Supplementary Materials

Supplementary Methods.

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Figure S5. Tumor Volume, Body Weight, and Blood Counts

Supplementary Methods

RNA-seq data collection

RNA from A673 and TTC-466 cells treated with vehicle (0.3% DMSO) or 2 μM HCI2509 or stably infected and selected for expression of a control Luc-RNAi or the EF-2-RNAi or the EWS/ERG-RNAi was extracted using the RNAeasy kit (Qiagen) with an on-column DNAse digestion protocol. Libraries for deep-sequencing were prepared according to the manufacturer's instructions (Illumina) and sequenced on an Illumina Hi-Seq with 50 cycles of single end reads. Sequences from HCI2509 in A673, EWS/ERG knockdown, and HCI2509 in TTC-466 were aligned to the human genome build hg19. Raw sequence reads can be found in the NCBI SRA under numbers SRA096343, SRA096347, SRA096354, respectively. The USeq analysis package was used to identify differentially expressed genes (useq.sourceforge.net). Significance parameters were set to a 2-fold, 3-fold or 4-fold change in expression and an FDR of 0.1 (10%) or 1.0×10^{-10} or 1.0×10^{-15} as indicated in the figure legends.

Table S3. Primer Sequences for qRT-PCR analysis from RNA.

qRT-PCR primers:

Gene	Forward	Reverse
NKX2-2	5’ CTACGACAGCAGCGACAACC 3’	5’ GCCTTGGAGAAAAGCACTCG 3’
CAV1	5’ ATCGACCTGGTCAACCGCGAC 3’	5’ CGAAGTAAATGCCCCAGATGA 3’
E2F1	5’ GCCACTGACTCTGCCACCATA 3’	5’ GGTGGGGAAAGGCTGATGAAC 3’
IGF1	5’ GAAGATGCACACCATGTCCTC 3’	5’ CTCCAGCCTCCTTAGATCACA 3’
GSTM4	5’ GCTGCCCTACTTGATTGATGG 3’	5’TGATTGGAGACGTCCATAGCC 3’
HMOX1	5’ AACTTTCAGAAGGGCCAGGT 3’	5’ GTAGACAGGGGCGAAGACTG 3’
IGFBP3	5’ CATCAAGAAAGGGCATGCTAA 3’	5’ CTACGGCAGGGACCATATTCT 3’
CDH1	5’ TGCCCAGAAAATGAAAAAGG 3’	5’ GTGTATGTGGCAATGCGTTC 3’
RUNX2	5’ CCTCGGAGAGGTACCAGATG 3’	5’ AAACTCTTGCCTCGTCCACT 3’

Figure S1: Transcriptional Profiling of HCI2509 in A673 and TTC-466

A,B) Cell viability assay showing the difference in HCI2509 sensitivity between (A) TTC-466 cells with control and EWS/ERG knockdown or (B) NIH 3T3 cells with control and EWS/FLI expression. The dose-response curves were determined after 96 hours of treatment and normalized to the vehicle controls. n=3 for each point. Error bars denote standard deviation. EC_{50} and 95% CI were determined using GraphPad Prism 6. Note the line for ERG-RNAi data in (A) is a connecting line, not a curve fit.

C) Venn diagram representations of the overlap between the EWS/FLI and EWS/ERG transcription profiles, both generated by RNA-seq. Chi-square determined p-values are indicated with the observed contingency tables shown.

D) Gene set enrichment analysis (GSEA) using genes regulated by EWS/FLI in A673 cells (RNA-seq) as the rank-ordered dataset and the EWS/ERG-up-regulated or the EWS/ERG-down-regulated genesets (RNA-seq). Normalized enrichment scores (NES) and p-values are shown.

E,F) Venn diagram representations generated from respective RNA-seq data sets using default cutoffs (2-fold change, FDR=10%). (E) represents the overlap between the HCI2509 and the EWS/FLI-knockdown transcription profiles, both generated in A673 cells; (F) the overlap between the HCI2509 and the EWS/ERG-knockdown transcription profiles, both generated in TTC-466 cells. Chi-square determined p-values are indicated with the observed contingency tables shown.

G,H) GSEA using genes directly regulated by EWS/FLI in A673 cells (ChIP-chip and RNA-seq

overlap) as the geneset and HCI2509 regulated genes in A673 cells (RNA-seq) as the rank-ordered dataset in (G) or the vorinostat regulated genes in A673 cells (microarray) as the rank-ordered dataset in (H). Normalized enrichment scores (NES) and p-values are shown.

I,J) Top ten categories from DAVID functional analysis of the (I) EWS/FLI up-/HCI2509 down- and EWS/FLI down-/HCI2509 up-regulated genesets and (J) EWS/ERG up-/HCI2509 down- and EWS/ERG down-/HCI2509 up-regulated genesets. The log transformed enrichment scores for each category are indicated on the x-axis.

K) Validation of *NKX2.2*, *CAV1*, *GSTM4*, *E2F1*, *IGF-1*, *RUNX2*, *IGFBP3*, *HMOX1* and *CDH1* as HCI2509 targets by qRT-PCR analysis using EWS-502, SK-ES-1, SK-N-MC, and TC-71 cells treated for 48 hours with vehicle or HCI2509 at $2xEC_{50}$. The p-value for each fold-change is < 0.05 (n=3). Individual p-values are reported in Supplementary Table S2.

Figure S2: Morphological Changes in A673 and TTC466

A) Whole-field immunofluorescence images of A673 cells treated with increasing doses of HCI2509 for 72 hours. Staining was performed for F-actin stress fibers (red – phalloidin) and for focal adhesions (green –paxillin), and nuclei (blue). HCI2509 induced a dose-dependent increase in the cell spreading and morphology.

B,C) Immunofluorescence images of A673 cells treated with either control siRNA or LSD1 siRNA 50 nM for 48 hours. Staining was performed for (B) LSD1 and (C) F-actin stress fibers. Measurements of LSD1 nuclear signal and cell area were performed on at least 6 fields for each transfection. Decrease in LSD1 nuclear signal correlated with more organized actin fibers and cell spreading.

(D) Whole-field and (E) close-up immunofluorescence images of TTC-466 cells treated with increasing doses of HCI2509 for 3 days. Staining was performed for F-actin stress fibers (red – phalloidin) and for focal adhesions (green – paxillin), and nuclei (blue). HCI2509 induced a dose-dependent increase in the cell spreading and morphology.

F) Measurement of cell area in pixels in phalloidin images shows a dose-dependent increase in cell spreading with HCI2509. TTC-466 cells were fixed and stained with phalloidin. Cell area was quantified as previously described (*39)*.

