Supplement Information

A.

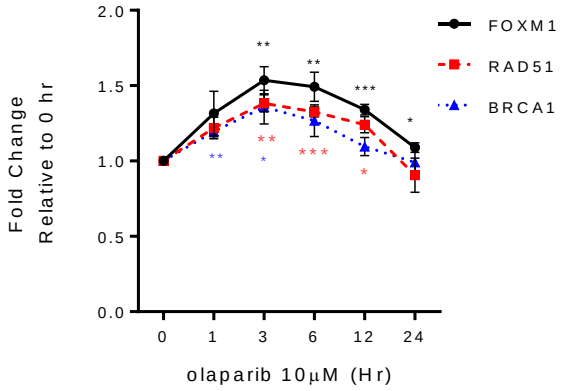


Fig S1. Olaparib increases BRCA1 and RAD51 protein level in ES-2 cells. (A) Quantification of protein level of FOXM1, RAD51 and BRCA1 after treatment with olaparib for 0, 1, 3, 6, 12 and 24 hours. Densitometry from 3 different western blots from 3 independent experiemnts. Data shown as Mean ± SEM. Statistics was performed with Student's t-test. *=p<0.05, **=p<0.01, ***=p<0.001.

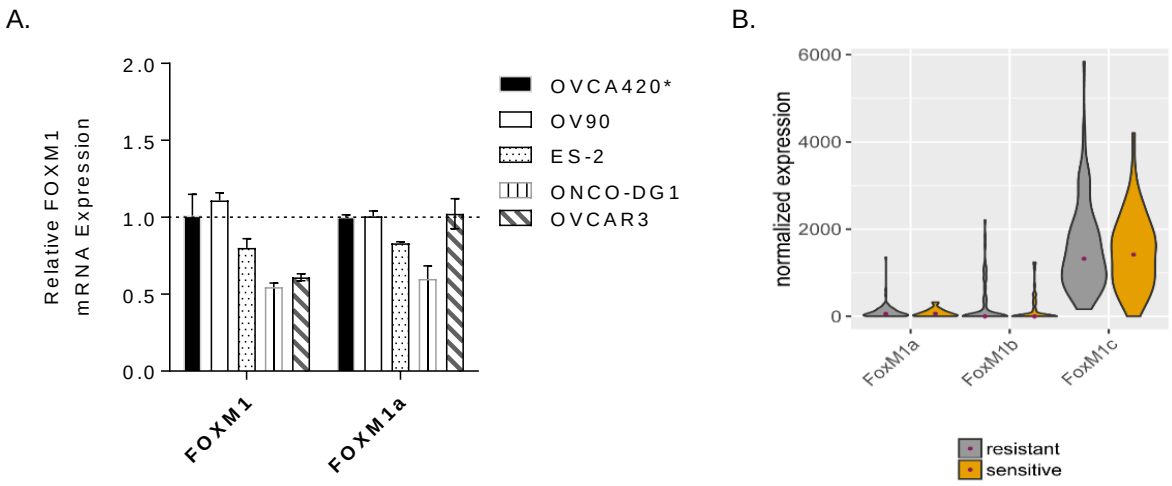
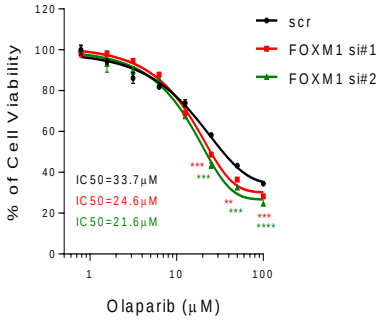
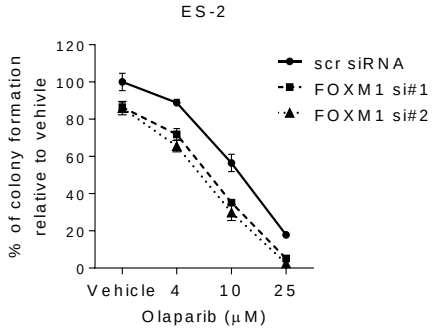


Fig S2. FOXM1c is the most abundantly expressed in ovarian cancer. (A) Quantification of *FOXM1* total mRNA and isoform a in multiple ovarian cancer cell lines by qRT-PCR. Data were shown as Mean ± SEM. (B) FOXM1c is the most abundantly expressed isoform in ovarian cancer patient tumor samples. Normalized expression of FOXM1a, FOXM1b and FOXM1c isoforms in chemotherapy treated patients from TCGA database.

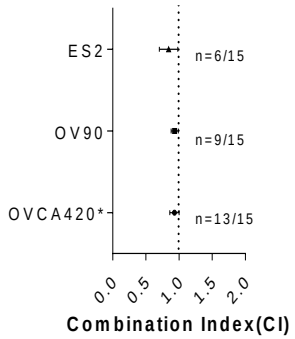
A.


scr
FOXM1 si#1
FOXM1 si#2
% of Cell Viability
IC50=33.7μM
IC50=24.6μM
IC50=21.6μM
Olaparib (μM)

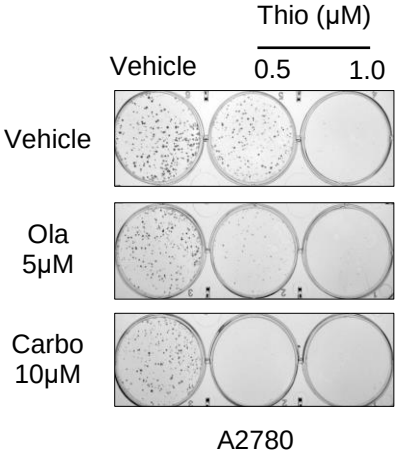
B.


ES-2
scr siRNA
FOXM1 si#1
FOXM1 si#2
% of colony formation relative to vehivle
Vehicle 4 10 25
Olaparib (μM)

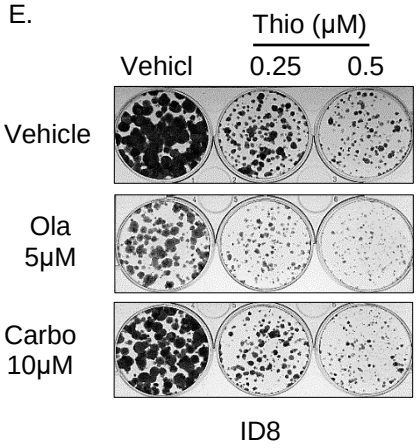
C.


ES2
OV90
OVCA420*
n=6/15
n=9/15
n=13/15
Combination Index(CI)

D.


Thio (μM)
Vehicle
0.5
1.0
Vehicle
Ola 5μM
Carbo 10μM
A2780

E.


Thio (μM)
Vehicl
0.25
0.5
Vehicle
Ola 5μM
Carbo 10μM
ID8

F.

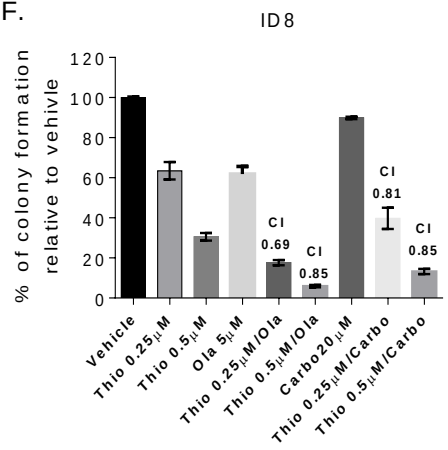

ID8
% of colony formation relative to vehivle
CI 0.69
CI 0.85
CI 0.81
CI 0.85
Vehicle
Thio 0.25μM
Thio 0.5μM
Ola 5μM
Thio 0.25μM/Ola
Thio 0.5μM/Ola
Carbo20μM
Thio 0.25μM/Carbo
Thio 0.5μM/Carbo

Fig S3. Thiostrepton synergizes with olaparib as well as with carboplatin in different ovarian cancer cells. (A-B) Cell survival curves and with scr siRNA or FOXM1 siRNA in ES-2 cells. ES-2 cells were transfected with scr siRNA or FOXM1 siRNAs and treated with increasing concentrations of olaparib for 72 hrs followed by SRB assays (A) and colony formation assays (B) Statistics was performed with Student's t-test. **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$. (C) Combination index between thiostrepton and olaparib in ES-2, OV90 and OVCA420* cells. Cells were seeded in 96-well plates and treated with different combinations of thiostrepton and olaparib for 3 days before SRB cell viability assay. Combination Index from combinations that gave effect between 0.2-0.8 were used. Data shown as mean with 95% confidence interval. The two drugs are synergistic if CI<1.0, additive if CI=1.0 and antagonistic if CI>1.0. (D) Thiostrepton enhanced effects of olaparib and carboplatin in A2780 cells. 500 cells/well in 6-well plates for colony formation assay. (E-F) Thiostrepton is synergistic with both olaparib and carboplatin in ID8 cell. 500 cells/well were seeded in 6-well plates for clonogenic assay (E). (F) After imaging, stained colonies were dissolved and measured fluorescent intensity on a plate reader, effects were shown as percentage of colony formation relative to vehicle group. Data shown as mean ± SEM. Combination index (CI) was calculated and shown on the graph.

