SUPPLEMENTARY MATERIALS

Targeting DNA repair pathway to synergize with trastuzumab deruxtecan in HER2-targeted antibody-drug conjugate-resistant HER2-overexpressing breast cancer

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Materials and Methods

Sulforhodamine B cell proliferation assay The anti-proliferation effects of HER2-ADC and selected inhibitors against HER2+ cells were assessed using sulforhodamine B staining assays. In brief, 1 to 3 × 10^3 cells/well were seeded in 12-well plates and treated the next day with HER2-ADC alone or HER2-ADC combined with BAY 1895344 for 7 days. The cells were then fixed with 5% trichloroacetic acid (Sigma-Aldrich) for 2 h and then stained with 0.03% sulforhodamine B solution (Sigma-Aldrich) for 30 min at room temperature. The stained cells were dissolved in 10 mM Tris buffer (Bio-Rad, Hercules, CA, USA). Optical density was determined at 480 (Ex)/590 (Em) nm using the VICTOR X3 plate reader (PerkinElmer, Waltham, MA, USA).

High-throughput RNAi screening

Non-biased kinome library RNAi screening was performed as described in previous studies (15,16). In brief, three unique siRNAs targeting one gene and a total of 2127 siRNAs targeting 709 kinase genes were selected from Ambion Silencer Select Human Genome siRNA Library V4 (Life Technologies) and administered into Greiner Bio-One CELLSTAR 384-Well plates (Thermo Fisher Scientific, Waltham, MA, USA). Seventy-five microliters of three pooled siRNAs (2 μM) for one gene were dispensed per well in quadruplicates. For an internal control, Silencer Select Negative Control No. 1 siRNA (Thermo Fisher Scientific), Silencer Select Positive Control PLK1 siRNA (Thermo Fisher Scientific), and no-

siRNA control (Thermo Fisher Scientific) were included in each plate. The positive control PLK1 siRNA was used to assess transfection efficiency, and the no-siRNA control was used to calculate cellular sensitivity to T-DXd. Lipofectamine RNAiMAX (0.05 µL/well; Invitrogen) in 10 µL of serum-free Opti-MEM was added to the plates, and then the plates were incubated at room temperature for 45 minutes. After incubation, SUM190-TDM1R or SUM190-TDXdR (300 cells) in 20 µL of complete media without antibiotics was added. The plates were sealed and incubated at 37 °C with 5% CO_2 for 48 h. A total of 28 plates were prepared for kinome screening, containing pooled siRNAs targeting 26 genes. At 48 h after treatment with pooled siRNAs, T-DXd at the final concentration of IC_{20} (0.3 nM) or vehicle (DMSO) in 30 µL of complete media was added to each well. The plates were then sealed and incubated at 37 °C with 5% CO_2 for 72 h. Following drug treatment, 35 µL of media was aspirated from each well, followed by the addition of ATPlite 1step (25 µL/well; PerkinElmer, Waltham, MA, USA). The plates were sealed and incubated at room temperature for 10 min, shaking at 1000 rpm on an orbital shaker. Luminescence readings were used as an indicator of cell viability. Luminescence readings for each treatment were averaged and normalized to the mean of the DMSO-treated no-siRNA negative control in the same plate to determine relative viability. PLK1-positive control and no-siRNA control were used to calculate each plate's z′-factor ($\boldsymbol{Z'}\textit{factor} = \frac{\mathbf{3 \times (\sigma p + \sigma n)}}{\mathbf{|\mu p - \mu n|}}$, where σp is the standard deviation of the positive control, σn is the standard deviation of the negative control, µp is the mean of the positive control, and µn is the mean of the negative control).

Fluorescence *in situ* hybridization (FISH) analysis

Fluorescence *in situ* assays were performed on the above cytological preparations using Her2 FISH probe and chromosome 17 centromeric probe (Empire Genomics Buffalo, NY, USA). The slides were hybridized with the FISH probes according to the manufacturer's instructions with slight modifications. Briefly, 2 µL of each of the two probes were mixed with 6 µl of the *in situ* hybridization buffer and applied to the slide, covered with a glass coverslip (22 × 22 mm), and sealed with rubber cement. The

slides were then denatured at 72-73 °C using the ThermoBrite system (Abbott Laboratories, Abbott Park, IL, USA) and incubated at 37 °C overnight. They were then washed using 2× saline-sodium citrate 45-70 °C for 1-2 min and counterstained with DAPI. The analysis and imaging were done using a Nikon Eclipse 80i microscope equipped with multiple filters.

Microarray analysis

Total RNA was extracted from parent and HER2-ADC–resistant cell lines using the TRIzol Reagent and Invitrogen Phasemaker Tubes (Thermo Fisher Scientific) and submitted to the Advanced Technology Genomics Core for microarray analysis using Affymetrix Clariom D Human Transcriptome arrays (Thermo Fisher Scientific). Transcriptome Analysis Console (TAC) Software (Thermo Fisher Scientific) was used to perform array quality control and data normalization, perform statistical tests for differential expression of genes or pathways of interest, interpret complex alternative splicing events, and obtain sequence information to design validation experiments. We also performed analysis using the oligo package of Statistical software R (version 4.1.3, https://www.r-project.org/). Normalization was performed using the RMA algorithm. Differential expression analysis was performed using a *t*-test between the contrasts of interest. The *p*-values obtained by multiple *t*-tests were corrected by the Benjamini-Hochberg method.

Droplet digital PCR (ddPCR) assay

The ddPCR assay was performed using the QX200 system (Bio-Rad) according to the manufacturer's recommendations. In brief, DNA isolation was performed using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific). The samples were processed for ddPCR in a blinded fashion. Restriction enzyme digestion of the genomic DNA with MSE I was performed prior to droplet generation. About 200 ng of DNA was used for restriction enzyme digestion in a final reaction volume of 10 µL. The amplification reaction comprised of the digested genomic DNA (3 µL), 4× ddPCR Multiplex Supermix for probes (Bio-Rad) and 20× copy number variation (CNV) assays in a final volume of 20 µL. The

ddPCR CNV assays were wet-lab validated in-house by the manufacturer and were used for both the target gene *HER2* (FAM) and for *RPP30* (HEX) as reference gene. The reaction mixtures were partitioned into an emulsion of approximately 20,000 droplets in oil by using 70 µL of droplet generation oil (Bio-Rad) and 20 µL of PCR reaction mix loaded into a disposable plastic cartridge (Bio-Rad) and placed in the droplet generator. After processing, the droplets obtained in a 40-µL volume from each sample were transferred to a 96-well PCR plate, and amplification was carried out using a T100 Thermal Cycler (Bio-Rad) with the following cycling conditions: DNA polymerase activation at 95 °C for 10 min followed by 40 cycles of PCR amplification (94 °C for 30 s and 60 °C for 60 s), and 98 °C for 10 min, 2 °C/s ramp rate at all steps. After PCR, the droplets were counted with the QX200 Droplet Reader using Bio-Rad QX Manager Standard Edition version 1.2 software. The *HER2*/*RPP30* copy number ratio was analyzed by calculating the copies per droplet from the Poisson distribution.

