Supplementary figure legends

Figure S1

(A) Expression of EWS/ATF1 in adult somatic cells. HA immunostaining was performed in histological sections of *EWS/ATF1*-inducible mice given 50 μg/ml doxycycline for 2 days. HA-positive cells were observed in the intestinal epithelium and hepatocytes whereas no staining was detected in the lung and kidney.

(B) Note that HA-positive cells are observed in fibrous tissue just above the skeletal muscles.

Figure S2

(A) The EWS/ATF1 expression in *EWS/ATF1*-induced tumors. Western blot analysis with anti-HA antibody and ATF1 antibody revealed the expression of EWS/ATF1 protein in these tumors. MP-CCS-SY, a human CCS cell lines expressing EWS/ATF1 was used as positive and negative control for ATF1 and HA immunoblot, respectively. Note that HA antibody-detectable fusion protein in *EWS/ATF1*-induced tumors has the same molecular size as the products detected by ATF1 antibody in *EWS/ATF1*-induced tumors.

(B) Kaplan-Meier plots showing the survival rates. The *EWS/ATF1*-induced (50 μg/ml doxycycline treatment) group included 39 *EWS/ATF1*-inducible animals. The control (no doxycycline treatment) group included 21 *EWS/ATF1* inducible animals. The statistical analysis was performed using Mantel Cox test.

(C) Multiple microscopic lesions in the lung. We performed detailed histological analysis of 9 mice that had been given doxycycline for more than 3 months to examine microscopic lesions. In 2 out of 9 cases, we observed multiple microscopic nodules in the lungs and lymph nodes of *EWS/ATF1*-induced mice. The microscopic nodules

contained neoplastic cells, which expressed EWS-ATF1fusion protein, suggesting that they may be metastatic lesions. Scale bar: 200 μm.

Figure S3

(A) A semi-quantitative RT-PCR analysis for *Wnt1* expression. Note that all the three X-gal positive tumors do not express *Wnt1*. RNAs from E10.5 mouse embryo were used as a positive control for *Wnt1* RT-PCR, and those from NIH3T3 were used as a negative control.

(B) As a positive control for X-gal staining, Whisker follicles from transgenic mice containing *Wnt1-Cre* and *Floxed-LacZ* reporter alleles were incubated in the X-gal solution. Neural crest-derived cells in the dermal papilla were stained by X-gal. Scale bars: 200 μm.

(C) *EWS/ATF1*-induced tumors without *Floxed-LacZ* reporter alleles were not stained by X-gal (b). Scale bars: 50 μm.

(D) All of *EWS/ATF1*-induced tumors containing *Wnt1-Cre* and *Floxed-LacZ* reporter alleles were positive for X-gal staining. (H); Head, (V); Trunk (ventral), (D); Trunk (dorsal), (L); Limb, (W); Whisker pad.

Figure S4

(A) A semi-quantitative RT-PCR analysis for *P0* (*Mpz*) expression.

EWS/ATF1-induced tumors did not express *P0*. RNAs from E10.5 mouse embryo were used as a positive control for the analysis, and those from NIH3T3 were used as a negative control.

(B) *EWS/ATF1*-induced tumors without *P0*-Cre alleles were negative for the EYFP signal.

(C) All *EWS/ATF1*-induced tumors revealed EYFP signals.

(D) Histological sections of EYFP-positive tumors were immunostained with anti-GFP antibody. Positive staining was confirmed in the tumor cells.

Figure S5

The effects of different concentrations of doxycycline (0 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.2 μg/ml, and 2.0 μg/ml) on the *EWS/ATF1* expressions in tumor cell line (G1297). Both the transcript (A) and protein (B) was induced in a dose-dependent manner by

doxycycline treatment. The transcript levels were normalized to the *β-actin* levels. Each bars represents the means ± SD (n=3).

(C) The *EWS/ATF-1* induced tumor cells (G1297) were cultured in the concentration of 0, 0.05 or 0.2 μg/ml doxycycline for 72 hrs, and were stained with DAPI (blue) and with an anti-HA-Tag antibody (red). Representative immunofluorescent images indicated that level of EWS/ATF1 in individual cell depends on different concentration of doxycyline. Immunofluorescence images were taken with the same exposure times. Images in insets were processed identically to enhance immunofluorescent signals using Adobe Photoshop software. Note that all cells show weak fluorescent signals by the exposure of lower concentration of doxycycline. Scale bars: 50 μm.

Figure S6

(A) The list represents probes with increased signals by the expression of *EWS/ATF1* for 3 hours in microarray analysis. The probe list was generated according to the following criteria: probes with increased signals (i) more than 2 folds at 3 hours with low-Dox treatment (0.05 μg/ml), (ii) more than 2.5 folds with high-Dox treatment (0.2 μg/ml) at both 3 hours and 48 hours were extracted.

(B) Over-represented TFBS (transcription factor-binding site) in upregulated genes by EWS/ATF1 was identified using CisView. Importantly, the CREB-binding site is the most represented site, suggesting that the direct binding of EWS/ATF1 activates the target genes.

