The combination of epigenetic drugs SAHA and HCI-2509 synergistically inhibits EWS-FLI1 and tumor growth in Ewing sarcoma

SUPPLEMENTARY MATERIALS

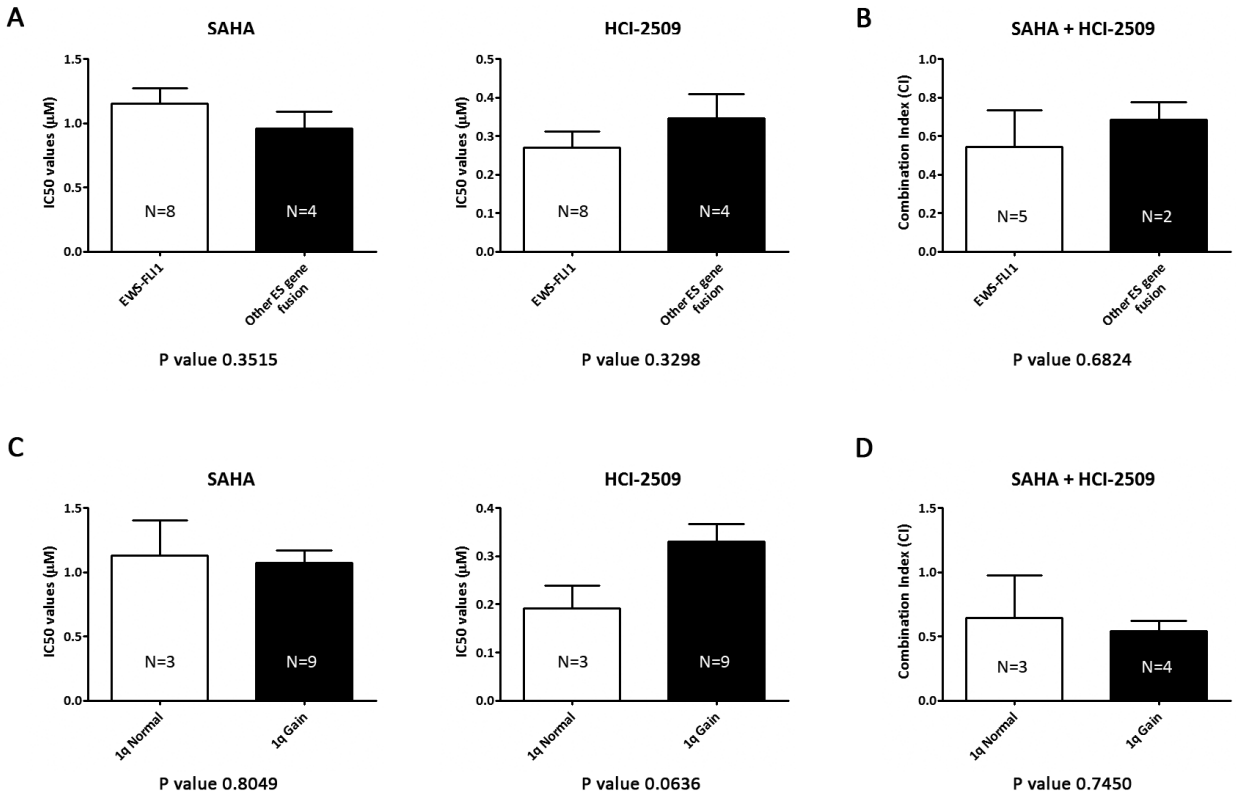
Supplementary Table 1: Taqman probes used for qRT-PCR

Genes	Brand	Reference	Amplicon length
CCND1	Thermo Fisher	Hs00765553_m1	57 bp
DKK1	Thermo Fisher	Hs00183740_m1	68 bp
EWSR1	Thermo Fisher	Hs01580530_gH	89 bp
EWS-FLI1	Thermo Fisher	Hs03024497_ft	80 bp
EZH2	Thermo Fisher	Hs00544830_m1	86 bp
TGFβR2	Thermo Fisher	Hs00234253_m1	70 bp
TPT1	Thermo Fisher	Hs02621289_g1	131 bp