Whole-genome sequencing analysis

Genomic DNA was extracted from parent and HER2-ADC–resistant cell lines using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) and submitted to Azenta (Burlington, MA, USA) for next-generation sequencing analysis using the Illumina HiSeq 4000 with 90 Gb coverage. In brief, genomic DNA was quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). NEBNext Ultra DNA Library Prep Kit for Illumina, clustering, and sequencing reagents were used throughout the process following the manufacturer's recommendations. Briefly, 500 ng of the genomic DNA was fragmented by acoustic shearing with a Covaris S220 instrument. Fragmented DNA was cleaned up and end repaired. Adapters were ligated after adenylation of the 3′ ends followed by enrichment by limited-cycle PCR. DNA libraries were validated using a High Sensitivity D1000 ScreenTape on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and were quantified using the Qubit 2.0 Fluorometer. The sequencing library was clustered onto a lane of an Illumina HiSeq 4000 flow cell. After clustering, the flow cell was loaded onto the Illumina HiSeq instrument according to the

manufacturer's instructions. The samples were sequenced using a 2 × 150 bp paired end configuration. Image analysis and base calling were conducted by the HiSeq Control Software. Raw sequence data (.bcl files) generated from Illumina HiSeq were converted into FASTQ files and de-multiplexed using Illumina bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

Karyotyping by G-banding and genomic instability analysis

Cells were cultured in completed media supplemented with 10% fetal bovine serum, and chromosome preparations were made following the standard air-drying technique. Aged slides were G-banded, using 0.05% trypsin EDTA solution (Thermo Fisher Scientific). G-banded metaphase spreads were photographed using a 80i Nikon Microscope and Applied Spectral Imaging Karyotyping system. A minimum of 10 metaphases were karyotyped. For genomic instability analysis, cytological preparations were stained in 4% Giemsa solution, and the slides were analyzed for chromosomal aberrations, including chromosome and chromatid breaks, fusions, fragments, and tetraploidy. A minimum of 35 metaphases/anaphases were analyzed per cell line.

Western blotting

Cells (3×10^5 cells/10 mL) were seeded in 6-cm plates overnight and the next day treated with T-DXd alone or in combination with BAY 1895344 for 48 h, and then total protein was extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientifc) complemented with phosphatase and protease inhibitors (Bimake). The protein samples were denatured by incubation with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) at 70 °C for 10 min, resolved by using NuPAGE 4–12% Bis-Tris Plus gel (Thermo Fisher Scientific), and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad). Following blocking with 4% bovine serum albumin, proteins of interest on the blots were probed using the primary antibodies. The secondary antibodies used were horseradish peroxidase–conjugated immunoglobulin G (Thermo Fisher Scientific) for chemiluminescence signal

detection (Thermo Fisher Scientific). The intensity of target proteins on the blots was captured using ImageQuant LAS 4000 imager (Cytiva, Marlborough, MA, USA).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Antiproliferation effect of T-DM1 and T-DXd in HER2-positive BC cell lines.

A, HER2 gene copy variation in HER2+ BC cell lines. TNBC cell line MDA-MB-231 used as negative control. Genomic DNA was used for ddPCR assay. **B**, T-DM1 and T-DXd inhibit the proliferation of HER2+ cell lines in dose-dependent manner. **C**. T-DM1 and T-DXd significantly reduced tumor volume n HER2+ BC cell xenograft models. SUM190 or HCC1954 cells were injected into mammary fat pad of nude mice, and the treatment was started when tumors averaged 200 mm^3. HER2-ADC (10 mg/kg) was administered one time on Day 0, and tumor size was monitored. IHC assay was used to to check the expression levels of HER2 and proliferation maker Ki-67 in tumor samples.

Supplementary Figure S2. A. Anti-HER2 did not alter *ERBB2* gene copy number in HER2+ BC cell lines. **B**. Transient differential expression of HER2 in SUM190 cell line. HER2-high and HER2-low populations were isolated by FACS sorting, cultured separately for 5 weeks, and then assessed for HER2 expression using FACS analysis. **C**. HER2-high and HER2-low SUM190 cells showed similar sensitivity to T-DXd treatment. SRB proliferation assay for 5 days.

Supplementary Figure S3. A. HER2-ADC cell lines do not show increase in genomic instability. 35 metaphases/anaphases were analyzed per cell line. **B**. Deletion of the amplified ERBB2 region was observed on chromosome 17 in SUM190-TDXdR cell line. Karyotyping assay. **C**. Intrinsic T-DXd resistant KPL4 cell line (KPL4-TDXdR) did not show reduced *ERBB2* gene copy numbers. Whole-genome sequencing analysis.

Supplementary Figure S4. A. Overexpression of HER2 did not increase the antiproliferation effect of T-DXd in T-DXd–resistant HER2+ BC cell lines. SUM190-TDXdR and HCC1954-TDXdR cells transfected with pcDNA3-HER2 plasmid and underwent SRB proliferation assay with T-DXd. **B**. Low HER2 expression is not related to T-DXd resistance. SUM190-TDM1R and HCC1954-TDM1R cells injected into mammary fat pad of nude mouse, and the treatment was started when tumors averaged 200 mm^3. T-DXd (10 mg/kg) was administered one time on Day 0, and tumor size was monitored. Data are presented as mean ± standard error **, $P < 0.01$, ****, $P < 0.0001$. **C**. Cells were treated with siRNA targeting EGR1 or SLC6A14 and underwent SRB proliferation assay with T-DXd.

Supplementary Figure S5. A. DNA repair pathway network analysis of microarray data from T-DXd–resistant cell lines using TAC software. **B** and **C**. Top 50 targets from kinome library RNAi screening using T-DXd–resistant cell lines. SUM190-TDM1R (B), SUM190-TDXdR (C).

