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Supplemental information

Cytoplasmic proteotoxicity regulates HRI-dependent phosphorylation of eIF2α via the Hsp70-Bag3 module

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Supporting Information

Supplement Tables:

Table S1. Pathways up- and downregulated by JG-98 in untransformed and transformed MCF10A cells. FDR values are shown for each pathway. Related to Figure 1.

Pathways upregulated	**Untransformed FDR-q.value**	**Transformed FDR-q.value**
HALLMARK_TNFA_SIGNALING_VIA_NFKB	0.005	0.00
HALLMARK_IL6_JAK_STAT3_SIGNALING	0.023	0.095
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0.035	0.006
HALLMARK_INFLAMMATORY_RESPONSE	0.143	0.062
HALLMARK_P53_PATHWAY	0.396	0.077
HALLMARK_APOPTOSIS	0.349	0.097
HALLMARK_HEME_METABOLISM	0.378	0.143
HALLMARK_PEROXISOME	0.617	-
HALLMARK_HEDGEHOG_SIGNALING	0.578	0.322
HALLMARK_KRAS_SIGNALING_DN	0.546	0.778
HALLMARK_KRAS_SIGNALING_UP	0.735	0.069
HALLMARK_TGF_BETA_SIGNALING	0.999	0.658
Pathways Downregulated	**Untransformed FDR-q.value**	**Transformed FDR-q.value**
HALLMARK_E2F_TARGETS	0.127	0.001
HALLMARK_G2M_CHECKPOINT	0.150	0.015
HALLMARK_MYC_TARGETS_V2	0.157	0.010

HALLMARK_MYC_TARGETS_V1	1.000	0.133
HALLMARK_TGF_BETA_SIGNALING	1.000	-

Table S2. Expression of genes belonging to different branches of the UPR following JG-98 treatment. Related to Figure 2.

		MCF10A/Her2		MCF10A/Control		
Ensembl_ID	Gene_ Symbols	Log2FC	adj.P.Val	Log2FC	adj.P.Val	UPR branch
ENSG00000044574	HSPA5	-0.0136	0.94133	-0.123	0.49058	IRE-1
ENSG00000178607	ERN1	0.82187	0.01346	0.4213	0.44783	IRE-1
ENSG00000100219	XBP1	0.55164	0.00148	0.32788	0.12712	IRE-1
ENSG00000118217	ATF6	0.16852	0.13802	0.08711	0.64384	ATF6
ENSG00000128965	CHAC1	4.02307	5.31E-05	2.71482	0.02402	eIF2α
ENSG00000175197	DDIT3	2.57308	8.31E-06	1.3092	0.03572	eIF2α
ENSG00000128272	ATF4	1.33301	9.97E-07	0.53568	0.01286	eIF2α

Table S3. List of siRNA sequences. Related to STAR methods section (Constructs and Oligonucleotides)

List	Sequence (5'-3')	Ref#
Bag3	GCAAAGAGGUGGAUUCUAA	D-011957-01-0005
CHOP-1	AAGAACCAGCAGAGGUCACUU	CTM-595156, Oligo ID: ALEIA-000013
Human HRI	CUGAUUAAGGGUGCAACUAUU	CTM-595153, Oligo ID: ALEIA-000007
Human HRI-2	Commercial siRNA	Horizon discovery J-005007-05-0002
Human GCN2	CACCGUCAAGAUUACGGACUU	CTM-595154, Oligo ID: ALEIA-000009
Control (Scrambled)	AGGUAGUGUAAUCGCCUUU	CTM-595155, Oligo ID: ALEIA-595155

Table S4. List of qPCR primers. Related to STAR methods section (Real Time PCR analysis).

List	Sequence (5'-3')	Ref#
M-Her2_fw	AACTGCAGTCAGTTCCTCCG	223747403
M-Her2_rev	GTGCTTGCCCCTCACATACT	223747404
huHRI_For	ACCCCGAATATGACGAATCTGA	226359069
huHRI_Rev	CAAGTGCTCCAGCAAAGAAAC	226359070
DDIT3_F	GAACGGCTCAAGCAGGAAATC	226024031
DDIT3_R	TTCACCATTCGGTCAATCAGAG	226024032
HuGCN2-F	TGGTAAACATCGGGCAAACTC	226265602
HuGCN2-R	GGACCCACTCATACAACAAGA	22626503
HuPERK1	ACGATGAGACAGAGTTGCGAC	226265600
HuPERK1-R	AATCCCACTGCTTTTTACCATGA	226265601
HuPKR-F	GCCGCTAAACTTGCATATCTTCA	229864192
HuPKR-R	TCACACGTAGTAGCAAAAGAACC	229864193
ACTB Human	CACCATTGGCAATGAGCGGTTC	225252514
ACTB Human-Rev	AGGTCTTTGCGGATGTCCACGT	225252513