Data is shown as scatter plot with mean plus standard deviation, and unpaired parametric t-test was used to determine p-values (* $p < 0.05$, *** $p < 0.0001$).

Figure S3: Effects of HCI2509 on Transformation, Methylation, and Apoptosis

A,B,C) Quantification of colonies formed by (A) EWS-502, (B) TC71, and (C) SK-ES-1 cells treated with either vehicle (0.3% DMSO) or varying doses of HCI2509. Error bars indicate SD of duplicate assays. EC_{50} values were determined using GraphPad Prism 6.

D) Representative western blot analysis of H3K4 and H3K9 methylation marks in A673 cells treated with either vehicle (0.3% DMSO) or 2 μM HCI2509 for 48 hours.

D,E,F) Cell viability and caspase activation at 0, 24, and 48 hours in (D) SK-N-MC, (E) TC71, and (F) SK-ES-1 cells treated with $2xEC_{50}$ HCI2509. Measurements were normalized to their respective vehicle (0.3% DMSO) sample at the appropriate time point. Error bars indicate SD (n=3).

Figure S4: Regulation of HMOX1

A) qRT-PCR for HMOX1 induction following treatment with candidate LSD1 inhibitors with respect to inhibitor biochemical potency against LSD1 in a biochemical assay (Cayman Chemical).

B) Western blot analysis to demonstrate expression of the RNAi-resistant 3x-FLAG tagged EWS/FLI, Δ22, or R2L2 cDNA constructs using an anti-FLAG antibody in A673 cells expressing a control shRNA (Luc) or an EWS/FLI shRNA. Tubulin was used as the loading control.

C) qRT-PCR analysis to assess level of knockdown of various co-repressors or *HMOX1* induction in A673 cells treated with either Luc-RNAi or RNAi for REST (REST p=3.53E-6, *HMOX1* p=1.92E-2), RCoR1 (RCoR1 p=1.18E-4, *HMOX1* p=3.67E-2), NCoR/SMRT (NCoR/SMRT p=2.60E-7, *HMOX1* p=5.85E-1), or Sin3A (Sin3A p=1.27E-6, *HMOX1* p=1.57E-1). Error bars indicate SD and p-values were determined using students t-test (n=3).

D) Western blot analysis for HMOX1 expression in A673 cells infected either with empty vector or an HA-tagged HMOX1 cDNA using an anti-HMOX1 antibody. Tubulin was used as a loading control.

E) Growth assays (3T5) for A673 cells described in (D). Student's t-test showed no significant difference in growth curves.

F) Quantification of colonies formed by A673 cells described in (D). Error bars indicate SD of duplicate assays.

Figure S5: Tumor Volume, Body Weight and Blood Counts

A) In vivo subcutaneous hind-flank xenograft studies measuring tumor volume for animals bearing tumors grown from (A) SK-ES-1 cells. The p-value was determined by 2-way ANOVA comparing the treatment curve to the vehicle curve. Individual tumor growth curves are shown for the vehicle-treated (blue) and HCI2509-treated (red) groups.

B,C,D) Body weight measurements for animals bearing tumors grown from (B) A673 cells, (C) SK-N-MC cells, and (D) SK-ES-1 cells. N=10 for all groups, with the exception of SK-N-MC HCI2509 treated group as noted. For body weights, the change in body weight normalized to day 0 was considered and a student's t-test was used to determine the p-value.

E) Blood counts for white blood cells (WBC), hematocrit (HCT), and platelets (PLT) from immunodeficient mice treated intraperitoneally either with vehicle or 40 mg/kg HCI2509 MWF for 24 days ± SD. Blood was drawn using a cheek draw and assayed at both day 0 and day 24. The normal range is reported.

Figure S1

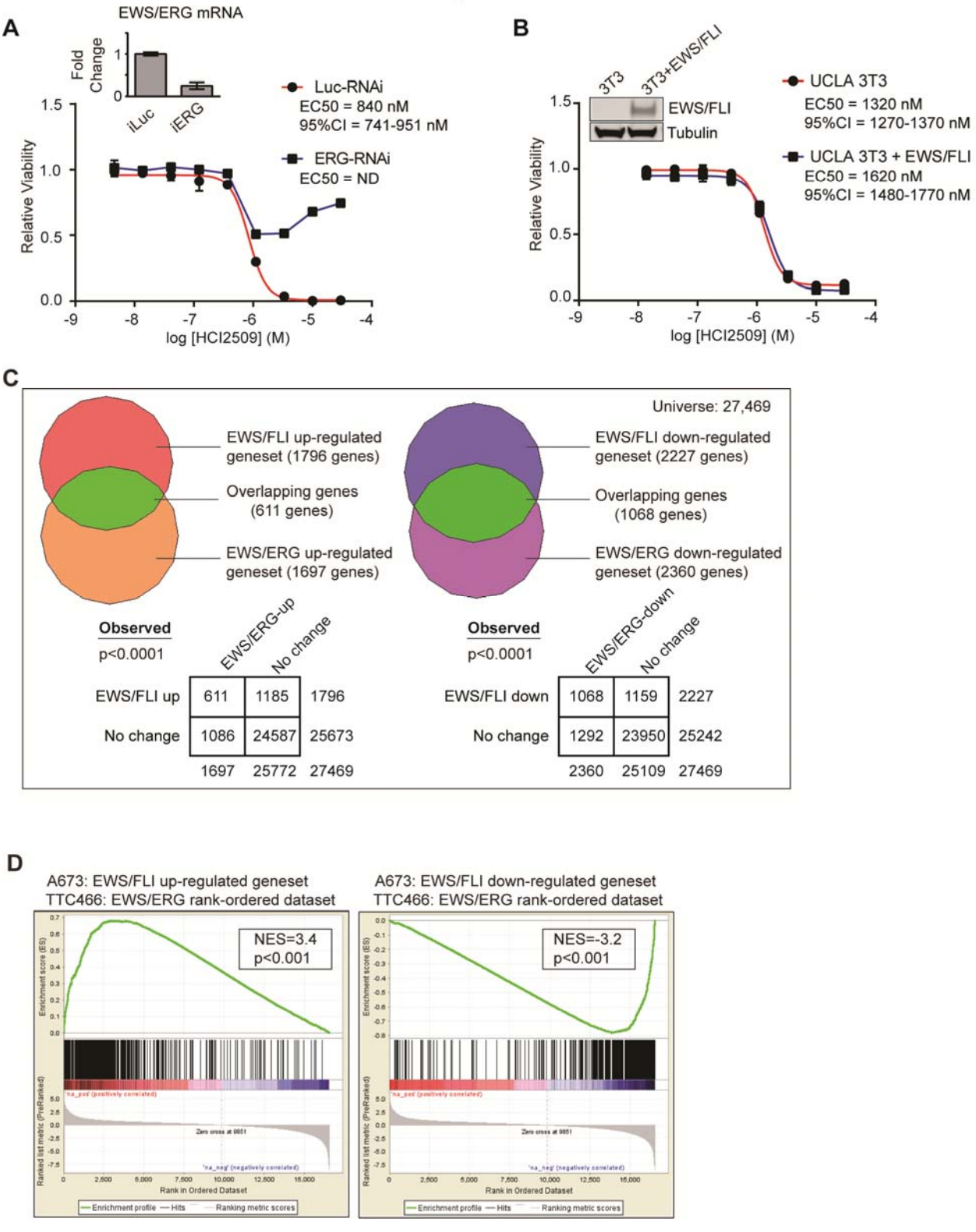
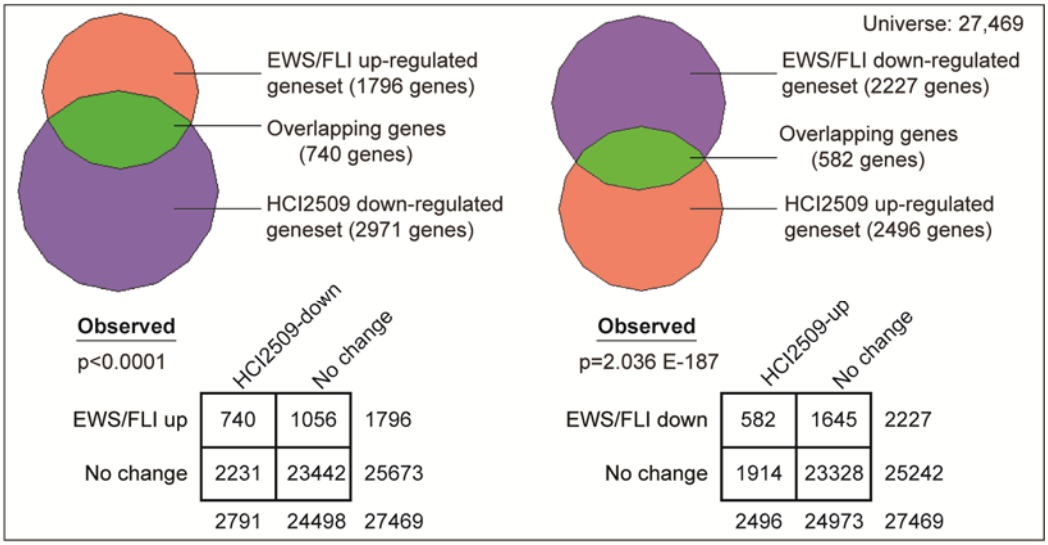