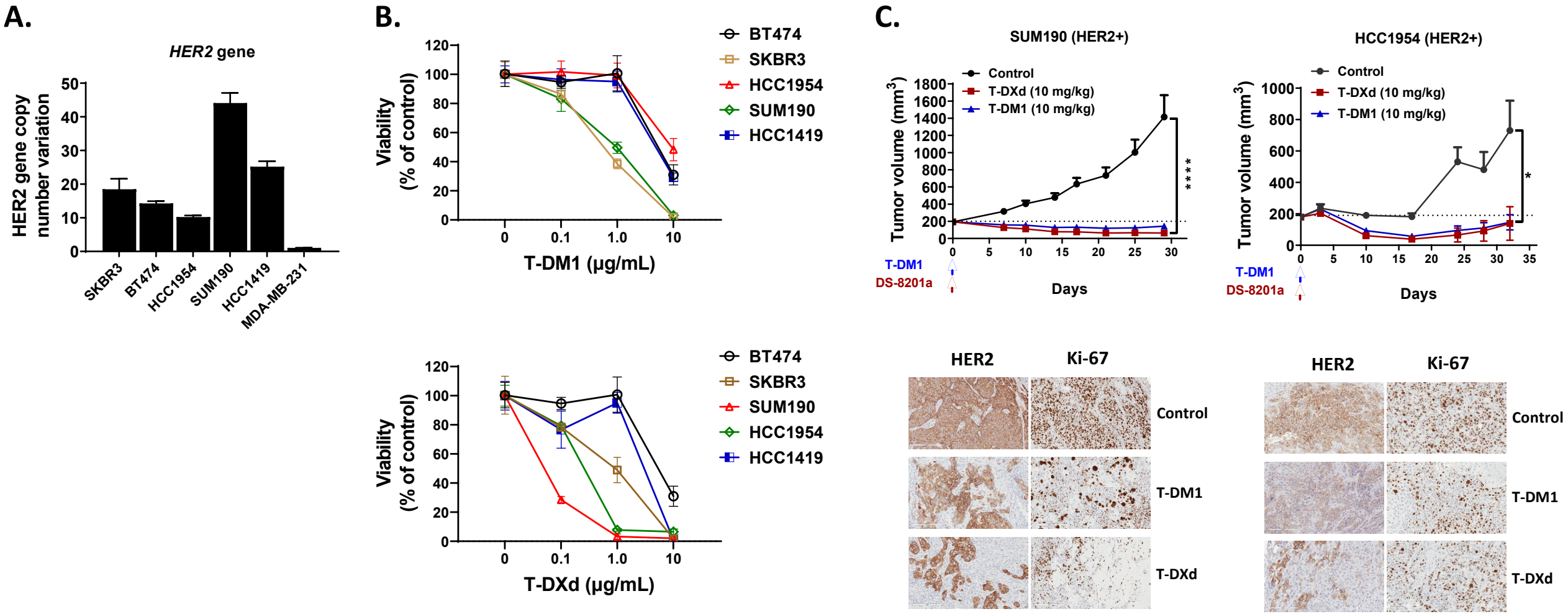
Supplementary Figure S6. DNA repair pathway–targeting drug enhances the efficacy of T-DXd in HER2-ADC–resistant HER2+ BC cell lines. SRB proliferation assay. **A**. SUM190, SUM190-TDM1R, SUM190-TDXdR cell lines. **B**. HCC1954, HCC1954-TDM1R, HCC1954-TDXdR cell lines.

Supplementary Figure S7. T-DXd and BAY 1895344 did not show toxicity in xenograft models. T-DXd was administrated one time on Day 0 via tail-vein injection. BAY 1895344 was administrated via oral gavage twice a day for 3 days weekly. Data are presented as mean ± standard deviation.

Supplementary Table S1. Patient characterization.

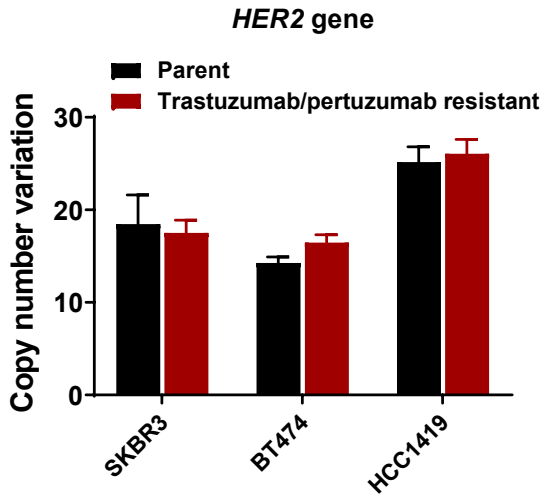
Patient characteristics		n (%)
Age		
	median, (range)	54 years (45-77 years)
Estrogen receptor		
	Positive	6 (60)
	Negative	4 (40)
Progesterone receptor		
	Positive	5 (50)
	Negative	5 (50)
HER2 expression before treatment		
	2+ (FISH+)	3 (30)
	3+	7 (70)
Specimen site		
	Primary	5 (50)
	Metastasis	5 (50)
Number of anti-HER2 therapy lines before post-phase biopsy		
	Chemotherapy plus anti-HER2	10 (100)
	Chemotherapy, anti-HER2, and T-DM1	5 (50)
Treatment		
	Chemotherapy	10 (100)
	Pertuzumab/trastuzumab	10 (100)
	T-DM1	5 (50)

FISH= fluorescence in situ hybridization



Supplemental Fig. 1

A.



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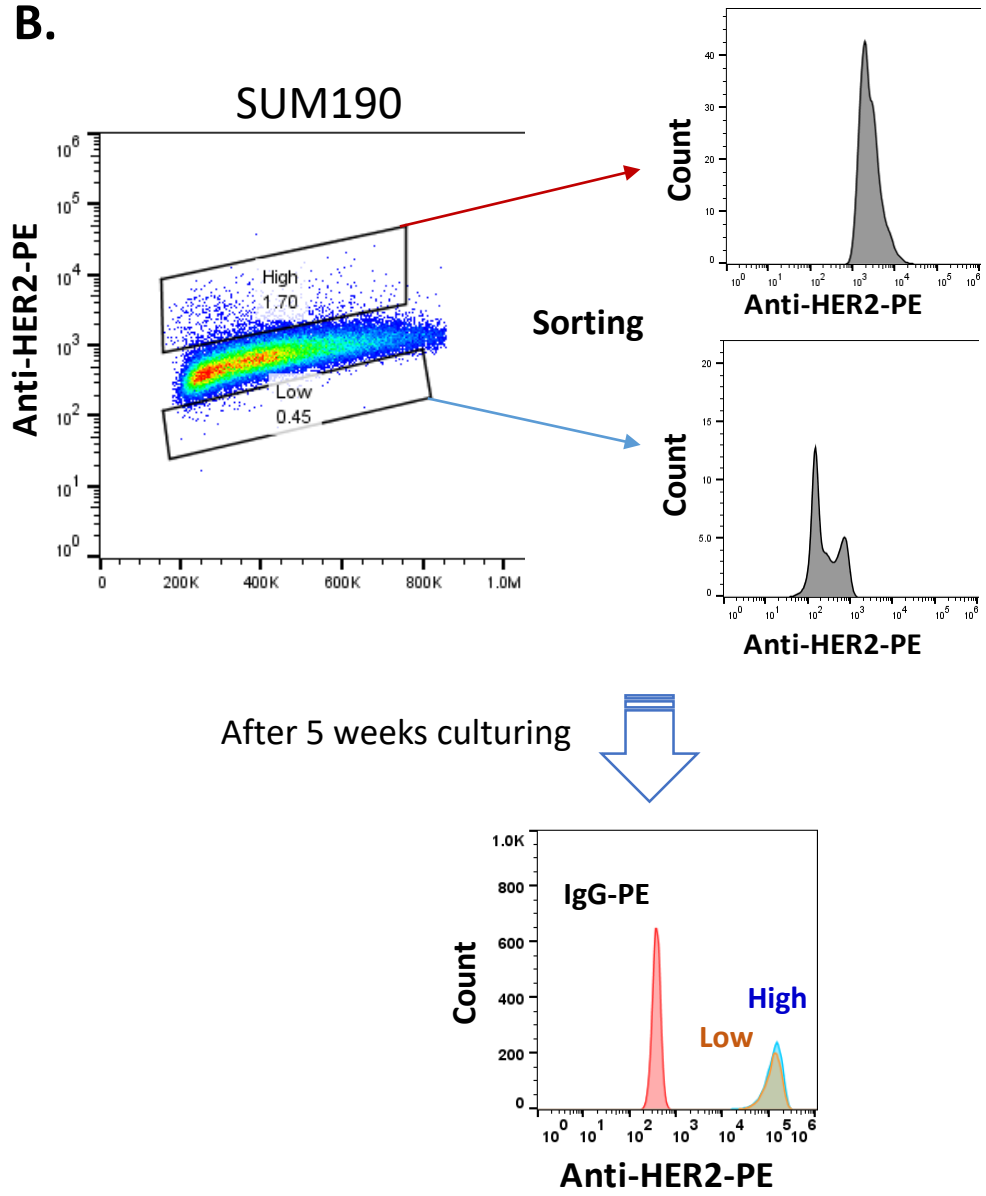