Supplementary Table 2: The clinical characteristics of the ES patients

Model code	Age at dx	Age at biopsy	Source of biopsy[1]	Primary tumor	Why biopsy	Fusion gene	STAG2 mutation	Patiet status[2]
HSJD-ES-001	17 y	21.7	Scapula	Scapula	Relapse	EWS-FLI1 type 2	mutated	DOD
HSJD-ES-004	10 y	18.0	Mediastinum	Vertebral body T12	Relapse	EWS-FLI1 type 1	Wild type	NED
HSJD-ES-006	13 y	13.7	Lung nodule	Fibula	Relapse	EWS-FLI1 type 1 ex 10-ex 5	Wild type	DOD
HSJD-ES-011	10 y	13.9	Pleura	n.a.	Relapse	EWS-FLI1 type 1	Not done	AWD

[1]Tumor tissues were collected with informed consent under an Institutional Review Board-approved protocol.
[2]DOD died of disease, NED no evidence of disease, AWD alive with disease.



E

HCI-2509 and SAHA inhibitory concentrations

HCI-2509 (iLSD1)

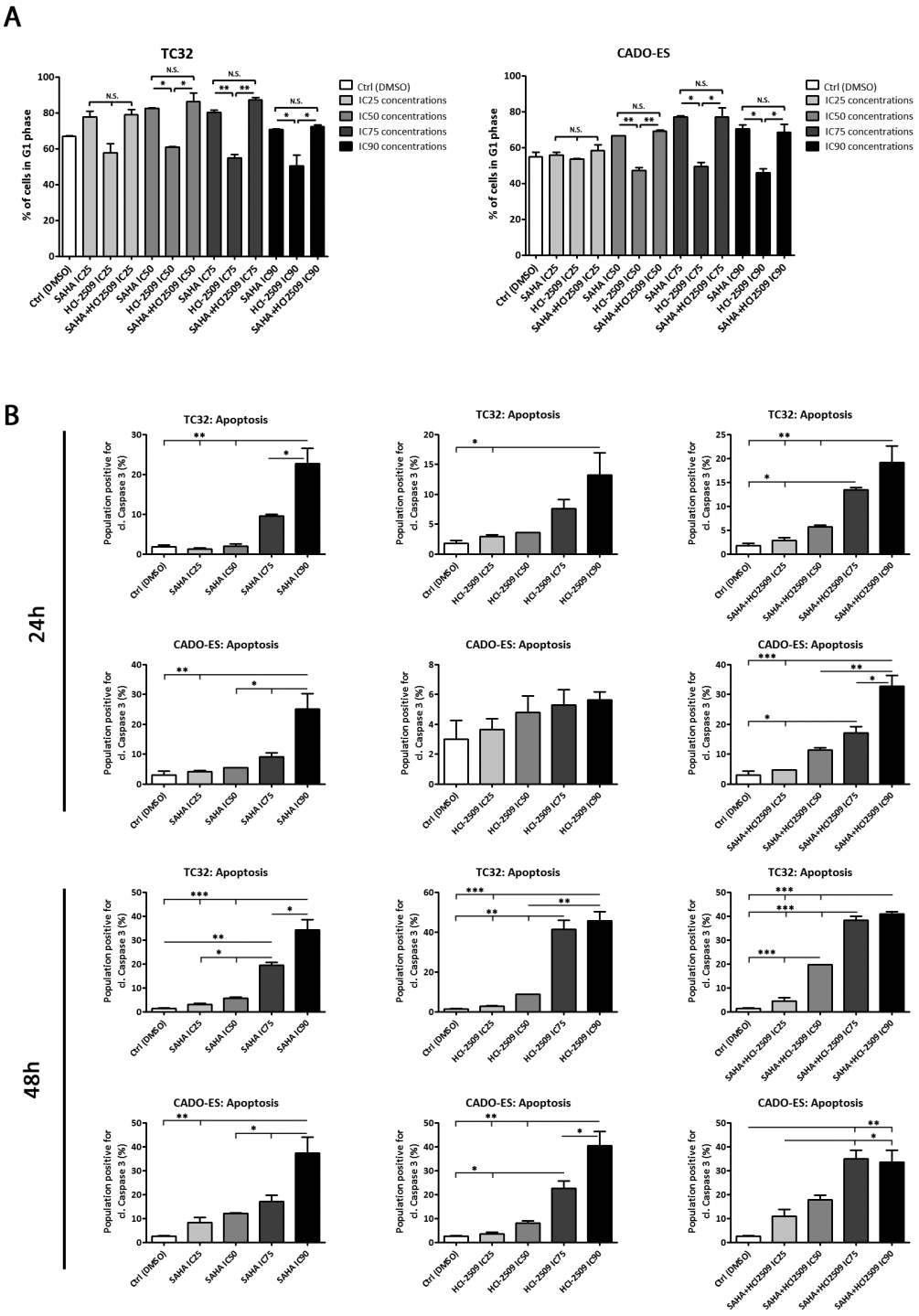
Cell Line	*IC25 (µM)*	*s.d.*	*IC50 (µM)*	*s.d.*	*IC75 (µM)*	*s.d.*	*IC90 (µM)*	*s.d.*
A673	0.029	0.022	0.097	0.052	0.372	0.036	1.951	0.381
CADO-ES	0.166	0.027	0.282	0.039	0.494	0.042	0.877	0.111
TC32	0.117	0.032	0.187	0.019	0.479	0.323	0.780	0.193

