

Supplemental Information and Figures for:

PACT suppresses PKR activation through dsRNA binding and dimerization, and is a therapeutic target for triple-negative breast cancer

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Supplemental Methods

Immunofluorescence

HCC1806 cells with expression of the indicated constructs were plated on glass coverslips (Corning) in 6-well dishes. After two days, the cells were washed in 1X PBS prior to fixation with 4% paraformaldehyde (Thermo-Scientific) in 1X PBS for 15 minutes at room temperature. Subsequently, the coverslips were washed in 1X PBS and the cells permeabilized with 0.15% Triton X-100 (Fisher) in 1X PBS. The coverslips were blocked in Protein Block (Aligent/Dako, X090930-2) prior to binding of primary antibodies for PKR (Cell Signaling Technology Cat# 3072, RRID:AB_2277600) and PACT (Santa Cruz Biotechnology Cat# sc-377103, RRID:AB_3665861), washing in 1X PBS and binding of secondary antibodies (Thermo Scientific, A21207 (RRID:AB_141637), A21203 (RRID:AB_2535789)). All antibodies were diluted in Antibody Diluent (Agilent/Dako, S302283-2). DAPI (Sigma-Aldrich) was added to the diluted secondary antibody during binding. After secondary antibody binding, the coverslips were washed in PBS and once in water before mounting on glass slides with Vectashield Antifade Mounting Media (Vector Laboratories). Fluorescence microscopy images were obtained with a DM6 B microscope (Leica) with a 20x objective and a DFC90000 GT monochrome digital camera (Leica). Fluorescence images were captured with Leica Application Suite X (Leica, RRID:SCR_013673) and resized and formatted with Fiji (RRID:SCR_002285).

Coding sequences for PACT constructs

>PACT_wobble_CDS

```
ATGTCCCAGAGCAGGCACCGCGCCGAGGCCCCCGCCGCTGGAGCGCGAGGACAGTGGGACCTTCAGTTT
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AAGAACATCCCAGTTTATGAATGTGAAAGATCTGATGTGCAAATACACGTGCCCACTTTACCTTCAGAGT
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CCAAATACAGATTACATCCAGCTGCTTAGTGAAATTGCCAAGGAACAAGGTTTTAATATAACATATTTGGAT
ATAGATGAACTGAGCGCCAATGGACAATATCAATGTCTTGCTGAACTGTCCACCAGCCCCATCACAGTCT
GTCATGGCTCCGGTATCTCCTGTGGCAATGCACAAAGTGATGCAGCTCACAATGCTTTGCAGTATTTAAAG
ATAATAGCAGAAAGAAAGTAA
```

-underlined bases are the target sites of sgPACT-1 and sgPACT-2, lowercase bases are wobble mutations to prevent base-pairing with the sgRNA or to disrupt the PAM (italicized)

>PACT_wobble_AA-mutant_CDS

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GTCATGGCTCCGGTATCTCCTGTGGCAATGCACAAAGTGATGCAGCTCACAATGCTTTGCAGTATTTAAAG
ATAATAGCAGAAAGAAAGTAA
```

-lowercase boldened bases are mutations to disrupt dsRNA binding

>PACT_wobble_EAA-dsRBD-mutant_CDS

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-lowercase boldened bases are mutations to disrupt dsRNA binding

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GCTAGAGTCATTTATGGAACTGGAAAGGGGGCATCAAAAAGCAAGCCAAAAGGAATGCTGCTGAGAAA
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GATGTACTTGGCATTCTTGAGGgaattcGTAA

-lowercase bases are the EcoRI site used to truncate PACT

>PACT_wobble_Δd3-GST_CDS

ATGTCCCAGAGCAGGCACCGCGCCGAGGCCCGCCGCTGGAGCGCGAGGACAGTGGGACCTTCAGTTT
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CGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCCAAAATTAGTTTGTTTTAAAAA
ACGTATTGAAGCTATCCACAAATTGATAAGTACTTGAATCCAGCAAGTATATAGCATGGCCTTTGCAGG
GCTGGCAAGCCACGTTTGGTGGTGGCGACCATCTCCAAAATGA

-lowercase bases are the EcoRI site used to truncate PACT and add GST

Amino acid sequences of PACT constructs

>PACT_WT

MSQSRHRAEAPPLEREDSGTFSLGKMITAKPGKTPIQVLHEYGMKTKNIPVYECERSDVQIHVPTFTFRVTVGD
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>PACT_AA-dsRBD-mutant

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HNALQYLKIIAERK

>PACT-EAA-dsRBD-mutant

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HNALQYLKIIAERK

>PACT- Δ d3

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>PACT- Δ d3-GST

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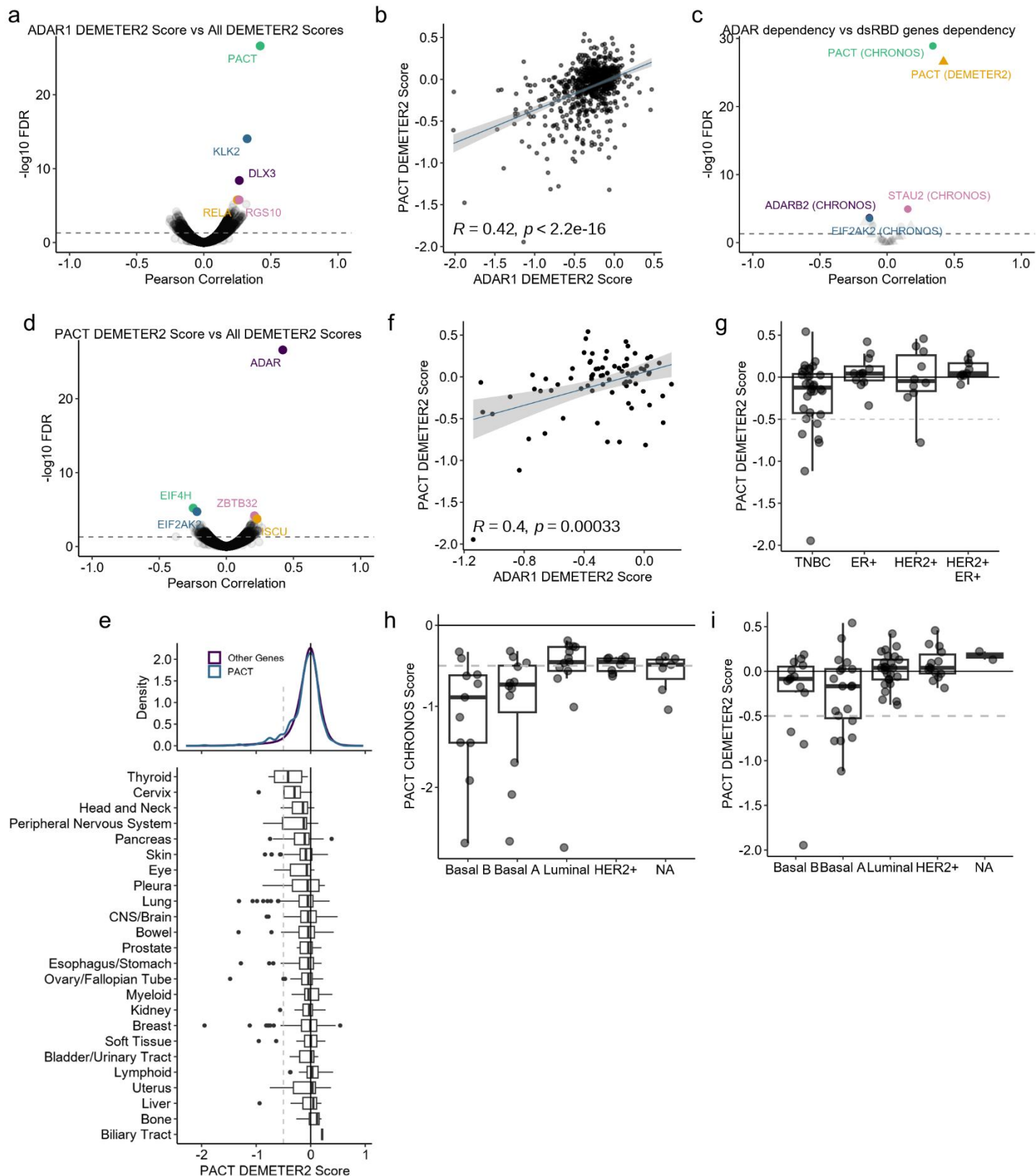


Figure S1

a and **d** Volcano plots of Pearson correlation coefficients and FDR corrected p-values for pairwise comparisons between ADAR1 DEMETER2 score (**a**) or PACT DEMETER2 score (**d**) and DEMETER2 scores for all genes in DepMap across all cell lines. **b** Correlation between PACT and ADAR1 DEMETER2 scores for all DepMap cell lines, Pearson correlation coefficient and p-value shown. **c** Volcano plot of Pearson correlation coefficients and FDR corrected p-values for pairwise comparisons between ADAR1 DEMETER2 or CHRONOS score and DEMETER2 or CHRONOS scores for all dsRBD containing genes in DepMap across all cell lines.

Figure S1 cont. **e** Top, density plot of DEMETER2 scores for PACT or all other genes. Bottom, boxplots for PACT DEMETER2 score by lineage. **f** Correlation between PACT and ADAR1 DEMETER2 scores for breast cancer cell lines, Pearson correlation coefficient and p-value shown. **g-i** Boxplot of PACT CHRONOS or DEMETER2 scores of breast cancer cell lines separated by subtype. All data shown from DepMap.

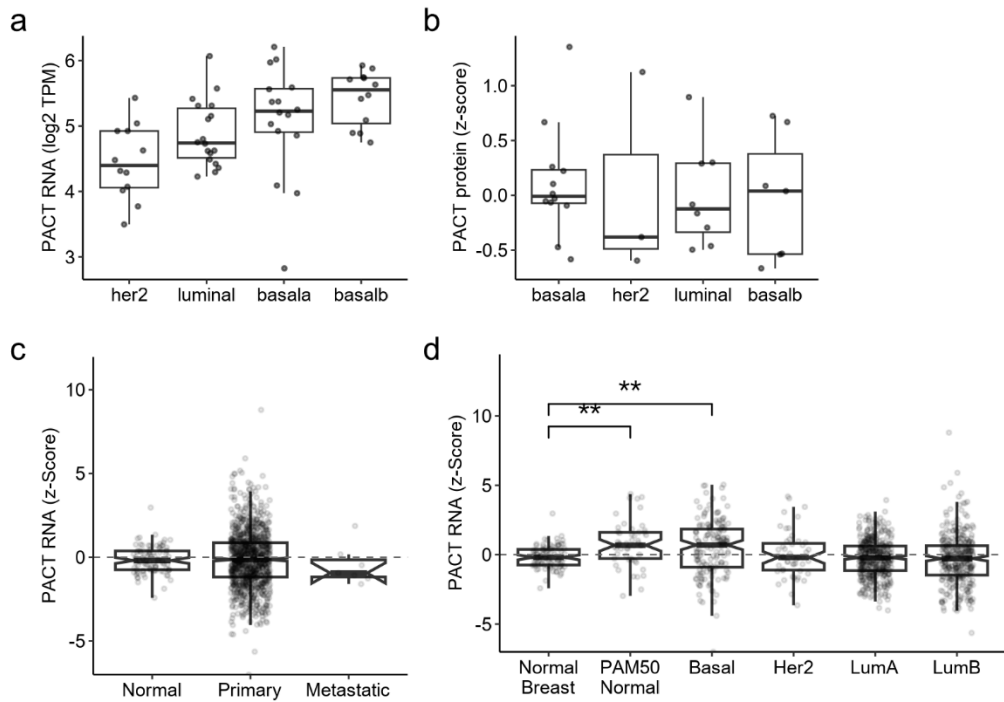


Figure S2

Expression of PACT at the RNA (a) or protein level (b) in breast cancer cell lines separated by subtype. c - d Expression of PACT at the RNA level in normal and breast tumor samples separated by subtypes or tumor location. For panels a-b, data from DepMap; c-d from TCGA.