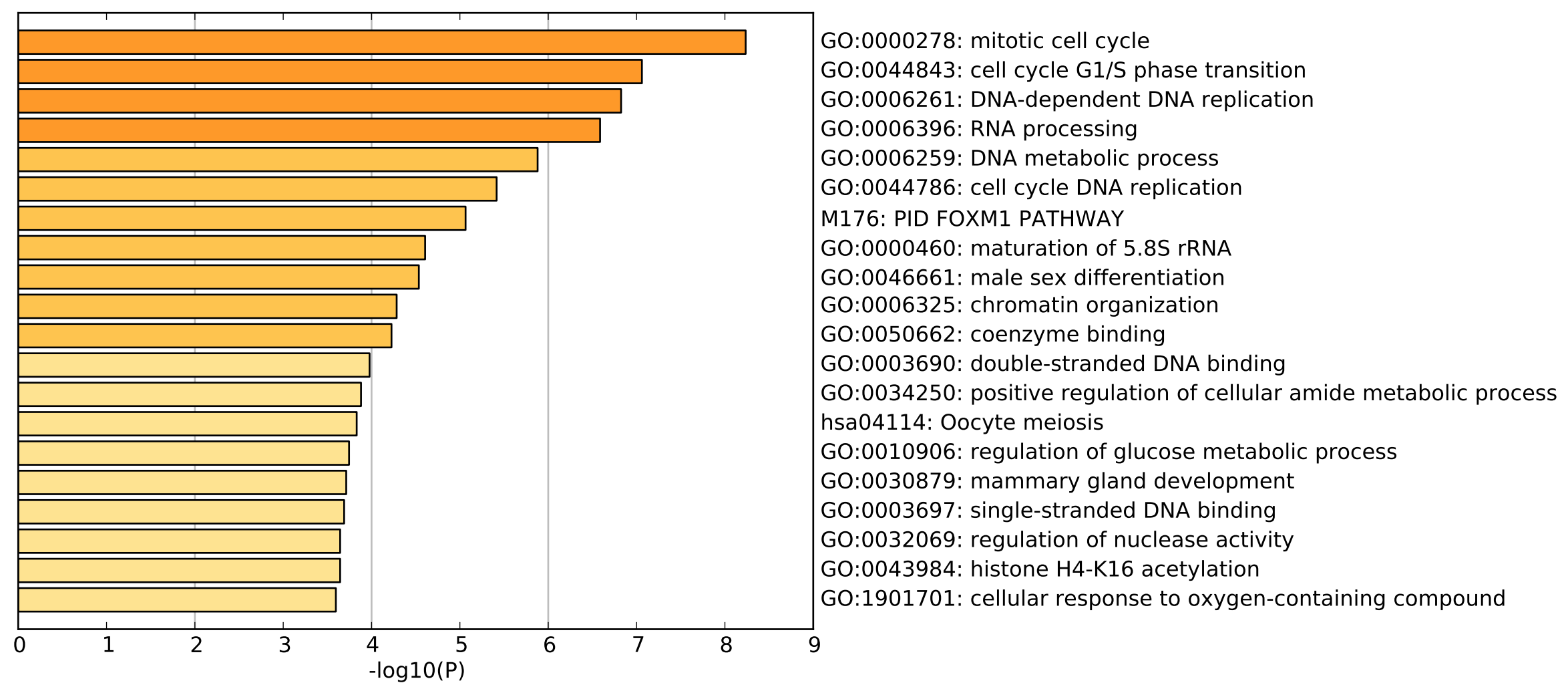
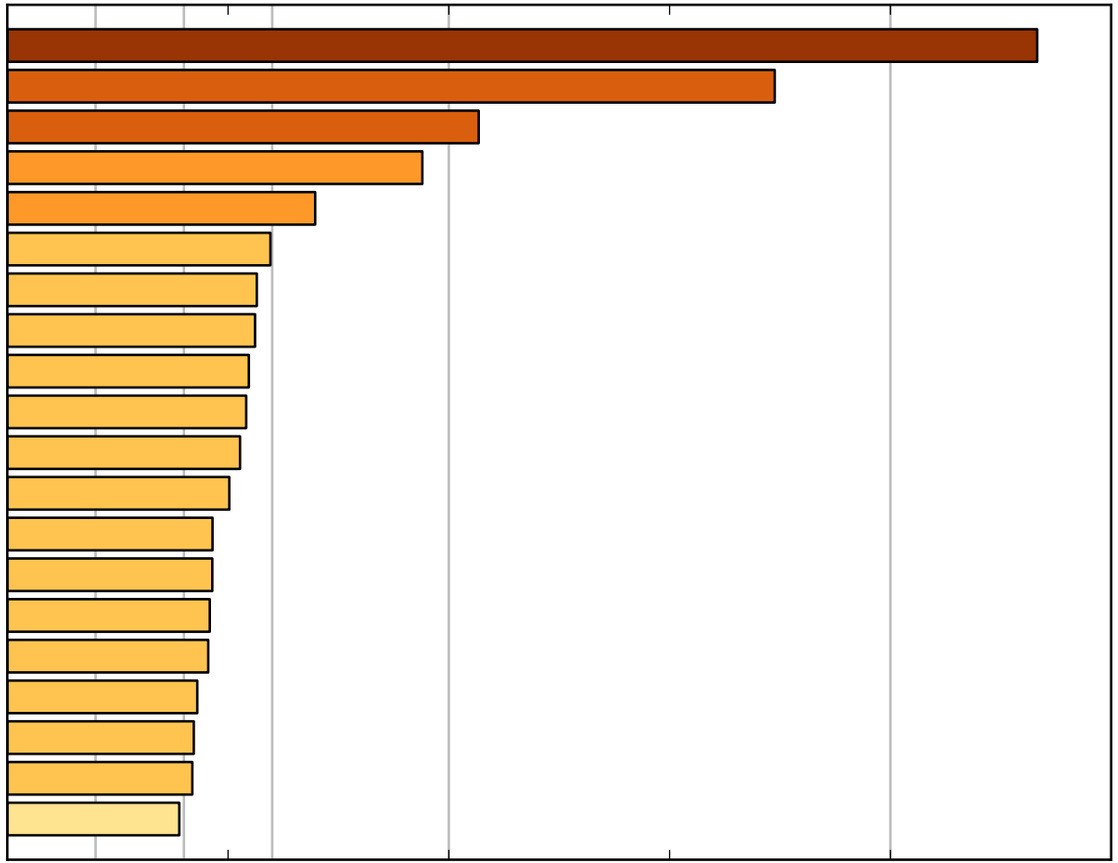

GO:0000278: mitotic cell cycle
GO:0044843: cell cycle G1/S phase transition
GO:0006261: DNA-dependent DNA replication
GO:0006396: RNA processing
GO:0006259: DNA metabolic process
GO:0044786: cell cycle DNA replication
M176: PID FOXM1 PATHWAY
GO:0000460: maturation of 5.8S rRNA
GO:0046661: male sex differentiation
GO:0006325: chromatin organization
GO:0050662: coenzyme binding
GO:0003690: double-stranded DNA binding
GO:0034250: positive regulation of cellular amide metabolic process
hsa04114: Oocyte meiosis
GO:0010906: regulation of glucose metabolic process
GO:0030879: mammary gland development
GO:0003697: single-stranded DNA binding
GO:0032069: regulation of nuclease activity
GO:0043984: histone H4-K16 acetylation
GO:1901701: cellular response to oxygen-containing compound
0
1
2
3
4
5
6
7
8
9
-log10(P)

Fig S4A. Enrichment analysis of genes that are downregulated by thiostrepton. The most significant gene ontology (GO) term associated with these genes is mitotic cell cycle. Also, these genes are also associated with FOXM1 pathway.



GO:0035966: response to topologically incorrect protein
ko04141: Protein processing in endoplasmic reticulum
GO:0010498: proteasomal protein catabolic process
GO:0006695: cholesterol biosynthetic process
GO:0036503: ERAD pathway
GO:0043618: regulation of transcription from RNA polymerase II promoter in response to stress
GO:1905897: regulation of response to endoplasmic reticulum stress
GO:0060548: negative regulation of cell death
GO:0016236: macroautophagy
GO:0051409: response to nitrosative stress
GO:0060590: ATPase regulator activity
GO:0006888: ER to Golgi vesicle-mediated transport
hsa04915: Estrogen signaling pathway
GO:0031669: cellular response to nutrient levels
GO:0045454: cell redox homeostasis
GO:1901698: response to nitrogen compound
GO:0044389: ubiquitin-like protein ligase binding
GO:0015918: sterol transport
GO:0071230: cellular response to amino acid stimulus
GO:0019725: cellular homeostasis

Fig S4B. Enrichment analysis of genes that are upregulated by thiostrepton. The most significant gene ontology (GO) term associated with these genes is response to topologically incorrect protein. Other GO terms associated with these genes are ERAD pathway and cell redox homeostasis.

