Supplemental Information and Figures for:

ZYS-1 is not an ADAR1 inhibitor

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Contents:

Supplemental Methods

Cell Culture

Cell lines (HEK293T (RRID: CVCL_0063), MDA-MB-453 (RRID: CVCL _0418), MDA-MB-468 (RRID: CVCL_0063), BT-549 (RRID: CVCL_1092), and HCC1806 (RRID: CVCL_1258)) were purchased from the American Type Culture Collection. STR profiling was used to authenticate the cell lines, which were obtained between 2023 and 2024. Mycoplasma contamination testing was performed using a PCR-based method. Cells used in experiments were under twenty-five passages. The cell line HEK293T was cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) with 10% fetal bovine serum (BioTechne), 1 mM sodium pyruvate (Hyclone), 2 mM glutamine (Hyclone), and 0.1 mM nonessential amino acids (Hyclone). HCC1806 cells were cultured in Roswell Park Memorial Institute 1640 media (RPMI) (Corning Cat# 10-041-CV) with 10% fetal bovine serum (BioTechne). The cell line BT-549 was cultured in RPMI as above, supplemented with recombinant insulin (Gibco) at 0.78 µg/mL. MDA-MB-453 and MDA-MB-468 cells were maintained in Leibowitz L-15 media (HyClone Cat# SH30525) with 10% fetal bovine serum (BioTechne). MDA-MB-453 and MDA-MB-468 were cultured at 37°C with atmospheric CO_2. All other cell lines were cultured at 37°C with 5% CO_2.

Viral Production and Transduction

Lentivirus was produced by branched polyethylenimine (~25,000 Da, Sigma-Aldrich) or LipoFexin (Lamda Biotech) transfection of HEK293T cells with pMD2.G (a gift from Didier Trono, Addgene plasmid #12259; RRID:Addgene_12259), psPAX2 (a gift from Didier Trono, Addgene plasmid #12260; RRID:Addgene_12260), and a transfer plasmid for the expression of the sgRNA or shRNA of interest. Lentivirus-containing media was collected 2 to 3 days after transfection and filtered through a 0.45 µm filter. Lentivirus transduction of cells was done in the presence of 10 µg/mL protamine sulfate (Sigma-Aldrich) or 8 µg/mL polybrene (Sigma-Aldrich). Cells were selected with puromycin at 2 µg/mL (Sigma-Aldrich) or 150 µg/mL hygromycin (InvivoGen) depending on the transfer plasmid.

Immunoblot

Cell pellets were lysed on ice in RIPA lysis buffer (1% Triton X-100 (Sigma-Aldrich), 50 mM Tris pH 7.4 (Ambion), 150 mM NaCl (Ambion), 0.1% sodium dodecyl sulfate (Promega) and 0.5% sodium deoxycholate (Sigma-Aldrich)) with 1x HALT Protease and Phosphatase Inhibitor (Pierce). To quantify protein concentration, the DC Assay kit (Bio-Rad) was used. The lysate supernatant was diluted in SDS Sample Buffer (125 mM Tris

pH 6.8, 30% glycerol, 10% sodium dodecyl sulfate, and 0.012% bromophenol blue) and boiled at 95 °C for 7 minutes. Between 25 and 40 micrograms of protein was loaded per lane onto 4-12% TGX Acrylamide Stain-Free gels (Bio-Rad). The Stain-Free gel was transferred onto a PVDF membrane (Bio-Rad) by TransBlot Turbo (Bio-Rad) and then blocked in either 5% milk or 5% bovine serum albumin in tris-buffered saline with tween before overnight primary antibody incubation. Following primary antibody binding and a horseradish-peroxidase conjugated secondary antibody (Jackson ImmunoResearch) incubation, Clarity Western ECL Substrate (Bio-Rad) was used for band detection via ChemiDoc (Bio-Rad). Visualized bands were then normalized to total protein using Image Lab (Bio-Rad) and the Stain-Free gel image, and the bands of interest were quantified.