A
EWS/ERG mRNA
Fold Change
iLuc
iERG
Luc-RNAi
EC50 = 840 nM
95%CI = 741-951 nM
ERG-RNAi
EC50 = ND
Relative Viability
log [HCI2509] (M)
B
3T3
3T3+EWS/FLI
EWS/FLI
Tubulin
UCLA 3T3
EC50 = 1320 nM
95%CI = 1270-1370 nM
UCLA 3T3 + EWS/FLI
EC50 = 1620 nM
95%CI = 1480-1770 nM
C
Universe: 27,469
EWS/FLI up-regulated geneset (1796 genes)
Overlapping genes (611 genes)
EWS/ERG up-regulated geneset (1697 genes)
EWS/FLI down-regulated geneset (2227 genes)
Overlapping genes (1068 genes)
EWS/ERG down-regulated geneset (2360 genes)
Observed
p<0.0001
EWS/ERG-up
No change
EWS/FLI up 611 1185 1796
No change 1086 24587 25673
1697 25772 27469
Observed
p<0.0001
EWS/ERG-down
No change
EWS/FLI down 1068 1159 2227
No change 1292 23950 25242
2360 25109 27469
D
A673: EWS/FLI up-regulated geneset
TTC466: EWS/ERG rank-ordered dataset
NES=3.4
p<0.001
A673: EWS/FLI down-regulated geneset
TTC466: EWS/ERG rank-ordered dataset
NES=-3.2
p<0.001
Enrichment score (ES)
Ranked list metric (PreRanked)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Figure S1

E



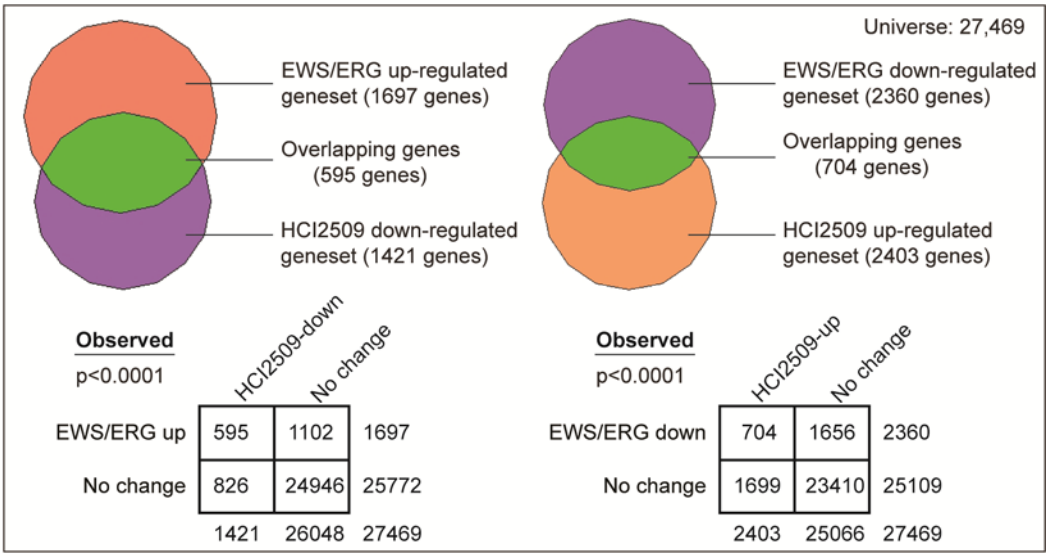
Observed p<0.0001

	HCI2509-down	No change	
EWS/FLI up	740	1056	1796
No change	2231	23442	25673
	2791	24498	27469

Observed p=2.036 E-187

	HCI2509-up	No change	
EWS/FLI down	582	1645	2227
No change	1914	23328	25242
	2496	24973	27469