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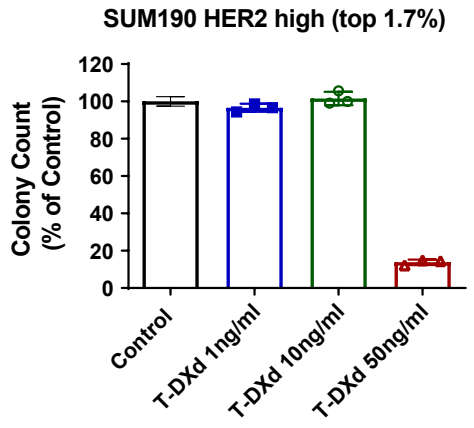
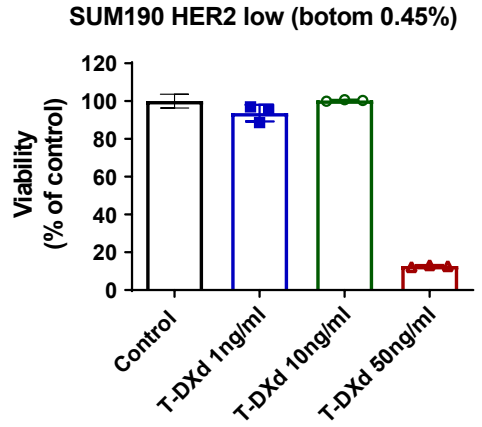
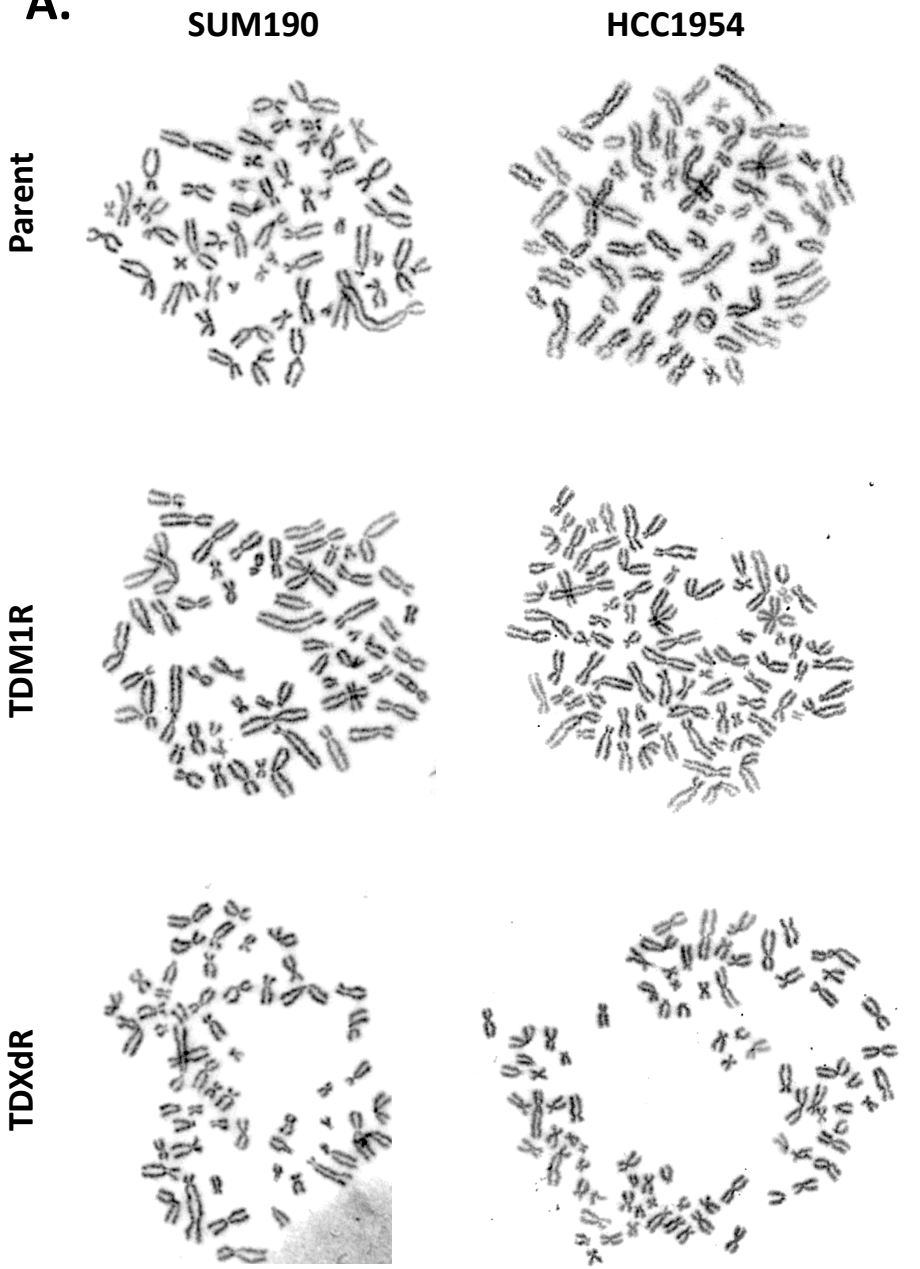




Supplemental Fig. 2

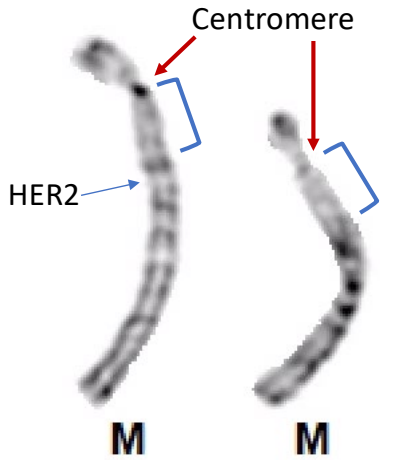
A.



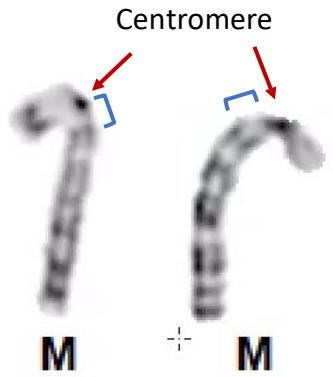
Sample	No. of meta. analysis	no. of normal looking meta	no. of aberr. meta	no. of cells with breaks	No. of cells with fusions	No. of tetra/polyp loid cells	No. of cells with C-anaphase morphology	Comment
SUM190	35	34	1	1	0	0	0	
SUM190-TDM1R	35	34	1	0	1	0	0	
SUM190-TDXdR	35	34	1	0	1	0	0	
HCC1954	35	31	4	2	3	1	0	1 cell with many aberrations
HCC1954-TDM1R	35	32	3	2	2	0	0	
HCC1954-TDXdR	35	32	3	1	2	0	0	

Supplemental Fig. 3

B.