5-HT$_{2C}$ sequences

DNA sequence (5'-3') encoding a portion of the **5-HT$_{2C}$** mRNA used for in vitro A-to-I editing assays

TAATACGACTCACTATAGTGGGTACGAATTCCCACTTACGTACAAGCTTACCTAGATATTTGTGCCCCGTCTGGATTTCTTTAGATGTT
TTATTTTCAACAGCGTCCATCATGCACCTCTGCGCTATATCGCTGGATCGGTATGTAGCAAT**A**CGTAATCCTATTGAGCATAGCCGTTT
CAATTCGCGGACTAAGGCCATCATGAAGATTGCTATTGTTTGGGCAATTTCTATAGGTAAATAAAACTTTTTGGCCATAAGAATTGCAG
CGGCTATGCTCAATACTTTCGGATTATGTACTGTGAACAACGTACAGACGTCGACTGGTAACATTTGCGTTTGATCGGGTTCTggtacc

RNA sequence (5'-3') for a portion of the **5-HT$_{2C}$** mRNA used for in vitro A-to-I editing assays

GUGGGUACGAAUUCCCACUUACGUACAAGCUUACCUAGAUAUUUGUGCCCCGUCUGGAUUUCUUUAGAUGUUUUAUUUUCAACAGCGUC
CAUCAUGCACCUCUGCGCUAUAUCGCUGGAUCGGUAUGUAGCAAU**A**CGUAAUCCUAUUGAGCAUAGCCGUUUCAAUUCGCGGACUAAGG
CCAUCAUGAAGAUUGCUAUUGUUUGGGCAAUUUCUAUAGGUAAAUAAAACUUUUUGGCCAUAAGAAUUGCAGCGGCUAUGCUCAAUACU
UUCGGAUUAUGUACUGUGAACAACGUACAGACGUCGACUGGUAACAUUUGCGUUUGAUCGGGUUCUG

-the underlined sequence is the T7 promoter
-the lowercase sequence is the KpnI site used to linearize the pTwist-5HT plasmid for in vitro transcription
-in both sequences above, the B-site adenosine is bold

Supplemental Table 1

sgRNA Sequences	
Name	**sgRNA sequence (5'-3')**
sgC1	GAGCCACATTAACCGGCCCT
sgADAR1-a	ACAATGGCCCCTCAAAAGCA

sgRNA Cloning Oligonucleotides	
sgADAR1-a_1	caccgACAATGGCCCCTCAAAAGCA
sgADAR1-a_2	aaacTGCTTTTGAGGGGCCATTGTc

shRNA Sequences	
Name	**shRNA sequence (5'-3')**
shSCR	CCTAAGGTTAAGTCGCCCTCG
shADAR1	GCCCACTGTTATCTTCACTTT

shRNA Cloning Oligonucleotides	
Name	**shRNA sequence (5'-3')**
shSCR_1	CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG
shSCR_2	AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGA
shADAR1_1	CCGGGCCCACTGTTATCTTCACTTTCTCGAGAAAGTGAAGATAACAGTGGGCTTTTTG
shADAR1_2	AATTCAAAAAGCCCACTGTTATCTTCACTTTCTCGAGAAAGTGAAGATAACAGTGGGC

Cloning Primers	
Name	**shRNA sequence (5'-3')**
tet-pLKO-puro_PstI_F	CCGCAAGCCCGGTGCCTGCAGACGCCCGCCCCACGAC
tet-pLKO-puro_PstI_R	GTCGTGGGGCGGGCGTCTGCAGGCACCGGGCTTGCGG
PuroR_F	CGAGTACAAGCCCACGGTG
Fluc_R_PstI	ACTACTGCAGTGTCGACTTAACGCGTGAAT

PCR Primers for human A-to-I Editing sites	
Name	**Sequence (5'-3')**
ZDHHC20_F	TGCTGTACTAGGAAATGACAGAGC
ZDHHC20_R	AACATTCTGTGATGCCTAATTTTG
BPNT1_F	TGCTGTGGGAGGCAAGTTAAC
BPNT1_R	GAGTCCGAGGCAGACAGATC
BPNT1_seq_F	GGAGTCTCGCTCTGTAGCCT
MRPS16_F	GAAATCGCACACTGAAATATCC
MRPS16_R	TTGACTCACAACCATTCTTAGGTC