F



Observed p<0.0001

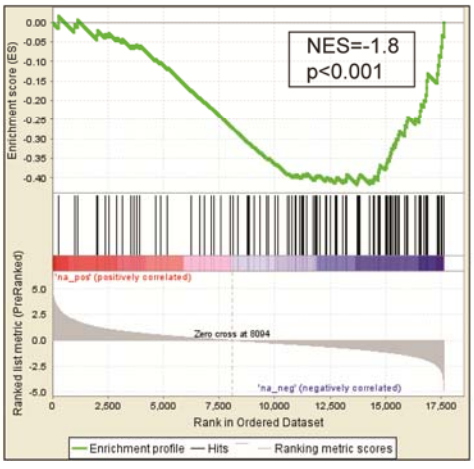
	HCI2509-down	No change	
EWS/ERG up	595	1102	1697
No change	826	24946	25772
	1421	26048	27469

Observed p<0.0001

	HCI2509-up	No change	
EWS/ERG down	704	1656	2360
No change	1699	23410	25109
	2403	25066	27469

G

A673: EWS/FLI direct up-regulated geneset
A673: HCI2509 rank-ordered dataset



A673: EWS/FLI direct down-regulated geneset
A673: HCI2509 rank-ordered dataset

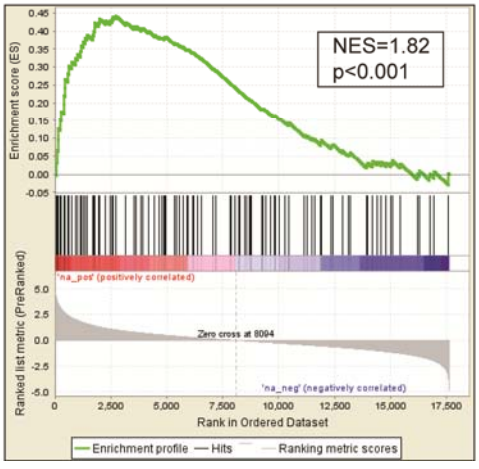
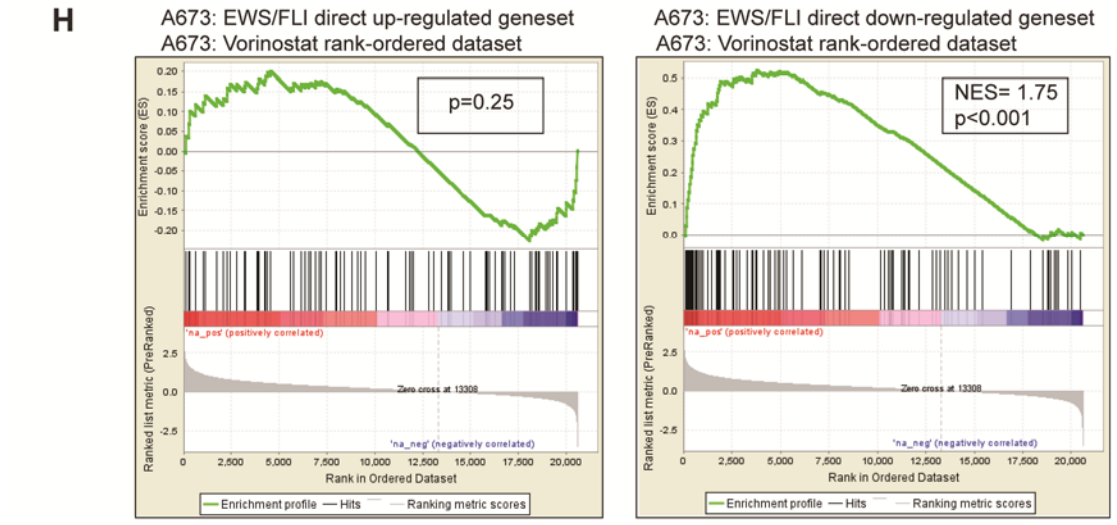
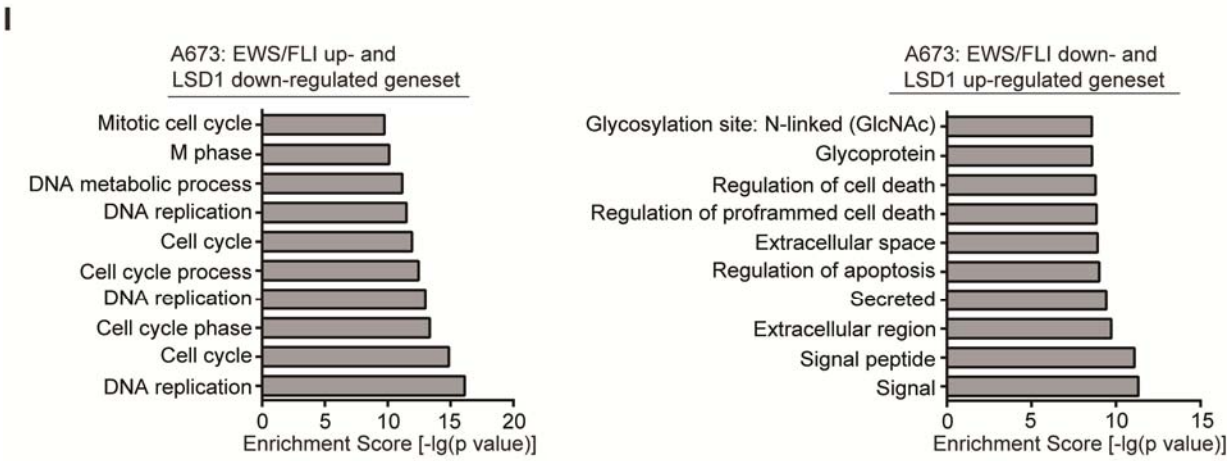


Figure S1


H
A673: EWS/FLI direct up-regulated geneset
A673: Vorinostat rank-ordered dataset
p=0.25
A673: EWS/FLI direct down-regulated geneset
A673: Vorinostat rank-ordered dataset
NES= 1.75
p<0.001
Enrichment score (ES)
Ranked list metric (PreRanked)
'na_pos' (positively correlated)
'na_neg' (negatively correlated)
Zero cross at 13308
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


I
A673: EWS/FLI up- and
LSD1 down-regulated geneset
Mitotic cell cycle
M phase
DNA metabolic process
DNA replication
Cell cycle
Cell cycle process
DNA replication
Cell cycle phase
Cell cycle
DNA replication
Enrichment Score [-lg(p value)]
A673: EWS/FLI down- and
LSD1 up-regulated geneset
Glycosylation site: N-linked (GlcNAc)
Glycoprotein
Regulation of cell death
Regulation of proframmed cell death
Extracellular space
Regulation of apoptosis
Secreted
Extracellular region
Signal peptide
Signal
Enrichment Score [-lg(p value)]

