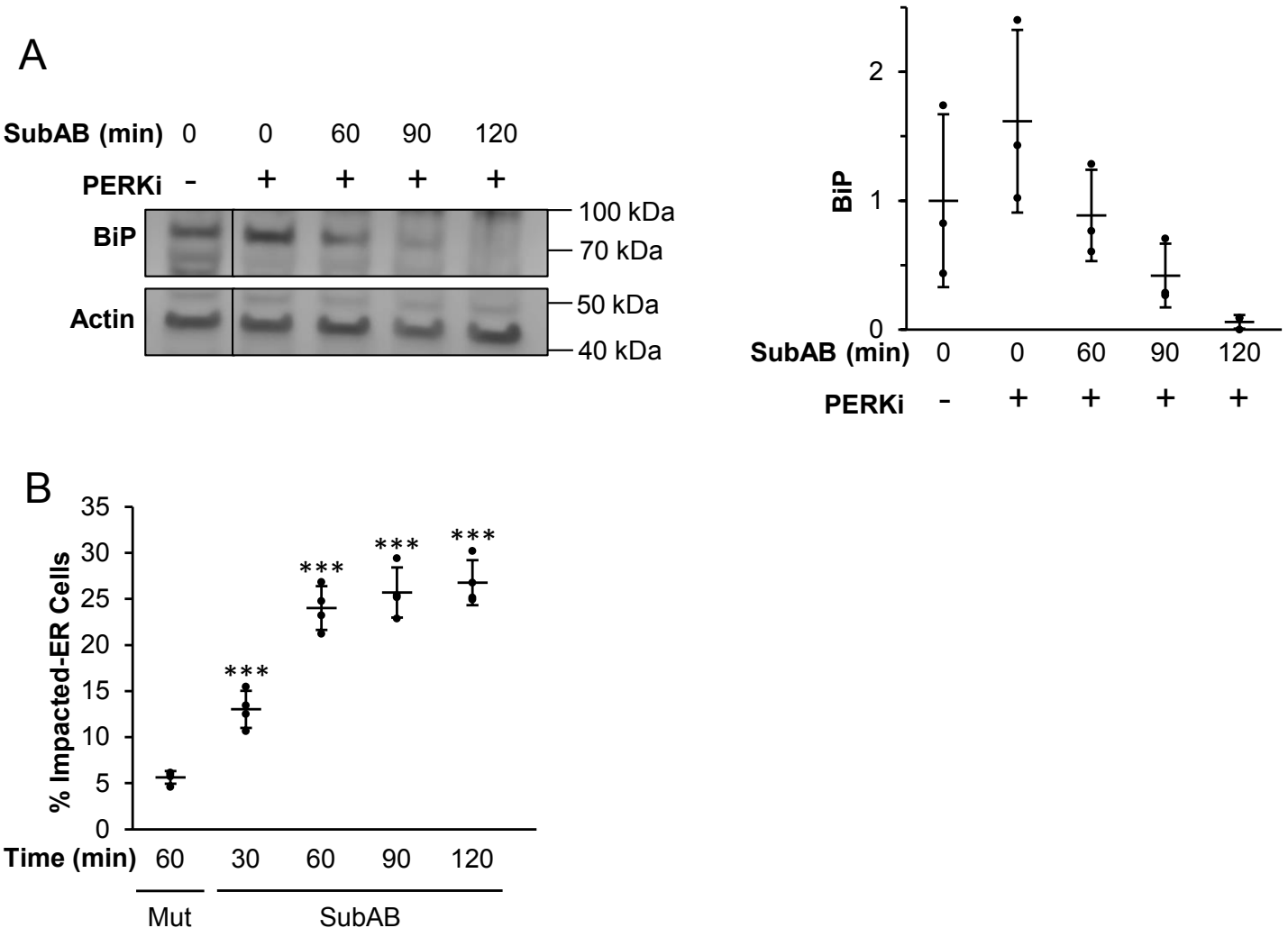


**Figure S6, related to Figure 6.**

A. *INS1* 832/13 cells treated for 24 hrs. with 1μM PERKi and treated as indicated with 0.5μg/mL Subtilase cytotoxin (SubAB). A representative blot is shown with quantification representing n=4 per treatment.

B. *INS1* 832/13 cells treated with SubAB in combination with PERKi. Proinsulin and DAPI were detected by immunocytochemistry, and Impacted-ER were counted as a frequency of total cells. Quantification represents n=4 per treatment. Statistical significance was calculated relative to the 60 minute Mutant control; ***$p<0.001$.

