

BIP Orchestrates Bidirectional ER Protein Trafficking via Co-Chaperone Complexes

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Running title: Bidirectional BIP Controls ER Protein Flux

Supplementary Materials and Methods:

Plasmids and Knockdown Experiments:

Plasmids expressing DNAJB12 and DNAJB14 were previously described by Dabsan et al. (Dabsan, Zur et al. 2025). Small interfering RNAs (siRNAs) targeting SGTA, DNAJB12, and DNAJB14 were utilized in accordance with the manufacturer's instructions (Santa Cruz Biotechnology) and fully described in (Dabsan, Zur et al. 2025). siRNAs targeting BIP/GRP78 were purchased from Santa Cruz Biotechnology (Catalog # sc-44261). CRISPR interference (CRISPRi)-mediated knockdown of DNAJB12, DNAJB14, and SGTA were described in (Dabsan, Zur et al. 2025).

CRISPRi-mediated knockdown of BIP. BIP knockdown was achieved using CRISPR interference (CRISPRi) following the protocol described by (Gilbert, Horlbeck et al. 2014), with minor modifications. Briefly, the dCas9-KRAB vector (Addgene plasmid #50917) was used to generate stable cells expressing dCas9-KRAB. Cells expressing dCas9-KRAB and a BIP-targeting single-guide RNA (Listed below) were thawed and cultured under standard conditions. The specific oligonucleotides used for BIP ((HSPA5-1)-(HSPA5-10)) knockdown were designed as described previously (Gilbert, Horlbeck et al. 2014):

HSPA5-1,GCACAGACAGATTGACCTATT
HSPA5-10,GACTTGCAGGCGGCAGGGGCC
HSPA5-2,GACTGCCTGCTGCTGCCCAAC
HSPA5-3,GGGGTGTTTCGCGAGTGTGAG
HSPA5-4,GACCTCACCGTCGCCTACT
HSPA5-5,GTATATAAGCCGAGTAGGCGA
HSPA5-6,GCCACGAACCAGGCGAAGGGC
HSPA5-7,GAAGGGCAGGTCTAGAAATAC
HSPA5-8,GCGACGGTGAGGTGACGC
HSPA5-9,GGGAGGGTATATAAGCCGAGT

Cytosolic BIP expression:

BIP was cloned by PCR using the oligonucleotides listed below. To generate the cytosolic BIP construct, the signal peptide (first 18 amino acids) and the ER retention motif (KDEL)

were removed. All constructs were subcloned into the pCDNA5/FRT/TO or pCDNA3.1 expression vectors. A FLAG tag was added at the end of the sequence before the KDEL.

ER BIP:

Forward Oligo BamHI-sp-BIP:

ACTGGATCCCAAGATGAAGCTCTCCCTGGTGGCCGCGATGCTGCT

Reverse Oligo FLAG-KDEL-NotI:

ATCAGCGGCCGCTGTCTACAACATCTTTCTTGTCGTCATCGTCTTTGTAGTCTT
CTGCTGTATCCTCTTCACCAGTTG

Cytosolic BIP:

Forward Oligo BamHI-BIP:

AACTGGATCCAAGATGGAGGAGGACAAGAAGGAGGACGTGGGCAC

Reverse Oligo FLAG-NotI:

GATCAGCGGCCGCTGTCTACTTGTCGTCATCGTCTTTGTAGTCTTCTGCTGTATCC
TCTTCACCAGTTGGGG

Construction of a Glycosylated PDIA1 Reporter: To generate glycosylated PDIA1, an artificial N-glycosylation site was introduced within a linker positioned immediately upstream of the KDEL sequence. Briefly, the PDIA1 open reading frame (ORF), including the signal sequence, was cloned into pcDNA3.1 using primers containing BamHI and NotI restriction sites. A FLAG tag was inserted followed by a six–amino acid linker harboring an N-glycosylation motif, the ER retention sequence KDEL, and a STOP codon.