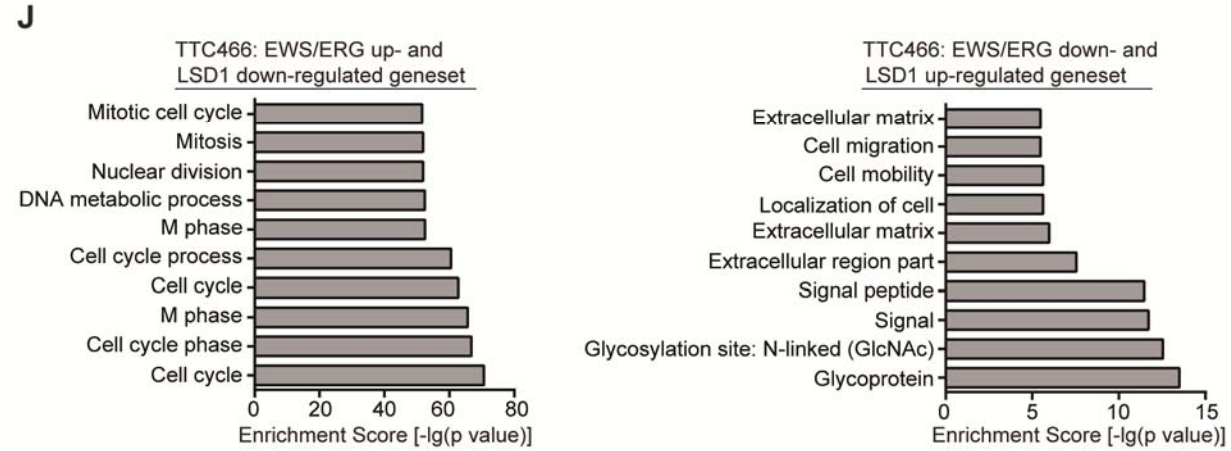

J
TTC466: EWS/ERG up- and
LSD1 down-regulated geneset
Mitotic cell cycle
Mitosis
Nuclear division
DNA metabolic process
M phase
Cell cycle process
Cell cycle
M phase
Cell cycle phase
Cell cycle
Enrichment Score [-lg(p value)]
TTC466: EWS/ERG down- and
LSD1 up-regulated geneset
Extracellular matrix
Cell migration
Cell mobility
Localization of cell
Extracellular matrix
Extracellular region part
Signal peptide
Signal
Glycosylation site: N-linked (GlcNAc)
Glycoprotein
Enrichment Score [-lg(p value)]

Figure S1

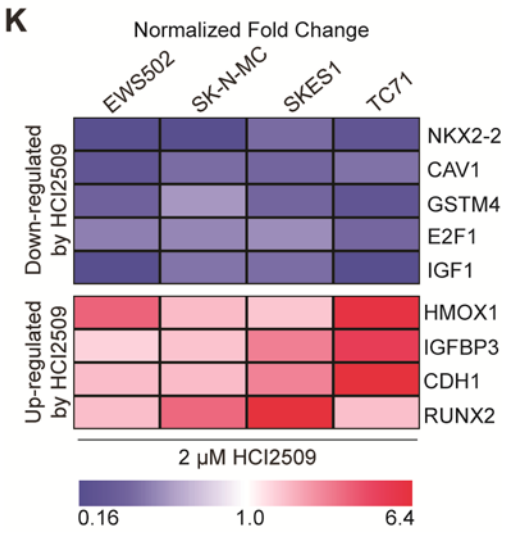

K
Normalized Fold Change
EWS502
SK-N-MC
SKES1
TC71
Down-regulated by HCI2509
NKX2-2
CAV1
GSTM4
E2F1
IGF1
Up-regulated by HCI2509
HMOX1
IGFBP3
CDH1
RUNX2
2 μM HCI2509
0.16
1.0
6.4