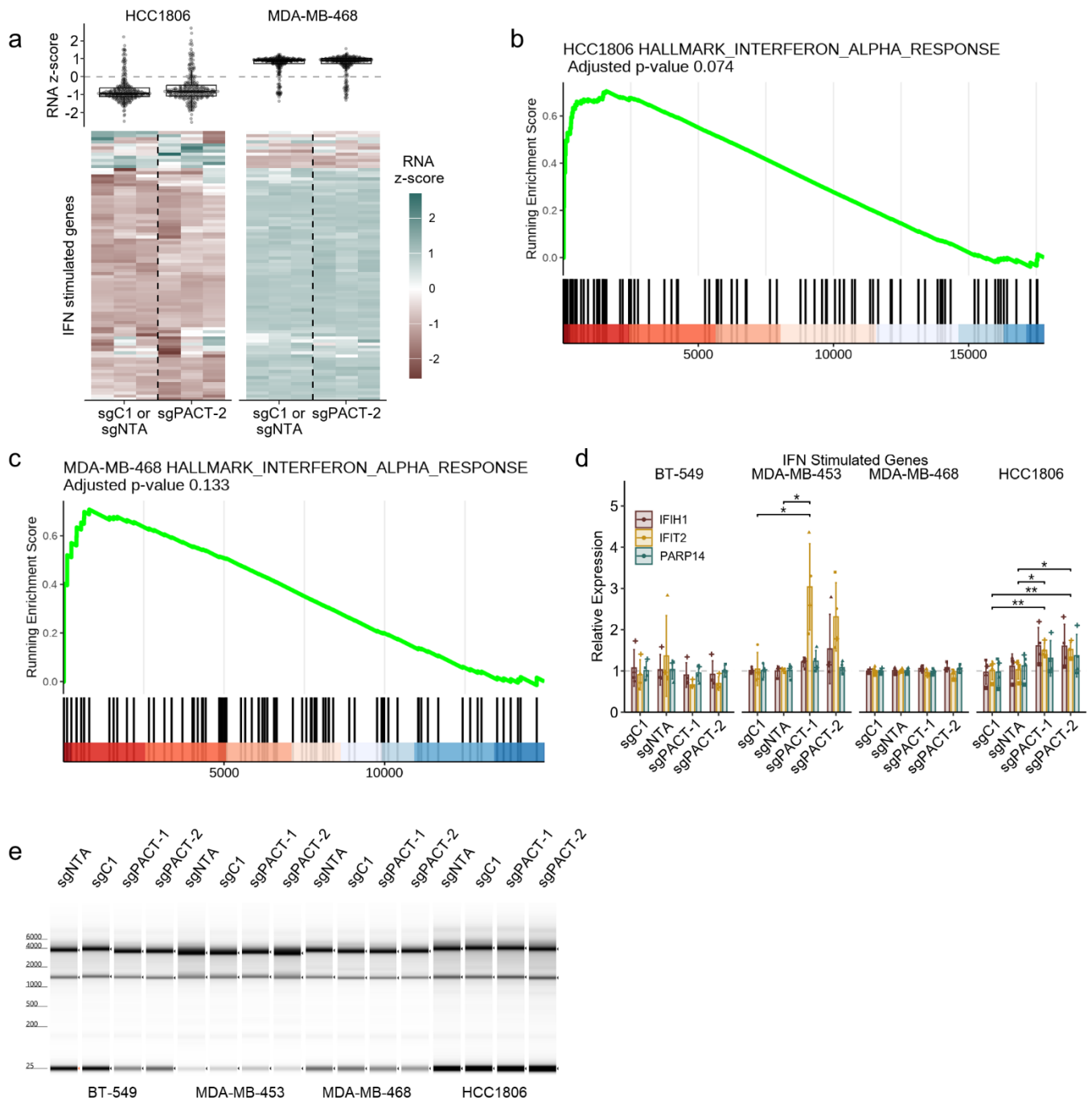


Figure S3:

a Heatmap for RNA expression of type I ISGs (genes belonging to the gene set: Hallmark interferon alpha response). Top panel, boxplot and overlaid 'quasirandom' plots for all ISGs in the heatmap below. The heatmap is clustered by gene (rows), the dendrogram has been omitted for brevity. **b-c** GSEA plots for 'Hallmark interferon alpha response' in HCC1806 and MDA-MB-468 PACT depleted versus control cells. **d** qRT-PCR for RNA expression for ISGs in PACT depleted and control cells. Bars represent the average of at least four biological replicates, error bars are +/- SD. **e** Representative pseudo-gel images of rRNA integrity for PACT depleted and control cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. P-values determined by one-way ANOVA with post-hoc Tukey

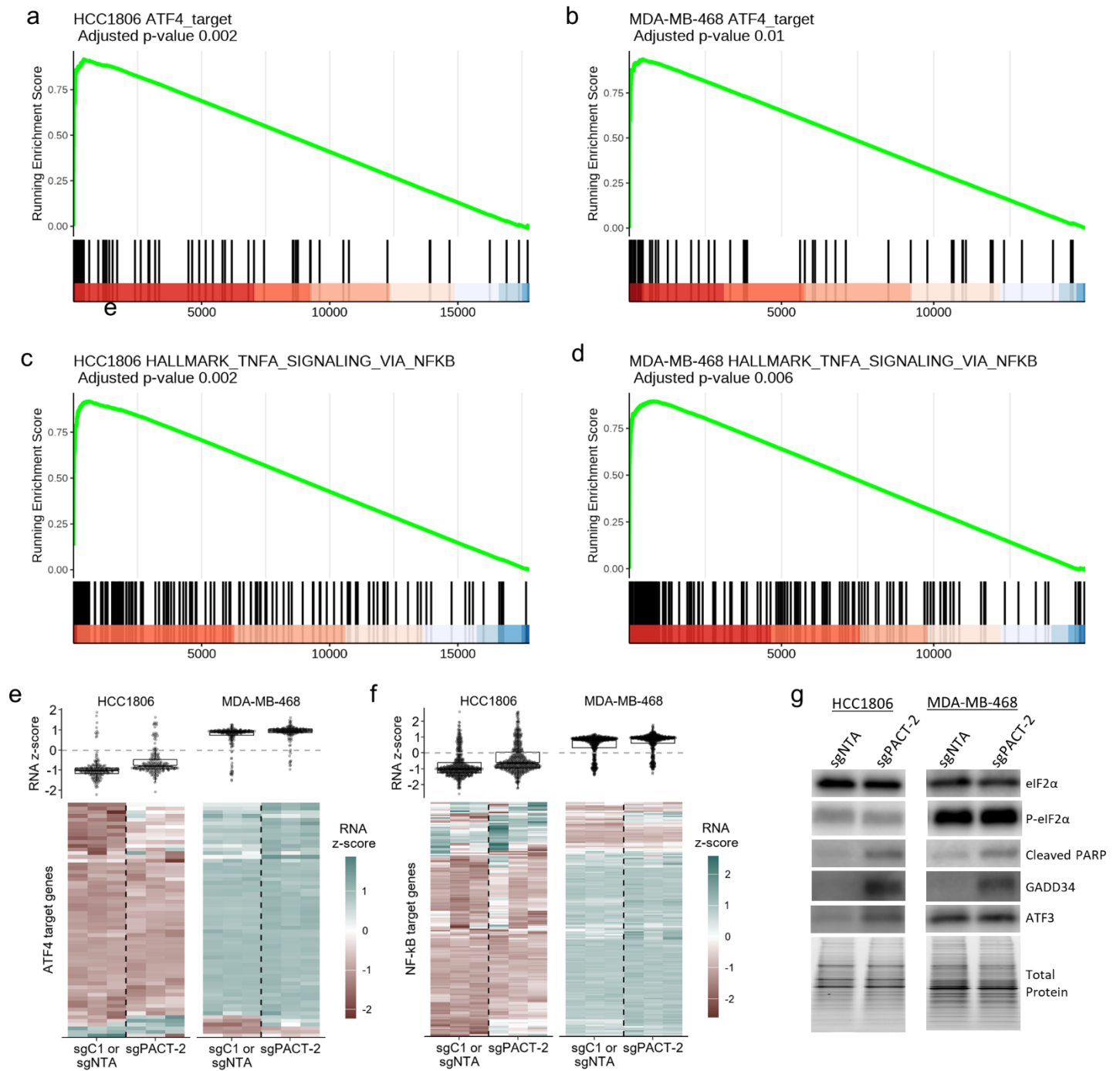


Figure S4

a-d GSEA plots for relevant gene sets. **e-f** Heatmaps for RNA expression of ATF4 or NF-κB target genes. Top panel, boxplot and overlaid 'quasirandom' plots for all genes in the heatmap below. The heatmap is clustered by gene (rows), the dendrogram has been omitted for brevity. **g** Representative immunoblot of PACT depleted and control cells. The blots for each cell line were performed simultaneously, but exposure times for some proteins vary between cell lines, as such bands should not be compared between cell lines. Total protein was imaged using a Stain-Free Gel and serves as a loading control.