DAVID Bioinformatics Resources 6.8
National Institute of Allergy and Infectious Diseases (NIAID), NIH

*** Welcome to DAVID 6.8 with updated Knowledgebase (more info). ***
*** If you are looking for DAVID 6.7, please visit our development site. ***

Functional Annotation Clustering

Help and Manual

Current Gene List: List_2
Current Background: Homo sapiens
375 DAVID IDs

Options **Classification Stringency** Custom
Rerun using options | Create Sublist

12 Cluster(s)

Download File

Annotation Cluster 1	**Enrichment Score: 3.74**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Cell cycle	RT			30	6.6E-6	4.2E-4
UP_KEYWORDS	Cell division	RT			20	7.9E-5	4.2E-3
UP_KEYWORDS	Mitosis	RT			16	8.1E-5	3.7E-3
GOTERM_BP_DIRECT	cell division	RT			19	2.0E-4	6.9E-2
GOTERM_BP_DIRECT	mitotic nuclear division	RT			11	2.5E-2	7.2E-1
Annotation Cluster 2	**Enrichment Score: 3.02**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Activator	RT			31	3.3E-6	2.6E-4
GOTERM_BP_DIRECT	positive regulation of transcription from RNA polymerase II promoter	RT			42	3.7E-6	8.1E-3
UP_KEYWORDS	Transcription regulation	RT			63	9.0E-4	2.2E-2
UP_KEYWORDS	Transcription	RT			64	1.1E-3	2.3E-2
UP_KEYWORDS	DNA-binding	RT			54	3.9E-3	6.7E-2
GOTERM_BP_DIRECT	transcription, DNA-templated	RT			51	2.9E-2	7.5E-1
GOTERM_BP_DIRECT	regulation of transcription, DNA-templated	RT			30	5.6E-1	1.0E0
Annotation Cluster 3	**Enrichment Score: 2.61**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Transferase	RT			51	3.8E-4	1.5E-2
UP_KEYWORDS	Kinase	RT			27	7.9E-4	2.1E-2
UP_KEYWORDS	ATP-binding	RT			38	1.0E-2	1.5E-1
UP_KEYWORDS	Nucleotide-binding	RT			46	1.2E-2	1.6E-1
Annotation Cluster 4	**Enrichment Score: 1.77**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Kinase	RT			27	7.9E-4	2.1E-2
GOTERM_BP_DIRECT	protein phosphorylation	RT			16	3.6E-2	7.7E-1
UP_KEYWORDS	Serine/threonine-protein kinase	RT			11	1.7E-1	6.5E-1
Annotation Cluster 5	**Enrichment Score: 1**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Zinc	RT			54	4.4E-2	3.7E-1
UP_KEYWORDS	Zinc-finger	RT			39	1.4E-1	6.0E-1
UP_KEYWORDS	Metal-binding	RT			74	1.5E-1	6.2E-1
Annotation Cluster 6	**Enrichment Score: 0.94**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	DNA repair	RT			10	8.2E-2	4.7E-1
UP_KEYWORDS	DNA damage	RT			11	1.0E-1	5.2E-1
GOTERM_BP_DIRECT	DNA repair	RT			8	1.8E-1	9.8E-1
Annotation Cluster 7	**Enrichment Score: 0.85**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	mRNA processing	RT			11	7.6E-2	4.6E-1
GOTERM_BP_DIRECT	mRNA splicing, via spliceosome	RT			8	1.5E-1	9.7E-1
UP_KEYWORDS	mRNA splicing	RT			8	1.9E-1	6.8E-1
UP_KEYWORDS	Spliceosome	RT			5	1.9E-1	6.7E-1
Annotation Cluster 8	**Enrichment Score: 0.32**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Innate immunity	RT			8	1.9E-1	6.8E-1
GOTERM_BP_DIRECT	innate immune response	RT			8	7.4E-1	1.0E0
UP_KEYWORDS	Immunity	RT			8	7.9E-1	9.9E-1
Annotation Cluster 9	**Enrichment Score: 0.27**				**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Postsynaptic cell membrane	RT			5	4.0E-1	8.8E-1

Annotation Cluster 1	**Enrichment Score: 3.74**			**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Cell junction	RT		14	4.3E-1	9.0E-1
UP_KEYWORDS	Synapse	RT		5	8.8E-1	1.0E0
Annotation Cluster 10	**Enrichment Score: 0.06**			**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	cell surface receptor signaling pathway	RT		6	6.3E-1	1.0E0
GOTERM_BP_DIRECT	G-protein coupled receptor signaling pathway	RT		12	9.7E-1	1.0E0
UP_KEYWORDS	Transducer	RT		9	9.9E-1	1.0E0
UP_KEYWORDS	G-protein coupled receptor	RT		8	9.9E-1	1.0E0
Annotation Cluster 11	**Enrichment Score: 0.05**			**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Membrane	RT		131	6.9E-1	9.8E-1
UP_KEYWORDS	Transmembrane	RT		89	9.5E-1	1.0E0
UP_KEYWORDS	Transmembrane helix	RT		88	9.6E-1	1.0E0
UP_KEYWORDS	Glycoprotein	RT		67	9.8E-1	1.0E0
Annotation Cluster 12	**Enrichment Score: 0.01**			**Count**	**P_Value**	**Benjamini**
UP_KEYWORDS	Disulfide bond	RT		50	9.7E-1	1.0E0
UP_KEYWORDS	Glycoprotein	RT		67	9.8E-1	1.0E0
UP_KEYWORDS	Signal	RT		57	9.9E-1	1.0E0

248 terms were not clustered.

2D View

Help

corresponding gene-term association positively reported corresponding gene-term association not reported yet

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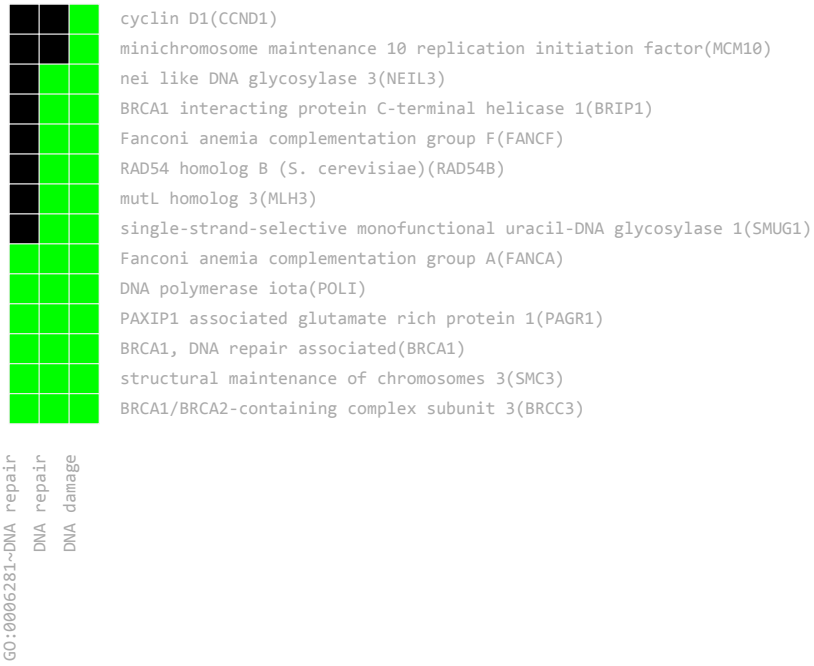
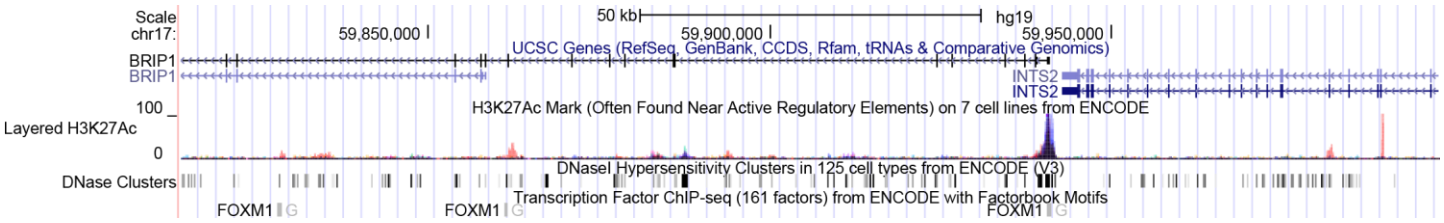
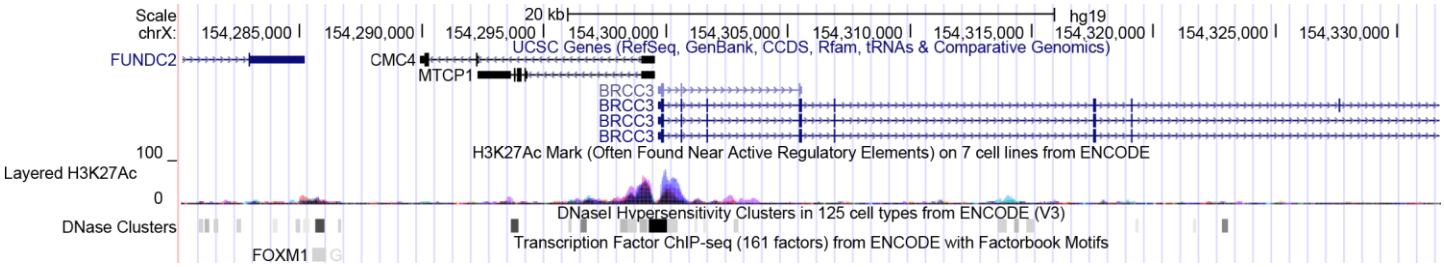


Fig S4C-S4D. Classification of gene function though DAVID bioinformatic program. One of the pathways that are enriched by these genes in DNA damage and repair pathway.