Figure S2

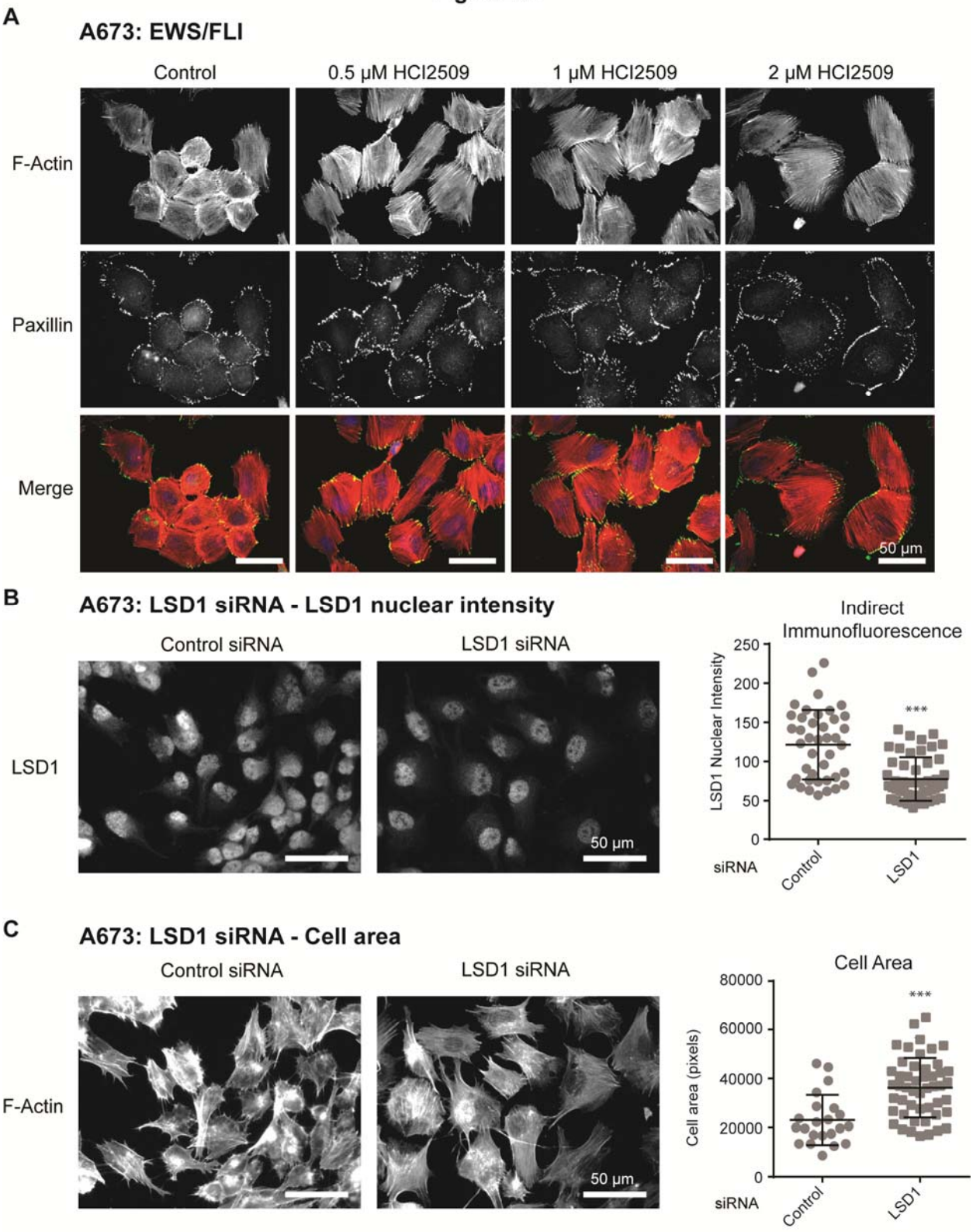

A
A673: EWS/FLI
Control
0.5 µM HCI2509
1 µM HCI2509
2 µM HCI2509
F-Actin
Paxillin
Merge
50 µm
B
A673: LSD1 siRNA - LSD1 nuclear intensity
Control siRNA
LSD1 siRNA
LSD1
50 µm
Indirect Immunofluorescence
LSD1 Nuclear Intensity
250
200
150
100
50
0
***
siRNA
Control
LSD1
C
A673: LSD1 siRNA - Cell area
Control siRNA
LSD1 siRNA
F-Actin
50 µm
Cell Area
Cell area (pixels)
80000
60000
40000
20000
0
***
siRNA
Control
LSD1

Figure S2

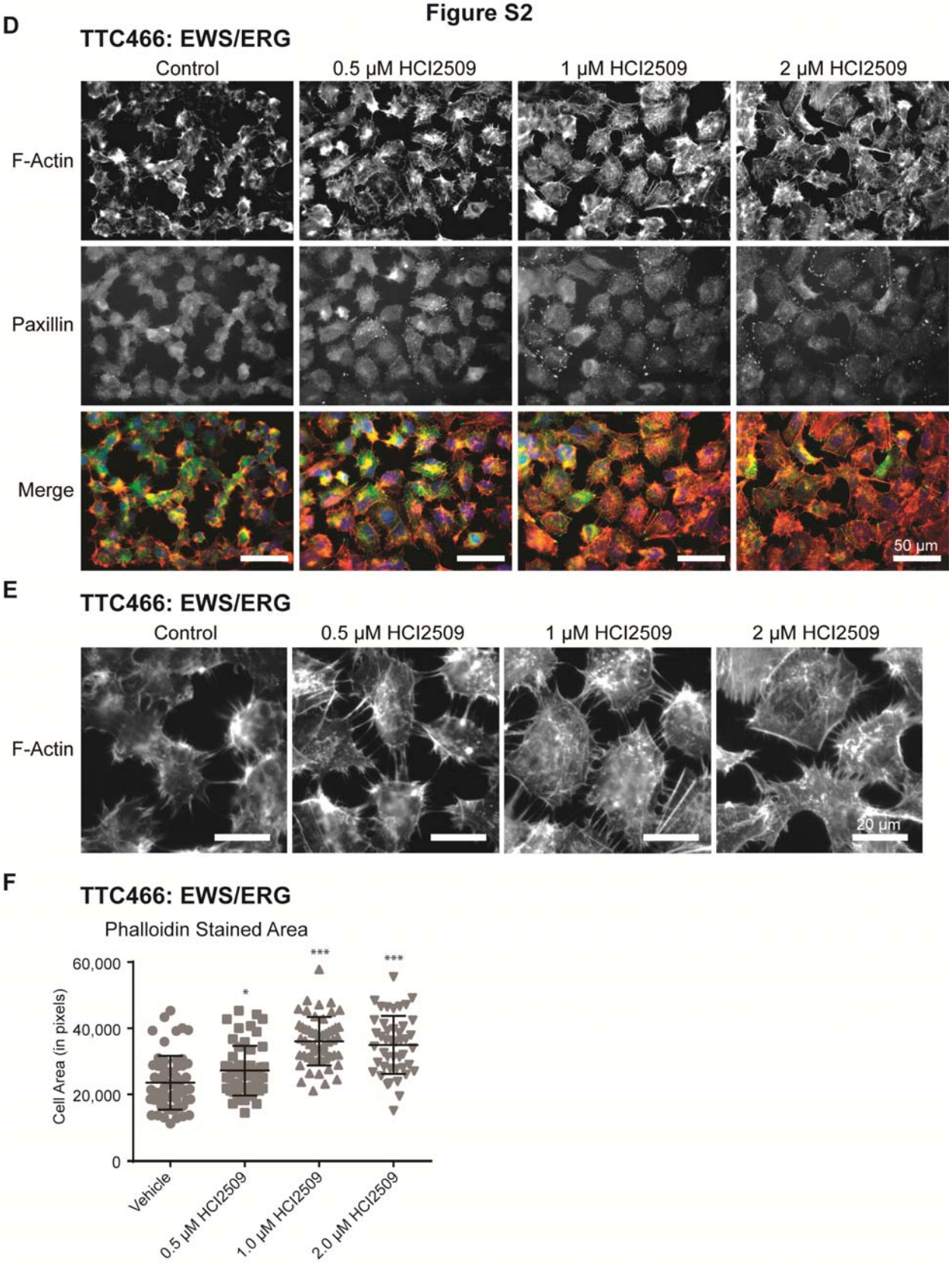

D
TTC466: EWS/ERG
Control
0.5 μM HCI2509
1 μM HCI2509
2 μM HCI2509
F-Actin
Paxillin
Merge
50 μm
E
TTC466: EWS/ERG
Control
0.5 μM HCI2509
1 μM HCI2509
2 μM HCI2509
F-Actin
20 μm
F
TTC466: EWS/ERG
Phalloidin Stained Area
Cell Area (in pixels)
60,000
40,000
20,000
0
*
***
***
Vehicle
0.5 μM HCI2509
1.0 μM HCI2509
2.0 μM HCI2509