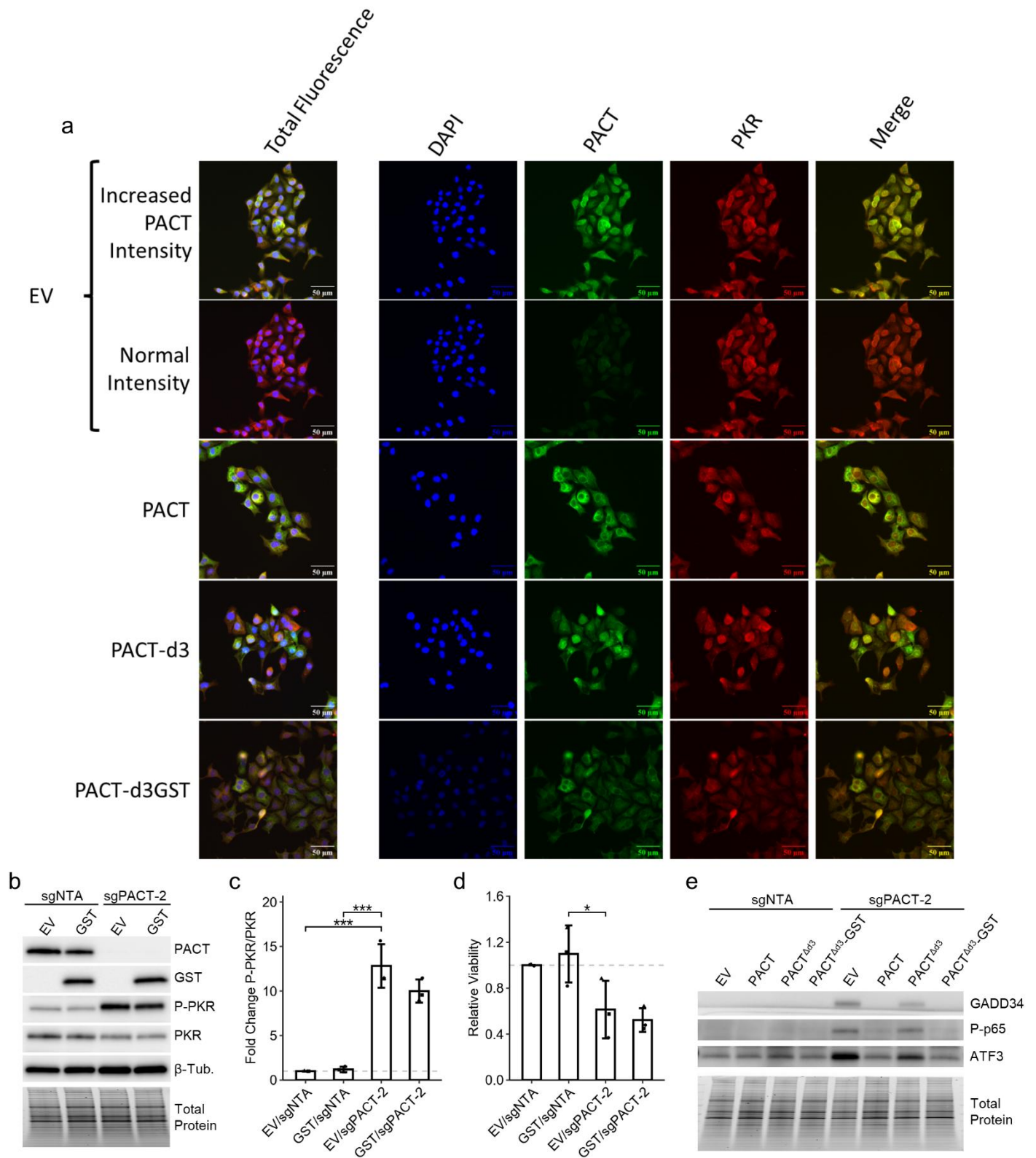


Figure S5

a Representative immunofluorescence micrographs for EV and PACT overexpression HCC1806 cells. For PACT, PACT- $\Delta d3$, PACT- $\Delta d3$ GST, the signal acquisition parameters were the same as EV – Normal Intensity. **b** Representative immunoblot for HCC1806 control (sgNTA) and PACT depleted (sgPACT-2), with (EV, empty vector) or GST overexpression. **c** Quantification of immunoblot in **b**. **d** Cell viability as assessed by CellTiter-Glo 2.0 for the same conditions as **b**. **e** Representative immunoblot for control (sgNTA) and PACT depleted (sgPACT-2), with (PACT, PACT $\Delta d3$ or PACT $\Delta d3$ -GST) or without (EV, empty vector) overexpression of WT or truncated PACT. Total protein was imaged using a Stain-Free Gel and serves as a loading control.

Figure S5 cont.

Bars represent the average of at least three replicates, error bars are +/- SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. P-values determined by Dunnett's test.

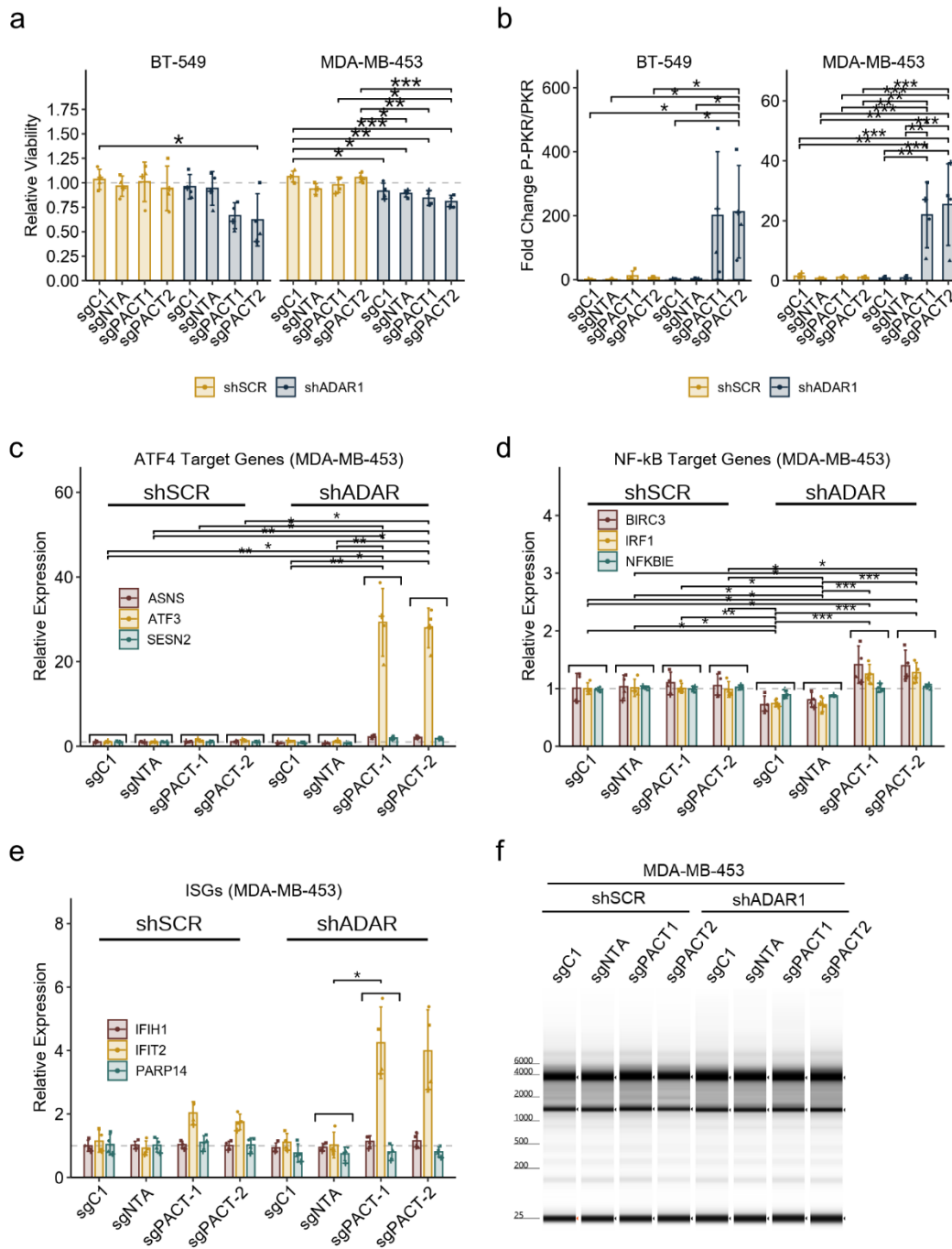


Figure S6

a Cell viability as assessed by CellTiter-Glo 2.0 for control (sgC1, sgNTA) and PACT depleted (sgPACT-1, sgPACT-2), with (shADAR1) or without (shSCR) knockdown of ADAR1. **b** Quantification of immunoblot for P-PKR/PKR in the same conditions as **a**. Panels **a** and **b** are shown in Figure 7b and 7c without p-values for comparison between sgNTA and sgC1 or sgPACT-1 and sgPACT-2, here all comparisons are shown. **c-e** qRT-PCR for RNA expression of ATF4 target genes (**c**), NF- κ B target genes (**d**) or ISGs (**e**) in control (sgC1, sgNTA) and PACT depleted (sgPACT-1, sgPACT-2) cells, with (shADAR1) or without (shSCR) knockdown of ADAR1. Panels **c** and **d** are shown without annotation of p-values in Figure 7d and 7e respectively. **f** Representative pseudo-gel images of rRNA integrity for the same conditions as **a**.