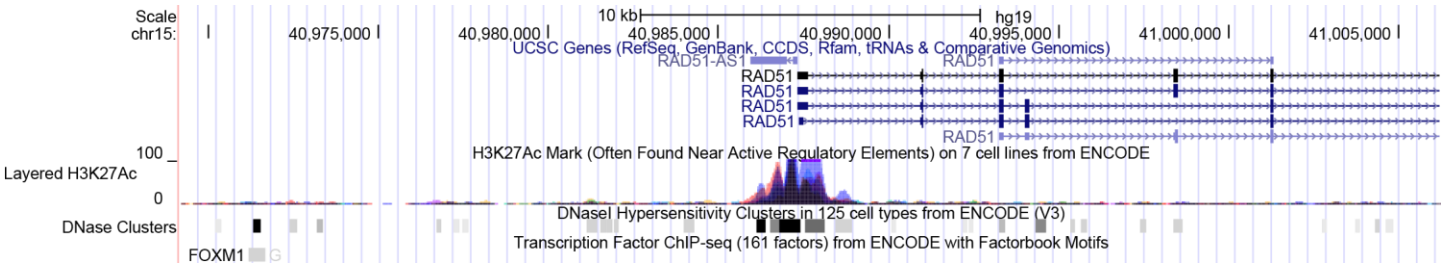
BRIP1



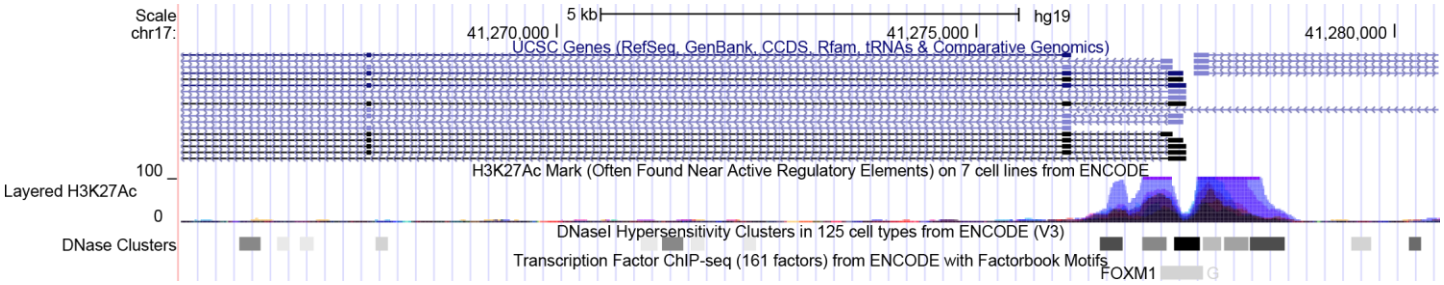
BRCC3



RAD51



BRCA1



FANCF

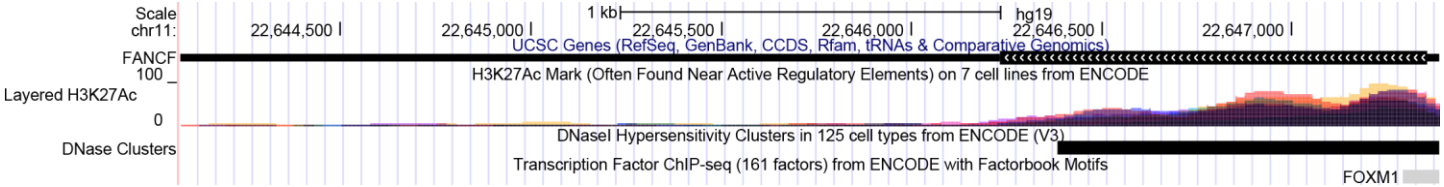
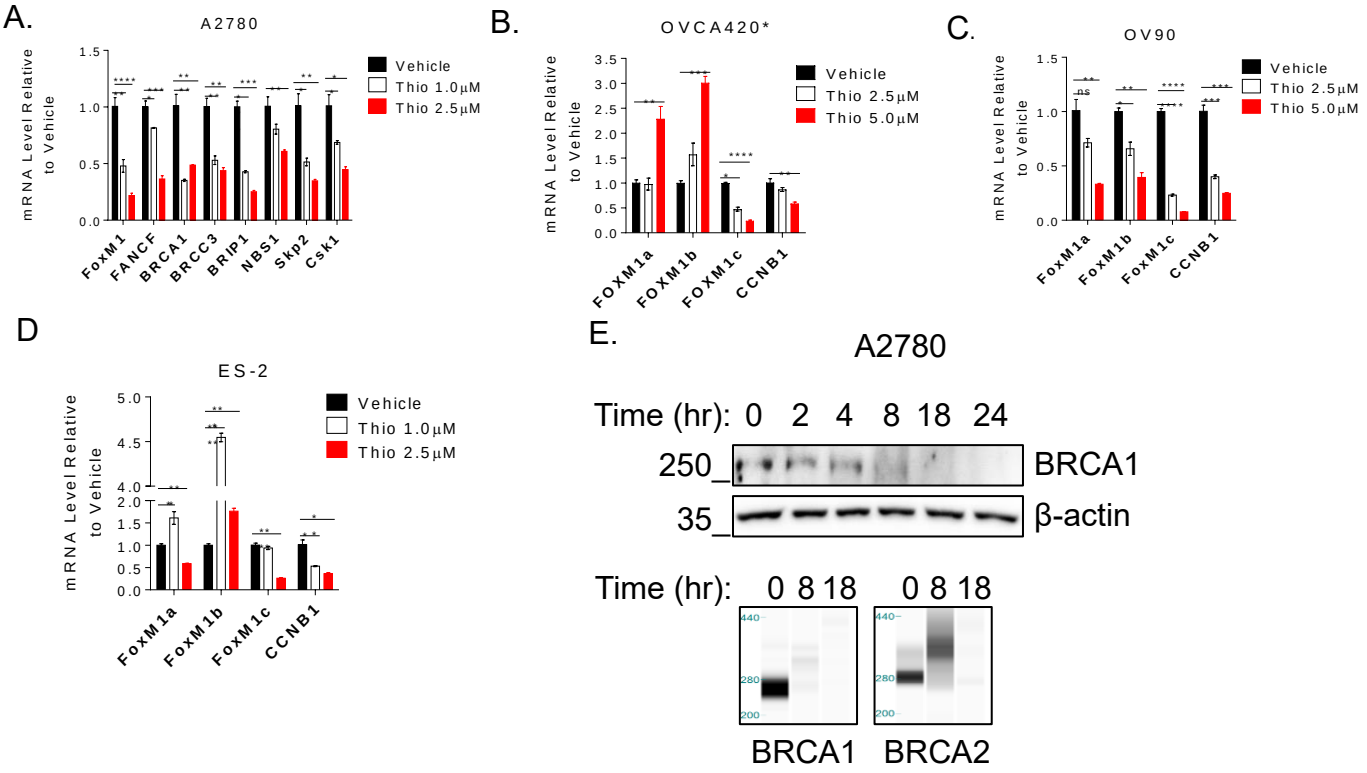
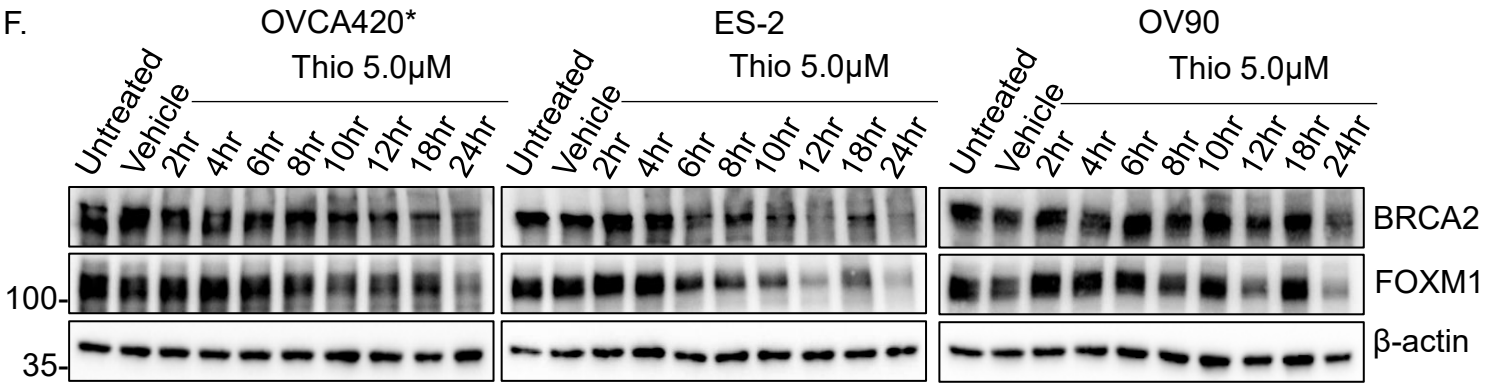
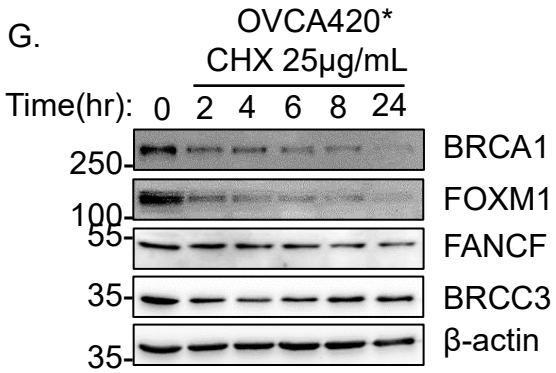
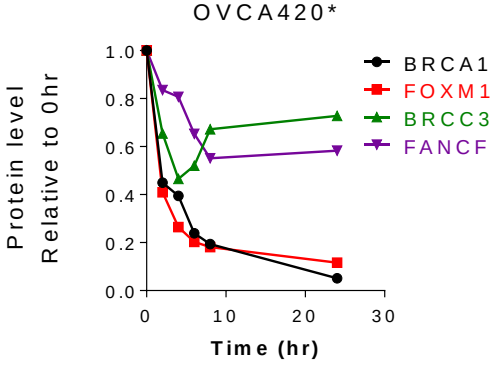


Fig S5. Analysis of ChIP-seq data set from ENCODE using UCSC Genome Browser indicates FOXM1 binding sites near the regulatory regions of DNA repair genes.