Signal Sequence	PDIA1-ORF	FLAG tag	6X Linker + Glyco site	KDEL	STOP codon
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Schematic showing the structure of PDIA1-Glyc

Supplement Table-S1

Antibody (Host)	Company/Cat. No.
DNAJB12 (Rabbit)	Ptg/16780
DNAJB14 (Rabbit)	Ptg/16501
SGTA (Rabbit)	Ptg/11019
SGTA (Mouse)	Ptg/ 60305-1-Ig
PDIA1 (Rabbit)	Ptg/11245-1-AP
PDIA1 (Mouse)	Ptg/66422-1-Ig
PDIA4 (Rabbit)	Ptg/14712-1-AP
PDIA4 (Mouse)	Ptg/66365-1-Ig
AGR2 (Rabbit)	Ptg/12275-1-AP
AGR2 (Mouse)	SantaCruz/sc-101211
AGR2 (Rat)	Biologend/943102
DNAJB11 (Rabbit)	Ptg/15484-1-AP
pan-p53 (DO-1) (Mouse)	SantaCruz/sc-126
p53 (Rabbit)	Ptg/ 10442-1-AP
p53 (Rabbit)	Ptg/60283
FLAG-DYKDDDDK (Mouse)	Ptg/66008-4
GADPH (G-9) (Mouse)	SantaCruz/sc-365062
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488	Invitrogen/ #A32723
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488	Invitrogen/ #A32731
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647	Invitrogen/ #A32733
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647	Invitrogen/ #A32728
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 555	Invitrogen/ #A32732
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Invitrogen/ #A-11006
Goat anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647	Invitrogen/ #A48265

Supplementary Table-S1: list of antibodies used in this study.

Supplementary Figure Legends

Figure S1: (A-C) IRE1 signaling arm: qPCR analysis of relative mRNA levels of XBP1s, BLOC1S, and GALNT1, respectively, in A549 cells treated with different concentrations of Tunicamycin (Tm-ng/mL), N=3. (D-F) PERK signaling arm: qPCR analysis of relative mRNA levels of HMOX1, GADD34, and CHOP, respectively, in A549 cells treated with different concentrations of Tm-ng/mL, N=3. (G-H) ATF6 signaling arm: qPCR analysis of relative mRNA levels of BIP and HERPUD1, respectively, in A549 cells treated with different concentrations of Tm-ng/mL, N=3. (I-K) qPCR of relative mRNA levels of BLOC1S, CHOP, and BIP in A549 cells treated with different concentrations of Thapsigargin (Tg-nM), N=3. (L-O) qPCR of relative mRNA levels of XBP1s, BLOC1S, GADD34, and BIP respectively in MCF-7 cells treated with different concentrations of Tm-ng/mL. (P-S) qPCR of relative mRNA levels of XBP1s, BLOC1S, GADD34, and BIP respectively in MCF-7 cells treated with different concentrations of Tg-nM. All experiments were done in biological triplicates, mean $\pm$ SD calculated using Prism 10 (GraphPad). (**p<0,01, ***p<0,001, *p<0.05).

Figure S2: (A) qPCR of relative mRNA levels of XBP1s in MCF-7 cells treated with IRE1 inhibitors -AMG-18 and MKC at a final concentration of 0.5 μ M and 5 μ M respectively, thapsigargin (Tg 15nM) and tunicamycin (Tm 100ng/mL), N=3. (B) Subcellular protein fractionation of DNAJB11, PDI, and PDIA4 in MCF-7 cells treated with Tm (100 and 150ng/mL) and Tg (Tg 15nM) in the presence of IRE1 inhibitors AMG-18 and MKC at a final concentration of 0.5 μ M and 5 μ M respectively. (C-E) Quantification of the refluxed ER proteins DNAJB11, PDIA1, and PDIA4, as shown in B, respectively. (F) Subcellular protein fractionation of DNAJB11, PDI, and PDIA4 in MEF IRE1^{+/+} and MEF IRE1^{-/-} cells treated with Tm (100ng/mL) and Tg (Tg 15nM). (G-I) Quantification of the refluxed ER proteins DNAJB11, PDI, and PDIA4, as shown in E, respectively. (J) Subcellular protein fractionation of DNAJB11 and PDIA4 in SGTA-silenced cells treated with Tm (100ng/mL) and 0.5 μ M AMG-18. (K-L) Quantification of the refluxed ER proteins DNAJB11, PDI, and PDIA4, as shown in J, respectively. (M) Representative immunoblot of GAPDH and SGTA in SGTA-silenced A549 cells. (N) Representative blot showing the interaction between the cytosolic SGTA and FLAG-tagged N-linked Glycosylated PDIA1 (PDI) treated with Endo

