Supplemental Methods:

IC_{50} Analysis

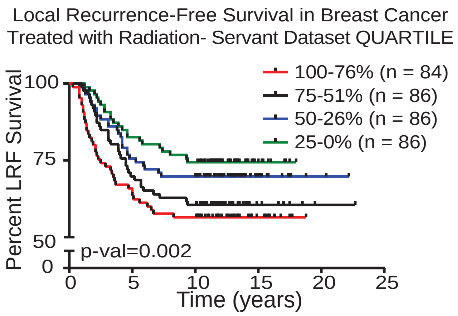
2,000 cells per well were plated in a 96 well plate and allowed to adhere overnight. Drug was added at varying concentrations (1nM-10µM) and the cells were incubated for an additional 72 hours. After 72 hours, AlamarBlue (Thermo-Fisher) was added at 1/10 the volume of media and incubated for 3 hours at 37˚C. The plate was read using a BioTek™ Cytation™ 3 Cell Imaging Multi-Mode Reader. A dose-response curved was made using GraphPad Prism 7 software.

Proliferation Assay

Cells were plated in 96 well plates (1,500 cells/well) and allowed to sit overnight. The following day cells were pretreated with DMSO or B909 for 1 hour before radiation (2 Gy). Cells were then placed in an IncuCyte® Machine which measured the confluence of the plate every 4 hours for ~4 days.

Supplemental Figure 1

S1A



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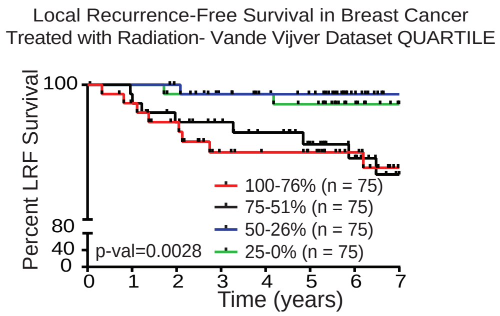

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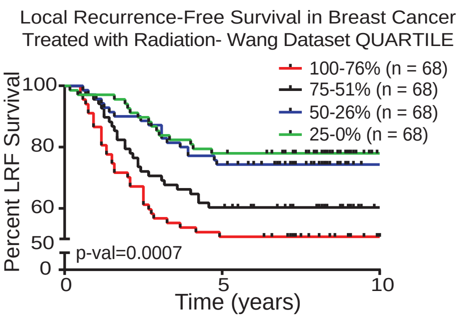

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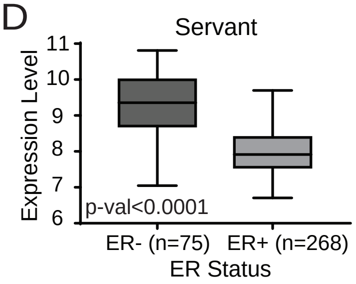

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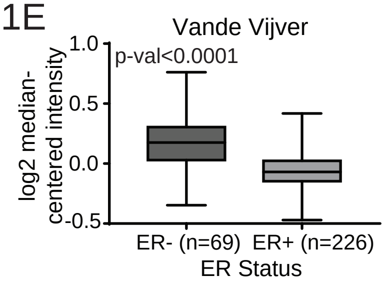

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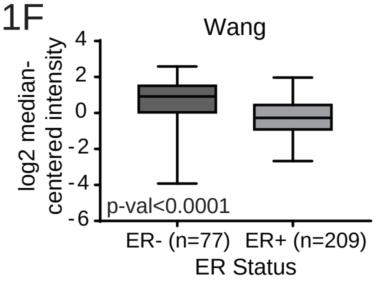

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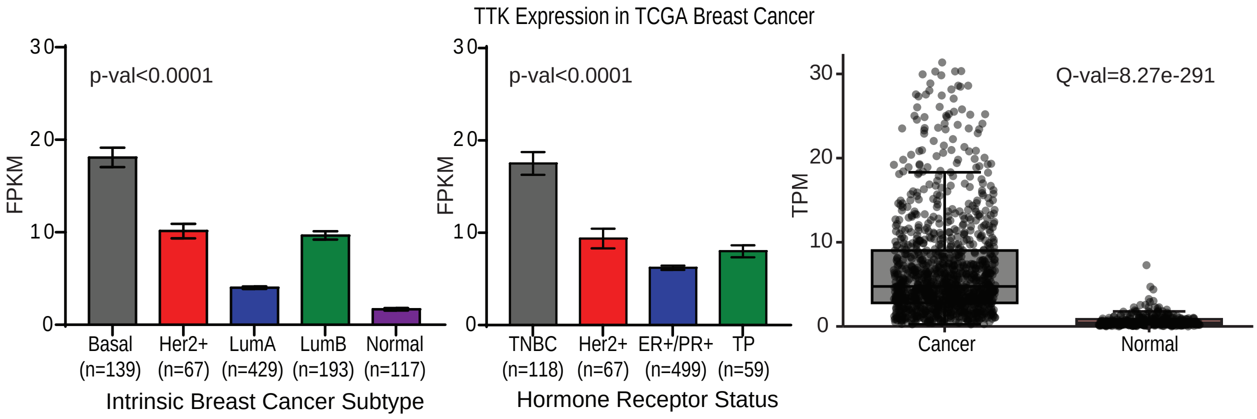