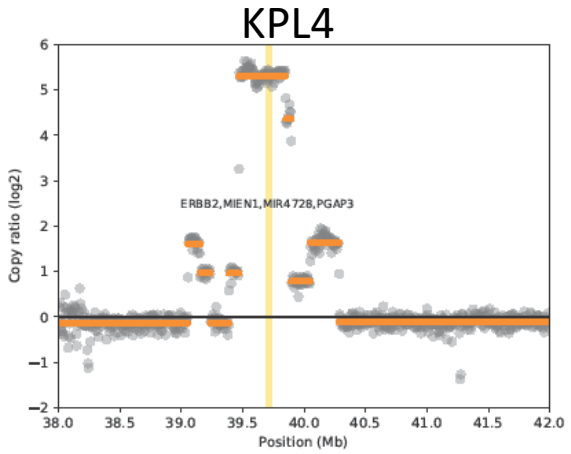


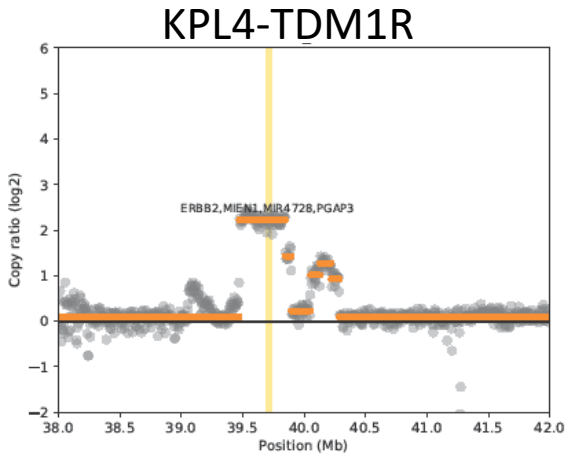
SUM190

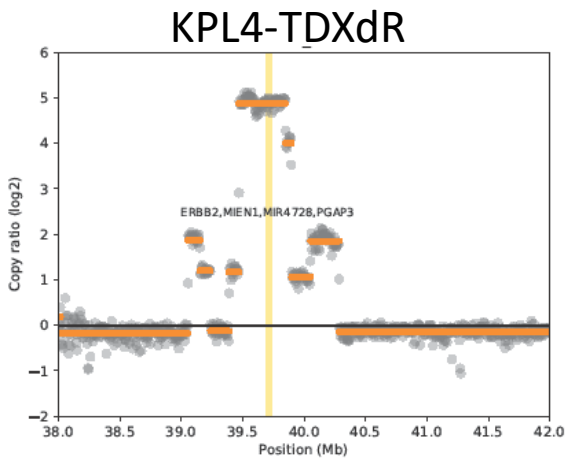


SUM190-TDXdR

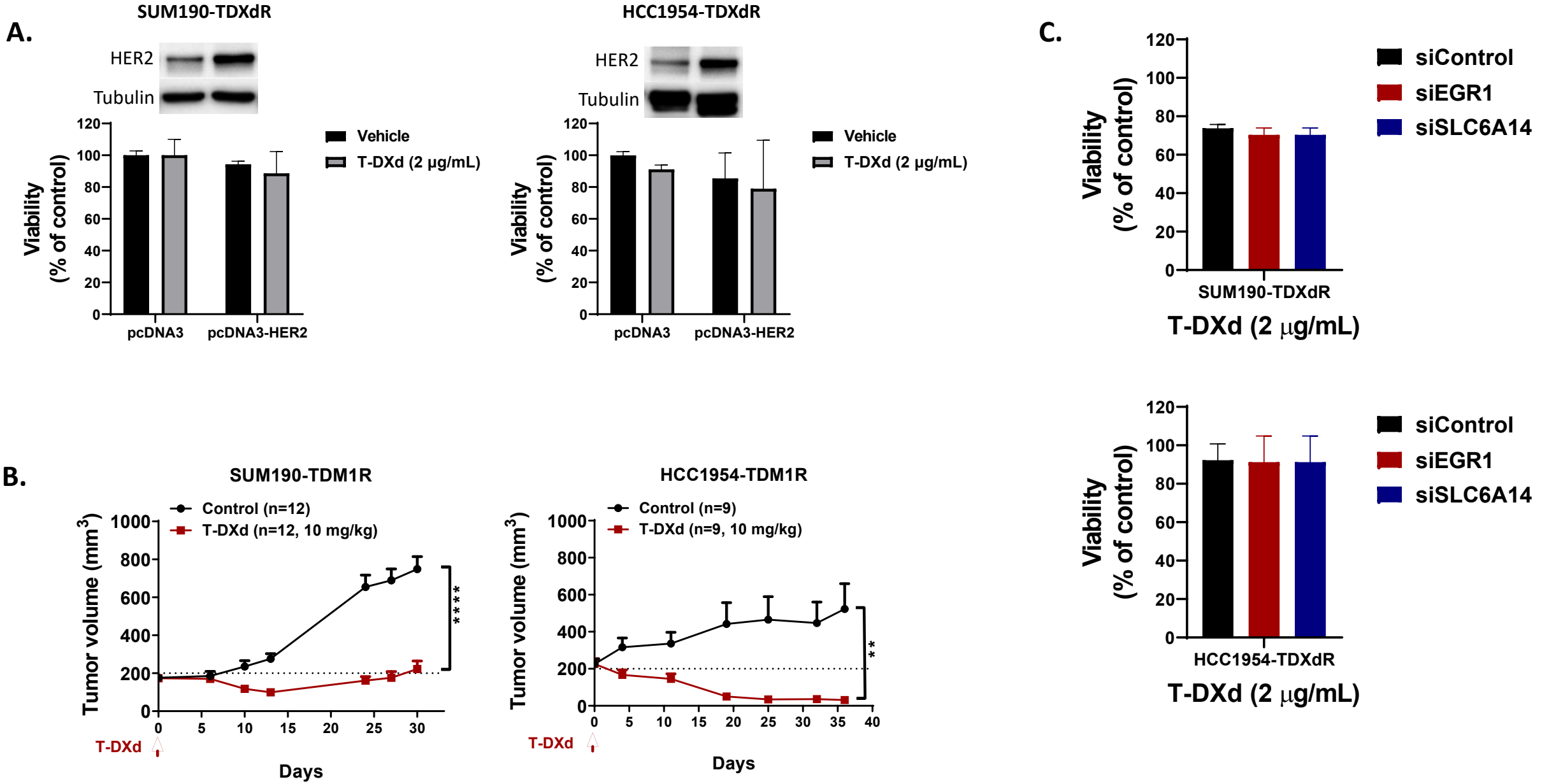
C.