SAHA

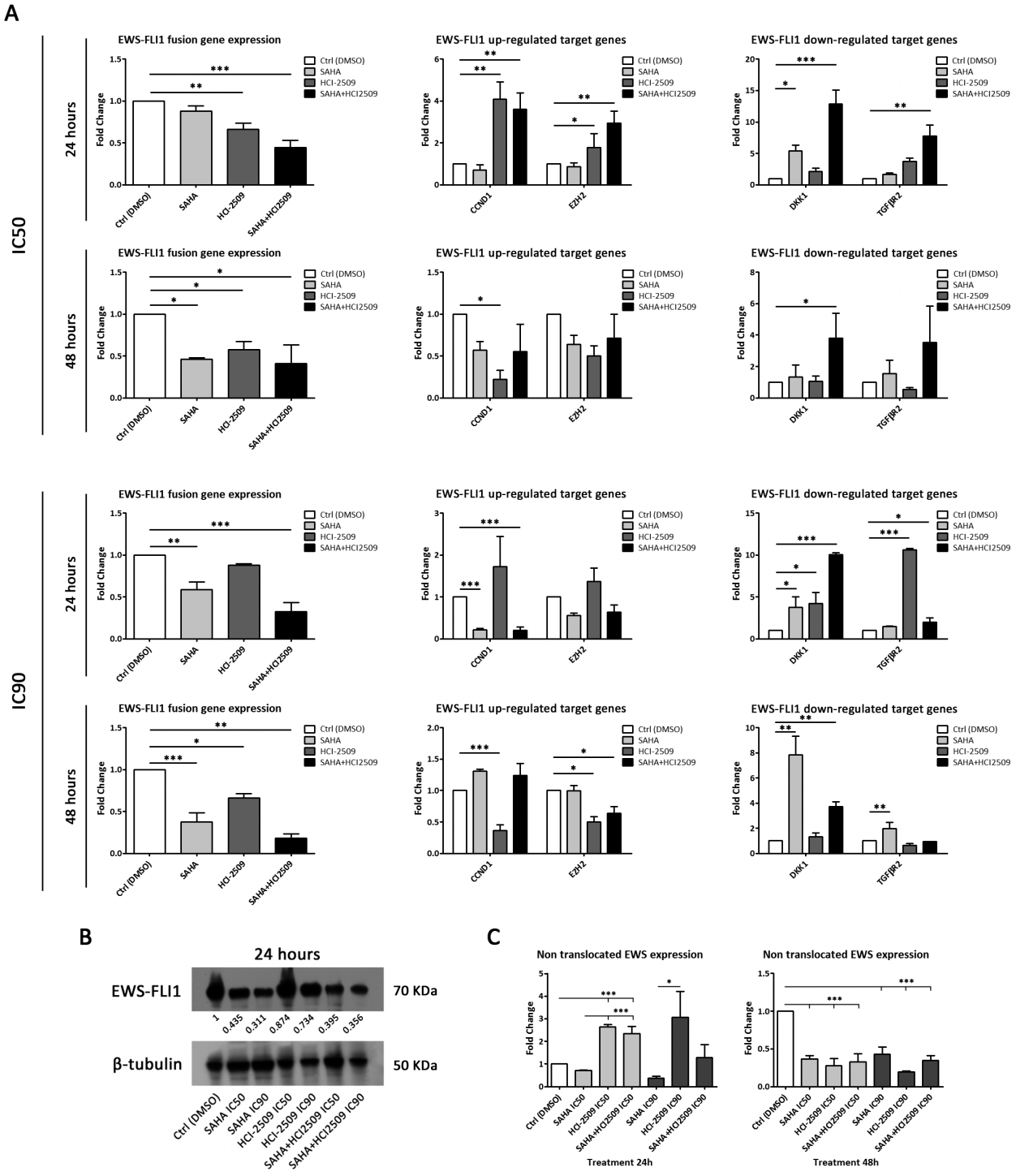
Cell Line	*IC25 (µM)*	*s.d.*	*IC50 (µM)*	*s.d.*	*IC75 (µM)*	*Cell Line*	*IC90 (µM)*	*s.d.*
A673	0.935	0.002	1.676	0.347	2.307	0.115	3.779	0.290
CADO-ES	0.604	0.118	1.089	0.168	1.634	0.286	2.755	0.568
TC32	0.902	0.123	1.553	0.160	2.495	0.253	4.432	0.505