(C) Hierarchical clustering analysis of microarray expression data for an *in vivo* tumor sample and *in vitro* tumor cell lines. Two hundred seventy four genes that are upregulated more than two-fold in doxycycline-treated tumor cell line in comparison with the untreated cell line were selected, and the hierarchical clustering analysis was performed on log2-transformed data using Pearson correlation. Purple, black and green indicate higher, identical and lower relative expression, respectively.

Figure S7

(A) *Mitf-M* is not detectable in tumors from the *EWS/ATF1*-induced mice.

(B) *Mitf-M* is not detectable in the *EWS/ATF1*-induced tumor cell line by RT-PCR. RNAs from B16F1 (mouse melanoma cell line) and NIH3T3 were used as a positive and negative control for *Mitf-M*, respectively.

(C) A real-time RT-PCR analysis of G1169 tumor cell line revealed a significant

upregulation of both *EWS/ATF1* and *Fos* at 3 hours after doxycycline exposure.

(D) A rapid and transient induction of Fos with serum stimulation in MEFs following phosphorylation of the ERK1/2. The serum-starved MEFs were stimulated by 30% FBS for 0, 0.5, 4, 8, 12, 24, and 48 hrs. The expression of Fos and p-ERK were analyzed by Western blot.

(E) The upregulation of Fos in *EWS/ATF1*-induced tumor is independent of an ERK pathway. The serum-starved *EWS/ATF1*-expressing (left) and non-expressing (right) tumor cells were stimulated 30% FBS for 0, 0.5, 4, 8, 12, 24, and 48 hrs

Figure S8

(A) The relative expression of *FOS* mRNA in CCS cell lines and WI38. Transcript levels were normalized to the *GAPDH* levels. Each bar represents the mean ± SD (n=3). WI38: a lung fibroblast cells, MP-CCS-SY and KAS: clear cell sarcoma cell lines.

(B) The relative expression of *FOS* mRNA in surgically-resected tumor samples including two samples of CCS tissues and other sarcomas. The transcript levels were normalized to the *GAPDH* levels. Each bar represents the mean ± SD (n=3).

(C) The relative expression of *MITF-M* mRNA in A375, CCS cell lines and WI38. A375: melanoma cell line. Transcript levels were normalized to the *GAPDH* levels. Each bar represents the mean ± SD (n=3).

Figure S9

(A) The expression of EWS/ATF1 in human CCS cell lines (MP-CCS-SY and KAS) was detected by Western Blot with anti-ATF1 antibody. Osteosarcoma cell lines (HOS and U2OS) were used as negative control cells.

(B) The detection of *EWS/ATF1* transcript in human CCS cell lines. A 246-bp product indicates *EWS/ATF1* type1 in MP-CCS-SY, and a 186-bp product indicates *EWS/ATF1* type2 in KAS.

(C) The transfection of a specific siRNA targeting the breakpoint of *EWS/ATF1*type1 fusion gene into CCS cell lines. The knockdown efficiency was measured quantitatively by real-time RT-PCR analysis. Note that the siRNA specifically suppressed the expression of *EWS/ATF1* type1 in MP-CCS-SY cells. si-E/A represents siRNA targeting *EWS/ATF1*. Numbers in parenthesis indicate the doses of siRNA (nM). The transcript levels were normalized to the *GAPDH* levels. Each bars

represents the means ± SD (N=4).

(D) Western blot analysis of EWS/ATF-1 protein in MP-CCS-SY at 48 hrs after transfection with the specific siRNA targeting the breakpoint of *EWS/ATF1* type1. *β*-Actin was used as a loading control. The bands of EWS/ATF1 were quantified, and their ratios relative to *β*-Actin were calculated.

(E) The specific siRNA targeting the breakpoint of *EWS/ATF1*type1 did not affect the expression of *EWS/ATF1* type2 in KAS. The transcript levels were normalized to the *GAPDH* levels. Each bar represents the mean ± SD (n=4).

(F) The specific siRNA targeting the breakpoint of *EWS/ATF1*type1 did not affect the expression of *ATF1* in KAS. The transcript levels were normalized to the *GAPDH* levels. Each bar represents the mean ± SD (n=4).

(G) Decreased expression of *FOS* mRNA is observed 48 hrs after transfection with the siRNA targeting the breakpoint of *EWS/ATF1* type 1 in MP-CCS-SY cells. The transcript levels were normalized to the *GAPDH* levels. Each bar represents the mean ± SD (n=4).

(H) The data represents *MITF-M* mRNA at 48 hr after transfection with the siRNA targeting the breakpoint of *EWS/ATF1* type 1 in MP-CCS-SY. The transcript levels

were normalized to the *GAPDH* levels. Each bars represents the means ± SD (N=4).

Figure S10

(A) G1297 cells were treated with siRNA targeting *Fos*. The expression of *Fos* and *EWS/ATF1* mRNA at 24 hr after transfection was measured by real-time RT-PCR analysis. The transcript levels were normalized to the *β-actin* levels. Each bars represents the means ± SD (N=4).

(B) siRNA targeting *Fos* efficiently reduced the expression of Fos protein. G1297 cells were harvested at 48 hr after transfection with siRNA and were analyzed by a Western blot. β-Actin was used as a loading control.