Supplement Figures:

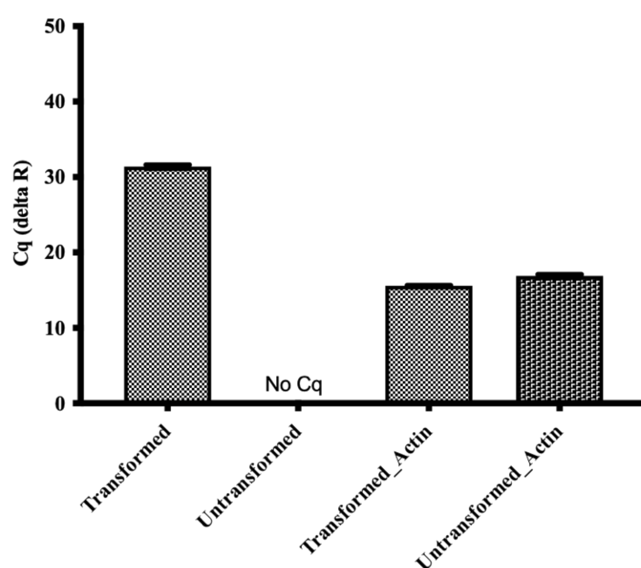

Figure S1. Expression levels of Her2 upon cell transformation. Related to Figure 1, qPCR was performed with Her2 primers. Graph represents the number of cycles on y-axis demonstrating the amplification of Her2. β-Actin was used as internal controls. Bar graph represents means ±SEM of triplicate experiments.

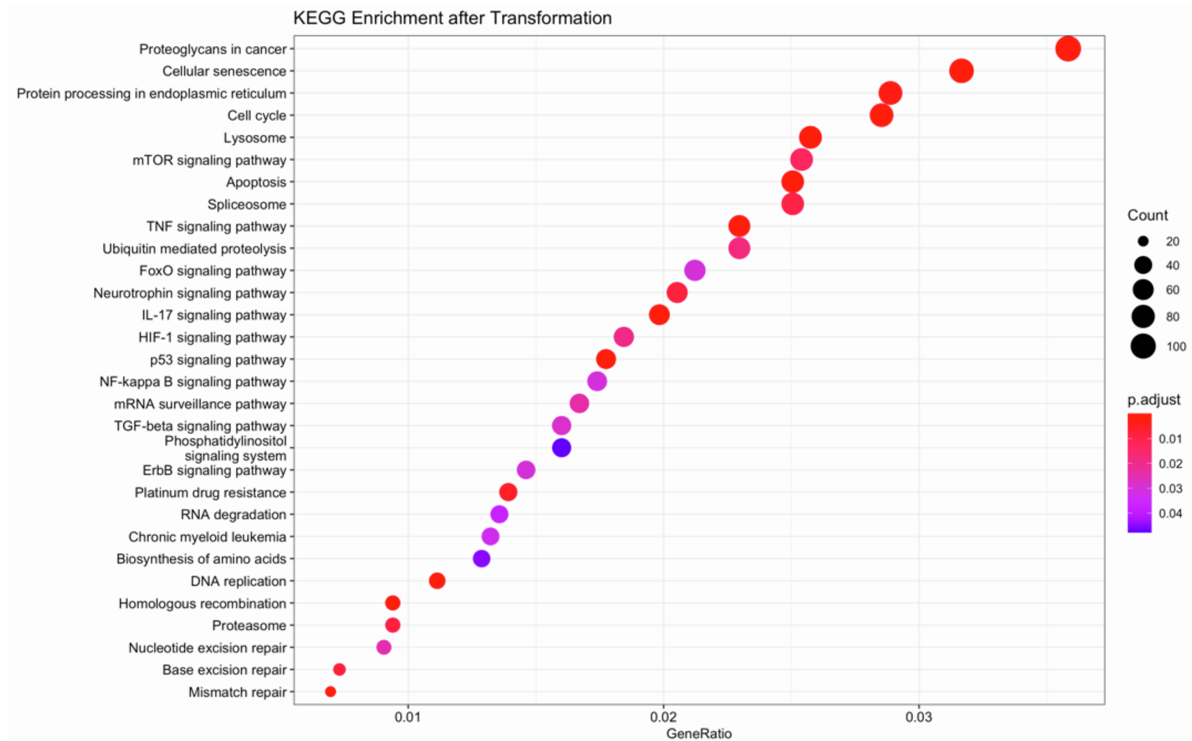

Figure S2. Enrichment of oncogenic signatures in transformed cells. Related to Figure 1, Transformed cells state was assessed by analysing differential expression of genes in transformed and untransformed cells. Enrichment of pathways (KEGG-Kyoto Encyclopedia of Genes and Genomes) such as m-TOR, HIF-1, TGF-beta, Phosphatidylinositol (PI3K), ErbB (Her2) shows the oncogenic signature in transformed cells. Count represents genes in the respective pathways.