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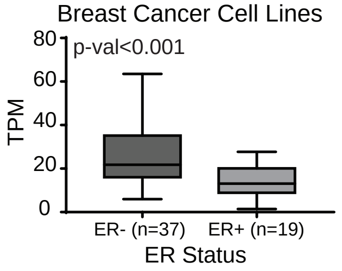

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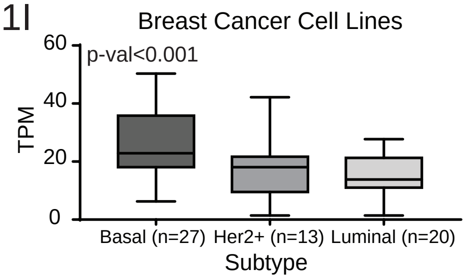

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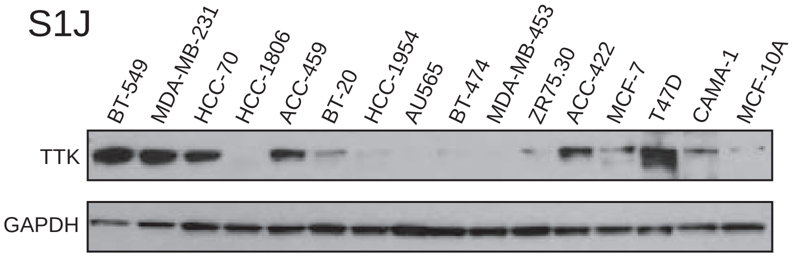
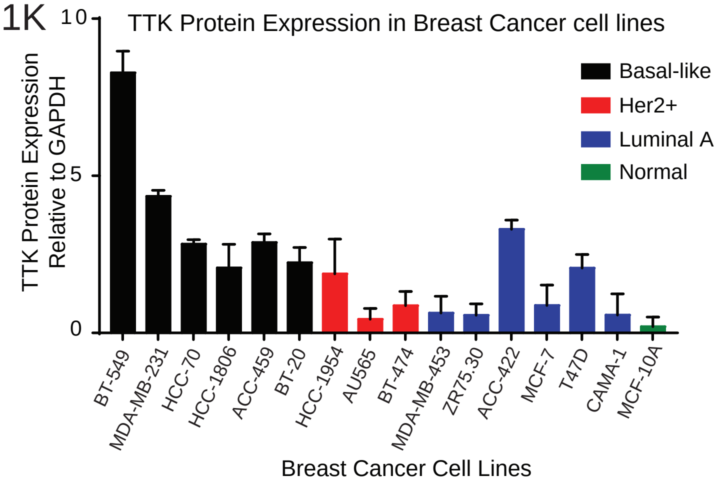


S1K



S1L

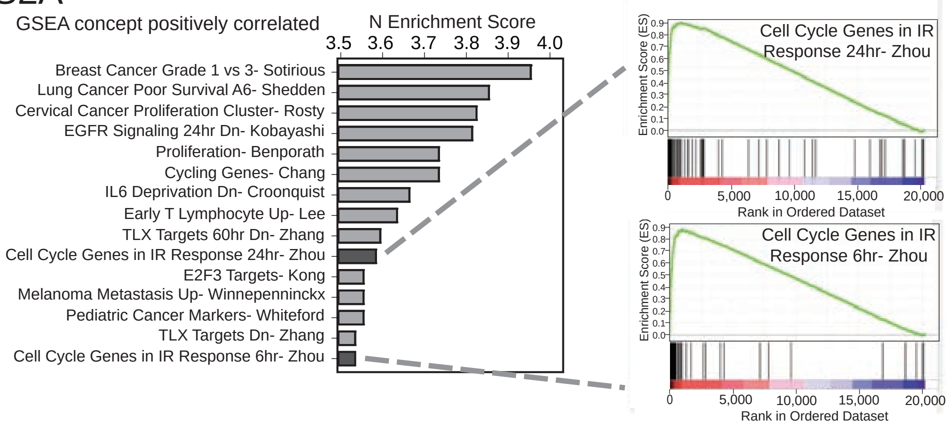
Cell Line Mutations

Cell Line	Mutations
MDA-MB-231	BRAF, CDKN2A, KRAS, NF2, TP53, PDGFRA
BT-549	PTEN, RB1, TP53
SUM-159	PIK3CA, TP53, HRAS
PDX-4664	EGFR, CK19, RPL39