Proliferation IC values of ES cell lines assayed for HCI-2509 or SAHA sensitivity and measured after 72h of drug exposition

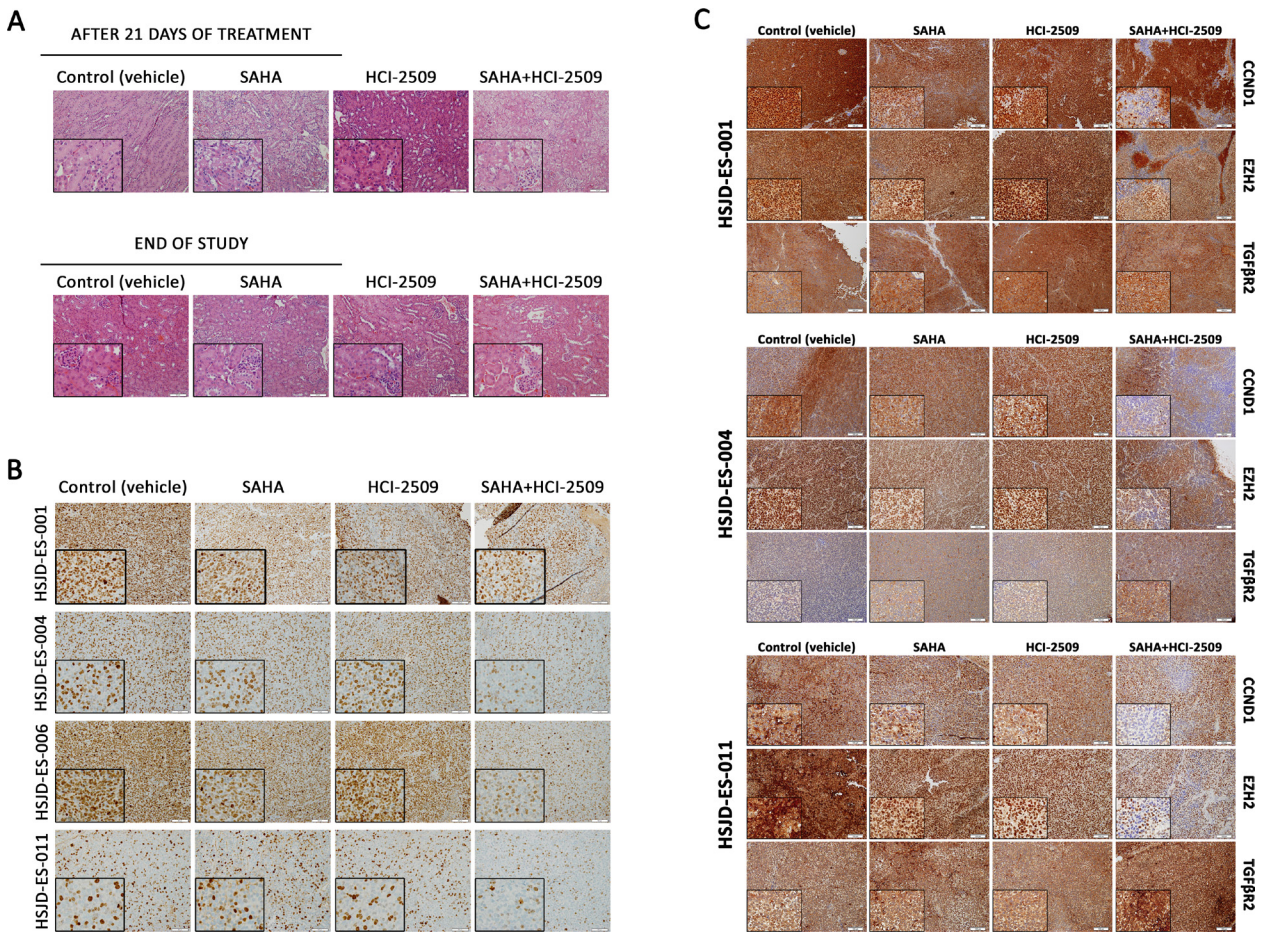
Supplementary Figure 1: Drug sensitivity is independent from ES fusion type or 1q status in ES cell lines. (**A**) Mean of SAHA and HCI-2509 IC50 values between *EWS-FLI1* fusion type-ES cell lines group and others fusion types-ES cell line group (*EWS-ERG* and *EWS-FEV*) are shown. (**B**) SAHA+HCI-2509 combination index (CI) of *EWS-FLI1* fusion type-ES cell lines group and others fusion types-ES cell lines group is shown. (**C**) Drug sensitivity (IC50 values) between 1q normal ES cell lines group and 1q gain ES cell line group. (**D**) SAHA+HCI-2509 combination index (CI) of 1q normal ES cell lines group and 1q gain ES cell lines group. Values shown are mean ± s.d. of three independent replicates. (**E**) IC25, IC50, IC75 and IC90 values of ES cell lines assayed for HCI-2509 or SAHA after 72 h exposition to the drugs are shown.