A.
A2780
mRNA Level Relative to Vehicle
Vehicle
Thio 1.0μM
Thio 2.5μM
FoxM1
FANCF
BRCA1
BRCC3
BRIP1
NBS1
Skp2
Csk1
B.
OVCA420*
mRNA Level Relative to Vehicle
Vehicle
Thio 2.5μM
Thio 5.0μM
FOXM1a
FOXM1b
FOXM1c
CCNB1
C.
OV90
mRNA Level Relative to Vehicle
Vehicle
Thio 2.5μM
Thio 5.0μM
FoxM1a
FoxM1b
FoxM1c
CCNB1
D
ES-2
mRNA Level Relative to Vehicle
Vehicle
Thio 1.0μM
Thio 2.5μM
FoxM1a
FoxM1b
FoxM1c
CCNB1
E.
A2780
Time (hr): 0 2 4 8 18 24
250
BRCA1
35
β-actin
Time (hr): 0 8 18 0 8 18
440
280
200
BRCA1
BRCA2


F.
OVCA420*
Thio 5.0μM
ES-2
Thio 5.0μM
OV90
Thio 5.0μM
Untreated
Vehicle
2hr
4hr
6hr
8hr
10hr
12hr
18hr
24hr
BRCA2
FOXM1
β-actin
100
35


G.
OVCA420*
CHX 25μg/mL
Time(hr): 0 2 4 6 8 24
250
100
55
35
35
BRCA1
FOXM1
FANCF
BRCC3
β-actin


H.
OVCA420*
Protein level Relative to 0hr
BRCA1
FOXM1
BRCC3
FANCF
Time (hr)

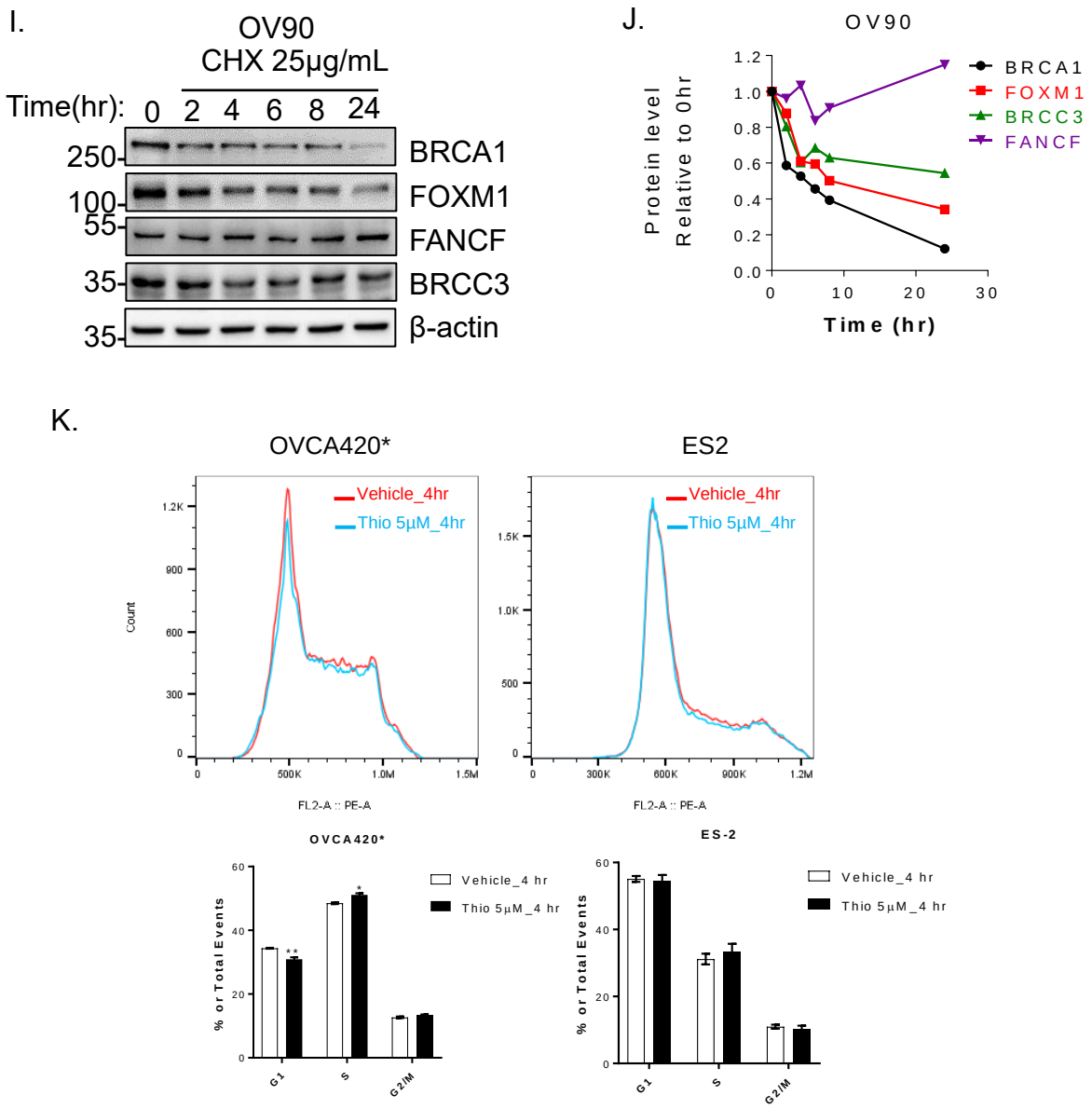
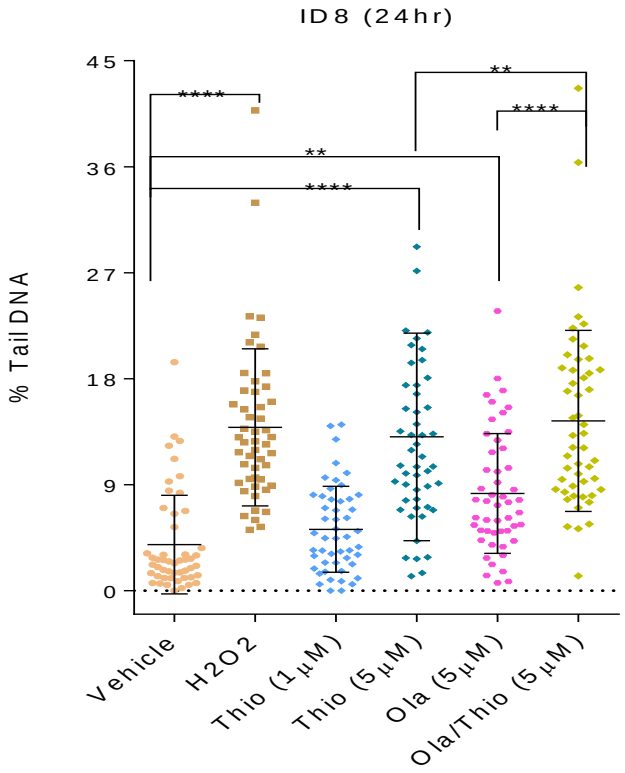


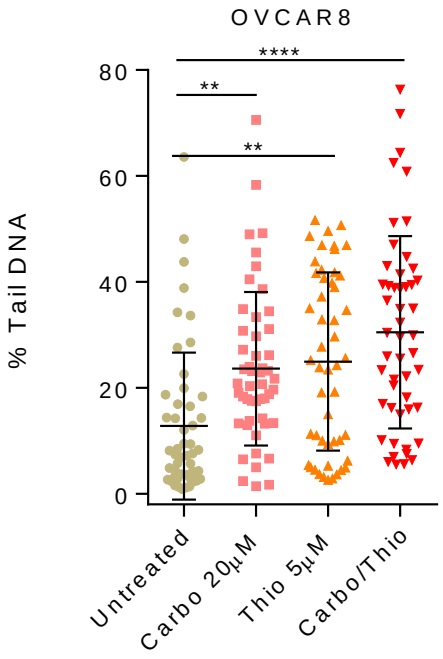
Fig S6. Thiostrepton downregulates all three FOXM1 isoforms and HR genes, including BRCA1 and BRCA2. (A) qRT-PCR analysis of FOXM1 and HR genes in A2780 cells after thiostrepton treatment. A2780 cells were treated with vehicle, 1.0 μM or 2.5 μM thiostrepton for 24 hours before total RNA extraction. (B-D) qRT-PCR of FOXM1 isoforms a, b, c and FOXM1 target gene CCNB1 after thiostrepton treatment. OVCA420* (B) and OV90 (C) cells were seeded in 6-well plates and treated with vehicle, 2.5 μM or 5.0 μM of thiostrepton for 24 hours before total RNA extraction. ES-2 cells (D) were treated with vehicle, 1.0 μM or 2.5 μM of thiostrepton for 24 hours. Statistics analysis was done with Student's t-test. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. (E) Western blot for BRCA1 and BRCA2 in A2780 cells. Cells were treated with 10 μM thiostrepton for 0, 2, 4, 8, 18 and 24 hours before collecting lysate for western blot. Upper blots: to detect BRCA1 levels for all time points after thiostrepton treatment. Equal amounts of total protein were loaded on to SDA-PAGE and β-actin was used as a loading control. Bottom

blots: to detect BRCA1 and BRCA2 levels, equal amounts of total protein for 0, 8 and 18 hour treatment samples were used to run western blot using Wes machine from ProteinSimple (CA, USA). (F) Immunoblot analysis of BRCA2 after thiostrepton treatment. OVCA420*, OV90, and ES-2 cells were treated with 5.0 µM thiostrepton for 0, 2, 4, 6, 8, 10, 12, 18 or 24 hours before immunoblotting. BRCA2 protein band is well above the 250kd protein marker, which is not indicated in the blot. (G-J) BRCC3 and FANCF have longer protein half-life while BRCA1 and FOXM1 have shorter half-life. (G, I) Immunoblotting of OVCA420* and OV90 cells respectively**.** Cells were treated with 25 µg/mL cycloheximide (CHX) and collected 0, 2, 4, 6, 8 or 24hrs later. (H, J) Densitometric analysis of protein levels for OVCA420* and OV90 cells respectively. (K) Cell cycle analysis with thiostrepton treatment. ES-2 and OVCA420* cells were treated with vehicle or 5 µM of thiostrepton for 4 hours and collected with trypsin, followed by ethanol fixation, RNase A treatment and Propidium Iodine staining before Flow Cytometry analysis. Line graphs show representative samples for each cell lines. Bar graphs show quantification of three replicates from one representative experiment. Similar results are observed from three independent experiments.