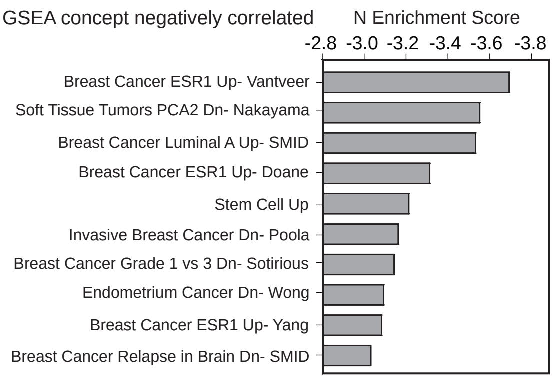
Supplemental Figure 1: TTK expression correlates with local recurrence-free survival (LRFS) in breast cancer (BC) and is overexpressed in estrogen receptor-negative (ER-) BC compared to estrogen receptor-positive (ER+) breast cancer. **A-C)** Kaplan Meier analysis by quartile expression demonstrates higher TTK expression correlates with decreased LRFS in the Servant (A), Vande Vijver (B), and Wang (C) datasets. **D-F)** TTK is overexpressed in ER- BC compared to ER+ BC in the Servant (D), Vande Vijver (E), and Wang (F) datasets. Log-rank (Mantel-Cox) test was used for analyses of survival curves. **G)** TTK is overexpressed in patients with TNBC/basal-like BC compared to other subtypes in the TCGA BC dataset. Additionally, TTK is overexpressed in BC compared to normal tissue. **H&I)** TTK is overexpressed in ER- BC cell lines compared to ER+ BC cell lines and TTK has highest expression in basal-like BC cell lines compared to other subtypes using CCLE data measured in transcripts per million (TPM). **J&K)** TTK protein expression is highest in MDA-MB-231 and BT-549 BC cell lines and TTK protein expression is highest in basal-like BC compared to other subtypes. **L)** Mutational landscapes of cell lines and PDX models used for *in vitro* and *in vivo* studies. Two-sided Student's *t*-test and one-way ANOVA were used for analyses. Error bars represent standard deviation.

Supplemental Figure 2

S2A



S2B



S2C

MDA-MB-231 shRNA Clonogenic Survival Assay

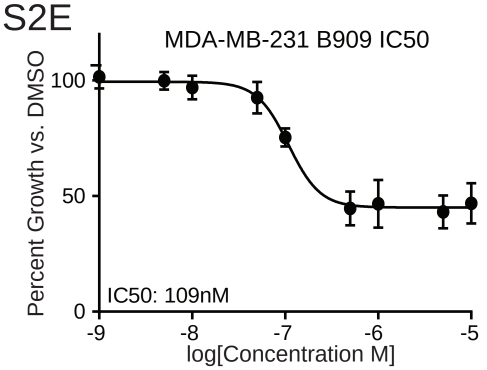
Treatment	Enhancement Ratio	Cytotoxicity
shControl No Dox	1.0	1.0
shControl Dox	0.98 ±0.04	1.00 ±0.05
shTTK#1 No Dox	1.0	1.0
shTTK#1 Dox	1.50 ±0.09	0.17 ±0.06
shTTK#2 No Dox	1.0	1.0
shTTK#2 Dox	1.23 ±0.02	0.73 ±0.24

S2D

BT-549 shRNA Clonogenic Survival Assay

Treatment	Enhancement Ratio	Cytotoxicity
shControl No Dox	1.0	1.0
shControl Dox	0.97 ±0.02	0.79 ±0.10
shTTK#1 No Dox	1.0	1.0
shTTK#1 Dox	1.23 ±0.02	0.91 ±0.25
shTTK#2 No Dox	1.0	1.0
shTTK#2 Dox	1.21 ±0.05	0.61 ±0.07

S2E



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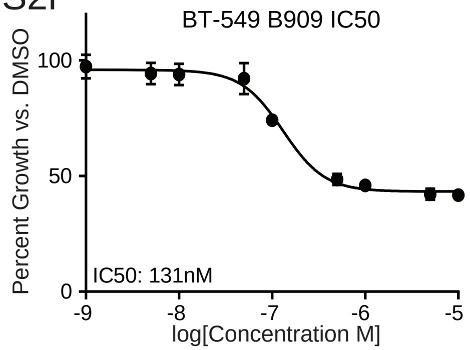

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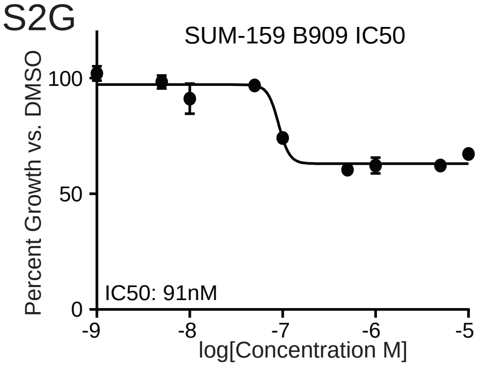
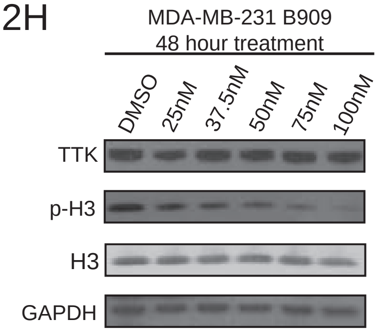


S2H



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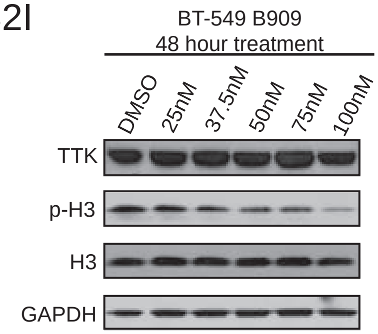