Primers for in vitro A-to-I editing	
Name	**Sequence (5'-3')**
RT_primer	gctataacgaatactcgagggctctgatgTACCAGAACCCGATCAAACGC
EA_F	CCCACTTACGTACAAGCTTACCTAG
EA_R	GCTATAACGAATACTCGAGG

qPCR Primers	
Name	**Sequence (5'-3')**
IRF1 qF	AGCAAGGCCAAGAGGAAGTC
IRF1 qR	ACTGTGTAGCTGCTGTGGTC
EEF1A1 qF	TGTCGTCATTGGACACGTAGA
EEF1A1 qR	ACGCTCGAGAGTGAGAGGCT
IFIH1 qF	AGTGATTCAGGCAACATGGG
IFIH1 qR	GCTGGGCAACTTCCATTTGG
PARP14 qF	GATGTTGCTGTTGTTACCTTTCA
PARP14 qR	CCAGGTGGCAGGTTTTCAA
IFIT2 qF	CTGGGGAAACTATGCCTGGG
IFIT2 qR	GTGTCCACCCTTCCTCACAG
HSPA5 qF	GAAAGAAGGTTACCCATGCAGT
HSPA5 qR	CAGGCCATAAGCAATAGCAGC

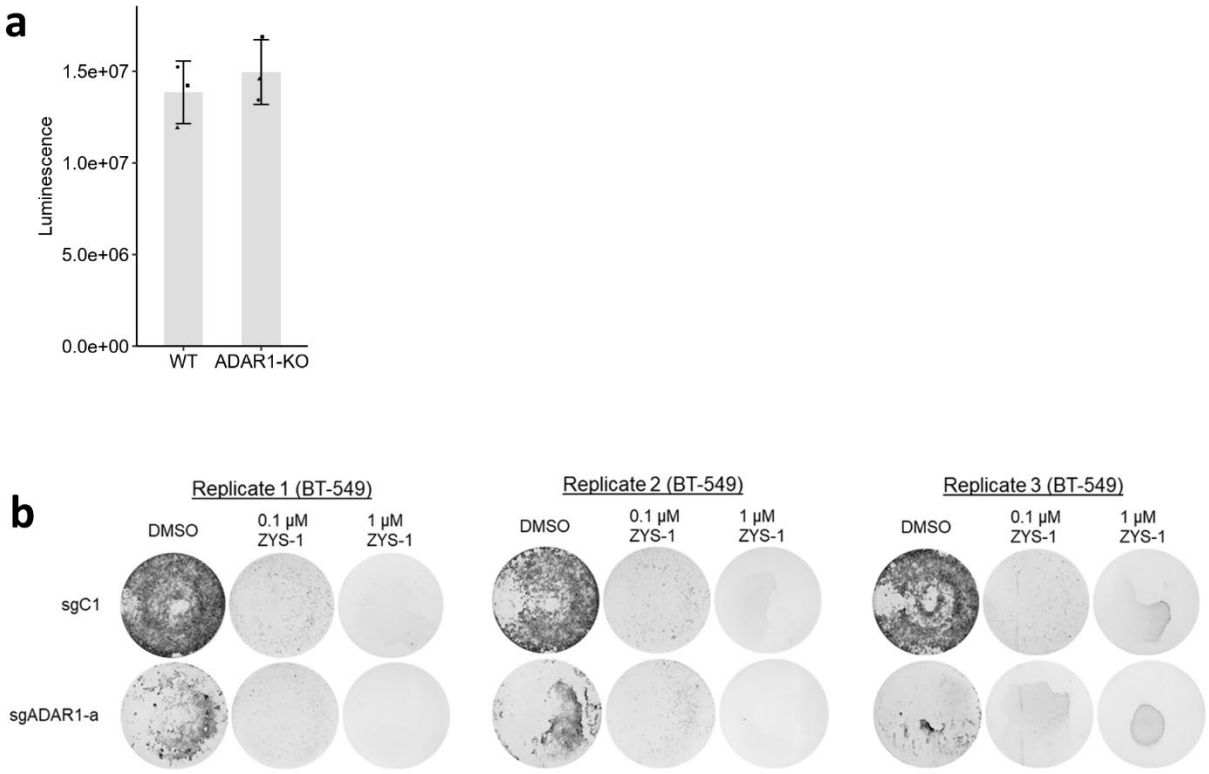


Figure S1

a Cell viability (luminescence) of DMSO-treated WT or ADAR1-KO BT-549 cells as assessed by CellTiter-Glo 2.0 assay. **b** Images of crystal violet-stained cells for three replicates of WT (sgC1) or ADAR1-depleted (sgADAR1-a) BT-549 cells following treatment with ZYS-1.