H. (O) Representative immunoblot of GAPDH, DNAJB12 and DNAJB14 in DNAJB12/14-silenced A549 cells. All experiments were done in biological triplicates, mean $\pm$ SD calculated using Prism 10 (GraphPad). (** $p < 0,001$, ** $p < 0,01$, * $p < 0.05$).

Figure S3: (A-C) qPCR analysis of XBP1s, BLOC1S, and GALNT10 in A549 cells overexpressing IRE1-wt vs IRE1-DN mutant. (D-F) qPCR of relative mRNA levels of XBP1s, BLOC1S, and GALNT10 respectively in HEK293T cells overexpressing the WT and DN mutant of IRE1 in the presence of 100ng/mL tunicamycin (Tm). (G-H) qPCR of relative mRNA levels of BIP, and GADD34 respectively in cells overexpressing the WT and DN mutant of IRE1 in the presence of 100ng/mL tunicamycin (Tm). (I-K) qPCR analysis of PERK, ATF6, and IRE1 target genes respectively in HEK293T cells treated with Tm (ng/mL). (L) Representative immunoblot of IRE1 levels in cells overexpressing the WT and DN IRE1 in different replicates (rep). (M) Subcellular protein fractionation of DNAJB11, PDI, and PDIA4 in HEK293T cells overexpressing the WT and DN mutant of IRE1 and treated with Tm. (N-P) Quantification of the refluxed ER proteins DNAJB11, PDI, and PDIA4, as shown in M, respectively. All experiments were done in biological triplicates, mean $\pm$ SD calculated using Prism 10 (GraphPad). (** $p < 0,001$, ** $p < 0,01$, * $p < 0.05$).

Figure S4: Inhibiting PERK does not affect ER to cytosol reflux. (A-C) qPCR of relative mRNA levels of HMOX1, GADD34, and CHOP respectively in A549 cells treated with Tm (ng/mL), and the PERK inhibitors - GSK2656157 (GSK1) or GSK2606414 (GSK2) at final concentration of 0.25 μ M. N=3. (D-F) qPCR of relative mRNA levels of HMOX1, GADD34, and CHOP respectively, in A549 cells treated with Tg (nM), and the PERK inhibitors - GSK2656157 (GSK1) or GSK2606414 (GSK2) at final concentration of 0.5 μ M, N=3. (G) Subcellular protein fractionation of DNAJB11, PDI, and PDIA4 in A549 cells treated with different concentrations of Tm, Tg, and the PERK inhibitors - GSK2656157 (GSK1) or GSK2606414 (GSK2) at final concentration of 0.5 μ M. (H-J) Quantification of the refluxed ER proteins DNAJB11, PDI, and PDIA4, as shown in G, respectively. (K-L) qPCR of relative mRNA levels of ATF6 and IRE1 target genes respectively in A549 cells treated with Tm, and the PERK inhibitors - GSK2656157

(GSK1) or GSK2606414 (GSK2) at final concentration of 0.25 μ M. N=3. All experiments were conducted in biological triplicates, mean $\pm$ SD calculated using Prism 10 (GraphPad). (** $p < 0.001$, * $p < 0.01$, * $p < 0.05$).