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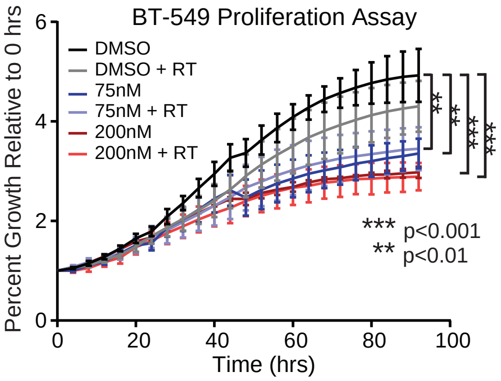


S2K

MDA-MB-231 B909 Clonogenic Survival Assay

Treatment	Enhancement Ratio	Cytotoxicity
DMSO	1.0	1.0
25nM	1.16 ±0.01	0.69 ±0.24
37.5nM	1.26 ±0.02	0.49 ±0.31
50nM	1.37 ±0.03	0.29 ±0.10

S2L

BT-549 B909 Clonogenic Survival Assay

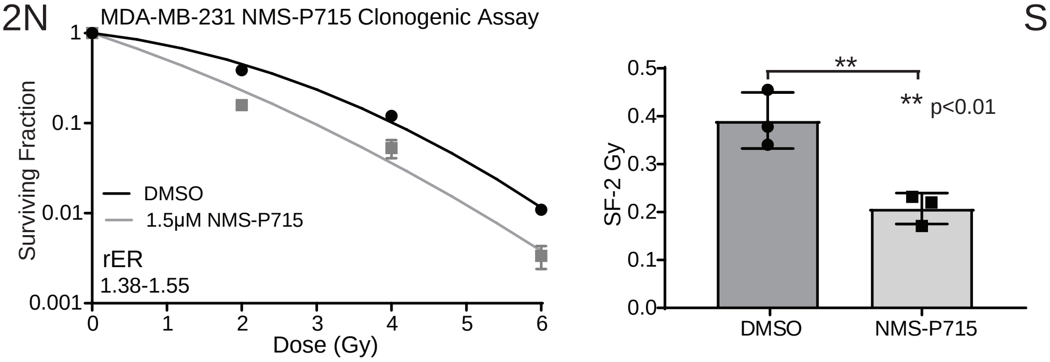
Treatment	Enhancement Ratio	Cytotoxicity
DMSO	1.0	1.0
25nM	1.14 ±0.03	0.71 ±0.19
37.5nM	1.20 ±0.07	0.87 ±0.13
50nM	1.32 ±0.07	0.41 ±0.06

S2M

SUM-159 B909 Clonogenic Survival Assay

Treatment	Enhancement Ratio	Cytotoxicity
DMSO	1.0	1.0
25nM	1.19 ±0.06	0.69 ±0.16
37.5nM	1.46 ±0.13	0.63 ±0.19
50nM	1.97 ±0.22	0.36 ±0.21

S2N



S2O

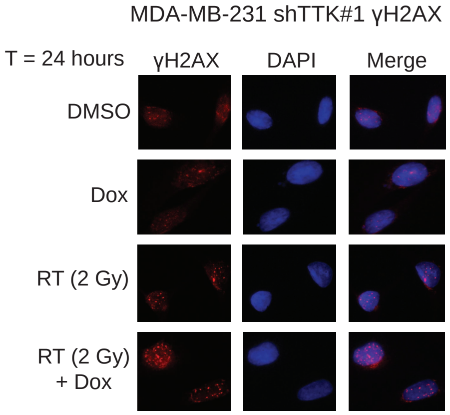
MDA-MB-231 NMS-P715 Clonogenic Survival Assay

Treatment	Enhancement Ratio	Cytotoxicity
DMSO	1.0	1.0
1.5μM	1.47 ±0.07	0.70 ±0.22

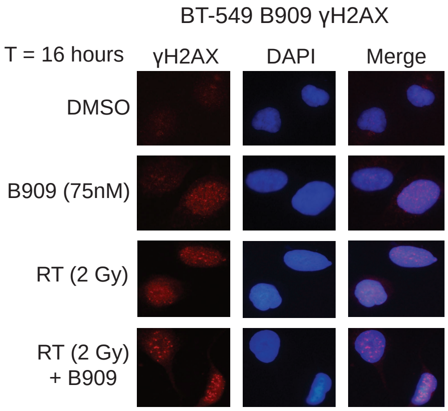
Supplemental Figure 2: Gene set enrichment analysis (GSEA) correlates TTK with radiation response and TTK inhibition radiosensitizes multiple basal-like breast cancer (BC) cell lines. **A)** GSEA identifies cell cycle genes at 6 and 24 hours as the top positively correlated concepts with TTK expression in TCGA BC dataset. **B)** GSEA concepts negatively correlated with TTK expression in the TCGA BC dataset. **C&D)** Summary of radiation enhancement ratios (rER) and cytotoxicity caused by TTK knockdown in MDA-MB-231 (C) and BT-549 (D) cells. **E-G)** IC50 of proliferation analyses of B909 in MDA-MB-231 (E) (109nM), BT-549 (F) (131nM), and SUM-159 (G) (91nM) cell lines. Error bars represent SEM. **H&I)** B909 reduces pH3 (Ser10) expression dose dependently in MDA-MB-231 (H) and BT-549 (I) cell lines. **J)** Proliferation assays of BT-549 cells treated with RT alone, B909 alone, or a combination treatment. Two-way ANOVA with Dunnett's Multiple Comparisons test was used to compare treatment groups. Data are a representation of 2-3 replicates. **K-M)** Radiation enhancement ratio (rER) and cytotoxicity of B909 clonogenic survival assays in MDA-MB-231 (J), BT-549 (K), and SUM-159 (L) cell lines. **N)** Clonogenic survival assay and SF-2 Gy in MDA-MB-231 cells using the TTK inhibitor NMS-P715 (1.5µM). **O)** Summary of radiation enhancement ratios (rER) and cytotoxicity caused by NMS-P715. Unless otherwise stated, data represent the mean of three independent experiments. Error bars represent SEM for clonogenic survival assays and proliferation assays and standard deviation for SF-2 Gy. A Two-sided Student's *t*-test was used for comparison. $^{**}p<0.01$, $^{***}p<0.001$