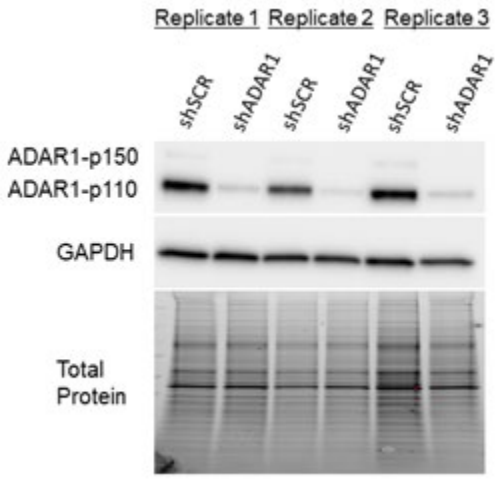


Figure S2

Immunoblot analysis of ADAR1 protein expression in control (shSCR) and ADAR1 knockdown (shADAR1) HCC1806 cells.

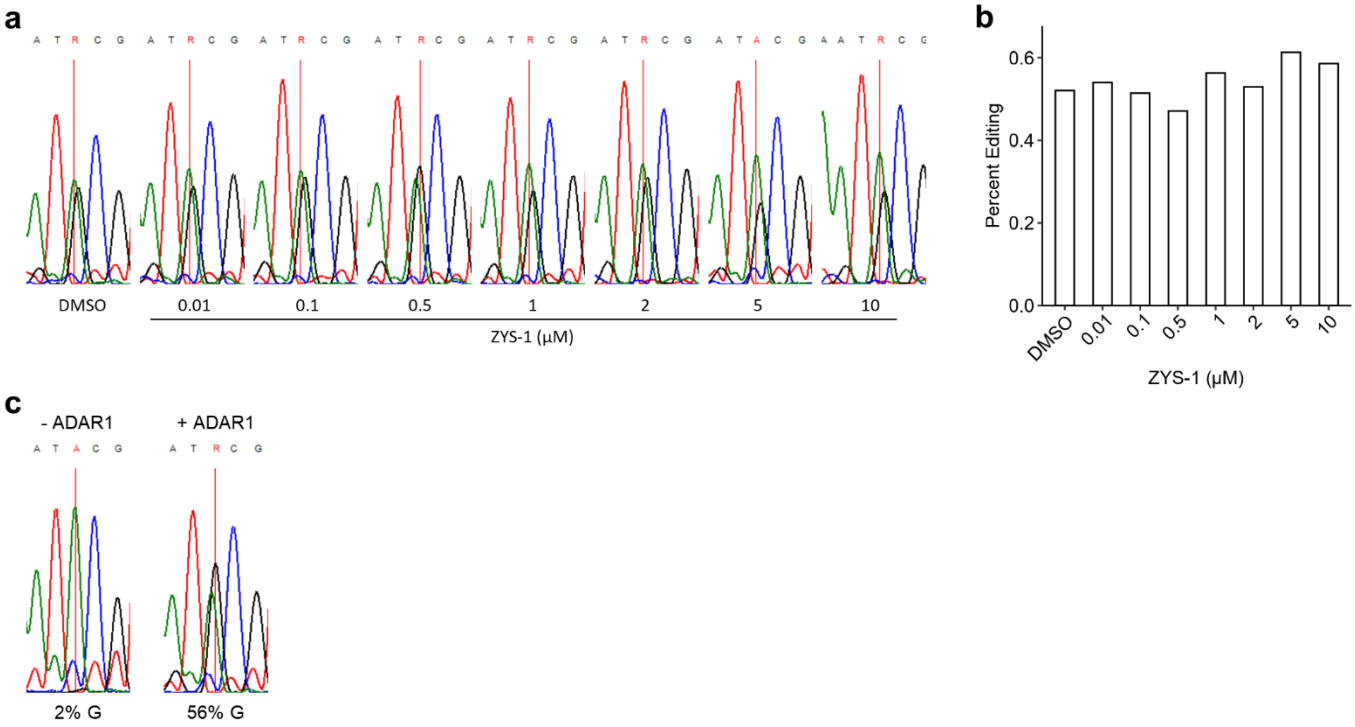


Figure S3

a Sanger sequencing trace of 5-HT_{2C} B-site resulting from the in vitro A-to-I editing assay with ZYS-1 as indicated. Recombinant ADAR1 used was from a different enzyme lot than that used to generate the results in Fig. 3i-j, and incubation time was shortened from an hour to 15 minutes. **b** Quantification of B-site