Figure S11

(A)(B)(C)(D) The treatment of CCS cell lines with two different siRNAs targeting *FOS* (si-FOS#1 for A and B, si-FOS#2 for C and D). MP-CCS-SY and KAS cells were transfected with the siRNA targeting *FOS* (10 nM and 20 nM), the control siRNA (20nM), or the mock, and harvested at 48 hr after transfection. The knockdown efficiency of *FOS* was measured quantitatively by real-time RT-PCR analysis. The

transcript levels were normalized to the *GAPDH* levels. Each bar represents the means ± SD (n=4).

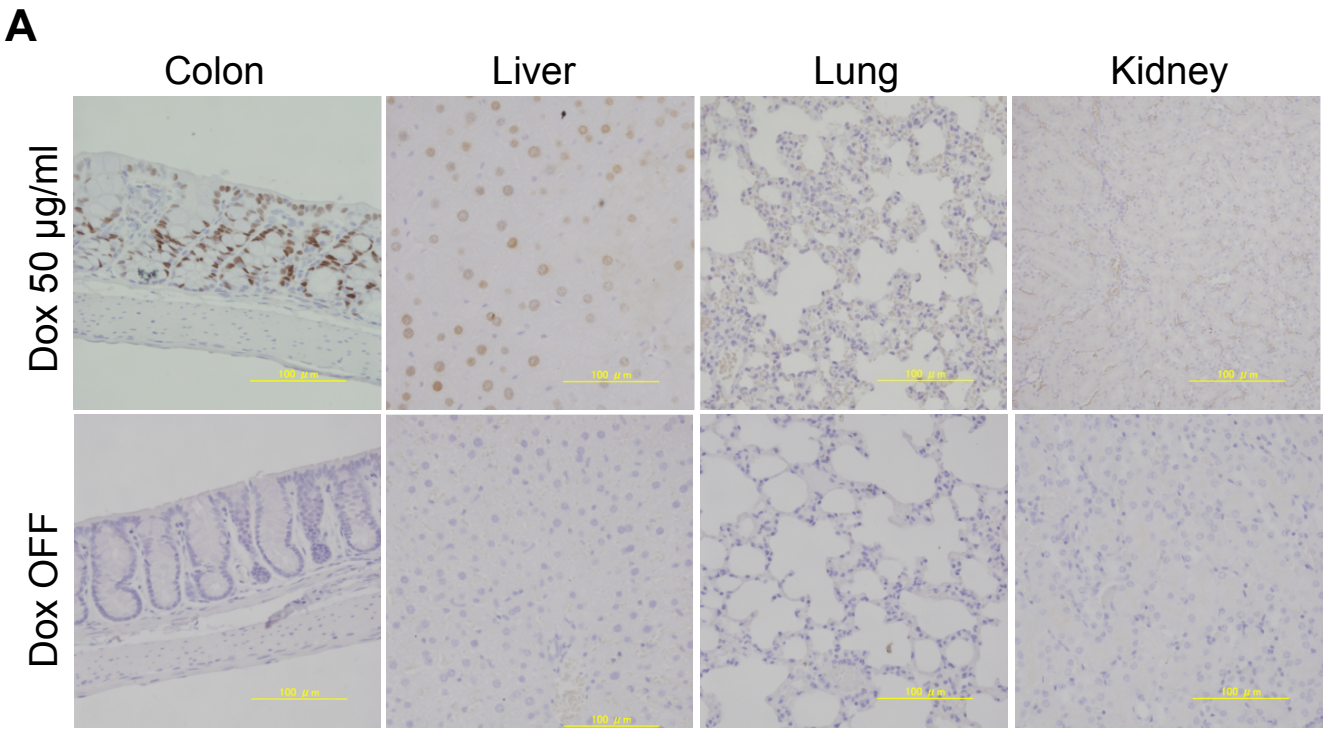
(E)(F) The effects of *FOS* knockdown on the growth of human CCS cell lines. Both MP-CCS-SY and KAS cells were treated with the siRNA#2 targeting *FOS* (10 nM and 20 nM), the control siRNA (20 nM), or lipofectamine alone (mock). Forty-eight and 96 hr later, the cell viability was determined by the WST-8 assay. The results represent the means ± SD (n=4). ***P < 0.001 vs. control siRNA and mock-treated cells.