Supplemental Figure 3

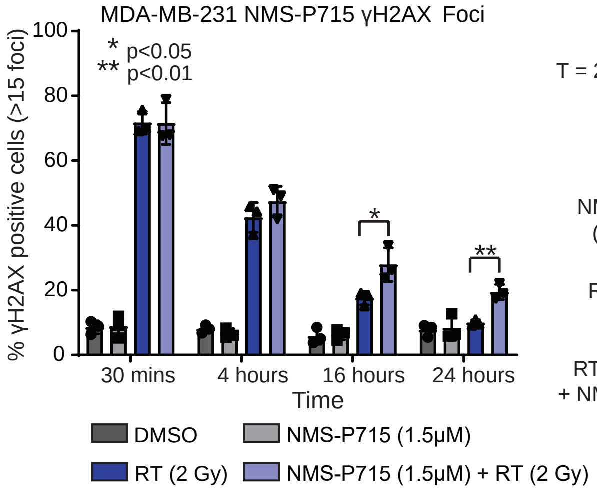
S3A


MDA-MB-231 shTTK#1 γH2AX

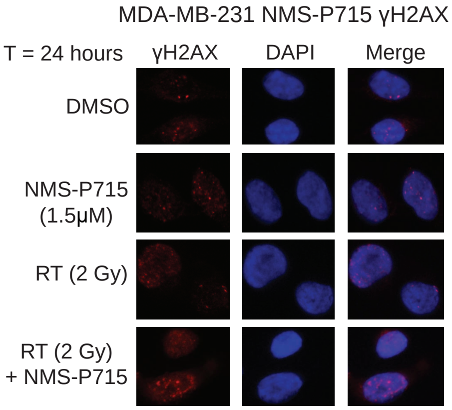
S3B


BT-549 B909 γH2AX

S3C


MDA-MB-231 NMS-P715 γH2AX Foci

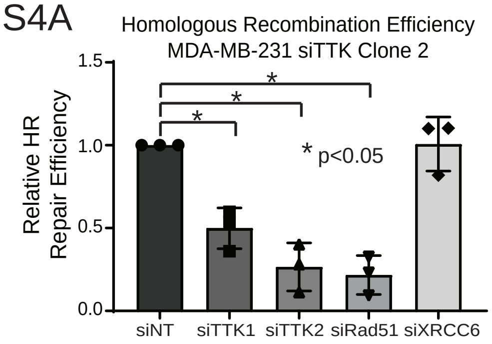
S3D


MDA-MB-231 NMS-P715 γH2AX

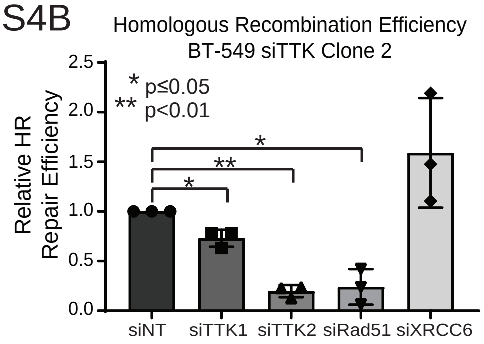
Supplemental Figure 3: Representative images of γH2AX foci. **A)** Representative images of γH2AX foci in MDA-MB-231 shTTK#1 cells 24 hours after radiation. **B)** Representative images of γH2AX foci in BT-549 cells treated with Bayer 1161909 at 16 hours after radiation. **C)** Combination treatment of TTK inhibition (NMS-P715) and RT lead to persistent double strand DNA damage over time. **D)** Representative images of γH2AX foci at 24 hours in MDA-MB-231 cells treated with NMS-P715. Data represent the mean of three independent experiments repeated in triplicate, with ~100 cells counted for each experiment, and error bars represent standard deviation. Two-sided Student's *t*-test was used for comparison. * $p < 0.05$, ** $p < 0.01$

Supplemental Figure 4

S4A



S4B



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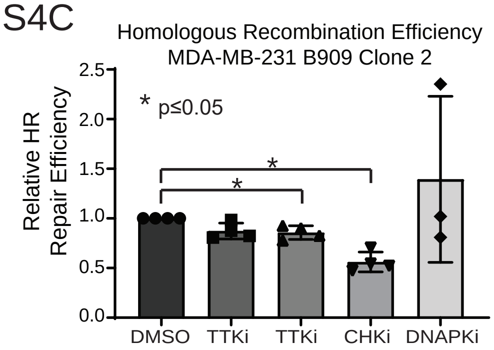