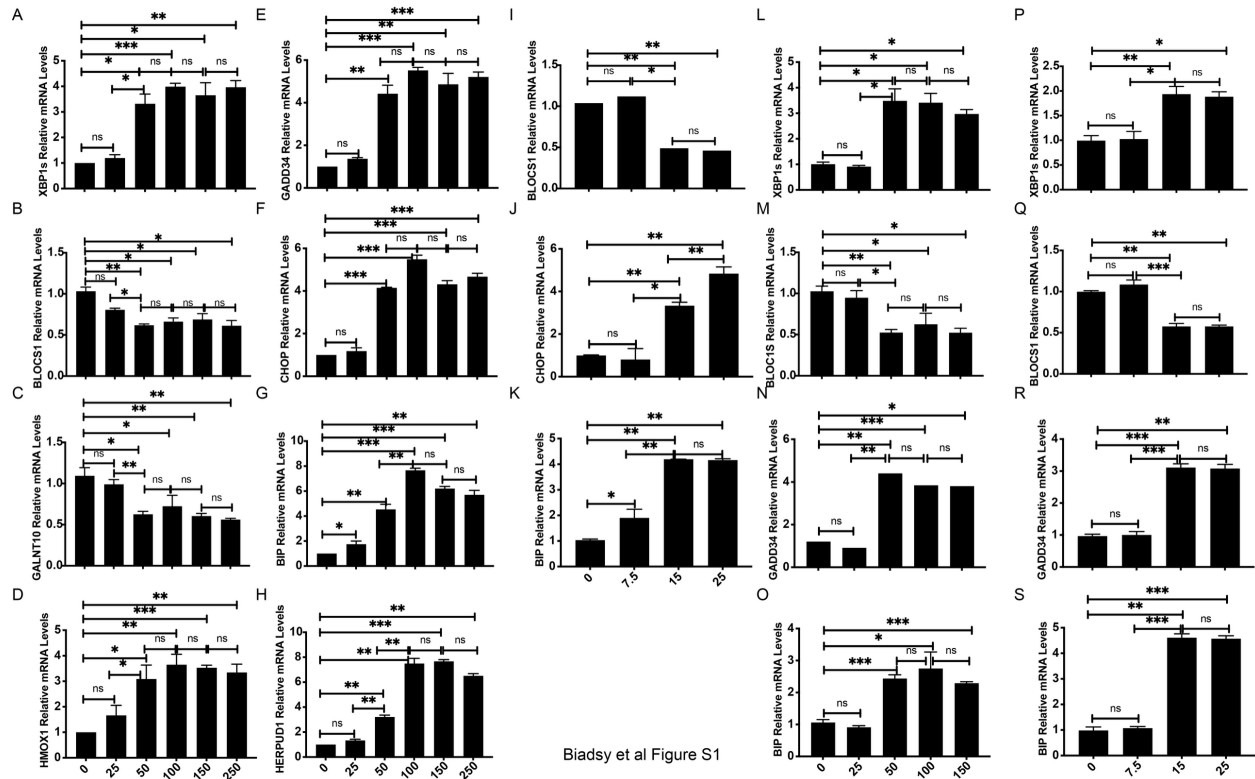
Figure S5: (A-B) qPCR analysis of BIP and HERPUD1 in A549 cells treated with tunicamycin (Tm-ng/mL) in the presence and absence of Ceapin-A7 at a final concentration of 3.5 μ M. (C-D) qPCR analysis of BIP and HERPUD1 in A549 cells treated with thapsigargin (Tg-nM) in the presence and absence of Ceapin-A7. (E-F) qPCR of relative mRNA levels of PERK and IRE1 target genes respectively in A549 cells treated with Tm, and the ATF6 inhibitor Ceapin-A7. (G) Subcellular protein fractionation of DNAJB11, PDI, and PDIA4 in MCF-7 cells treated with Tm, Tg, and Ceapin-A7 at a final concentration of 3.5 μ M. (H-J) Quantification of the refluxed ER proteins DNAJB11, PDI, and PDIA4 as shown in G, respectively. All experiments were conducted in biological triplicates, mean $\pm$ SD calculated using Prism 10 (GraphPad). (** $p < 0.001$, * $p < 0.01$, * $p < 0.05$).