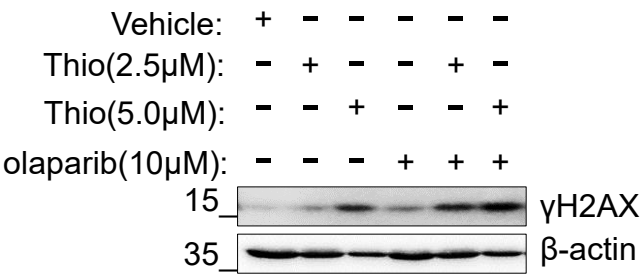
A.


ID8 (24hr)
% Tail DNA
45
36
27
18
9
0
****
**
****
**
****
Vehicle
H2O2
Thio (1μM)
Thio (5μM)
Ola (5μM)
Ola/Thio (5μM)

B.


OVCAR8
% Tail DNA
80
60
40
20
0
****
**
**
Untreated
Carbo 20μM
Thio 5μM
Carbo/Thio

C.


OV90
Vehicle: + - - - - -
Thio(2.5μM): - + - - + -
Thio(5.0μM): - - + - - +
olaparib(10μM): - - - + + +
15
γH2AX
35
β-actin

D.

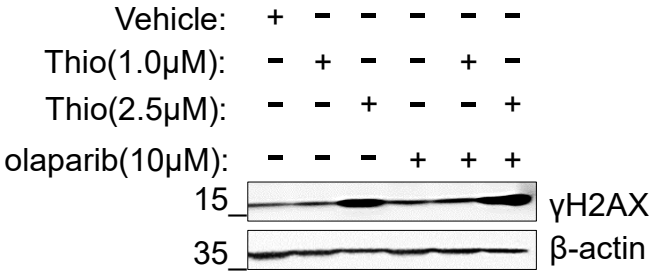
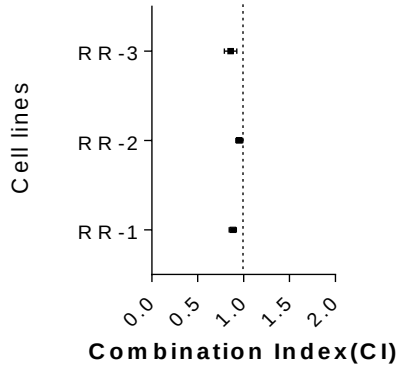

ES-2
Vehicle: + - - - - -
Thio(1.0μM): - + - - + -
Thio(2.5μM): - - + - - +
olaparib(10μM): - - - + + +
15
γH2AX
35
β-actin

Fig S7. Thiostrepton enhances DNA damage when combined with olaparib and carboplatin. (A) Neural comet assay in mouse ovarian cancer cell ID8. ID8 cells were treated with Vehicle, H_2O_2, 1 μM or 5 μM thiostrepton, 5 μM olaparib or combination of 5 μM and 5 μM thiostrepton for 24 hours. H_2O_2 treatment served as a positive control. (B) Neutral comet assay in OVCAR8 cell. OVCAR8 cells were treated with 20 μM carboplatin, 5 μM thiostrepton or combination of 20 μM carboplatin and 5 μM thiostrepton for 24 hours before comet assay. Data were shown as mean with SD. Each dot represents one comet. Statistics analysis was done with one-way ANOVA. Difference with $p \leq 0.05$ was considered to be significant. **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. (C-D) Immunoblotting to measure γH2AX after thiostrepton and/or olaparib treatment in OV90 and ES-2 cells respectively. (C) OV90 cells were treated with 2.5 μM or 5.0 μM thiostrepton alone or in combination with 10 μM olaparib for 48 hours and harvested for western blot analysis and blotted with antibody against γH2AX. β-actin was used as loading control. (D) ES-2 cells were treated with 1.0 μM or 2.5 μM thiostrepton alone or in combination with 10 μM olaparib for 48 hours.

A.



B.

Combination Index				
	Thio 0.3μM	Thio 0.75μM	Thio 1.0μM	
Ruc 5μM	1.163	0.841	0.891	RR-1
Ruc 10μM	1.025	0.862	0.867	
Ruc 20μM	0.989	0.886	0.88	

Combination Index				
	Thio 0.3μM	Thio 0.75μM	Thio 1.0μM	
Ruc 5μM	0.659	0.868	0.897	RR-2
Ruc 10μM	0.642	0.832	0.921	
Ruc 20μM	0.913	1.005	1.029	

C.

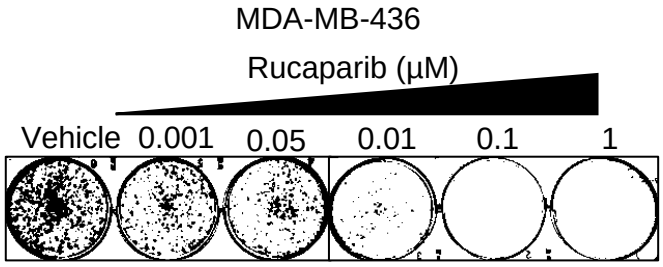


Fig S8. Thiostrepton and rucaparib have synergistic effects in resistant cells. (A) Synergistic effect between thiostrepton and rucaparib in rucaparib resistant cells. 3 different rucaparib resistant cells derived from breast cancer cell MDA-MB-436, RR-1, RR-2 and RR-3 were seeded in 96 well plates and treated with combinations of thiostrepton and rucaparib and incubated for 5 days before SRB assay. Combination index was calculated as described in supplement materials and methods. Data shown as mean with 95% confidence intervals. Only used CIs from combinations that gave effects of 0.2-0.8. The two drugs are synergistic if CI<1.0, additive if CI=1.0 and antagonistic if CI>1.0. (B) Combination index (CI) for different combination of thiostrepton (thio) and rucaparib (Ruc). (C) Parental MDA-MB-436 cell is sensitive to rucaparib. Cells were seeded in 6 well plate and treated with increasing concentrations of rucaparib for 3 days and allow colonies to form for another 15 days. Colonies were stained with SRB and imaged.

Supplemental Experimental Procedures

TCGA data analysis

The data used for the violin plot were RNA sequencing datasets (RNAseqV2level3) from high-grade serous ovarian cancer and were downloaded from the TCGA Research Network Data Portal (http://tcga_data.nci.nih.gov/tcga, 04/01/2016). To distinguish between sensitive and resistant tumor samples, clinical data associated with these samples were downloaded from cBioPortal (http://www.cbioportal.org/data_sets.jsp, 04/01/2016). Chemotherapy sensitive tumor samples are defined as those with disease-free survival for over 24 months (55 samples). Resistant tumor samples are defined as those with disease-free survival for less than 12 months. Normalized expression was extracted from the isoforms for FOXM1 and plotted in R with the R package 'ggplot2' using the plot function geom_violin (version 2.1.0, https://cran.r-project.org/src/contrib/Archive/ggplot2/).

Cell lines and cell culture

ES-2 and A2780 cells were maintained in MCDB105 and M199 (1:1) (Sigma, USA) containing 5% FBS (Sigma), OV90 cells were maintained in MCDB105 and M199 (1:1) with 15% FBS. OVCA420* cells were cultured in DMEM (Sigma and Caisson Labs, USA) supplemented with 10% FBS. ID8 cells (mouse epithelial ovarian cancer cell line obtained from Dr. Kathy Roby, Kansas University Medical Center) were maintained in DMEM (high glucose, Sigma) containing 4% fetal bovine serum and ITS (5 mg/mL insulin, 5 mg/mL transferrin, and 5 ng/mL sodium selenite). MDA-MB-436 and its derivative rucaparib resistant cells RR-1, RR-2, RR-3 were kind gifts from Dr. Neil Johnson laboratory at Fox Chase Cancer Center and were cultured in RPMI1640 (Sigma and Caisson Labs) supplemented with 10% FBS. All the media were supplemented with 100 units/MI penicillin and 100 µg/mL streptomycin. All cell lines were subjected to cell line identity confirmation.

Antibodies and Compounds

Rabbit polyclonal anti-FOXM1 antibody (C-20, sc-502), rabbit polyclonal anti-BRCA1 antibody (C-20, sc-642), mouse monoclonal anti-FANCF antibody (D-2, sc-271952) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-BRCC3 antibody (GTX31765) were from GeneTex (Irvine, CA, USA). Rabbit polyclonal anti-BRCA2 antibody (ab123491) was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal anti-beta actin antibody (A1978) was from Sigma-Aldrich (St Louis, MO, USA). For Secondary antibodies, horse anti-mouse IgG-HRP antibody (7076S) was purchased from Cell signaling Technologies, Goat anti-rabbit IgG-HRP antibody (sc-2030) was from Santa Cruz Biotechnology.