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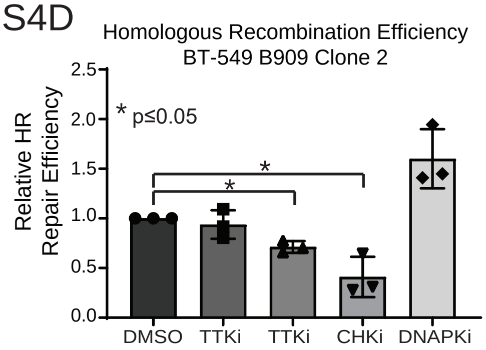

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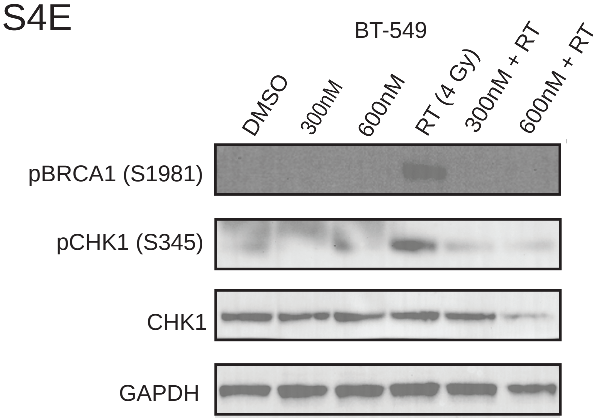

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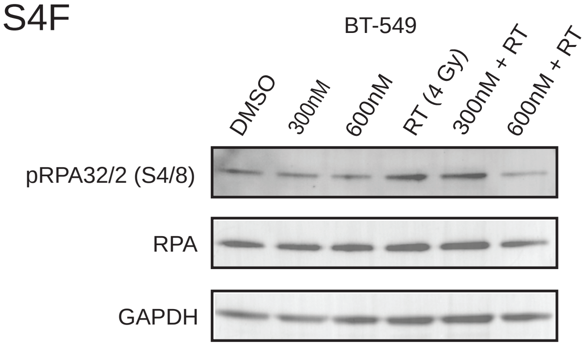
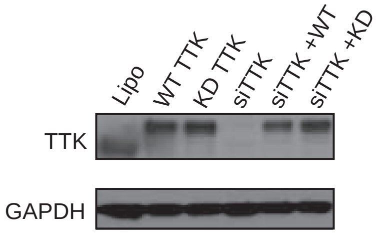


Supplemental Figure 4: Homologous recombination (HR) efficiency is reduced by TTK inhibition in a second stable HR specific reporter clone and through western blot analysis. **A&B)** Knockdown of TTK by siRNA significantly reduces HR efficiency in MDA-MB-231 (A) and BT-549 (B) cells. **C&D)** TTK inhibition by Bayer 1161909 reduces HR efficiency in MDA-MB-231 (C) and BT-549 (D) cells. Data represent the mean of 3-4 independent experiments and error bars represent standard deviation. **E)** TTK inhibition reduces phospho-BRCA1 and phospho-CHK1 after RT compared to RT alone in BT-549 cells. Western blots represent two independent experiments. **F)** TTK inhibition reduces phospho-RPA after RT compared to RT alone in and BT-549 cells. Western blots represent two independent experiments. One-way ANOVA with Dunnett's multiple comparisons test was used for comparison. $^*p \leq 0.05$, $^{**}p < 0.01$.

Supplemental Figure 5

S5A

MDA-MB-231



S5B

MDA-MB-231 TTK Rescue Clonogenic Survival Assay

Treatment	Enhancement Ratio	Cytotoxicity
Lipo	1.0	1.0
siTTK	1.19 ±0.06	0.69 ±0.16
siTTK + WT TTK	1.46 ±0.13	0.63 ±0.19
siTTK + KD TTK	1.97 ±0.22	0.36 ±0.21

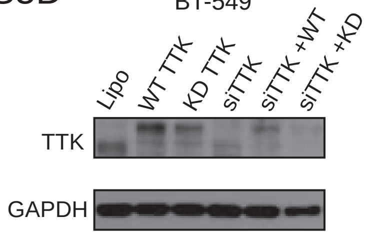
S5C

SUM-159 TTK Rescue Clonogenic Survival Assay

Treatment	Enhancement Ratio	Cytotoxicity
Lipo	1.0	1.0
siTTK	1.48 ±0.15	0.15 ±0.11
siTTK + WT TTK	1.03 ±0.09	0.45 ±0.18
siTTK + KD TTK	1.29 ±0.09	0.41 ±0.15

S5D

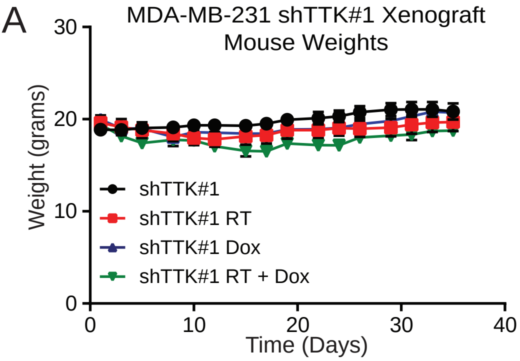
BT-549



Supplemental Figure 5: TTK rescue representative western blots and clonogenic assay cytotoxicity information. **A)** Representative western blot of TTK knockdown by siRNA and overexpression of wild-type (WT) or kinase dead (KD) TTK. **B&C)** Cytotoxicity and radiation enhancement ratio information for MDA-MB-231 (B) and SUM-159 (C) clonogenic assays. D) Representative western blot of TTK knockdown by siRNA and overexpression of WT or KD TTK in the BT-549 cell line. Western blots are representative of duplicate experiments.