percent editing using Sanger sequencing results shown in **a**. **c** Sanger sequencing trace of the 5-HT_{2C} B-site with or without the addition of ADAR1 to the in vitro A-to-I editing assay.

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

FIGURE 1f.

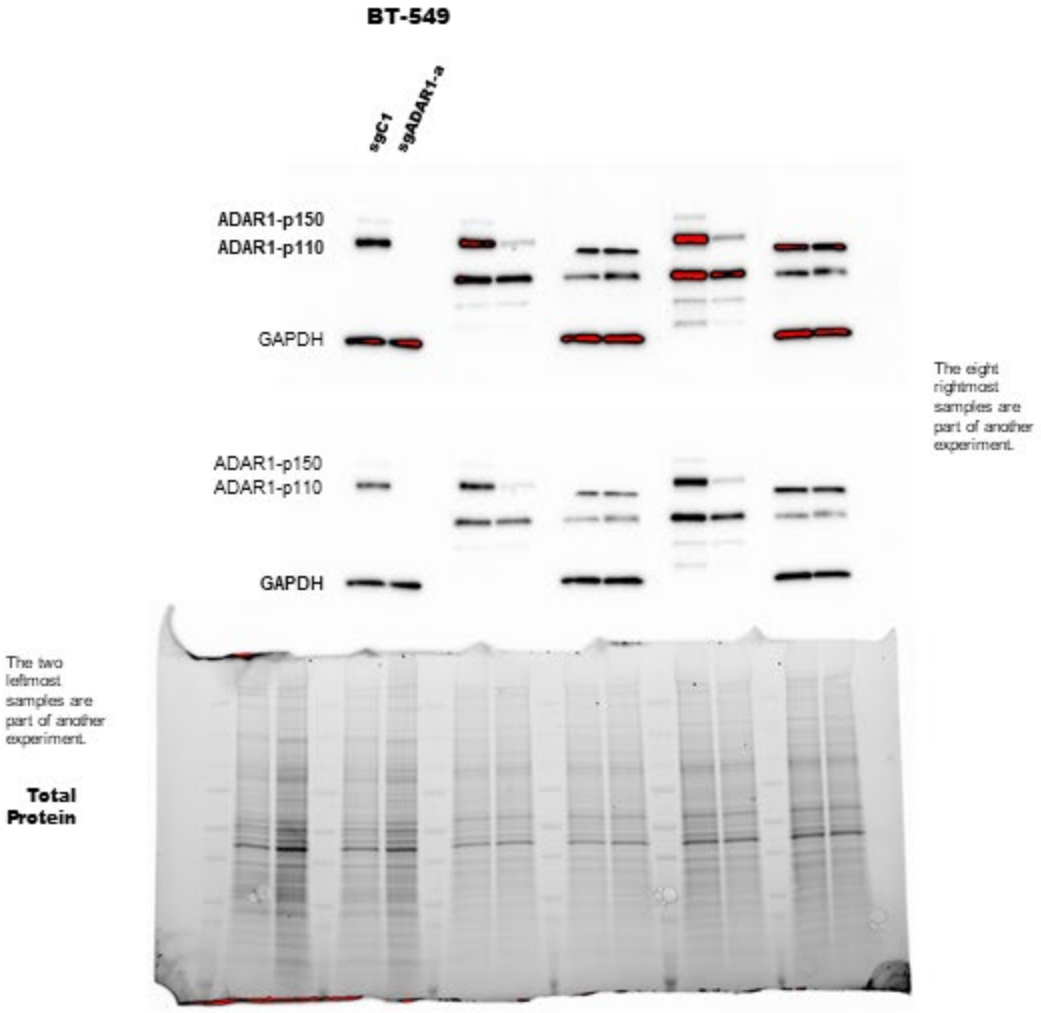

BT-549
sgC1
sgADAR1-a
ADAR1-p150
ADAR1-p110
GAPDH
ADAR1-p150
ADAR1-p110
GAPDH
The eight rightmost samples are part of another experiment.
The two leftmost samples are part of another experiment.
Total Protein

FIGURE 2a (HCC1806).