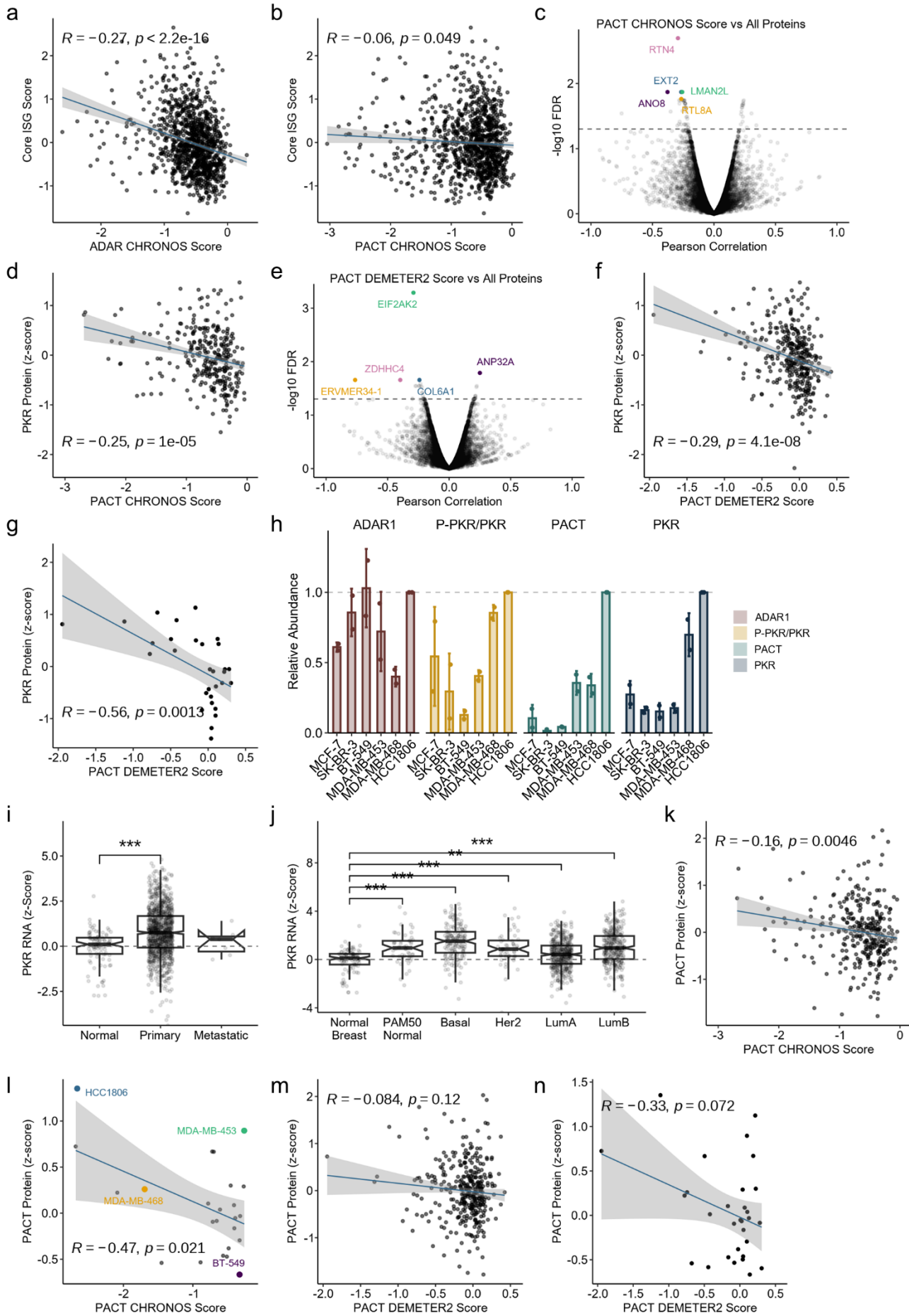


Figure S7

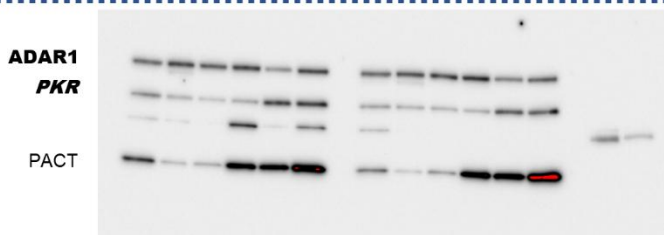
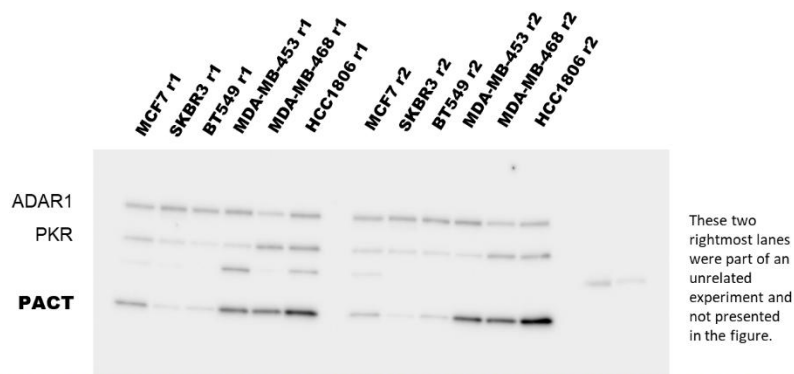
Figure S7 continued

a – b Scatter plots comparing ISG expression (Core ISG Score, described previously (Kung et al. 2021)) and either ADAR1-dependency score (ADAR CHRONOS Score, **a**) or PACT-dependency score (PACT CHRONOS Score, **b**) for all cancer cell lines in DepMap. **c** and **e** Volcano plots of Pearson correlation coefficients and FDR corrected p-values for pairwise comparisons between PACT CHRONOS score (**c**) or PACT DEMETER2 score (**e**) and protein abundance for all proteins in DepMap across all cell lines. **d, f** and **g** Scatter plots comparing PKR protein abundance and PACT-dependency score for all cell lines in DepMap (**d** and **f**) or only breast cancer cell lines (**g**). **h** Quantification of immunoblot in Figure 8e. **i** and **j** PKR RNA expression in normal human breast and breast tumors, data from TCGA. **k-n**, Scatter plots comparing PACT protein abundance and PACT-dependency score for all cell lines in DepMap (**k** and **m**) or only breast cancer cell lines (**l** and **n**). For all scatter plots, the Pearson correlation coefficient and p-values are shown. For panels **i-j**, data from TCGA; all other panels (except **h**) DepMap.

Below are uncropped blots for the main and supplemental figures. Some blots have multiple exposures. The exposure used for each protein in the main or supplemental figure is labeled in bold. Stain-free gel images for total protein are included and annotated to describe for which blots they correspond.

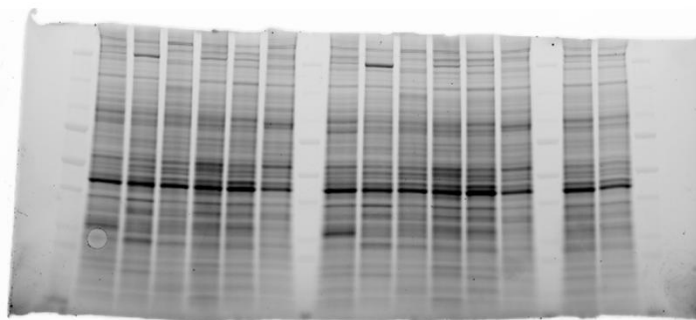
FIGURE 2c/8e

Figure 2a

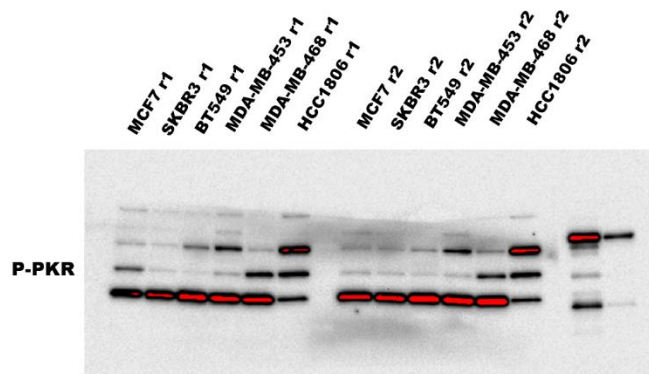


The immunoblot here is used for ADAR1 in 2a and PKR in 9e. Both were quantified using the gel below.

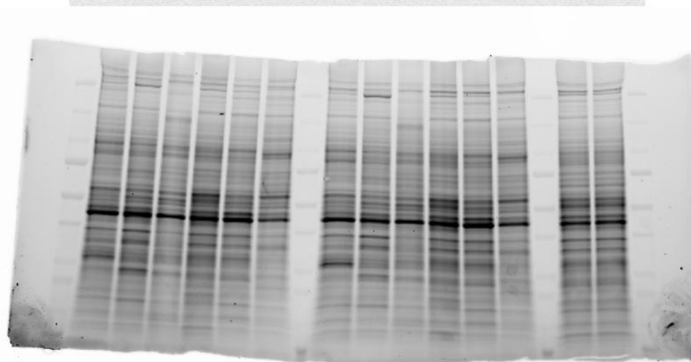
Total Protein



This gel is used for the normalization of ADAR1, PKR, and PACT.



Total Protein



This gel is used for the normalization of P-PKR

Figure 9e

FIGURE 3a
(independent cell
lines).

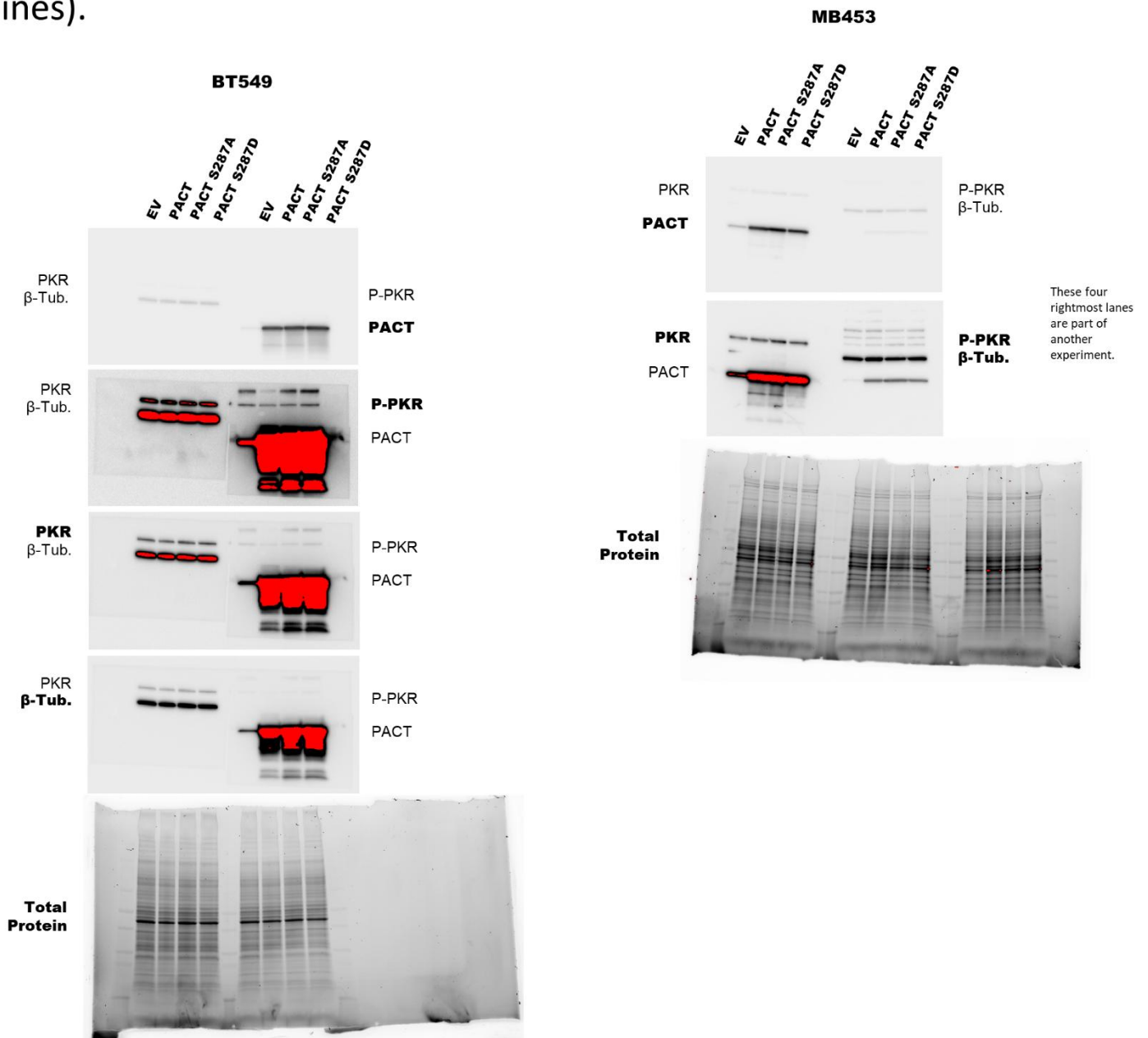
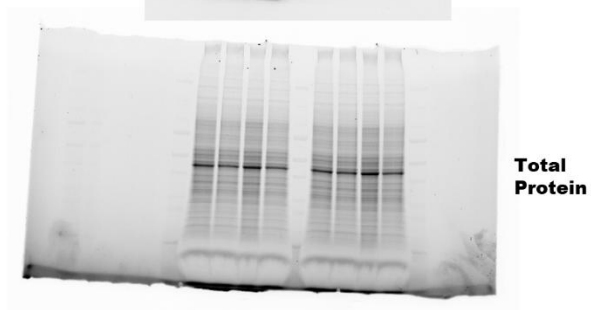
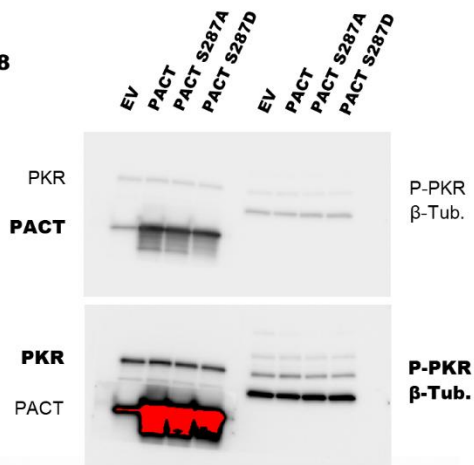


FIGURE 3a-(dependent cell lines).