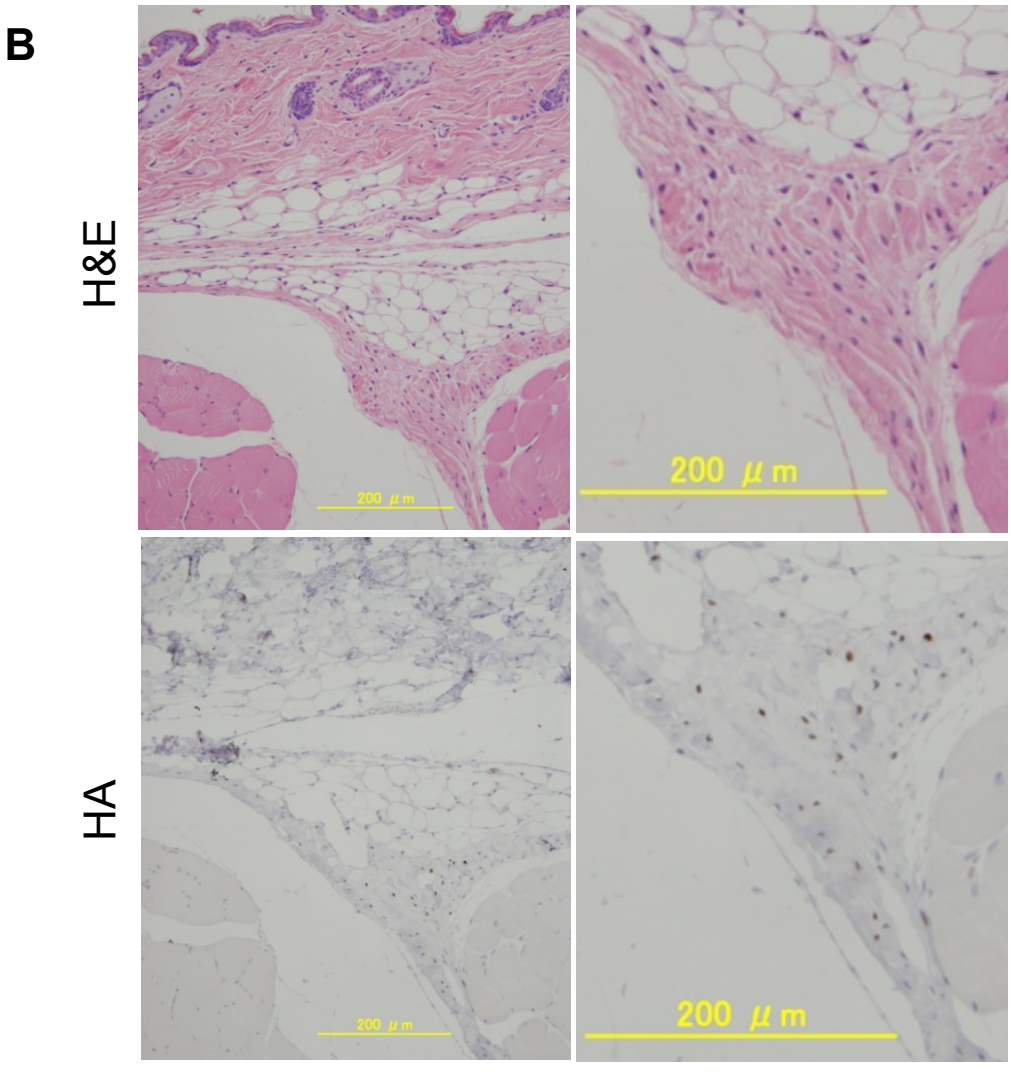
Figure S12

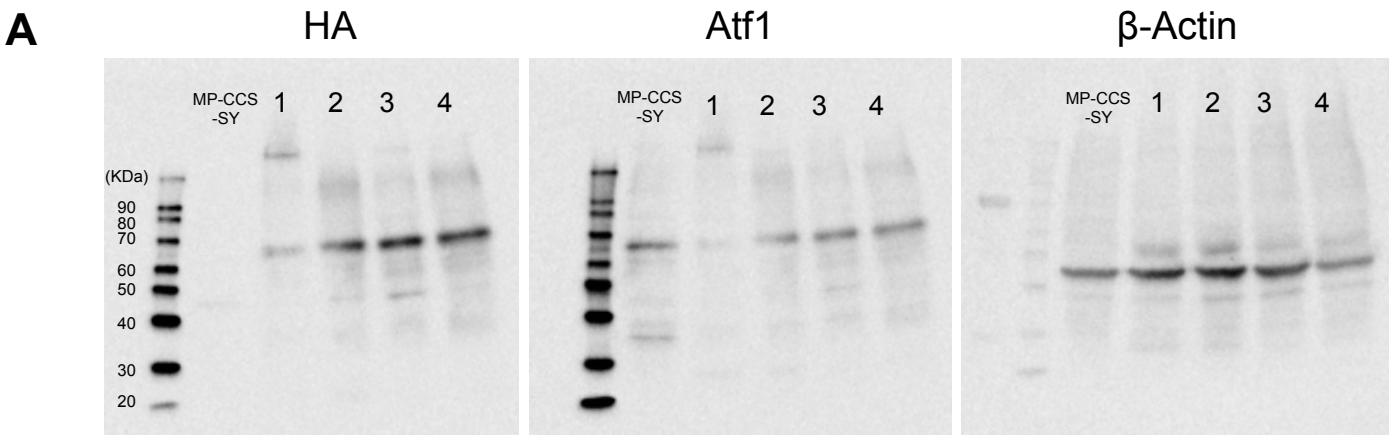
(A) (B) (C) The effects of a c-FOS/AP-1 inhibitor on the growth of human CCS lines. 3-{5-[4-(Cyclopentyloxy)-2-hydroxybenzoyl] -2-[(3-hydroxy - 1,2-benzisoxazol -6-yl) methoxy] phenyl} propionic acid (T-5224) has been developed as a novel c-FOS/AP-1 inhibitor (*Nat Biotechnol* 26:817–823, 2008). We treated human CCS cell lines, MP-CCS-SY and KAS, and the control WI38 with T-5224 to examine the therapeutic effect of blocking the FOS pathway on growth of human CCS. T-5224 inhibited the cell growth of both CCS cell lines more efficiently than those of WI38. T-5224 was synthesized by Toyama Chemical and dissolved in DMSO for the *in vitro* study.