Olaparib (AZD2281, Ku-0059436) was purchased from Selleckchem. Olaparib stock solutions were made with DMSO at 50 mM and stored at -80°C. Micelle-encapsulated

thiostrepton were used (formulated as describe below). Cycloheximide was from Sigma-Aldrich.

Immunoblotting

Cells were washed at least twice with PBS at the end of treatments if applicable, and then lysed with appropriate volume of 1X electrophoresis sample buffer (Bio-Rad Laboratories, CA, USA) with 5% β-mercaptoethanol (Sigma-Aldrich). The cell lysates were then boiled at 95 °C for 5 minutes before using. Immunoblotting procedures were performed as previously described (Zhang et al., 2014). For apoptosis marker checking, cells were collected at the end of treatments, and total proteins were extracted using RIPA buffer (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in 1X PBS) containing protease/phosphatase inhibitor cocktail (Roche). BCA protein assay reagent kit (Pierce) was used to determine protein concentrations. Equal amounts of total proteins were loaded for SDS-PAGE and transferred onto PVDF membranes (GE healthcare). The densitometric analysis was performed with Image J software (NIH).

Real-time quantitative PCR (RT-qPCR)

The total RNA were extracted with the Trizol reagent (15596-028, Invitrogen) according to the manufacturer's mannual. The cDNA was synthesized using SuperScript II reverse transcriptase (180604014, Invitrogen) with 1 μg of total RNA in a 20μL reaction. The resulting cDNA was diluted 1:20 in nuclease-free water and 1 μL was used per qPCR reaction with triplicates. qPCR was carried out using Power SYBR Green PCR Master Mix (4367659, Thermo Fisher Scientific) on a CFX384 Real-Time PCR Detection System (Bio-Rad) including a non-template negative control. Amplification of GAPDH or 18S rRNA was used to normalize the level of mRNA expression. Primers used in the assays are shown in the Supplemental Table S1.

Cytotoxicity assay using Sulforhodamine B (SRB) and drug synergy studies

SRB assays were performed as previously described(Vichai and Kirtikara, 2006) with modifications shown below. For OVCA420*, OV90 and ES-2, 3000 cells were seeded in 96-well plates, and treated with drugs at least 12hours after seeding. Then the cells were incubated for another 3 days. For MDA-MB-436 rucaparib resistant cells RR-1, RR-2 and RR-3, 5000 cells were seeded and incubated for 5 days after drug treatment. Dose response curves were fitted and the IC50 for each drug was determined using GraphPad Prism 6 four parameters. All curves were constrained with 100% on top. Synergy was determined by calculating the combination index (CI) obtained from the plate reading. CI was calculated based on dividing the expected effect by the observed effect.

Colony formation assay

For A2780 and ID8 cells, 1000 cells/well were seeded in 6-well plates, and for MDA-MB-436 rucaparib resistant cells RR-1 and RR-2 and 3, seeded 2000 or 3000 cells per well in 6-well plates. The cells were treated with drugs at least 12hours after seeding

and further incubated for another 3-5 days before changing to fresh media. The medium was changed every 2-3 days allowing colonies to form. At the end of experiments, SRB assay was performed to stain the colonies which were imaged with Molecular Imager ChemiDoc MP System (Bio-Rad). The colonies were further dissolved and measured with a plate reader. Analysis of colonies were performed in GraphPad Prism 6.

Neutral Comet assay

Neutral comet assay was performed according to Tregvigen Instructions for Comet Assay with modifications. Briefly, ID8 and OVCAR8 cells were treated with vehicle or appropriate drug(s) for 24 hours. Cell suspension with $1X10^5$ cells/mL was mixed with molten LMA agarose (Trevigen) at a ratio of 1:10 and transferred onto Comet Slide (Trvigen). After cooling slides at 4°C for 10 minutes, slides were immersed in Lysis solution (Trevigen, 4250-050-01) at 4°C for 1 hour and immersed in 50 mL of 1X Neutral Electrophoresis Buffer (0.5 mM Tris Base and 1.5 mM Sodium Acetate) for 30 minutes in 4 °C. 1 hour electrophoresis was performed at 4°C using Wide Mini-Sub Cell GT Horizontal Electrophoresis System (Bio-Rad) and applied voltage at 17 volts (1 volt per cm). Slides were then immersed into DNA Precipitation Solution (1M NH_4Ac and 82.27% ethanol) for 30 minutes at room temperature followed by 30 minutes incubation in 70% ethanol. Then let slides dry at 37°C for 10-15 minutes and stained with SYBR Green I and viewed slides and imaged comets using ZIESS fluorescent microscope (10X). Comets were analyzed using free comet assay software from Casp Lab (1.2.3beta2 version).

Cell cycle analysis

Cell cycle analysis was performed as described before (Bastola et al., 2016). Briefly, 350,000 cells were seeded in 6-well plates and treated with vehicle or 5 µM thiostrepton for 4 hours. Cells were then collected using trypsin, washed with ice-cold PBS, resuspended in 300 µL of ice-cold PBS and added equal volume of 95% ethanol, followed by overnight fixation at 4 ℃ in dark. Cells were then collected, washed twice with ice-cold PBS incubate with RNase A at 37℃ for 1 hr before staining with propidium iodide (PI). Flow cytometry was performed with BD AccuriTM C6 Flow Cytometer (BD Biosciences).

Formulation of micelle-encapsulated thiostrepton

<u>Materials.</u>

N-(Carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (Sunbright® DSPE-PEG_{2000}) was purchased from NOF America Corporation. HEPES (99% for biochemistry) was purchased from Acros Organics. Chloroform (Certified ACS), acetonitrile (HPLC Grade) and dimethyl sulfoxide (DMSO, HPLC Grade) were obtained from Fisher Scientific.

<u>Methods.</u>

Preparation of thiostrepton loaded DSPE-PEG$_{2000}$ micelles

Thiostrepton loaded micelles were prepared by a modified version of the thin film hydration method described elsewhere (Wang and Gartel, 2011). Briefly, 333 mg of thiostrepton and 1.69 g of DSPE-PEG$_{2000}$ were weighed in a 1 L flask and dissolved in 333 ml of chloroform. The flask was lightly stirred for 5 minutes and bath sonicated for 1 minute or until completely dissolved and homogenous. A thin film of the drug-polymer mixture was formed by rotary evaporation of the organic solvent under reduced pressure at 30°C. Any remaining solvent was removed by placing the flask under high vacuum (< 2 mbar) overnight. The resulting film was then hydrated using 27.75 mL of PBS, pH 7.4. The flask was stirred for 1 minute and bath sonicated for 5 minutes until a homogenous solution was obtained. Un-encapsulated thiostrepton was removed by centrifugation at 3000g for 3 minutes followed by removal of the supernatant and storage of the final formulation at 4°C.

Drug loading, encapsulation efficiency and physical characterization of micelle formulation

Thiostrepton loading and encapsulation efficiency within the DSPE-PEG$_{2000}$ formulation were characterized by RP-HPLC on a Shimadzu 2010CHT using a Supelco Discovery HS C18 column (15cm x 4.6mm, 3µm). The mobile phase consisted of A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) with a linear gradient of 20%-90% B over 13 minutes at 1 mL/min. UV absorption was measured at 275 nm. Test samples were diluted with DMSO to the linear range of an AUC standard curve of thiostrepton in DMSO. Encapsulation efficiency (EE%) and drug loading (DL) were calculated by the following equation:

EE% = (Weight of thiostrepton in micelle formulation supernatant / Weight of thiostrepton added to mixture) X 100

DL = (Concentration of thiostrepton fed to final formulation) x (EE%)

Dynamic light scattering (DLS) and phase analysis light scattering (PALS) were used to measure the hydrodynamic diameter and zeta potential of the DSPE-PEG$_{2000}$ micelles loaded with thiostrepton using a Malvern Zetasizer Helix at 25°C. All sizing measurements were repeated eight times with error reported as standard deviation.

Results.

Thiostrepton encapsulation and micelle characterization

Thiostrepton, a highly hydrophobic drug, was successfully encapsulated and solubilized into a micellar core using the amphiphilic polymer DSPE-PEG$_{2000}$. An encapsulated drug concentration of 9 mg/ml with an encapsulation efficiency of 75% (Fig S9) was obtained following dispersion of the polymer-drug film in PBS.

Dynamic light scattering of the resulting micelles using the intensity based method resulted in two distinct populations with an average effective hydrodynamic diameter (Z-

average) of 67.81 +/- 7.46 nm (Fig S10A). Particle diameter determined using a multi-modal size distribution analysis on a number basis which filters out trace quantities of large particles that contribute unequally in an intensity based analysis was determined to be 11.35 +/- 2.5 nm (Fig S10B). Zeta potential of the particles were measured at -4.45 +/- 0.513 mV (Table S1).