MB468



HCC1806

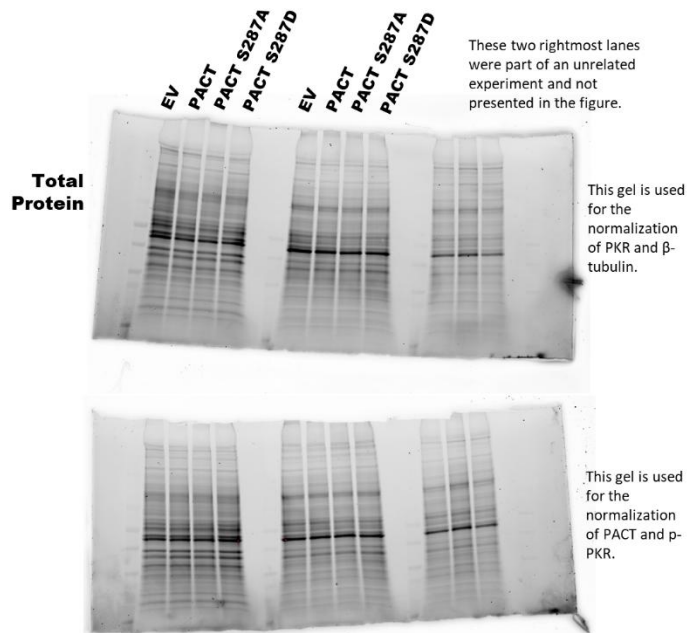
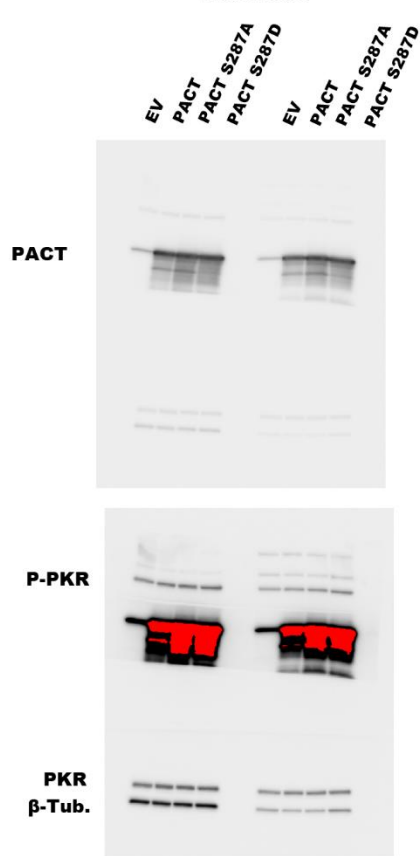


FIGURE 4b-(independent).

This gel and immunoblot is the same as that used in Figure 8a

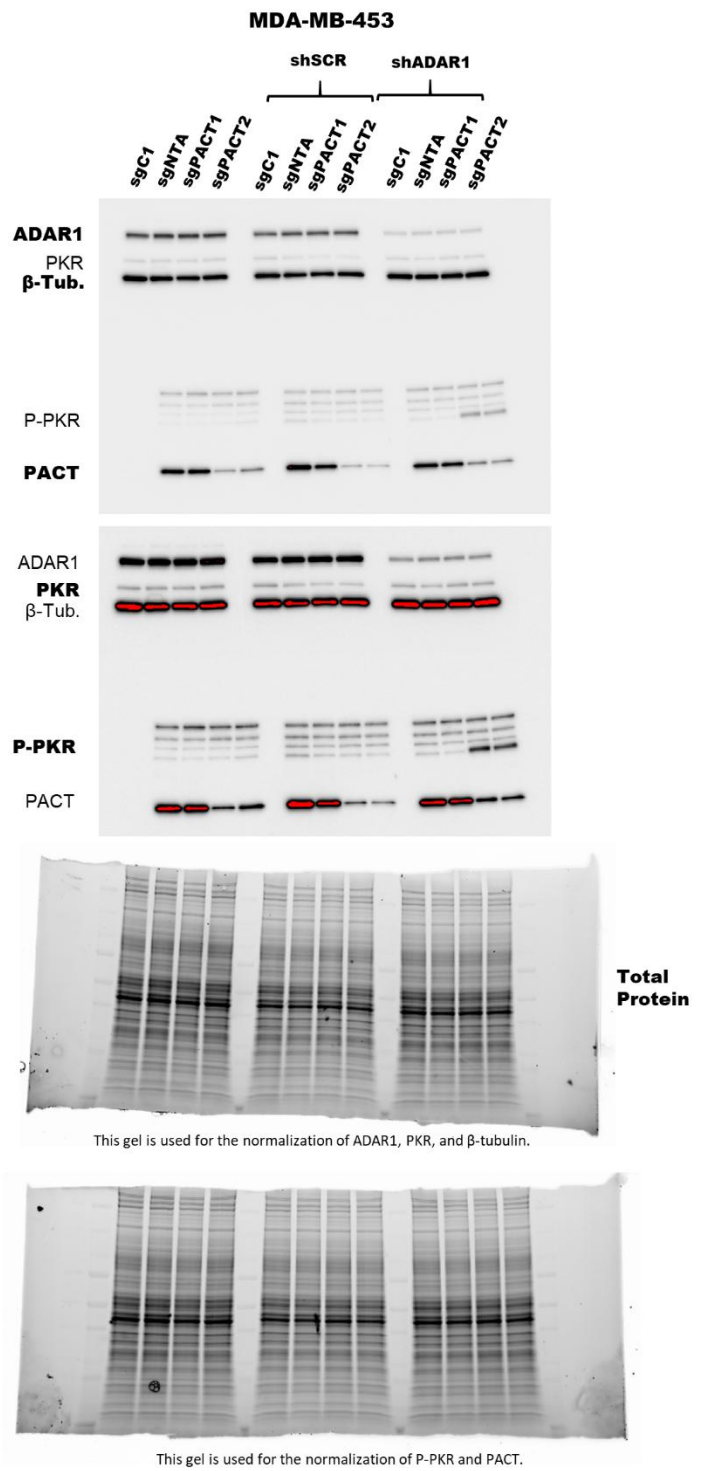
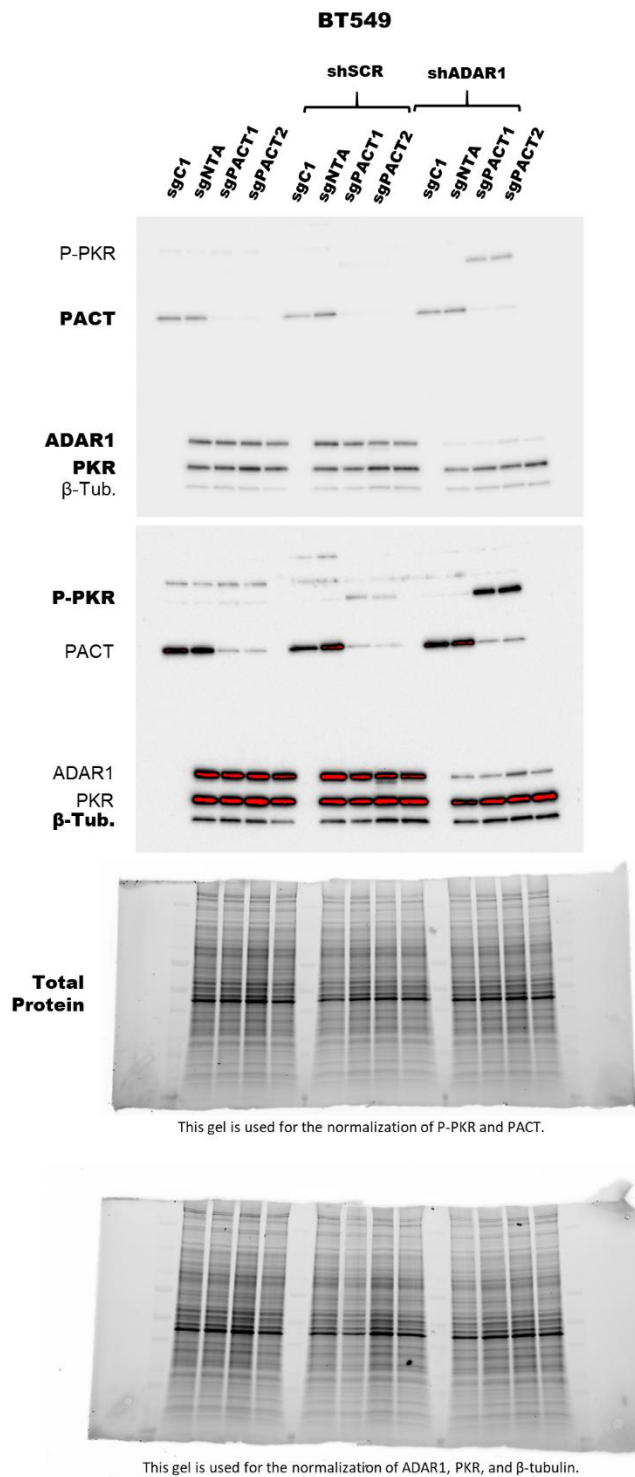


FIGURE 4b-(dependent)

The leftmost replicate is presented in the main figure.