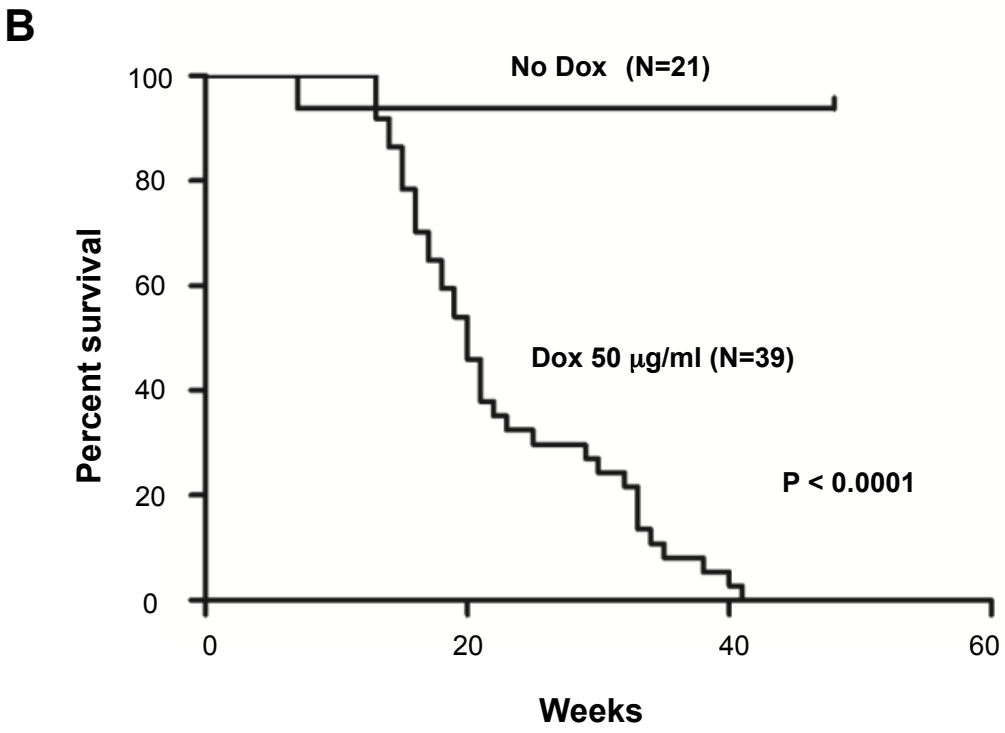
MP-CCS-SY, KAS and WI38 cells were treated with T-5224 (50, 60, 70, or 80 μM) or DMSO. Twenty-four and 48 hrs later, the cell viability was determined by the WST-8 assay. The results represent the means ± SD (n=8). *P <0.05 and ***P < 0.001 vs. DMSO-treated cells.

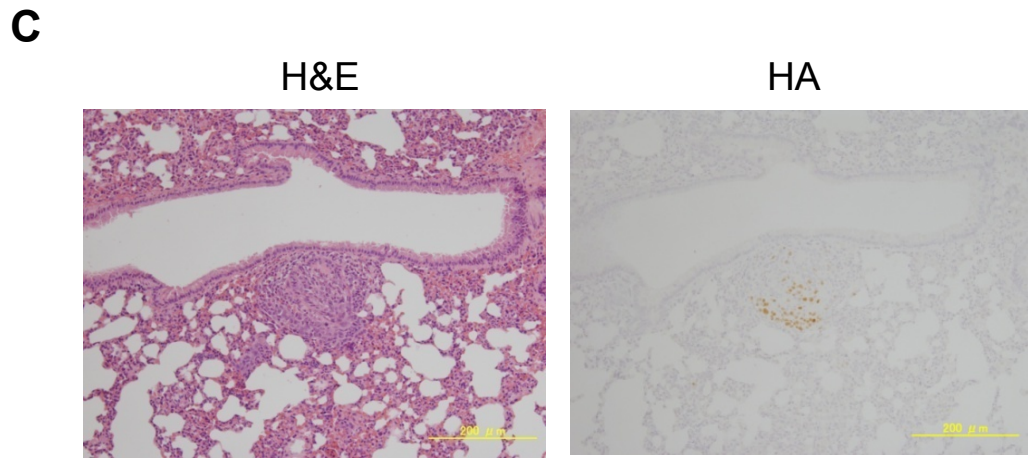
(D) *In vivo* effect of T-5224 administration on tumor cell growth using xenograft model transplanted with *EWS/ATF1*-induced tumor cell line. The tumor cell growth tended to be suppressed by the oral administration of T-5224 *in vivo*, although it did not reach statistical difference. A total of 5.0×10^6 MP-CCS-SY cells in 0.1 mL of serum-free RPMI1640 were inoculated subcuaneously through a 26-gauge needle into the posterior flank of male 8-week-old BALB/c athymic (nu/nu) nude mice. Once tumors had reached a volume of 100 mm^3 (Day 0), the tumor-bearing nude mice were treated with T-5224 (3 mg/kg or 30 mg/kg) or polyvinylpyrrolidone (vehicle) orally once per day. Tumor growth was measured every 7 days for a period of up to 6 weeks. The results represent the mean tumor volume ± SE (n = 6).