Figure S3

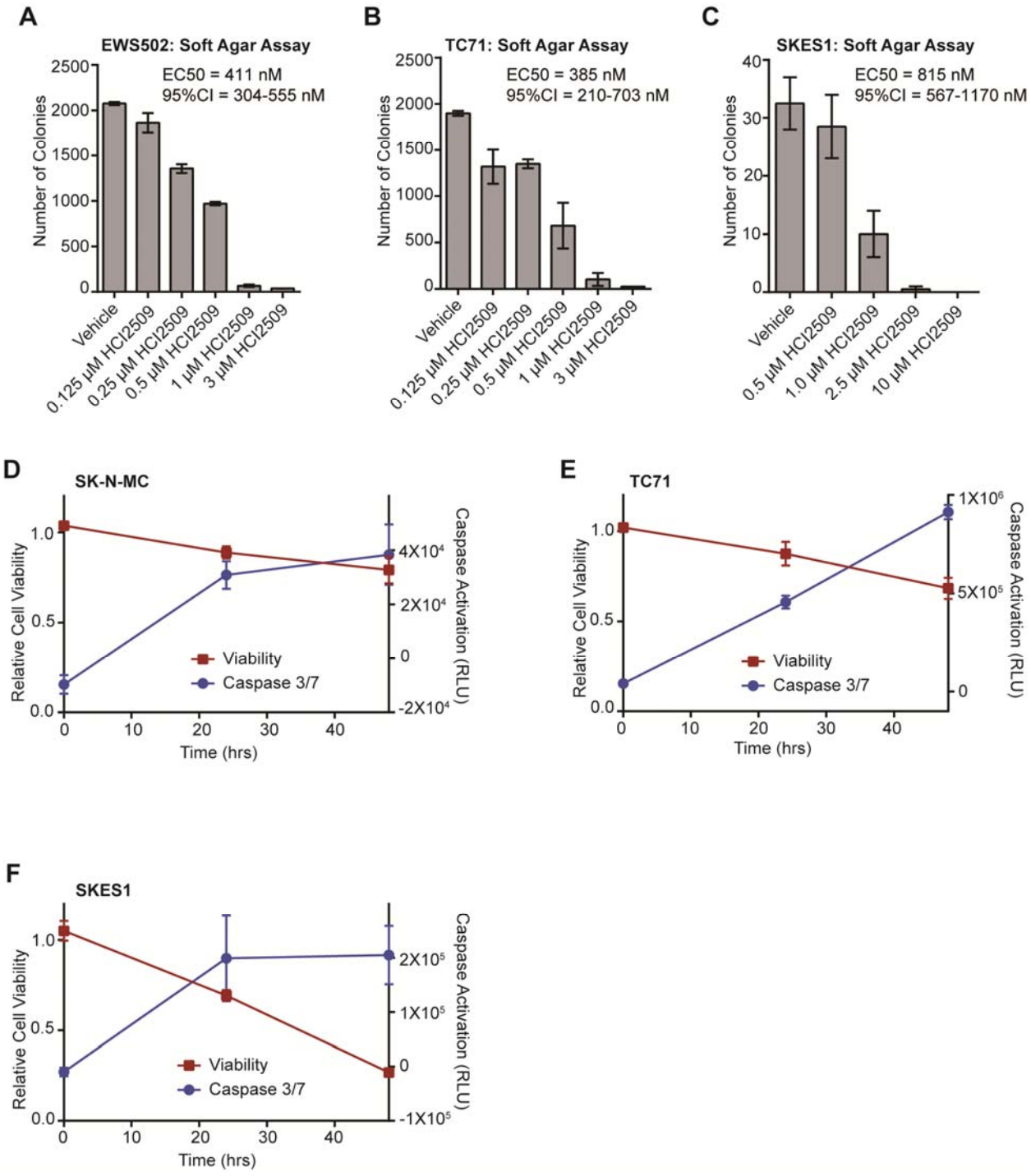

A
EWS502: Soft Agar Assay
EC50 = 411 nM
95%CI = 304-555 nM
Number of Colonies
2500
2000
1500
1000
500
0
Vehicle
0.125 μM HCl2509
0.25 μM HCl2509
0.5 μM HCl2509
1 μM HCl2509
3 μM HCl2509
B
TC71: Soft Agar Assay
EC50 = 385 nM
95%CI = 210-703 nM
Number of Colonies
2500
2000
1500
1000
500
0
Vehicle
0.125 μM HCl2509
0.25 μM HCl2509
0.5 μM HCl2509
1 μM HCl2509
3 μM HCl2509
C
SKES1: Soft Agar Assay
EC50 = 815 nM
95%CI = 567-1170 nM
Number of Colonies
40
30
20
10
0
Vehicle
0.5 μM HCl2509
1.0 μM HCl2509
2.5 μM HCl2509
10 μM HCl2509
D
SK-N-MC
Relative Cell Viability
1.0
0.5
0.0
Caspase Activation (RLU)
4X10⁴
2X10⁴
0
-2X10⁴
Viability
Caspase 3/7
0
10
20
30
40
Time (hrs)
E
TC71
Relative Cell Viability
1.0
0.5
0.0
Caspase Activation (RLU)
1X10⁶
5X10⁵
0
Viability
Caspase 3/7
0
10
20
30
40
Time (hrs)
F
SKES1
Relative Cell Viability
1.0
0.5
0.0
Caspase Activation (RLU)
2X10⁵
1X10⁵
0
-1X10⁵
Viability
Caspase 3/7
0
10
20
30
40
Time (hrs)