Figure S6: (A–C) qPCR analysis of the UPR target genes XBP1s, GADD34, and HERPUD1 in A549 cells overexpressing DNAJB12-WT or DNAJB12-H139Q. (D) qPCR analysis of the UPR target gene XBP1s in BIP-silenced A549 cells. (E–G) qPCR analysis of relative mRNA levels of XBP1s, GADD34, and HERPUD1, respectively, in BIP-silenced A549 cells overexpressing DNAJB12-WT or the DNAJB12-HPD mutant. (H) Representative immunoblot showing subcellular fractionation of cytosolic BIP (c-BIP) and ER-targeted BIP (er-BIP) in cytosolic and membranous fractions. (I) Quantification of BIP levels in A549 cells following BIP silencing using siRNA (si-BIP) or CRISPRi-mediated knockdown (BIP-KD). (J) Subcellular fractionation of DNAJB11, PDI, and PDIA4 in A549 cells overexpressing cytosolic or ER-localized BIP together with IRE1-WT, following BIP knockdown using siRNA. (K) Subcellular fractionation of PDI and PDIA4 in A549 cells overexpressing cytosolic or ER-localized BIP together with IRE1-WT, following BIP knockdown using CRISPRi. (L–N) Quantification of refluxed ER proteins DNAJB11, PDI and PDIA4 corresponding to panel J. (O) Wild-type p53 transcriptional activity in BIP-silenced A549 cells treated with DMSO, etoposide, tunicamycin, or the indicated combinations.

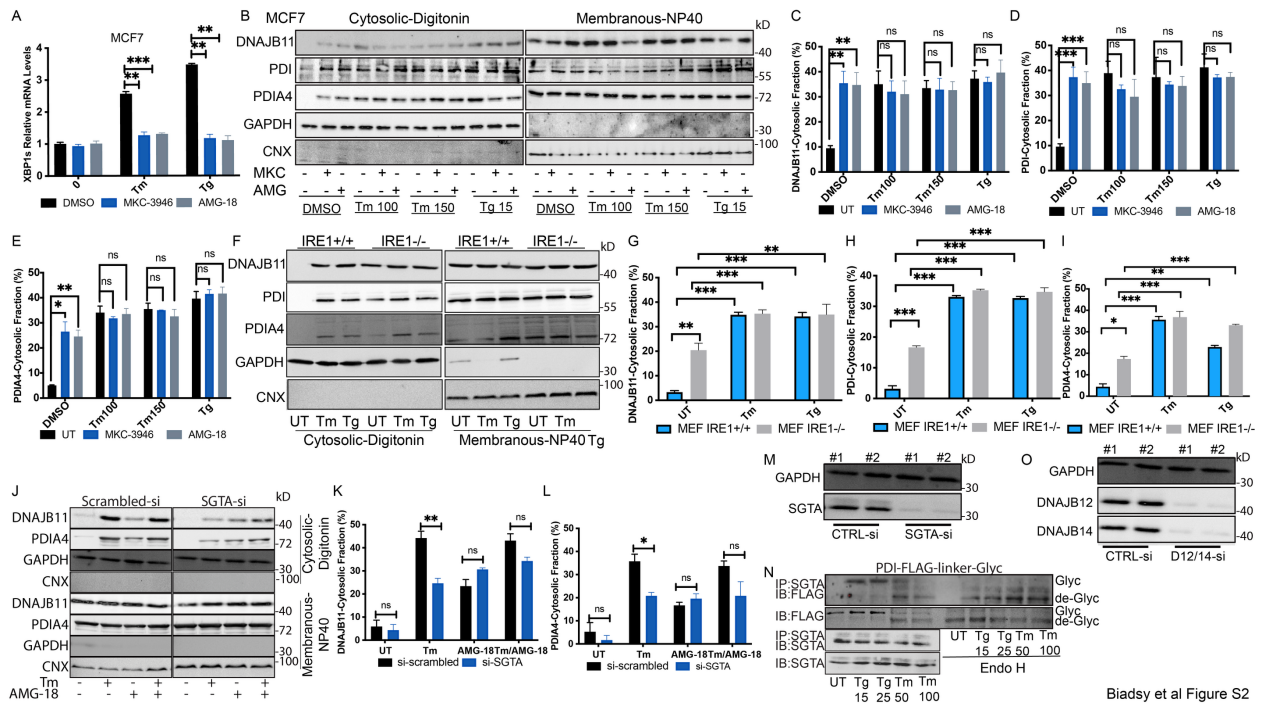
Supplementary References:

Dabsan, S., G. Zur, N. Abu-Freha, S. Sofer, I. Grossman-Haham, A. Gilad and A. Igbaria (2025). "Cytosolic and endoplasmic reticulum chaperones inhibit wt-p53 to increase cancer cells' survival by refluxing ER-proteins to the cytosol." Elife **14**.