Supplemental Figure 6

S6A

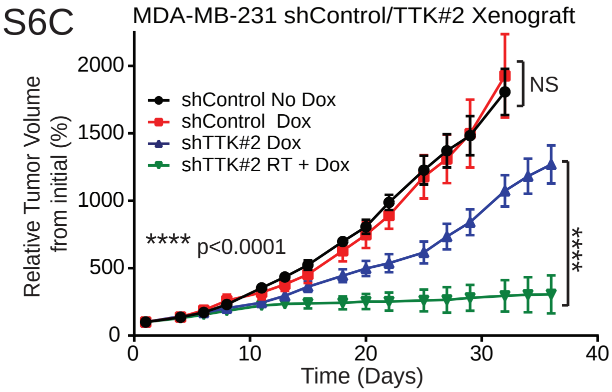


S6B

MDA-MB-231 shTTK#1 Xenograft
Fractional Tumor Volume (FTV)

			Combination		
Day	Dox	RT	Expected	Observed	Ratio
10	0.79	0.95	0.75	0.79	0.96
22	0.60	0.58	0.34	0.37	0.94
31	0.51	0.40	0.21	0.23	0.89
33	0.51	0.38	0.19	0.21	0.91
35	0.50	0.35	0.17	0.20	0.92

S6C



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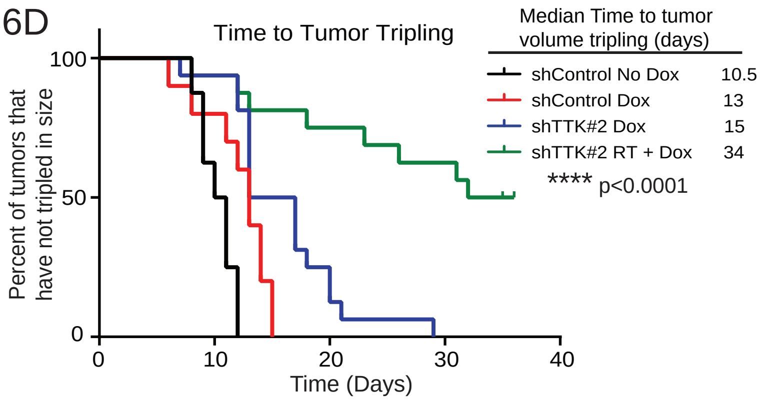
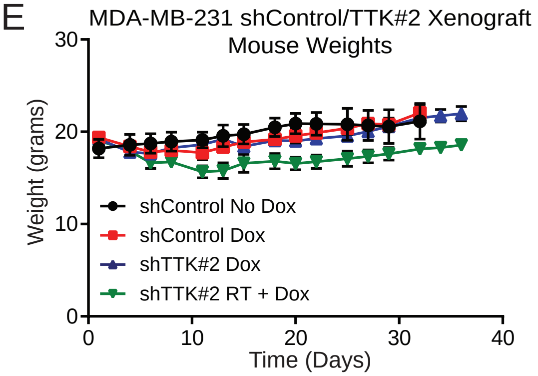
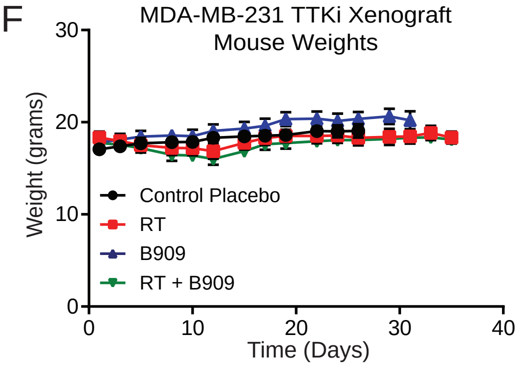


S6E



S6F

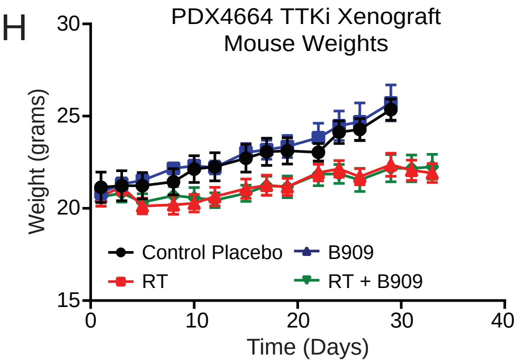


S6G

MDA-MB-231 B909 Xenograft
Fractional Tumor Volume (FTV)