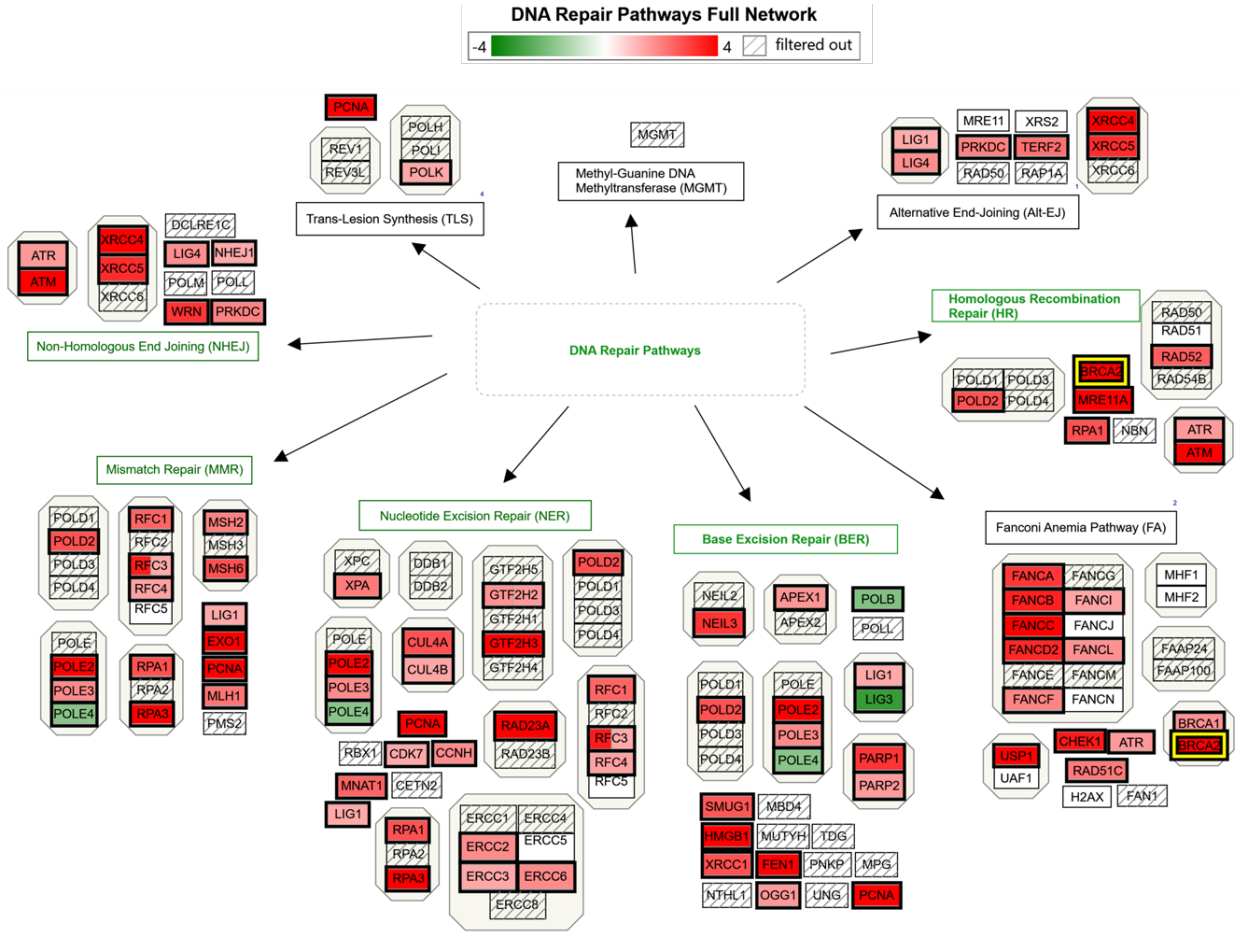
Supplemental Fig. 3



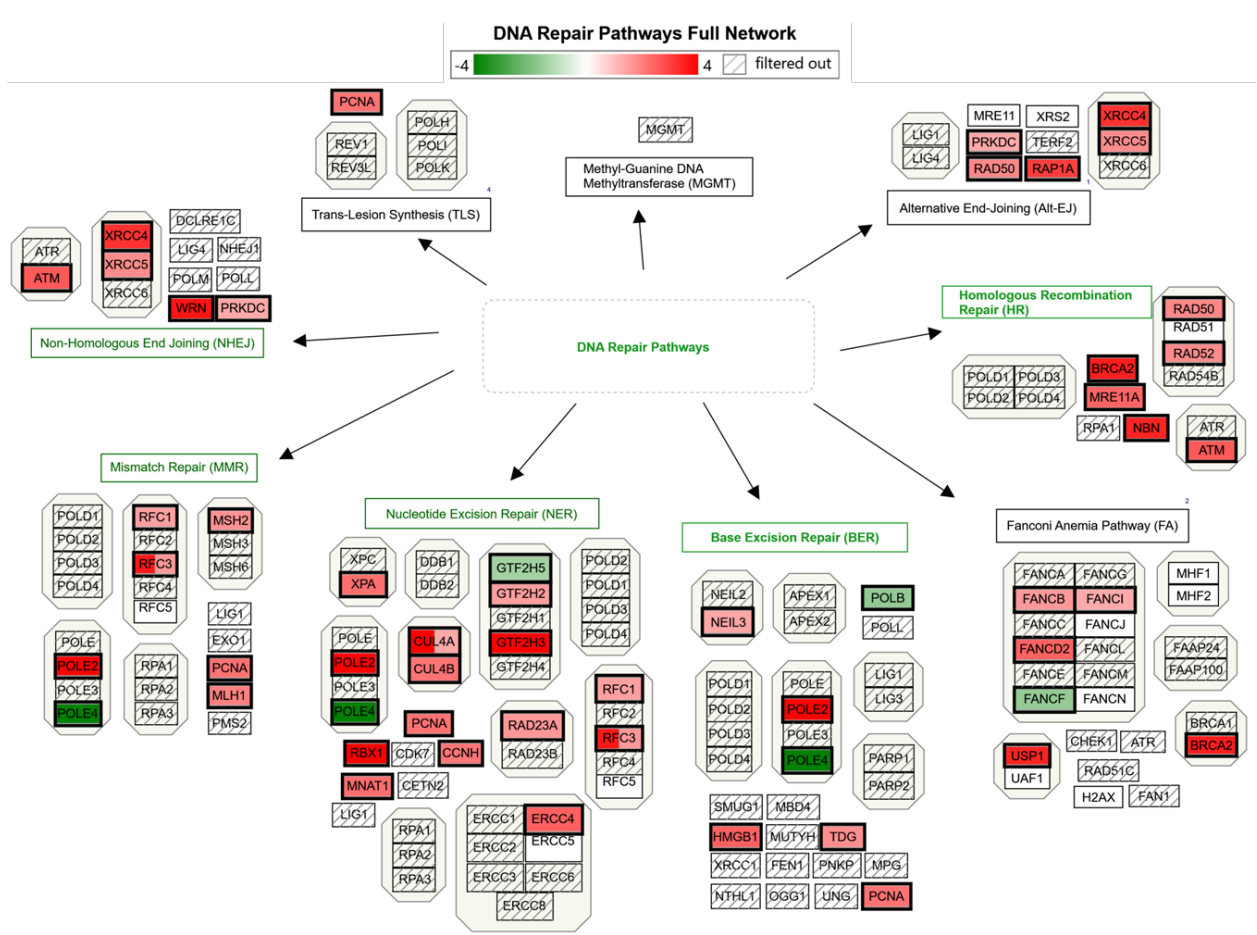
Supplemental Fig. 4

A.