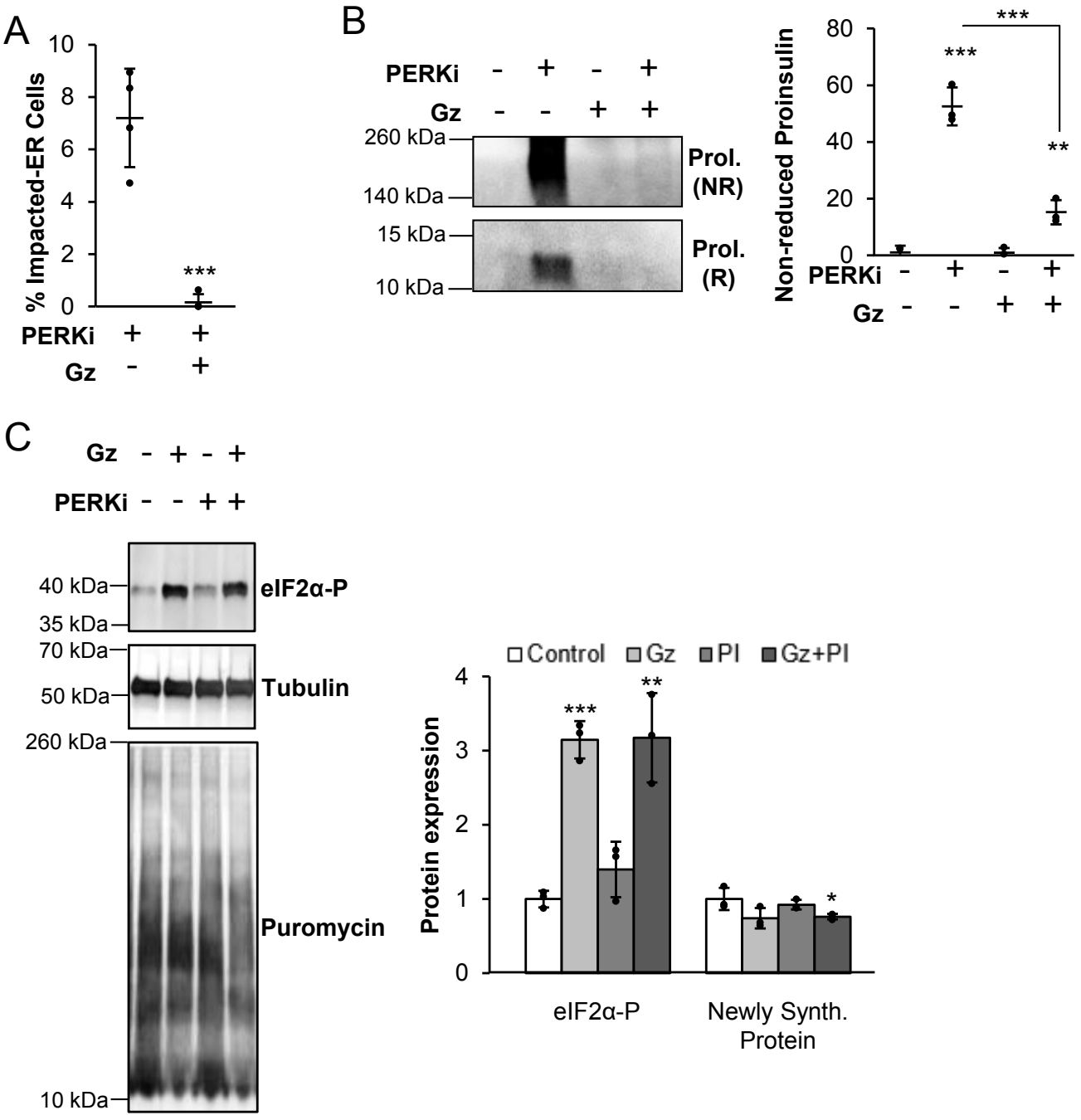


**Figure S7.**

A. INS1 832/13 cells treated for 24 hrs. with 500nM PERKi and 50μM Gz. Quantification represents n=4 per treatment. Statistical significance is calculated relative to PERKi treatment; ***p<0.001.

B. INS1 832/13 cells treated for 24 hrs. with 500nM PERKi and 50μM Gz. HMW protein aggregates were pelleted and electrophoresed as described in Fig. 1G. Representative blots are shown with quantification representing n=3 per treatment. Statistical significance is calculated relative to the DMSO control or as indicated; **p<0.01, ***p<0.001.

C. MIN6 cells treated for 1 hr. with 500nM PERKi and 50μM Gz. Puromycin (10μg/mL) was added 15 minutes prior to harvest to measure newly synthesized protein. A representative blot is shown with quantification representing n=3 per treatment. Statistical significance is calculated relative to the DMSO control; *p≤0.05, **p<0.01, ***p<0.001.

**SUPPLEMENTARY TABLES**

TABLE S1: Mouse primers used for qPCR

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *Actin* | 5′-GCCCTGAGGCTCTTTTCC-3′ | 5′-TGCCACAGGATTCCATACCC-3′ |
| *Gapdh* | 5′-GGAGCGAGACCCCACTAACA-3′ | 5′-ACATACTCAGCACCGGCCTC-3′ |
| *Erp57* | 5′-GGCGGATGCAACATATCACC-3’ | 5′-TGTGGTTCGTACTGTCCCCC-3’ |
| *Bip* | 5′-GCTTCGTGTCTCCTCCTGAC-3′ | 5′-TAGGAGTCCAGCAACAGGCT-3′ |
| *Erp72* | 5′-TTCCACGTGATGGATGTTCAG-3′ | 5′-AGTCTTACGATGGCCCACCA-3′ |
| *Ero1B* | 5’-TGATTCGCAGGACCACTTTTG-3’ | 5’-TAGCCAGTGTACCGTTCCGG-3’ |
| *Pdi* | 5’-TTGCTGGCAGCAGAGGCTAT-3’ | 5’-GACCACCCCATCTTTGTCCA-3’ |