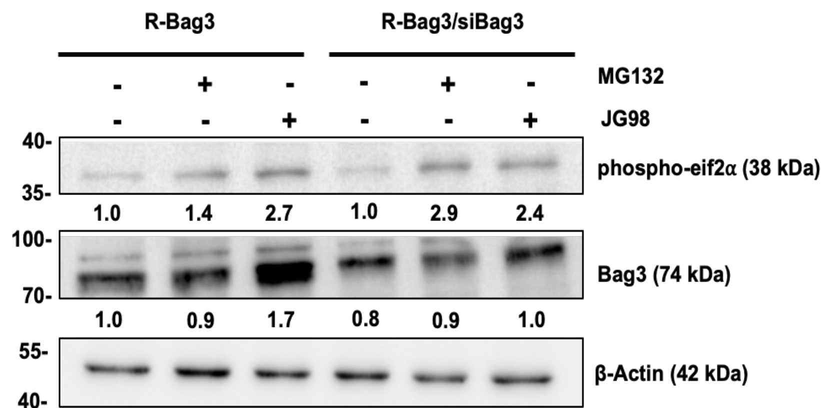

Figure S3. Expression of siRNA-resistant Bag3 reverses effects of the siRNA on suppression of phosphorylation of eIF2α in response to JG-98 and MG132. Related to Figure 2, MCF-10A cells expressing Bag3 resistant to siBag3 were transfected with siBag3 or siControl and further treated with JG98. Quantification of the blot was performed using ImageJ and OD ratios for each protein compared to the reference after normalization is added below each blot.

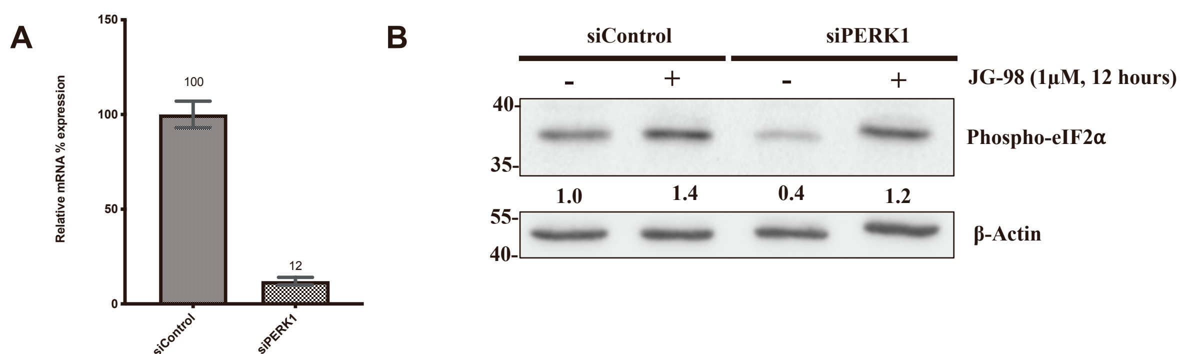

Figure S4. Depletion of PERK1 does not suppresses phosphorylation of eIF2α in response to JG-98. Related to Figure 2. **(A)** Efficiency of PERK1 depletion following siRNA treatment. Levels of PERK1 mRNA were quantified by qPCR**.** Bar graph represents means ±SEM of triplicate experiments. **(B)** Depletion of PERK1 did not suppress phosphorylation of eIF2α in presence of JG-98. Cells were transfected with siPERK1 or siControl and further treated by JG-98 (1uM, 12 hours) or left untreated. Levels of phospho-eIF2α were determined in cell

lysates by immunoblotting with the corresponding antibody. Quantification of the blot was performed using ImageJ and OD ratios for each protein compared to the reference after normalization is added below each blot.