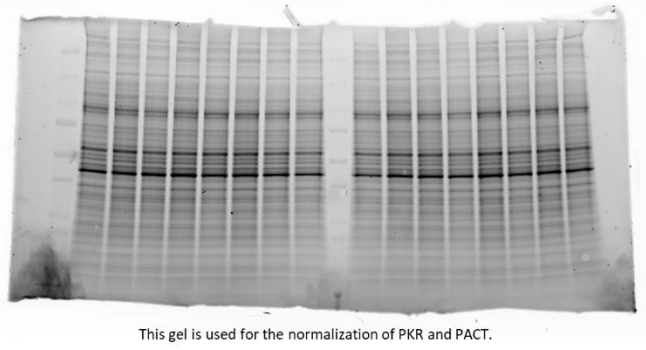
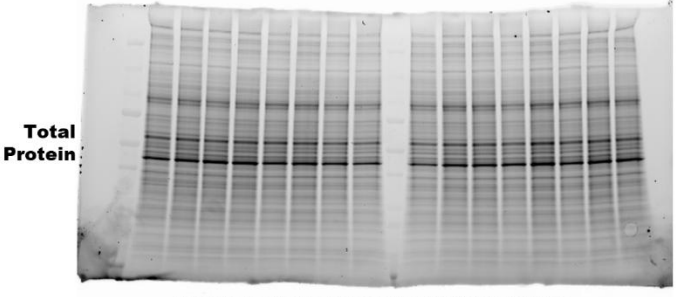
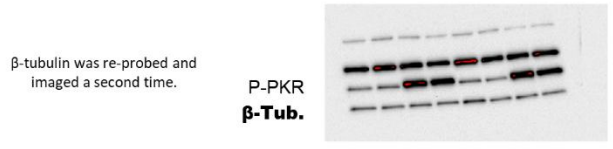
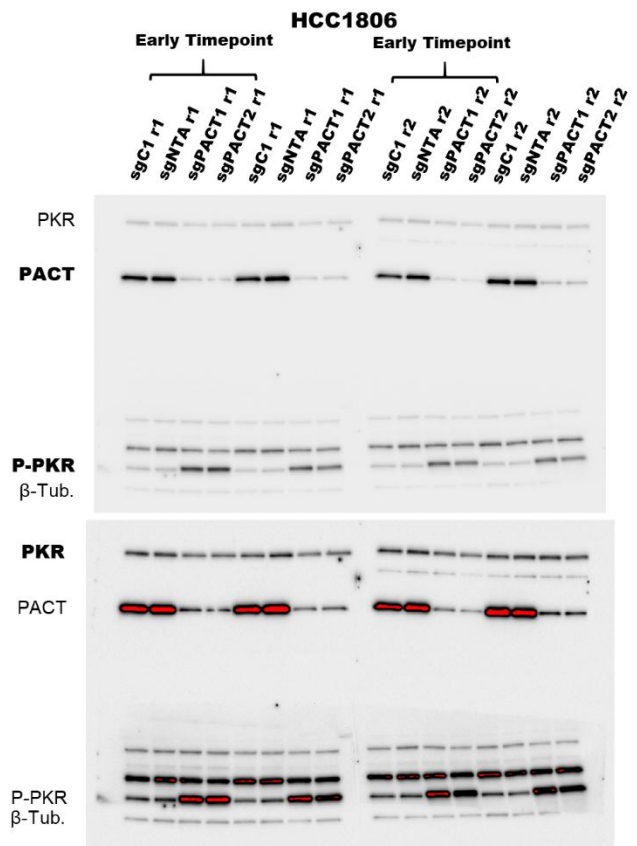
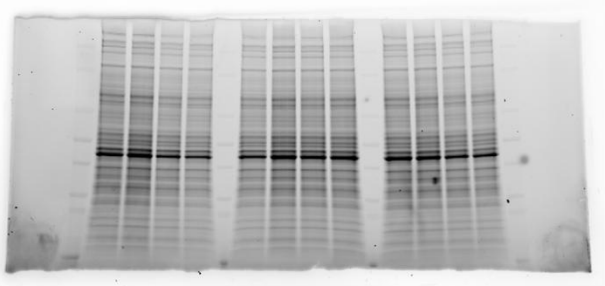
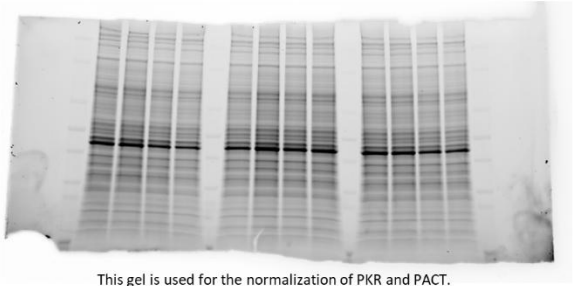
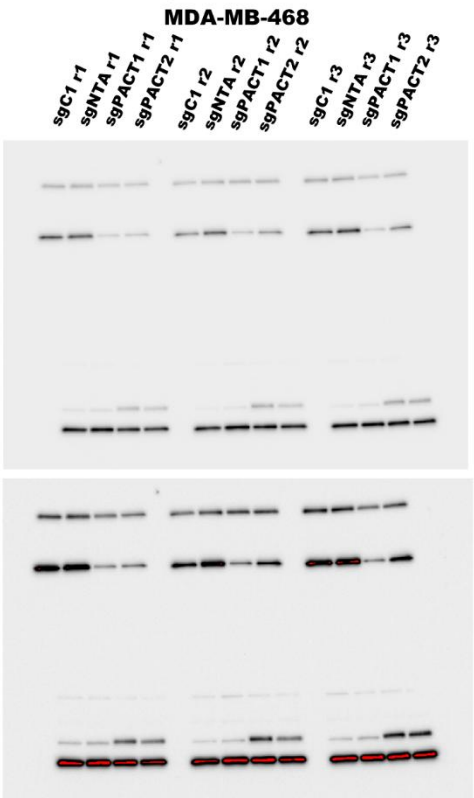
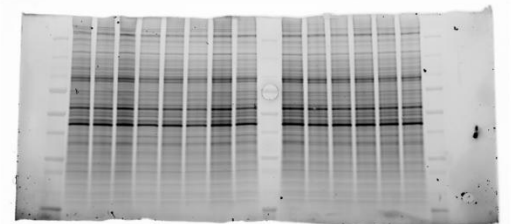
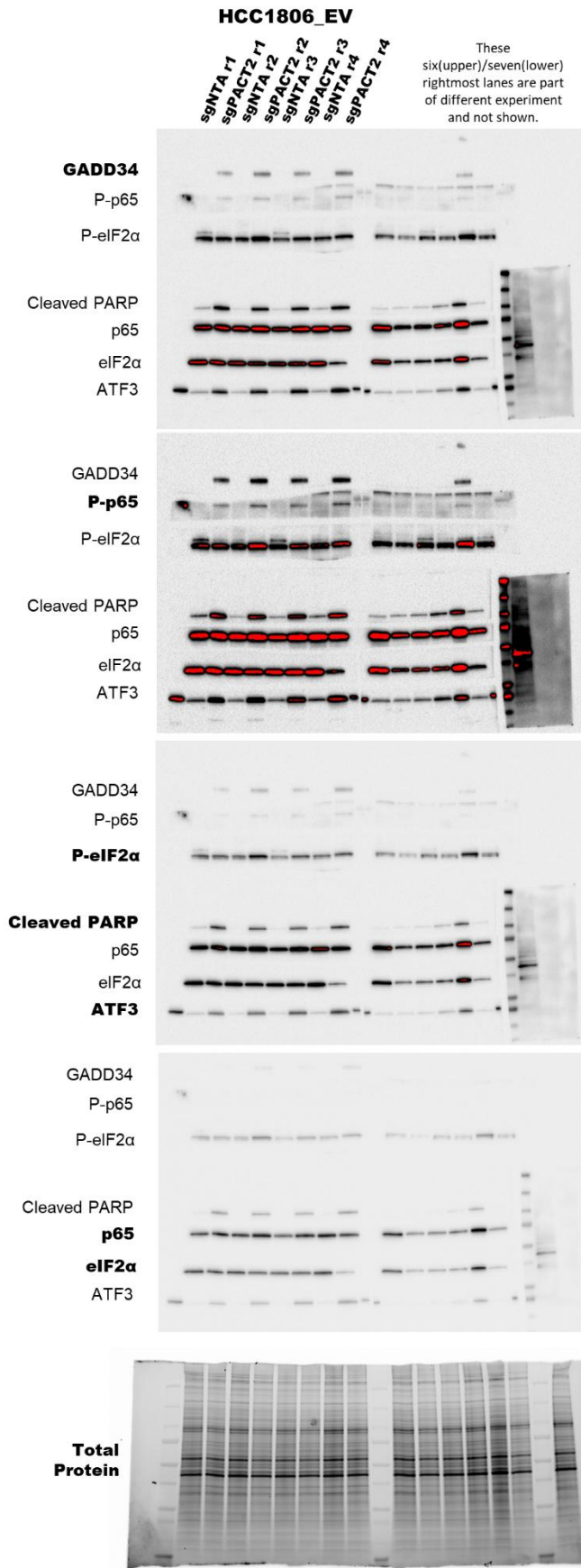


FIGURE 5e.



This gel is used for the normalization of GADD34, P-p65, and P-eIF2α.

This gel is used for the normalization of Cleaved PARP, p65, eIF2α, and ATF3.

FIGURE 5h.