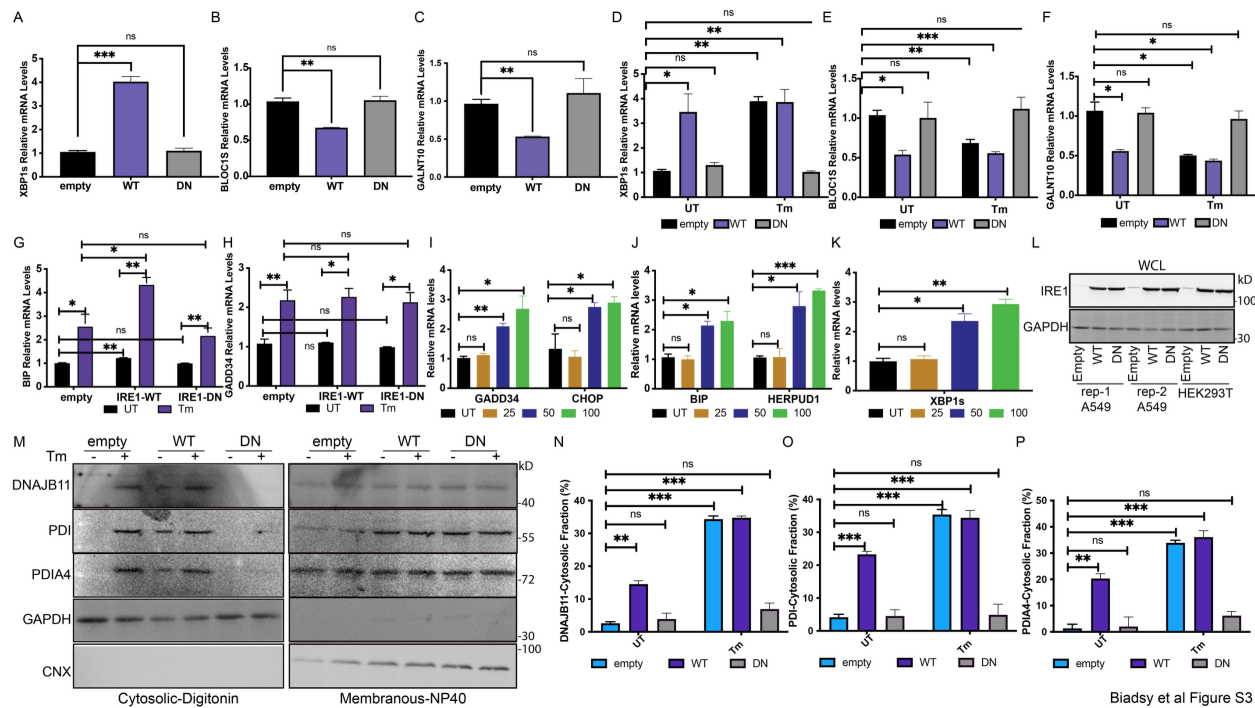
Gilbert, L. A., M. A. Horlbeck, B. Adamson, J. E. Villalta, Y. Chen, E. H. Whitehead, C. Guimaraes, B. Panning, H. L. Ploegh, M. C. Bassik, L. S. Qi, M. Kampmann and J. S. Weissman (2014). "Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation." Cell **159**(3): 647-661.