SUM190-TDXdR



HCC1954-TDXdR



Supplemental Fig. 5

B.

SUM190-TDM1R

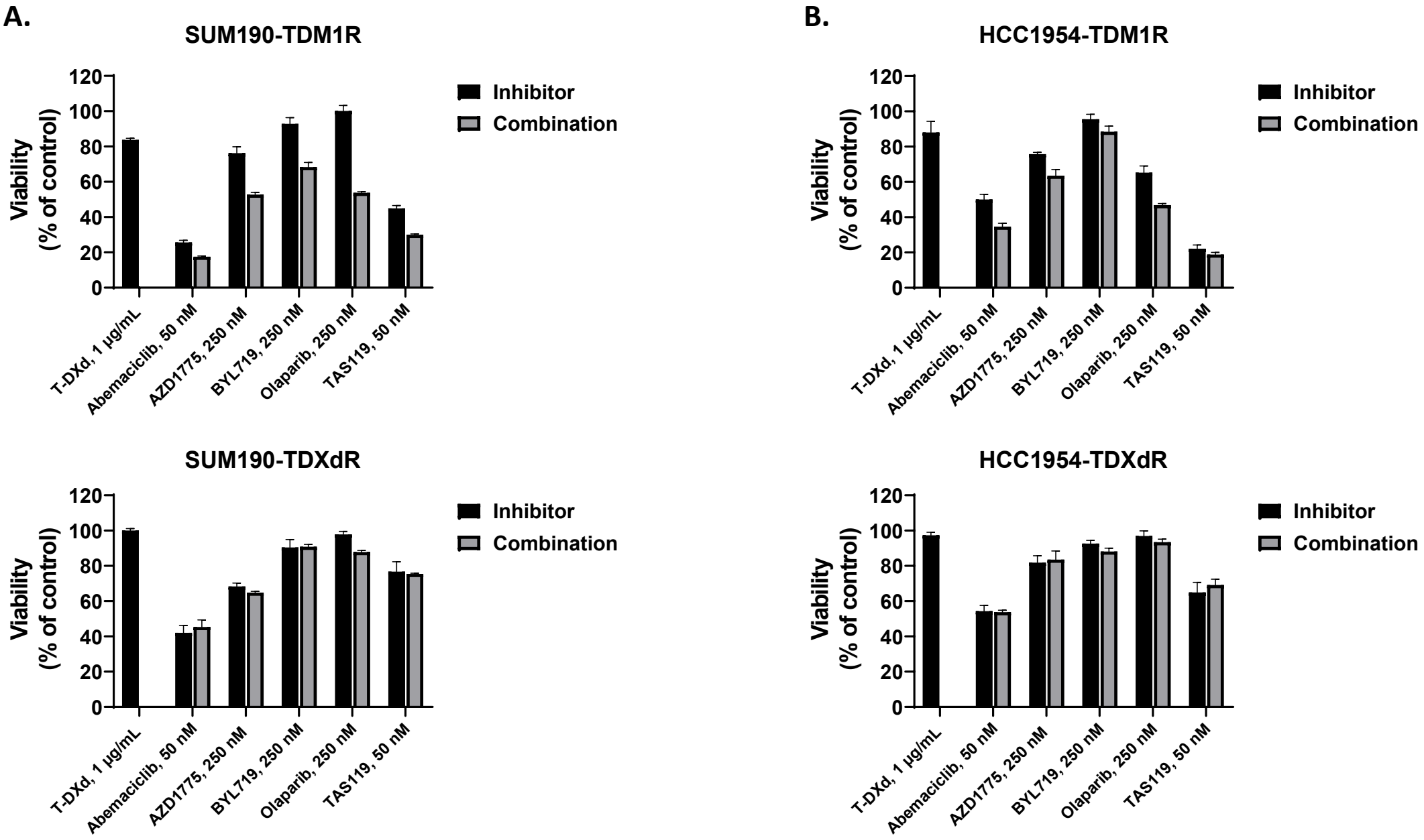
Ranking	Gene symbol	Accession	Ranking	Gene symbol	Accession
1	CSNK1E	NM_001894	26	LATS1	NM_004690
2	ASP	NM_031916	27	CAMK4	NM_001744
3	MGC42105	NM_153361	28	CARD10	NM_014550
4	AK2	NM_001625	29	MGC16169	NM_033115
5	FLJ11856	NM_024531	30	KSR2	NM_173598
6	AK3L1	NM_016282	31	PHKA1	NM_002637
7	RPS6KA6	NM_014496	32	ATR	NM_001184
8	PFKM	NM_000289	33	PDK4	NM_002612
9	AK7	NM_152327	34	CKM	NM_001824
10	PI4KII	NM_018425	35	AGTR2	NM_000686
11	CCL4	NM_002984	36	CDK7	NM_001799
12	PANK1	NM_138316	37	EDG1	NM_001400
13	AK1	NM_000476	38	KIT	NM_000222
14	FLJ31393	NM_153024	39	MIDORI	NM_020778
15	TLK1	NM_012290	40	IHPK2	NM_016291
16	C9ORF96	XM_291304	41	CHRM2	NM_000739
17	ATM	NM_138293	42	PSKH1	NM_006742
18	MPP2	NM_005374	43	PRKCI	NM_002740
19	TXNDC3	NM_016616	44	PRKG2	NM_006259
20	CSNK1G2	NM_001319	45	EIF2AK3	NM_004836
21	LIM	NM_006457	46	TRIB3	NM_021158
22	DGKD	NM_003648	47	GAP43	NM_002045
23	MGC5601	NM_025247	48	ERN1	NM_001433
24	DAPK3	NM_001348	49	PDK3	NM_005391
25	GMFB	NM_004124	50	BTK	NM_000061

C.