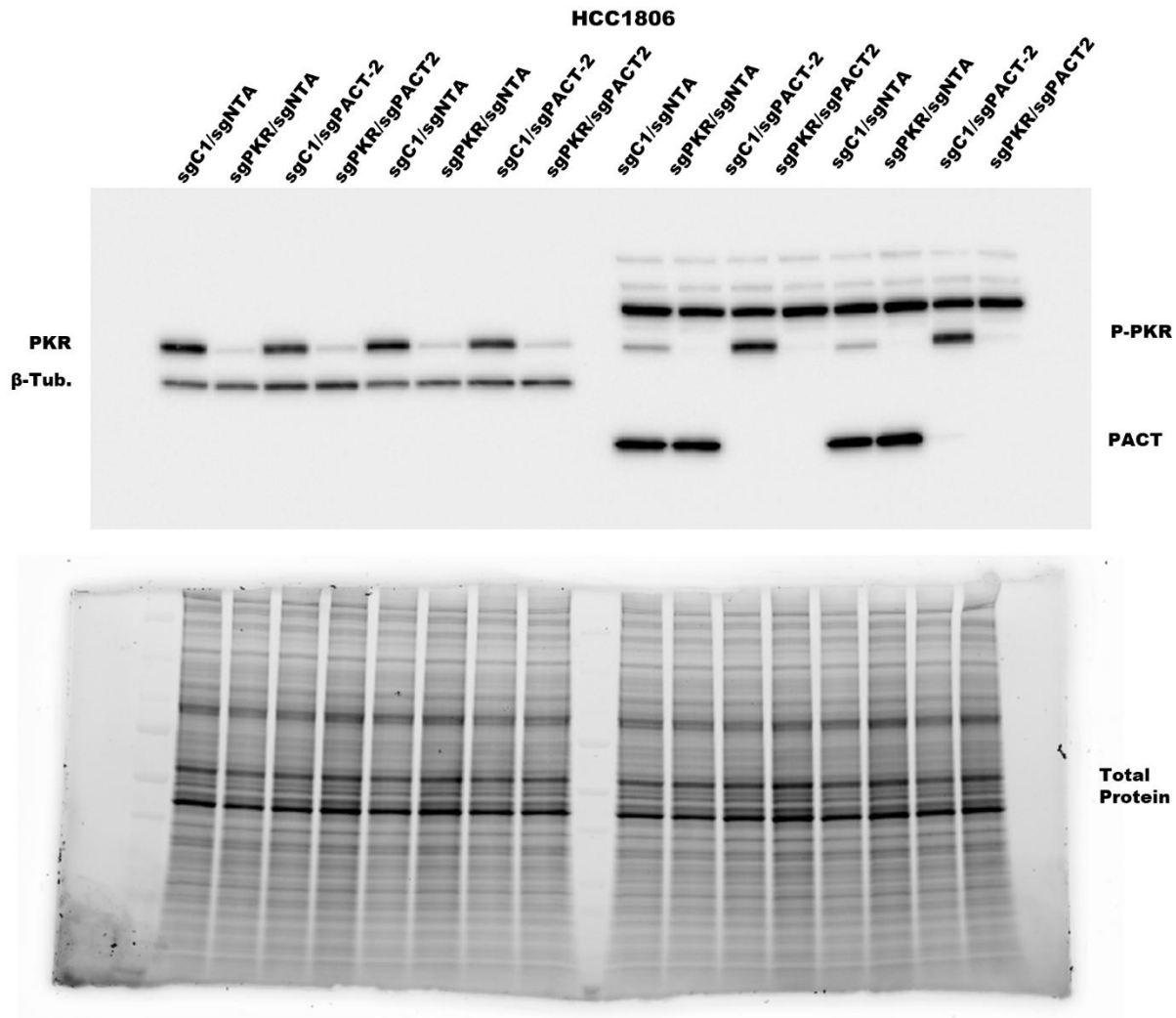
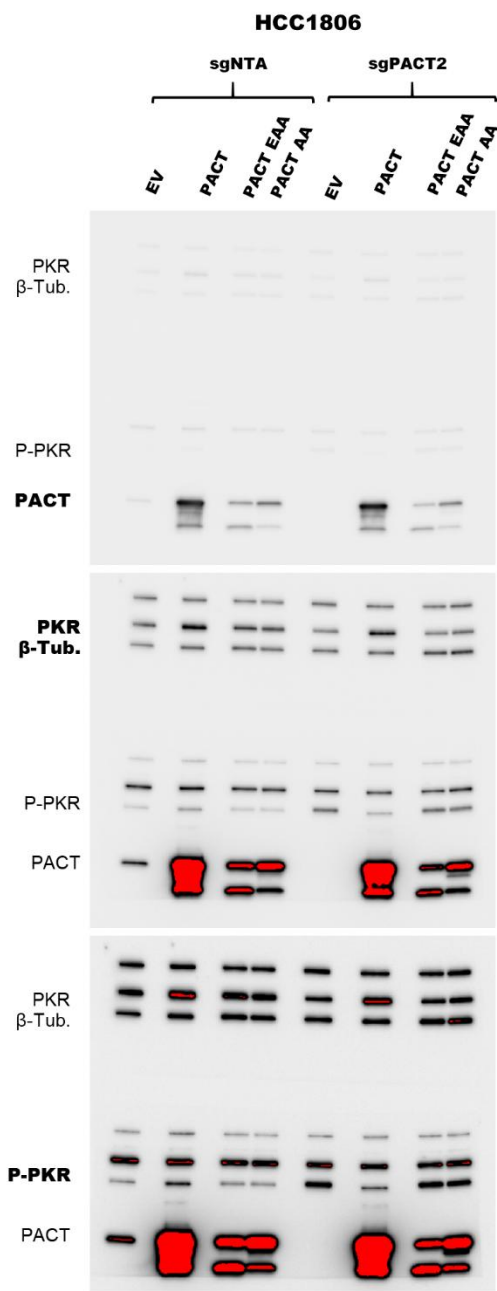
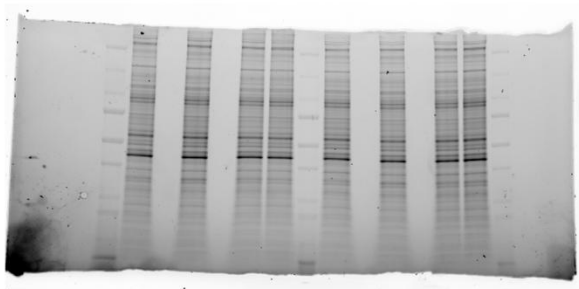


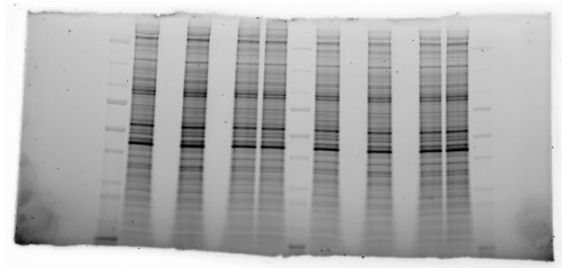
FIGURE 6b.



Total Protein



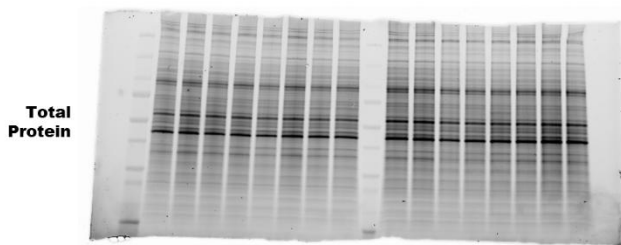
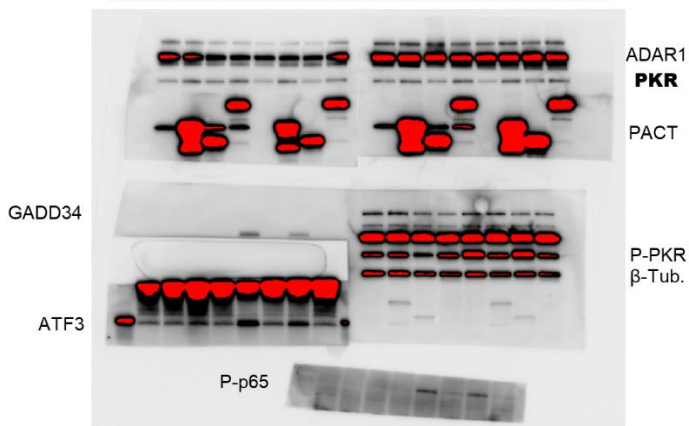
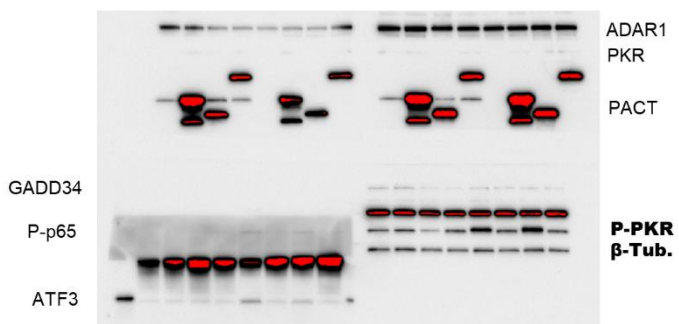
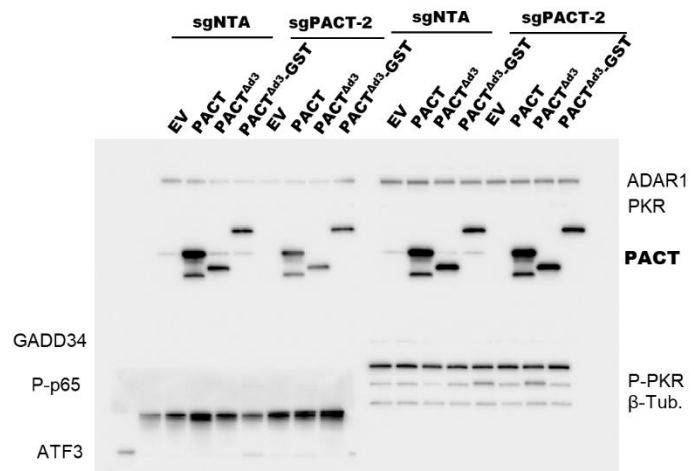
This gel is used for the normalization of PKR and β -tubulin.



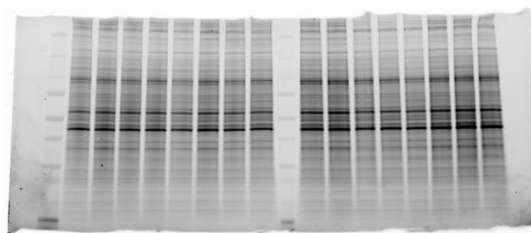
This gel is used for the normalization of P-PKR and PACT.

FIGURE 6f.

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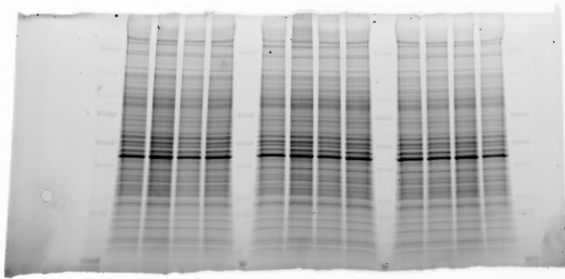
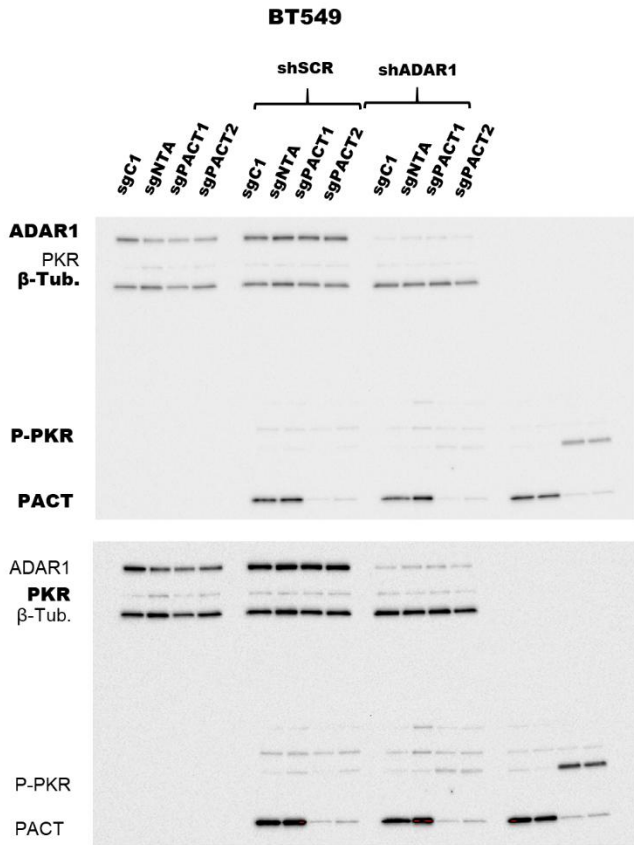


This gel is used for the normalization GADD34, P-p65, ATF3, P-PKR, and β-tubulin.

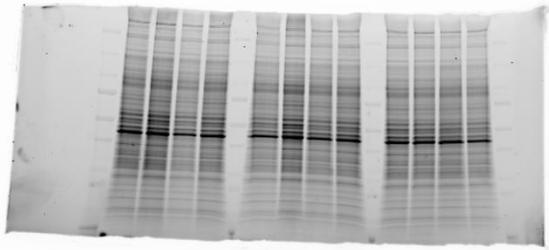


This gel is used for the normalization of ADAR, PKR, and PACT.

FIGURE 7a.