A
Colon
Liver
Lung
Kidney
Dox 50 µg/ml
Dox OFF

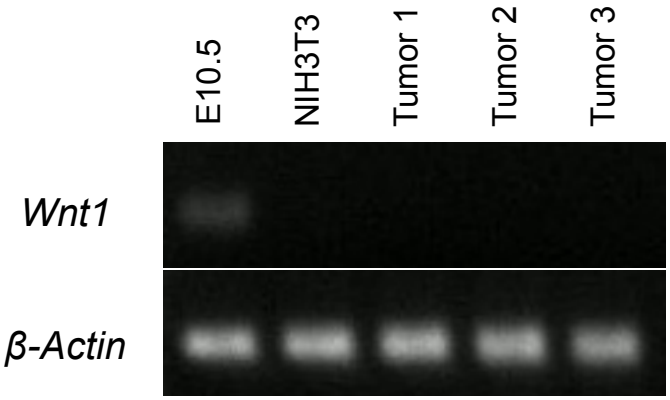

B
H&E
HA
200 µm
200 µm


A
HA
Atf1
β-Actin
MP-CCS -SY
1
2
3
4
(KDa)
90
80
70
60
50
40
30
20

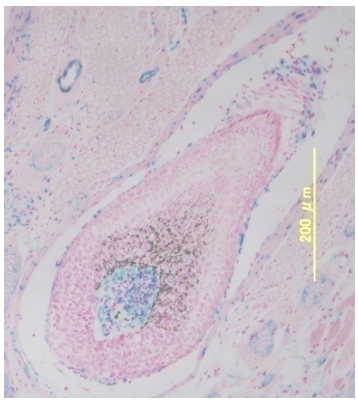

B
No Dox (N=21)
Dox 50 μg/ml (N=39)
P < 0.0001
Percent survival
100
80
60
40
20
0
0
20
40
60
Weeks