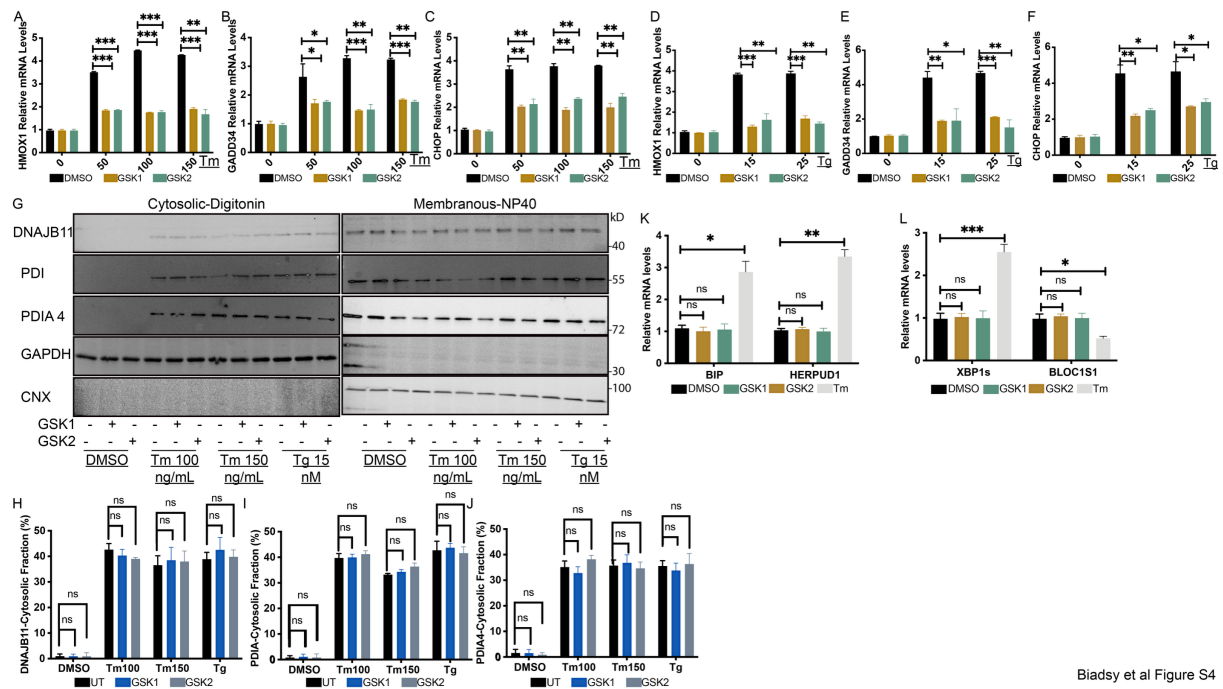


Biadys et al Figure S1

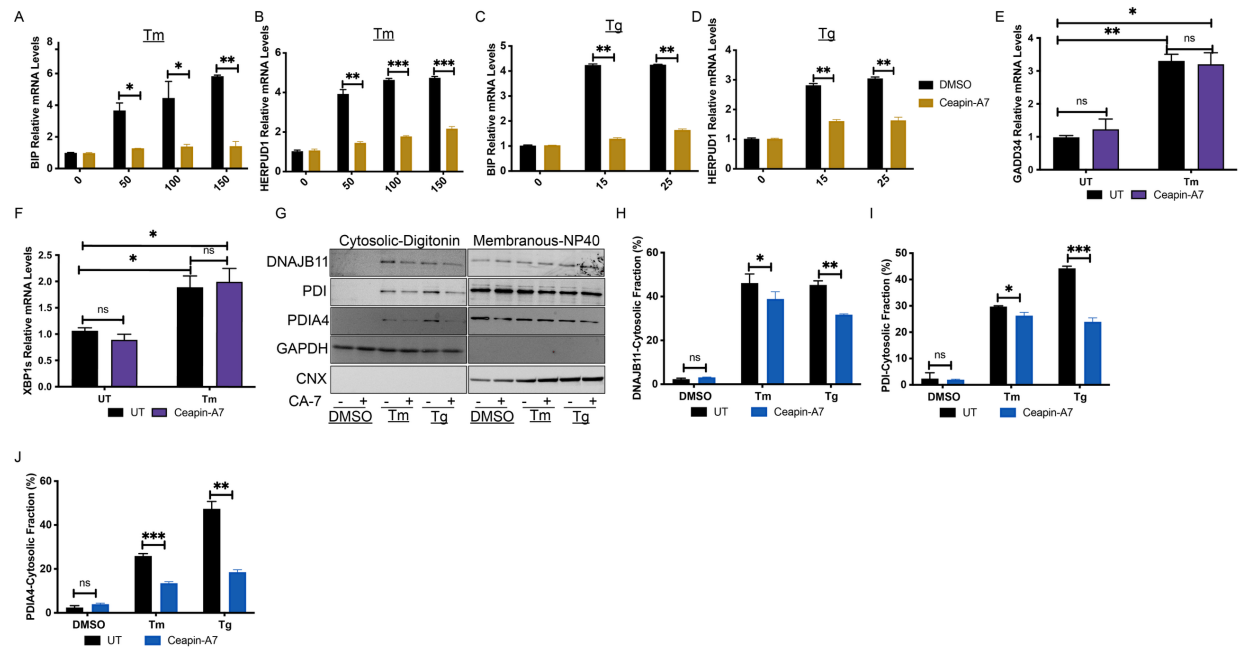


Biadys et al Figure S2