Figure S4

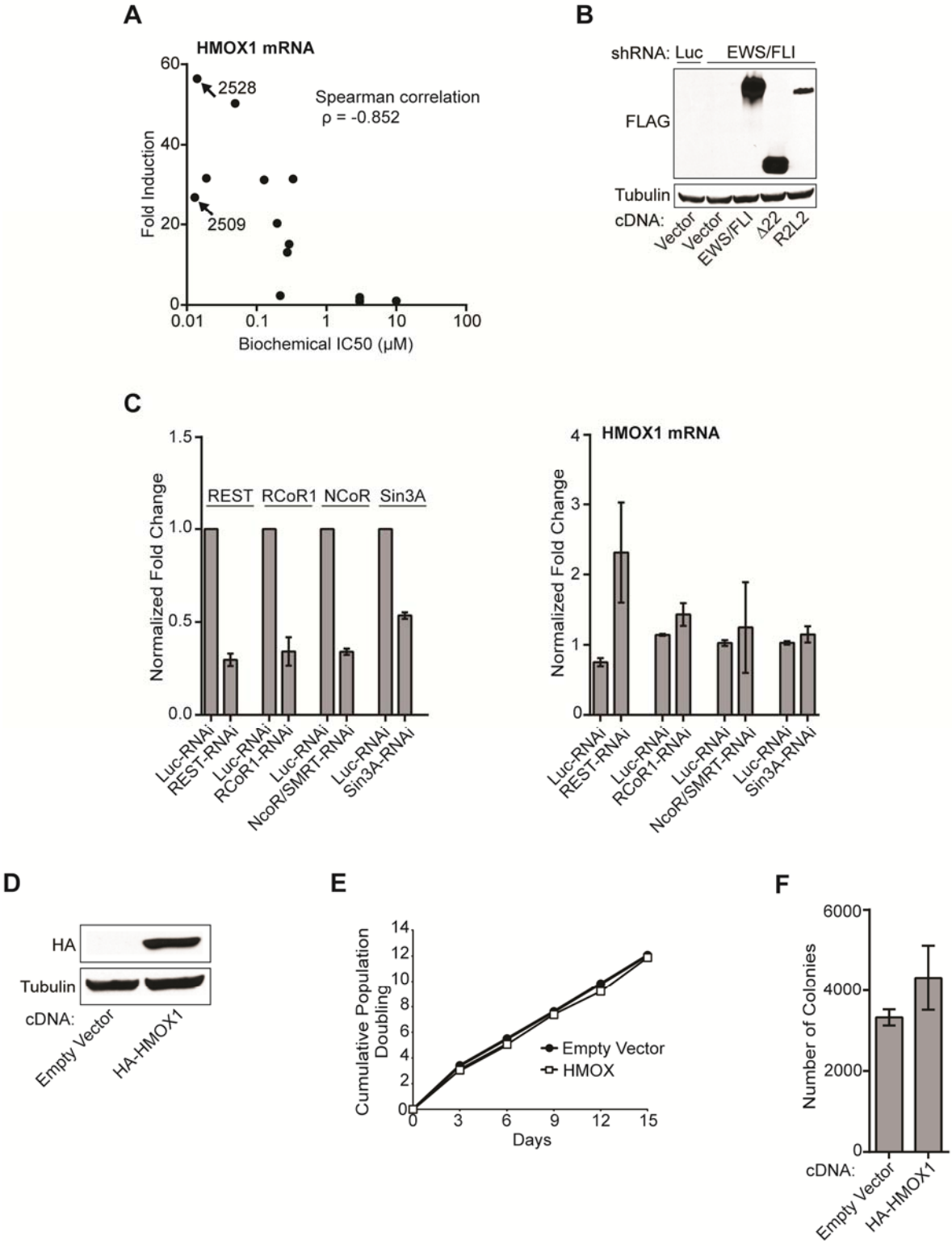
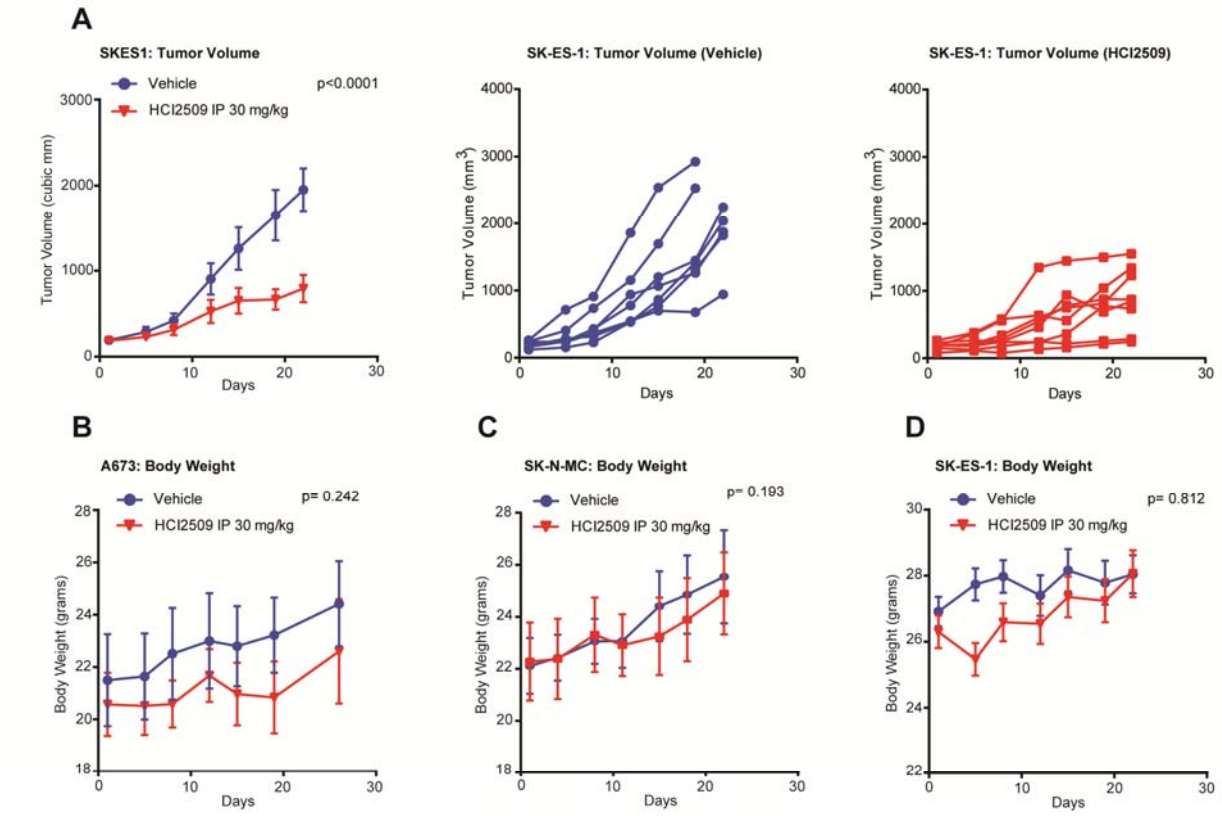

A
HMOX1 mRNA
2528
2509
Spearman correlation
ρ = -0.852
Fold Induction
Biochemical IC50 (μM)
B
shRNA: Luc
EWS/FLI
FLAG
Tubulin
cDNA:
Vector
Vector
EWS/FLI
Δ22
R2L2
C
REST
RCoR1
NCoR
Sin3A
Normalized Fold Change
Luc-RNAi
REST-RNAi
RCoR1-RNAi
NcoR/SMRT-RNAi
Sin3A-RNAi
HMOX1 mRNA
D
HA
Tubulin
cDNA:
Empty Vector
HA-HMOX1
E
Cumulative Population Doubling
Empty Vector
HMOX
Days
F
Number of Colonies
cDNA:
Empty Vector
HA-HMOX1

Figure S5



E

Measurement	Vehicle Day 0	Vehicle Day 24	2509 Day 0	2509 Day 24	Normal Range
WBC (x 10^3/μL)	6.53 ± 1.06	7.75 ± 4.13	9.15 ± 0.92	9.64 ± 3.25	2.6-10.1
HCT (%)	39.60 ± 6.76	42.75 ± 11.27	42.90 ± 12.44	44.20 ± 11.86	32.8-48.0
PLT (x 10^3/μL)	230.75 ± 55.09	214.00 ± 61.97	177.75 ± 45.02	195.00 ± 45.02	250-1540