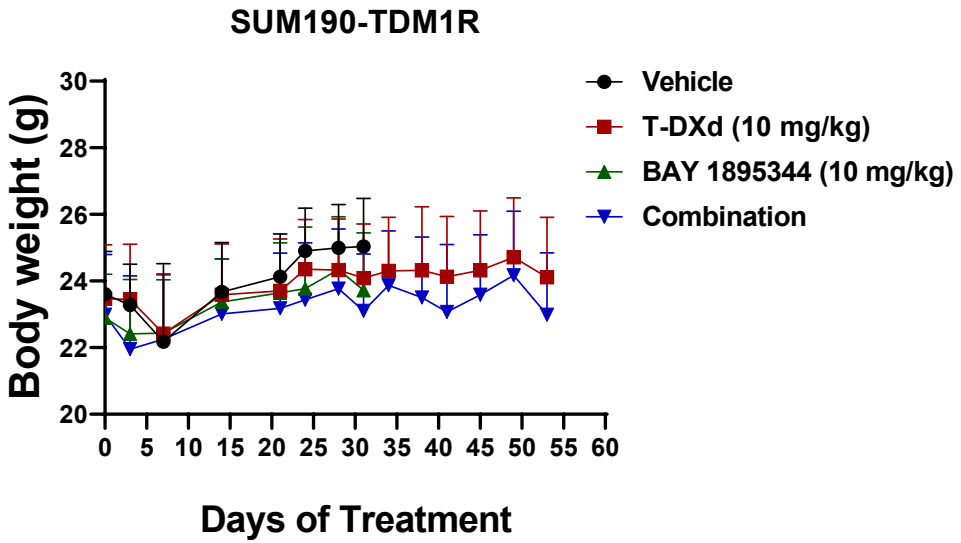
SUM190-TDXdR

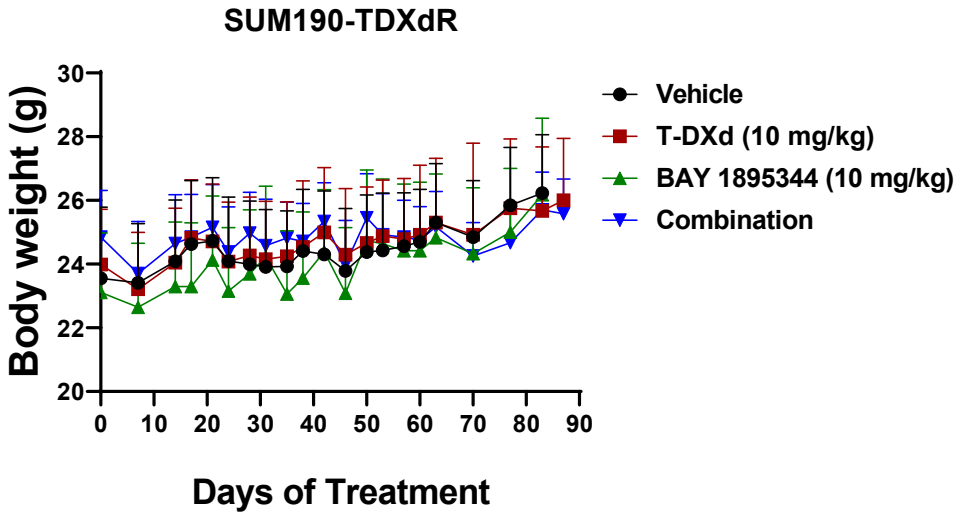
Ranking	Gene symbol	Accession	Ranking	Gene symbol	Accession
1	PRPSAP2	NM_002767	26	MAPK12	NM_002969
2	AKAP14	NM_178813	27	DGKQ	NM_001347
3	CERK	NM_022766	28	SRPK2	NM_182691
4	ADPGK	NM_031284	29	DYRK4	NM_003845
5	ROS1	NM_002944	30	PIP4K2A	NM_005028
6	NMRK1	NM_017881	31	DYRK3	NM_003582
7	PLK2	NM_006622	32	CAMK1D	NM_153498
8	LIMK1	NM_002314	33	PAPSS2	NM_004670
9	PAK2	XM_001126110	34	PHKG2	NM_000294
10	BMP2K	NM_198892	35	SGK1	NM_005627
11	MPP3	NM_001932	36	PRKG1	NM_006258
12	CDK4	NM_000075	37	AKAP8	NM_005858
13	MPP5	NM_022474	38	ATR	NM_001184
14	MPP7	NM_173496	39	BRSK2	NM_003957
15	PRKACA	NM_207518	40	ULK4	XM_929989
16	LY6G5B	NM_021221	41	PIP5K1C	NM_012398
17	PRKCB	NM_212535	42	CIB4	NM_001029881
18	LRPPRC	NM_133259	43	PASK	NM_015148
19	STK25	NM_006374	44	MAGI1	NM_173515
20	TGFBR1	NM_004612	45	ERBB4	NM_005235
21	STK32A	NM_145001	46	MAP3K1	XM_042066
22	NIM1	NM_153361	47	CASK	NM_003688
23	PDGFRB	NM_002609	48	TAOK3	NM_016281
24	TTBK2	NM_173500	49	MAP3K10	NM_002446
25	TRRAP	NM_003496	50	PIK3CD	NM_005026

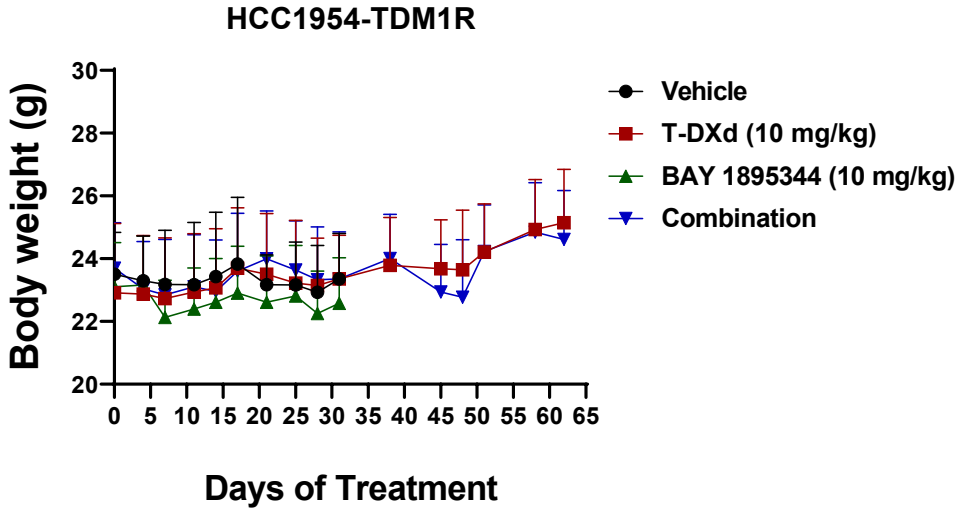
Supplemental Fig. 5

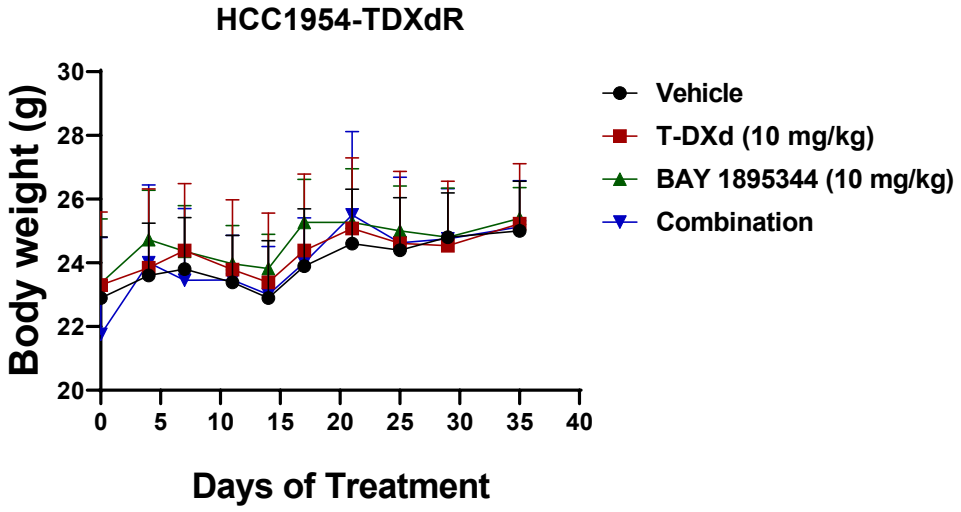


Supplemental Fig. 6









Supplemental Fig. 7