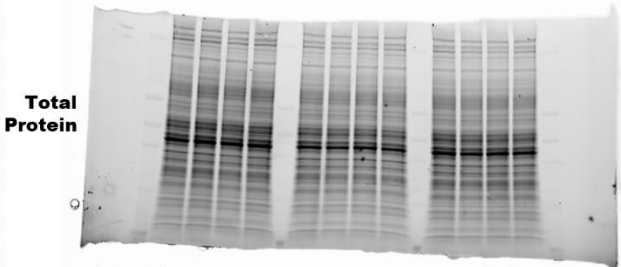
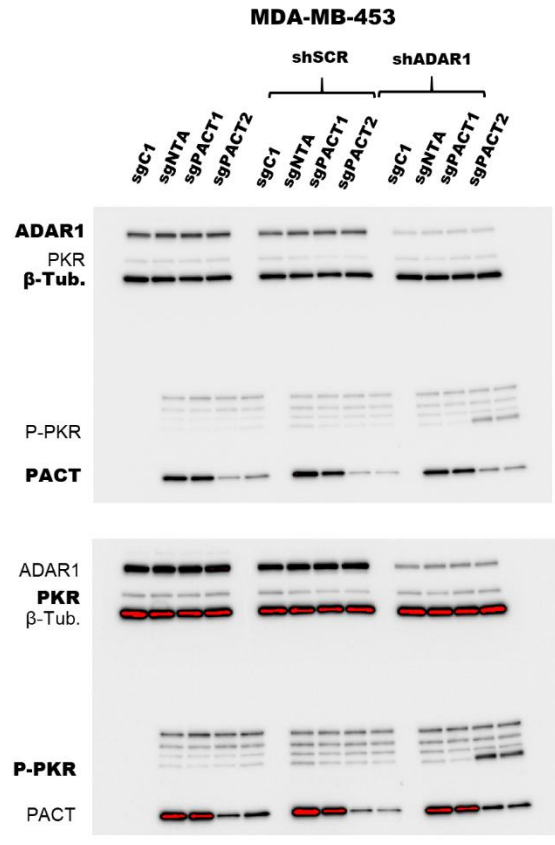


This gel is used for the normalization of ADAR1, PKR, and β-tubulin.

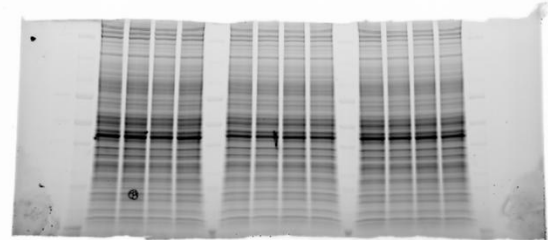


This gel is used for the normalization of P-PKR and PACT.

This gel and immunoblot is the same as that used in Figure 4b



This gel is used for the normalization of ADAR1, PKR, and β-tubulin.



This gel is used for the normalization of P-PKR and PACT.

FIGURE 7f.

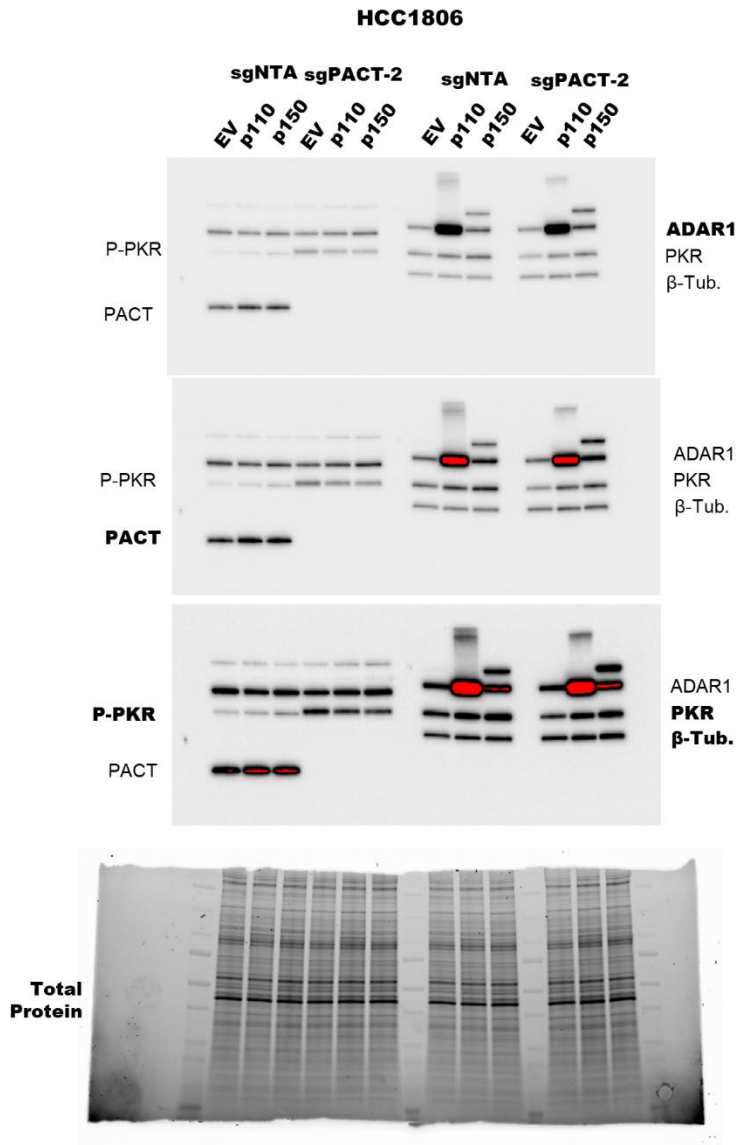
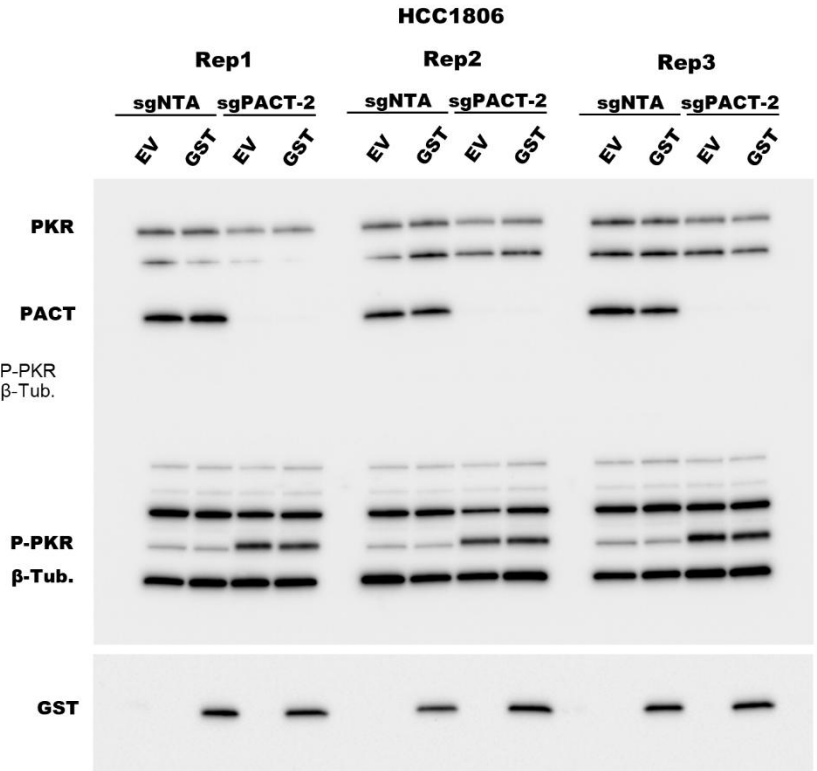
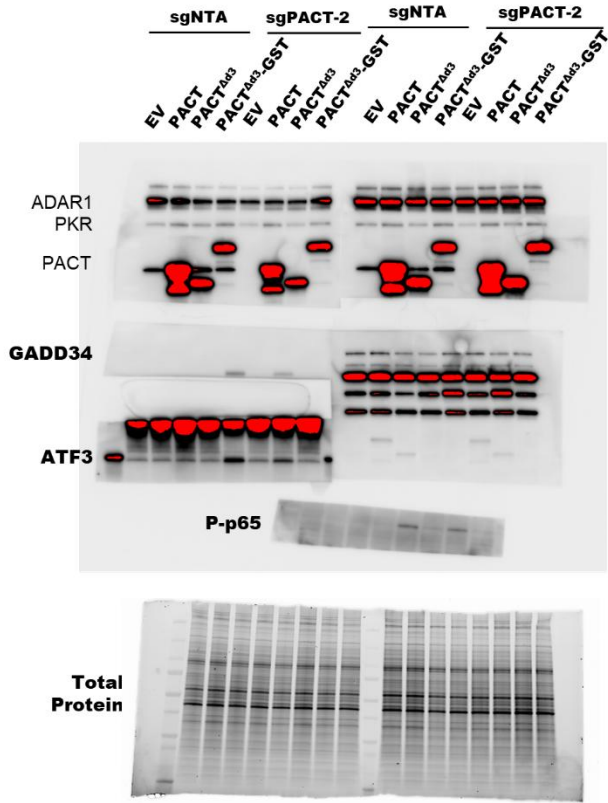


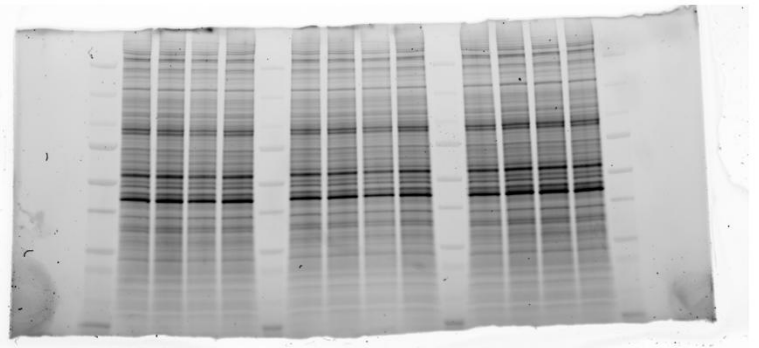
FIGURE S5b-c.

Blots from Figure S5b are shared with Figure 6f

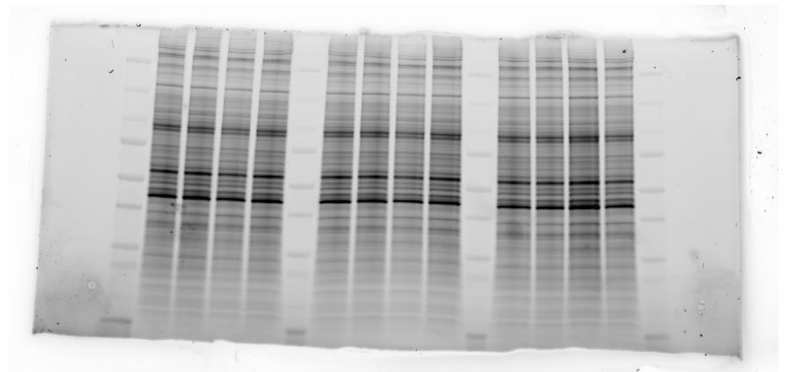
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Total Protein



This gel is used for the normalization of P-PKR, β -tubulin, and GST.



This gel is used for the normalization of PKR and PACT.