C
H&E
HA

A



B



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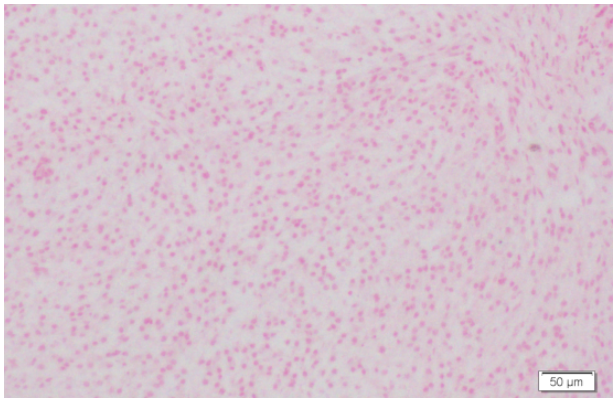
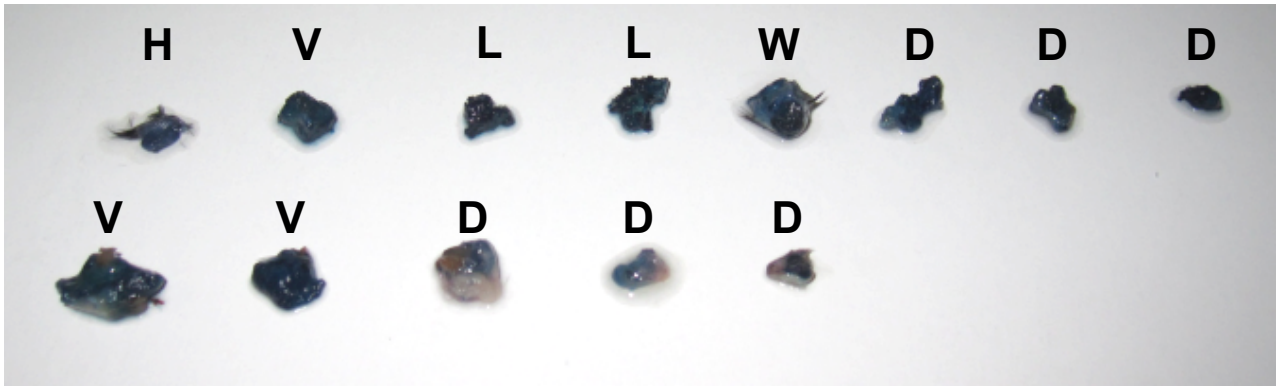
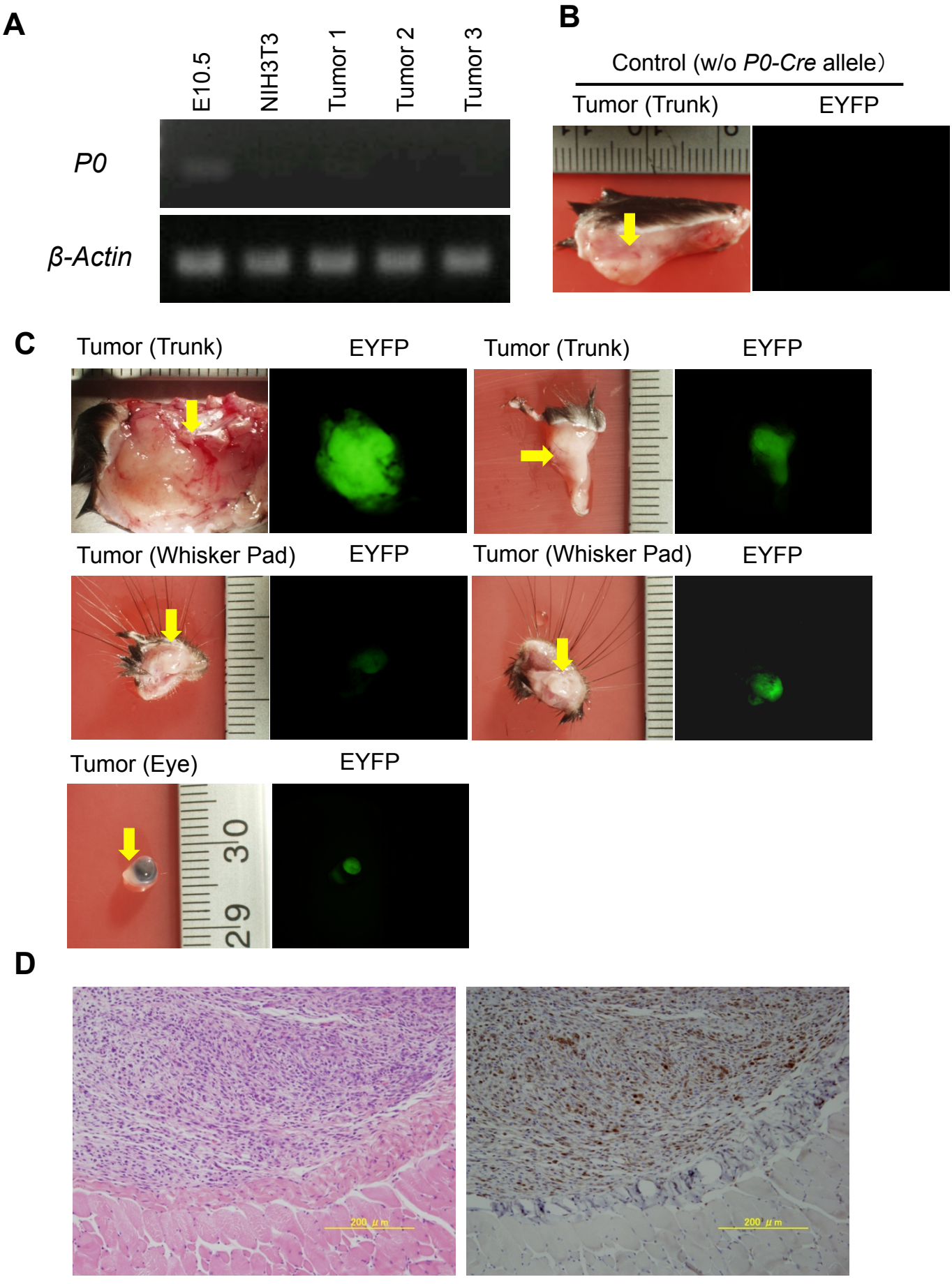