TABLE S2: Rat primers used for qPCR

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *Actin* | 5′-ATCCTGGCCTCACTGTCCAC-3′ | 5′-CTAGAAGCATTTGCGGTGCA-3′ |
| *Gapdh* | 5′-CACCACCAACTGCTTAGCCC-3′ | 5′-TGGCATGGACTGTGGTCATG-3′ |
| *Erp72* | 5′-TGGCATGGACTGTGGTCATG-3′ | 5′-TCATGGTAAGGTGCCGAGG-3′ |
| *Chop* | 5’-CCGGTCCAATTACAGTCATGG-3’ | 5’-TCTCAAAGGCGAAAGGCAGA-3’ |
| *Gadd34* | 5’-CTACAGCCCCTTCACCTGCA-3’ | 5’-CACCACCTCCCCAACTTTCTT-3’ |
| *Xbp1-spliced* | 5’-ATCCATGGGAAGATGTTCTGG-3’ | 5’-CTGAGTCCGAATCAGGTGCAG-3’ |
| *Xbp1-total* | 5’-CCCTTCTCCCTTCAGCGAC-3’ | 5’-CGTTGGCAAAAGTGTCCTCC-3’ |
| *Bip* | 5’-AACCAAGACATTTGCCCCAG-3’ | 5’-GCATGGGTGACCTTCTTTCC-3’ |
| *Pdi* | 5’-GGACTCAAGCGAAGTGACGG-3’ | 5’-TCTGCTGCCAGCAAGAACTG-3’ |
| *Erp57* | 5’-CGAAAACTTCGAGAGTCGCG-3’ | 5’-GCAAGCCTCTTGCAATGTCC-3’ |
| *Ero1B* | 5’-TCTCCTGCTGCACAGTATGTGG-3’ | 5’-AGTGTACCGTTCCGGGTTCA-3’ |
| *Atf6* | 5’-CCAGCAGAAAACCCGCATT-3’ | 5’-GAATTCGAGCCCTGTTCCAG-3’ |

**SUPPLEMENTARY EXPERIMENTAL PROCEDURES:**

*TEM* —Pancreas tissues was isolated and initially fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate buffer. Secondary fixation was with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were then stained with 2% uranyl acetate and dehydrated through an ethanol and acetone series. The samples were infiltrated and embedded in Spurr’s resin over several days and polymerized at 60°C. Transmission electron microscopy (TEM) samples were sectioned using a diamond knife (70 nm thick) and stained with uranyl acetate and lead citrate. Images were captured on a JEOL 1200 EXII TEM microscope.

*The SUnSET method* — In order to quantify new global and proinsulin synthesis as previously described (73,74), 10μg/ml puromycin was added 15 minutes before cells were harvested. Western blot was performed as described below and proteins were probed with mouse anti-puromycin primary antibody overnight at 4°C. After washing with TBST, IR800 anti-mouse secondary antibody was used (1:20000) for visualization of newly synthesized protein using a LI-COR Odyssey scanner and quantified using NIH ImageJ software.

*Isolation of high molecular weight proinsulin aggregates*— Cells were washed in ice-cold 1X PBS containing 2mM EDTA and 10mM N-ethylmaleamide (Sigma). Cells were then lysed on ice for 10 minutes in an ice-cold buffer containing TBSN (20mM HEPES pH 7.5, 150mM NaCl. 1% Triton X100, 1mM EDTA) and wash buffer mixed at ratio of 2:5 to which 1X Protease Inhibitor Cocktail (Sigma) was added. Lysates were centrifuged through a QiaShredder (Qiagen) for 2 minutes at 10,0000 x *g*, 4ºC to break up DNA. Total protein quantification of the lysates was measured via Bradford assay and loading

volumes were adjusted accordingly. Equal lysates were pelleted through a 50% sucrose cushion (20mM HEPES pH7.5, 100mM NaCl, 50% sucrose w/v) at 100,000 x *g* for 45 minutes at 4$^{o}$C in a Beckman Optimax TLX benchtop ultracentrifuge using a TLA 120.2 rotor. Care was taken to mix the lysates with any material that might have precipitated prior to differential centrifugation to ensure that insoluble aggregates were uniformly layered onto the sucrose cushion. Pellets were washed twice with RIPA buffer followed by centrifugation at 10,000rpm for 10 minutes at 4$^{o}$C and then dissolved in 1X urea-PAGE sample buffer (9.6 M urea, 12% glycerol, 1.36% SDS, 40 mM Tris, pH 6.8) with or without beta-mercaptoethanol. All samples were boiled for 5-7 minutes prior to loading on 4-12% Novex NuPage Bis-Tris Midi Gels (Thermo-Fisher).