			Combination		
Day	RT	B909	Expected	Observed	Ratio
8	0.77	0.80	0.62	0.69	0.90
15	0.52	0.67	0.35	0.42	0.83
22	0.38	0.74	0.28	0.25	1.12
24	0.37	0.78	0.29	0.23	1.24
26	0.35	0.76	0.27	0.21	1.28

S6H



S6I

PDX4664 B909 Xenograft
Fractional Tumor Volume (FTV)

			Combination		
Day	RT	B909	Expected	Observed	Ratio
8	0.73	0.74	0.54	0.59	0.92
15	0.50	0.72	0.36	0.34	1.07
22	0.39	0.85	0.33	0.23	1.43
24	0.36	0.89	0.32	0.21	1.52
26	0.34	0.88	0.30	0.19	1.63

Supplemental Figure 6: *In vivo* studies additional information. **A)** MDA-MB-231 shTTK#1 mouse weights from *in vivo* study. **B)** Additive/synergistic analysis for MDA-MB-231 shTTK#1 *in vivo* study using fractional tumor volume (FTV) method. **C)** In a second dox inducible MDA-MB-231 shTTK cell line (shTTK#2), tumor growth is inhibited by a combination of dox and RT compared to dox alone, while dox has no effect on shControl cells. Two-sided Student's *t*-test was used for analyses. Error bars represent standard error of the mean. **D)** Combination treatment (dox + RT) leads to increased time to tumor tripling, while dox alone has no effect on shControl cells (n=16 tumors per group). Log-rank (Mantel-Cox) tests were used for analyses. **E)** Mouse weights from MDA-MB-231 shControl/shTTK#2 *in vivo* study. **F)** Mouse weights from MDA-MB-231 Bayer 1161909 (B909) *in vivo* study. **G)** Additive/synergistic analysis for MDA-MB-231 B909 *in vivo* study using the FTV method. **H)** Mouse weights from the PDX4664 orthotopic study. **I)** Additive/synergistic analysis for PDX4664 B909 *in vivo* study using the FTV method. ****p<0.0001.

Supplemental Table 1:

Antibody	**Company**	**Number**	**Clone**	**Dilution/Conc. Used/Sequence**
TTK	Santa Cruz	376842	D-8	1:1,000
RAD51	Santa Cruz	8349	H-92	1:2,000
GAPDH	Cell Signaling	2118	14C10	1:10,000
Histone 3	Cell Signaling	9715S	polyclonal	1:1,000
p-Histone 3 (Ser10)	Cell Signaling	9701S	polyclonal	1:1,000
Mouse Secondary	Cell Signaling	7076s	polyclonal	1:10,000
Rabbit Secondary	Cell Signaling	7074s	polyclonal	1:10,000
pRPA S4/8	Bethyl Lab	BL-165-5F1	BL-165-5F1	1:1,000
RPA	Cell Signaling	52448	polyclonal	1:1,000
pKu80 T714	Invitrogen	38118	polyclonal	1:1,000
Ku80	Cell Signaling	2180	C48E7	1:1,000
pChk1 S345	Cell Signaling	2341S	polyclonal	1:1,000
Chk1	Santa Cruz	8408	G-4	1:1,000
pBRCA1 S1524	Cell Signaling	9009S	polyclonal	1:1,000
BRCA1	Santa Cruz	6954	D-9	1:1,000
γH2AX (Ser139)	Millipore	3076468	JBW301	1:2,000
Goat anti-Mouse IgG Alexa Fluor 594	ThermoFisher	A-11005	polyclonal	1:2,000
RAD51	Santa Cruz	8349	H-92	1:300
Goat anit-Rabbit Secondary Alexa Fluor 488	ThermoFisher	A-11008	polyclonal	1:2,000
DAPI ProLong Gold Antifade Mountant	ThermoFisher	P36931		Manufacturer's Instructions
Drugs/Compounds				
Bayer 1161909 (Empesertib)	MedChemExpress	HY-12858		Varied
NMS-P715	MedChemExpress	HY-12382		1.5µM
AZD7762	Gift from Morgan Lab			100nM
NU7441	Selleckchem	Ku-57788		1.5µM
Doxycycline hyclate	Sigma	D9891		2.0ug/mL
G418 (Geneticin)	ThermoFisher	10131035		300ug/mL
siRNA/shRNA				
siTTK#1	Dharmacon			TGAACAAAGTGAGAGACAT
siTTK#2	Dharmacon			TTGGACTGTTATACTCTTGAA
siRAD51	Dharmacon	J-003530-10		
siXRCC6	Dharmacon	L-005084-00		SMARTpool
ON-TARGETplus Non-targeting Control Pool	Dharmacon	D-001810-10		Pooled
TRIPZ Inducible Lentiviral shRNA (TTK)	Dharmacon	RHS4696-201906503		TTCTTTTCATTTAACACCT
Primers - NHEJ Reporter Systen				
Rejoined F	IDT			GCTGGTTTAGTGAACCGTCAG
Rejoined R	IDT			GCTGAACTTGTGGCCGTTTA
Uncut F	IDT			TACATCAATGGGCGTGGATA
Uncut R	IDT			AAGTCCCGTTGATTTTGGTG

Supplemental Table 1: Extended information for reagents used.