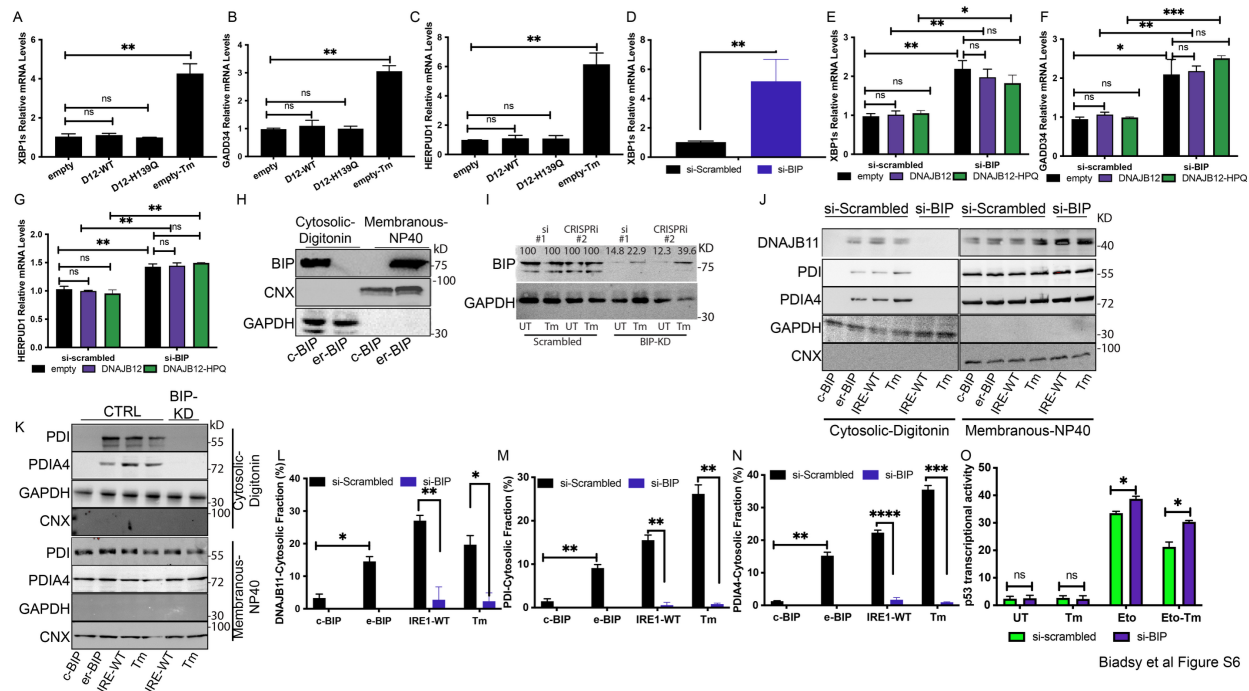




Biadsky et al Figure S4



Biadysy et al Figure S5



Bladys et al Figure S6