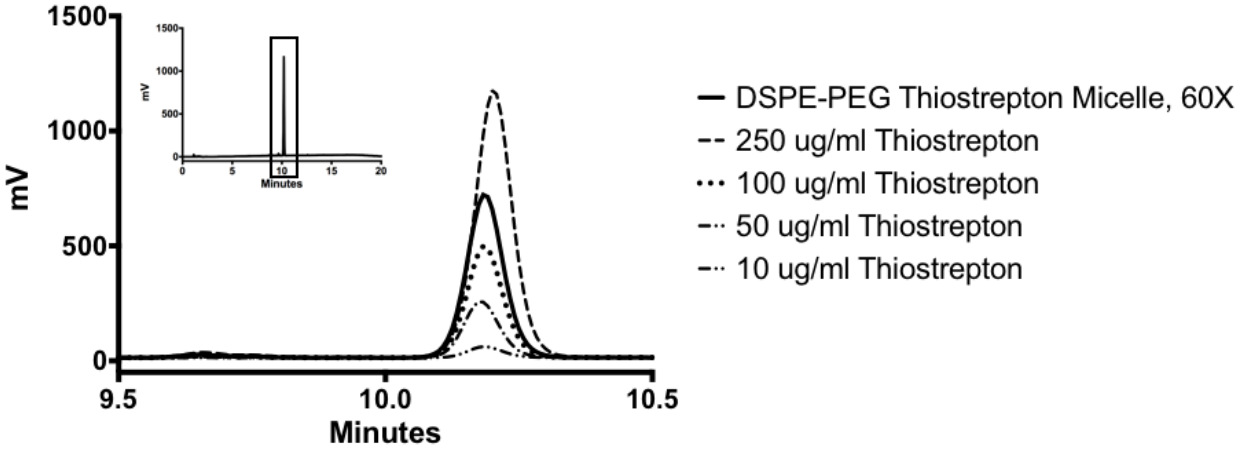


Figure S9. Representative chromatograph of DSPE-PEG$_{2000}$ thiostrepton micelle versus thiostrepton standards.

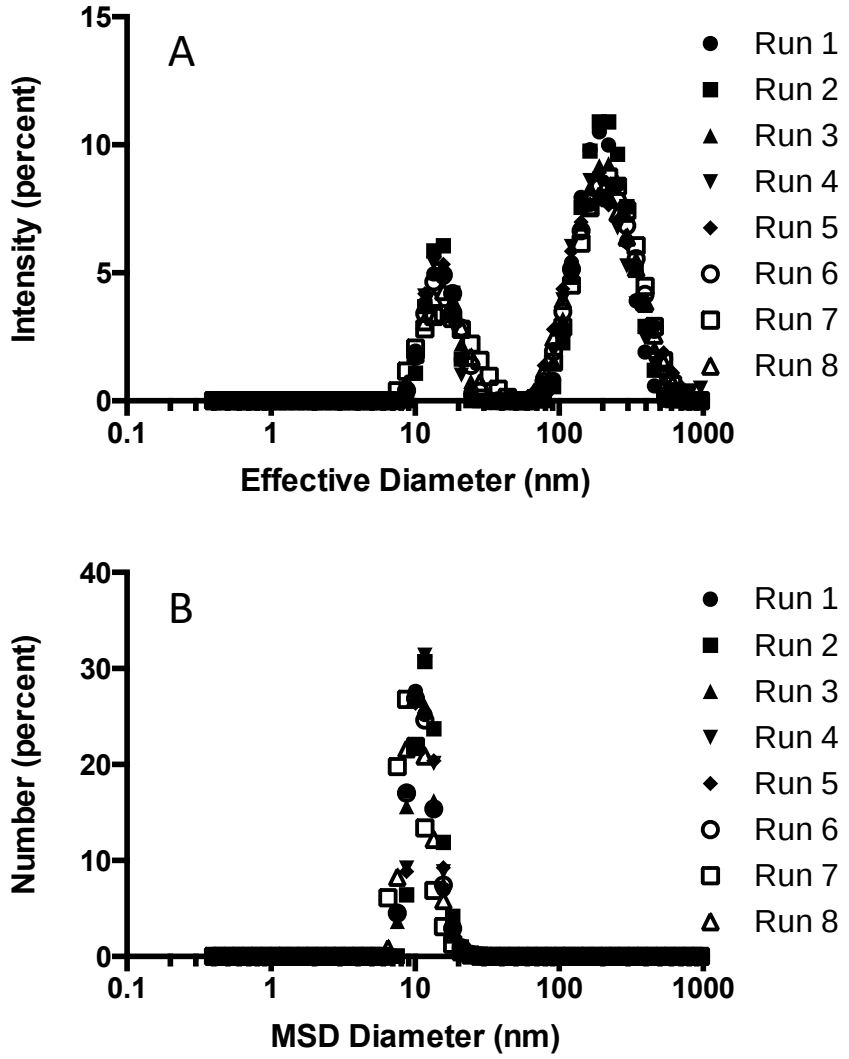


Figure S10. Particle sizing using dynamic light scattering (DLS). Effective hydrodynamic diameter using scattering intensity (A) and the number based multi-modal size distribution (B).

Sample	Zeta Potential (mV)	Standard Deviation (n=6)
DSPE-PEG$_{2000}$ – Thiostrepton Micelle	-4.45	0.513

Table S1. Micelle zeta potential

Table S2. Real-Time PCR primers (5'-3')

Gene	Forward	Reverse
FOXM1(total)	5'AGAATTGTCACCTGGAGCAG	5'TTCCTCTCAGTGCTGTTGATG
FOXM1A	5'GGTACACCCATCACCAGCTT	5'ATGGGTCTCGCTAAGTGTGG
FOXM1B	5'CGTGGATTGAGGACCACTTT	5'TCGGTCGTTTCTGCTGCTT
FOXM1C	5'CCCGAGCACTTGGAATCAC	5'TCCTCAGCTAGCAGCACCTT
CCNB1	5'GGCTTTCTCTGATGTAATTCTTGC	5'GTATTTTGGTCTGACTGCTTGC
FANCF	5'GCATTTGGGTTGGAACTGAG	5'CTTCAAAATCTCCATCCTGCG
BRCA1	5'TAATGCTATGCAGAAAATCTTAGAG	5'TACTTTCTTGTAGGCTCCTTTTGG
BRCA2	5'TTCATGGAGCAGAACTGGTG	5'AGGAAAAGGTCTAGGGTCAGG
BRCC3	5'CCTCATGTCACTATCGGGAAAG	5'GGATCTTGGTTACTGAGTCCAG
BRIP1	5'GCTTAGCCTTACTTTGTTCTGC	5'TTTCACTTACGCCCTCATCTG
NBS1	5'AGACCAACTCCATCAGAAACTAC	5'AATGAGGGTGTAGCAGGTTG
CsK1	5GAATGGAGGAATCTTGGCGTT	5'TCTTTGGTTTCTTGGGTAGTGGG
Skp2	5'CTGGGTGTTCTGGATTCTCTG	5'GCTGGGTGATGGTCTCTG
DDIT3	5'GTACCTATGTTTCACCTCCTGG	5'TGGAATCTGGAGAGTGAGGG
DDIT4	5'GTTTGACCGCTCCACGAG	5'GTGTTCATCCTCAGGGTCAC
GADD45A	5'GGAGAGCAGAAGACCGAAAG	5'AGGCACAACACCACGTTATC
BCL-2	5'GTGGATGACTGAGTACCTGAAC	5'GCCAGGAGAAATCAAACAGAGG
GAPDH	5'GAAACTGTGGCGTGATGGC	5'CACCACTGACACGTTGGCAG
18S rRNA	5'GCCCGAAGCGTTTACTTTGA	5'TCCATTATTCCTAGCTGCGGTATC
CCNB1 FOXM1 BS	5' CGCGATCGCCCTGGAAACGCA	5'CCCAGCAGAAACCAACAGCCGT
BRCA1 FOXM1 BS	5'CAAGGTACAATCAGAGGATGGG	5'TCCTCTTCCGTCTCTTTCCT
RAD51 FOXM1 BS	5'ACCAGGCAGAGAATCTTGTTC	5'TTCAAGTCTAACCCAGTGCAG
FANCF FOXM1 BS	5'AAGGCCCTACTTCCGCTTTC	5'CACGGATAAAGACGCTGGGA
RAD51D FOXM1 BS	5'CACATTCGGCCTCTACCTTC	5'TTGGAACGGAAGCTGGC
FANCD2 FOXM1 BS	5'TTGGCGTCACGTCATGG	5'CCACTTACTCACCGAGAAGC

Table S3. Datasets from Connectivity Map for cDNA array analysis

Scan ID	Batch	Condition	Arraytype
5500024030700072107988.A06	1	DMSO	HT_HG-U133A
5500024030700072107988.B05	1	DMSO	HT_HG-U133A
5500024030700072107988.D04	1	DMSO	HT_HG-U133A
5500024030700072107988.G02	1	DMSO	HT_HG-U133A
5500024030700072107988.H01	1	DMSO	HT_HG-U133A
5500024030700072107988.H02.MCF7.656.thio	1	Thiostrepton	HT_HG-U133A
5500024031723100807770.A12	2	DMSO	HT_HG-U133A
5500024031723100807770.B11	2	DMSO	HT_HG-U133A
5500024031723100807770.D10	2	DMSO	HT_HG-U133A
5500024031723100807770.G08	2	DMSO	HT_HG-U133A
5500024031723100807770.H07	2	DMSO	HT_HG-U133A
5500024031723100807770.H08.MCF7.705.thio	2	Thiostrepton	HT_HG-U133A
Data can be downloaded from Connectivity Map 02 using .cel file download utility.			
https://portals.broadinstitute.org/cmap/fileDownloadServlet?servletAction=input			

Supplemental References

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Vichai, V., and Kirtikara, K. (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature protocols *1*, 1112-1116.

Wang, M., and Gartel, A.L. (2011). Micelle-encapsulated thiostrepton as an effective nanomedicine for inhibiting tumor growth and for suppressing FOXM1 in human xenografts. Molecular cancer therapeutics *10*, 2287-2297.

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