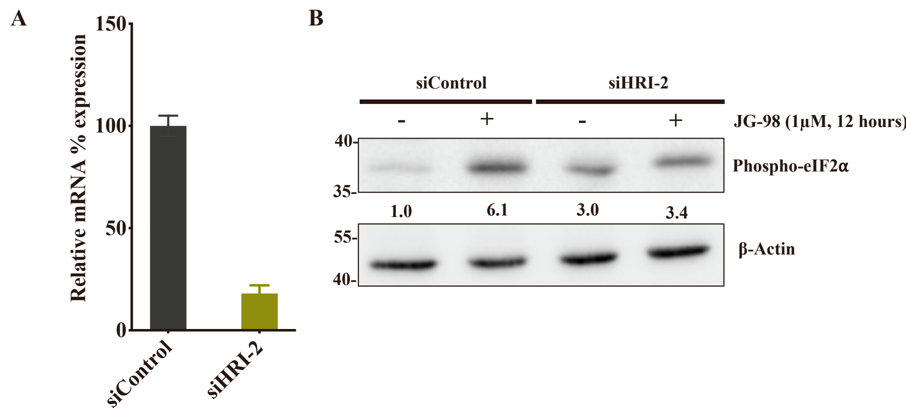

Figure S5. Depletion of HRI with a distinct siRNA (siHRI-2) also suppresses phosphorylation of eIF2α in response to JG-98. Related to Figure 2, (A) Depletion of HRI measured by qPCR. Bar graph represents means ±SEM of triplicate experiments. (B) Effect of siHRI-2 on p-eIF2α. Quantification of the blot was performed using ImageJ and OD ratios for each protein compared to the reference after normalization is added below each blot.

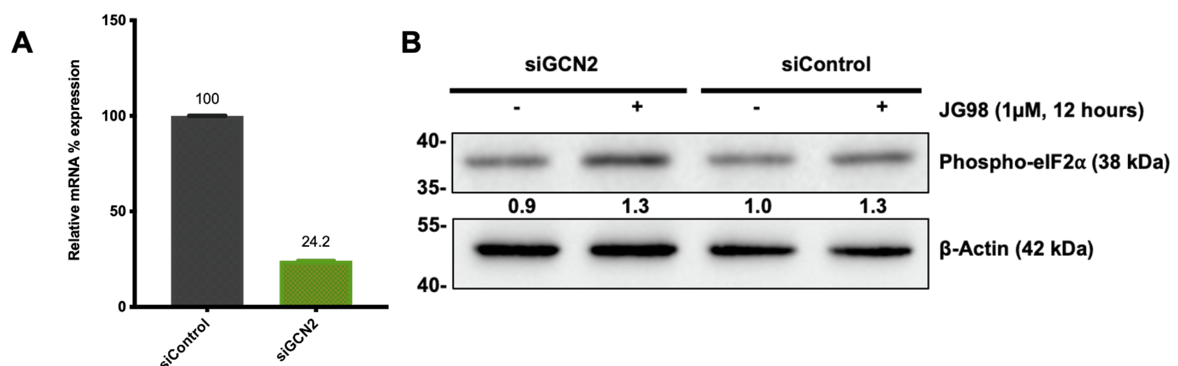

Figure S6. Depletion of GCN2 does not suppress phosphorylation of eIF2α in response to JG-98. Related to Figure 2, (A) GCN2 depletion was done by the corresponding siRNA for 48 hours, followed by qPCR. Bar graph represents means ±SEM of triplicate experiments. (B) Depletion of GCN2 shows no impact on eIF2α phosphorylation in presence of JG-98.

Quantification of the blot was performed using ImageJ and OD ratios for each protein compared to the reference after normalization is added below each blot.

Figure S7. Depletion of PKR does not suppress phosphorylation of eIF2α in response to JG-98. Related to Figure 2, **(A)** PKR depletion was done by the corresponding siRNA for 48 hours, followed by qPCR. Bar graph represents means ±SEM of triplicate experiments. **(B)** Depletion of PKR shows no impact on eIF2α phosphorylation in presence of JG-98. Quantification of the blot was performed using ImageJ and OD ratios for each protein compared to the reference after normalization is added below each blot.

Figure S8. Bag3 silencing reduces the level of HRI. Related to Figure 3, **(A)** Cells were treated with siBag3 or left untreated. Ubiquitinated proteins were pulled down from these cells extracts using ubiquilin-1 affinity column (see Materials and Methods) and HRI levels in the pulldown were measured by immunoblotting. Depletion of Bag3 reduces the level of HRI with polyubiquitinated proteins, **(B)** Silencing levels of the Bag3 in pull down experiment. Quantification of the blot was performed using ImageJ and OD ratios for each protein compared to the reference after normalization is added below each blot.