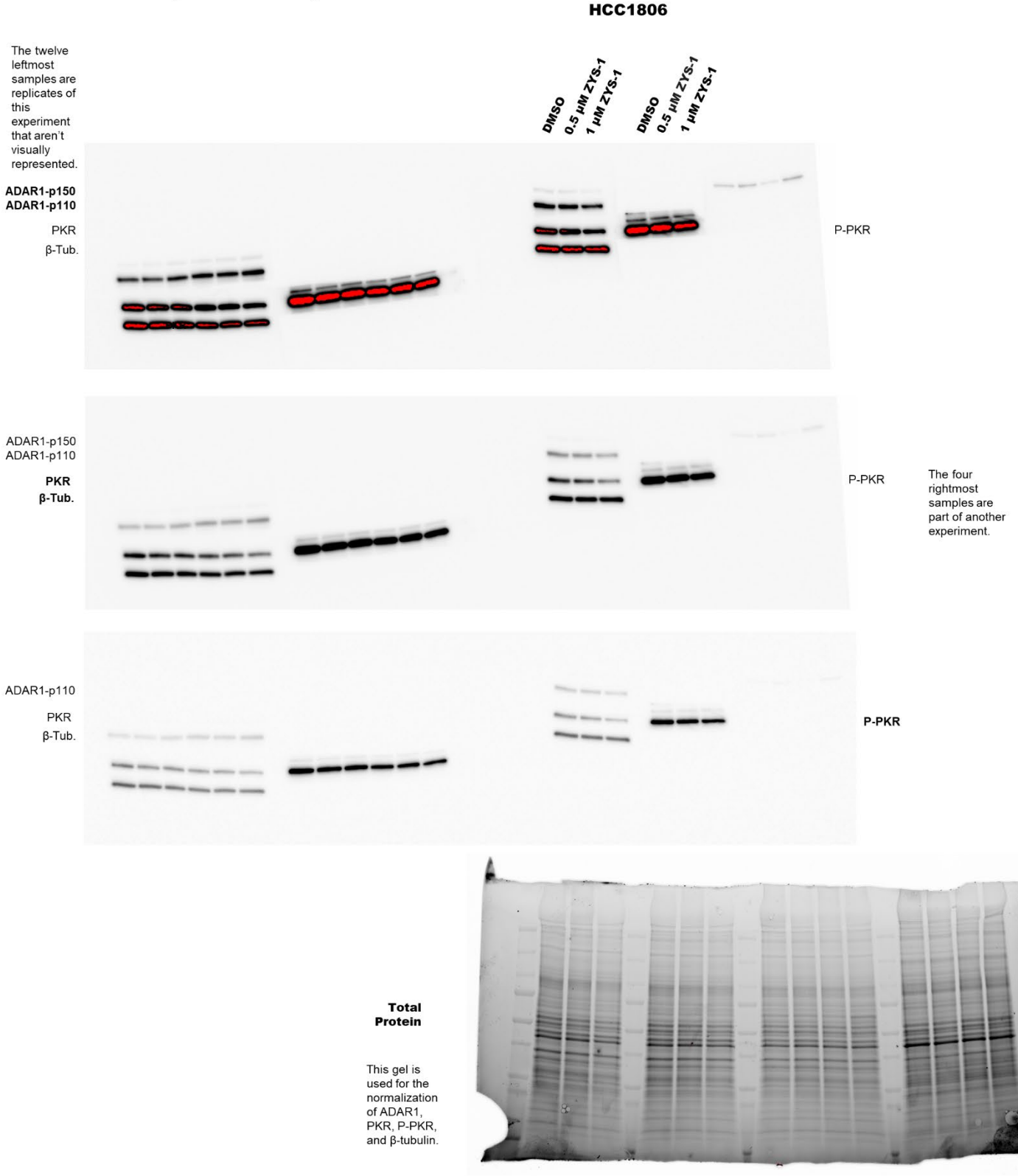
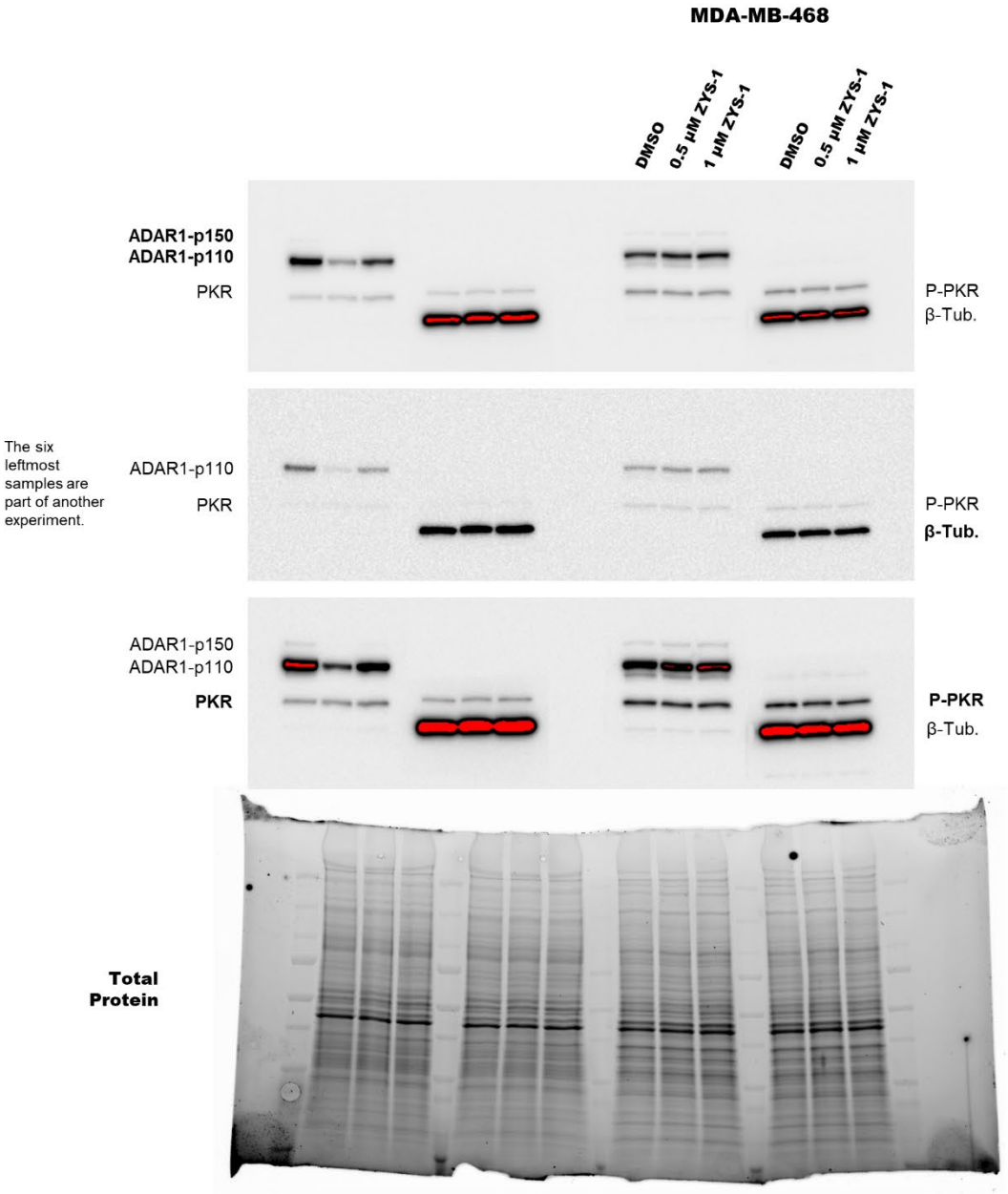


FIGURE 2a (MDA-MB-468).



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

SUPPLEMENTAL FIGURE 2