Supplementary Figure 2: SAHA+HCI-2509 combination altered cell cycle progression and induced apoptosis. (**A**) Quantification of cells in G1 phase of cell cycle after SAHA, HCI-2509 and combination treatment are shown in TC32 and CADO-ES cell lines. (**B**) Apoptosis induction detailed analysis of population positive for cleaved Caspase 3 in TC32 and CADO-ES cell lines after 24 h (upper panel) and 48 h (lower panel) of SAHA, HCI-2509, alone or in combination, at different concentrations. In all cases, mean ± s.d. of three biological independent replicates. Statistical tests: significant analysis of variance, Tukey post-hoc test <0.001 (***), 0.01 (**), and 0.05 (*).



Supplementary Figure 3: SAHA, HCI-2509, and SAHA+HCI-2509 treatment inhibited EWS-FLI1 expression in the A673 cell line. (**A**) mRNA expression analysis by RT-qPCR of EWS-FLI1 (left column), and EWS-FLI1-induced/repressed target genes (middle/right column) after 24 h and 48 h of SAHA, HCI-2509 and combination treatment at IC50 (upper panel) and IC90 concentrations (lower panel) in A673 cell line. (**B**) Immunoblot of EWS-FLI1 protein expression after 24h of SAHA, HCI-2509 and combination treatment at IC50 and IC90 concentrations in A673 cell line is shown. Relative quantification is showed respect to the control (DMSO). (**C**) mRNA expression analysis by RT-qPCR of non-translocated EWSR1 after 24 h (left panel) and 48 h (right panel) of SAHA, HCI-2509 and combination treatment at IC50 and IC90 concentrations in TC32 cell line. Mean ± s.d. of three biological independent replicates is shown. Statistical tests: significant analysis of variance, Tukey post-hoc test <0.001 (***), 0.01 (**), and 0.05 (*).



Supplementary Figure 4: Histopathological study of PDX treated tumor samples. (**A**) Morphological study of mice kidney tissue (hematoxylin and eosin staining) showed intracellular vacuolization induced by SAHA treatment in these samples. (**B**) Immunohistochemical staining of Ki67-positively labelled nuclei after 21 days of SAHA, HCI-2509, and combination treatment (20× and 40× magnifications). (**C**) Immunohistochemical staining of EWS-FLI1 targets CCND1, EZH2 and TGFβR2 in ES-PDX tumor samples treated in monotherapy or combination (20× and 40× magnifications).