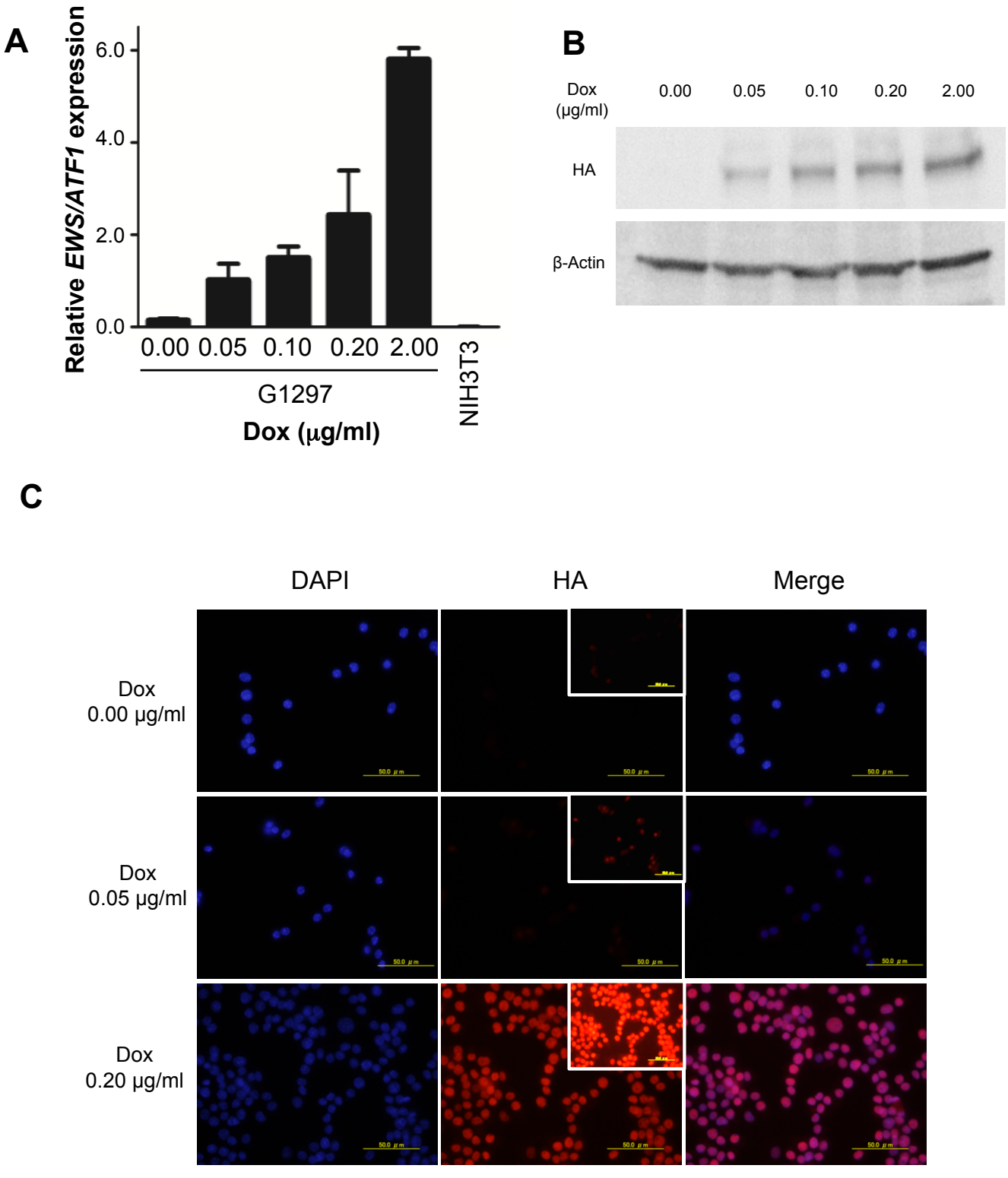
D



H; Head
V; Trunk (ventral)
D; Trunk (dorsal)

L; Limb
W; Wisker pad


A
E10.5
NIH3T3
Tumor 1
Tumor 2
Tumor 3
P0
β-Actin
B
Control (w/o P0-Cre allele)
Tumor (Trunk)
EYFP
C
Tumor (Trunk)
EYFP
Tumor (Trunk)
EYFP
Tumor (Whisker Pad)
EYFP
Tumor (Whisker Pad)
EYFP
Tumor (Eye)
EYFP
D
200 μm
200 μm


A
Relative EWS/ATF1 expression
6.0
4.0
2.0
0.0
0.00 0.05 0.10 0.20 2.00
G1297
NIH3T3
Dox (μg/ml)
B
Dox (μg/ml)
0.00 0.05 0.10 0.20 2.00
HA
β-Actin
C
DAPI
HA
Merge
Dox 0.00 μg/ml
Dox 0.05 μg/ml
Dox 0.20 μg/ml

A

Probes upregulated by *EWS/ATF1*

			Fold increase		
			3 hours		48 hours
	ProbeName	GeneSymbol	Dox 0.05 μg/ml	Dox 0.20 μg/ml	Dox 0.20 μg/ml
1	A_30_P01033111		5.299945631	17.29894991	38.3053092
2	A_52_P262219	Fos	2.677049321	16.16371033	21.78060329
3	A_51_P128397	Nr4a3	2.77773125	14.17645641	65.1136396
4	A_52_P70796	Cxcr5	3.152491723	9.951895317	5.417339496
5	A_55_P1994997	Arhgap11a	2.041232747	7.049414437	19.42250597
6	A_30_P01028855		2.391728019	6.743827606	16.96975126
7	A_52_P366803	Cyp3a44	2.039737394	5.793960368	927.7118676
8	A_55_P2000454		4.379586269	5.036767519	2.566768042
9	A_55_P2130501	LOC100044727	2.553299679	4.799061949	3.253528982
10	A_55_P2096947	Ereg	3.839845189	4.576025297	3.695173596
11	A_30_P01025610		2.773827028	4.342631732	5.218874578
12	A_51_P308796	Fosl1	6.512172105	3.578250715	8.27275716
13	A_52_P257625	Esm1	3.252877084	3.488860534	78.22959638
14	A_66_P121459	Cenpa	3.634817998	3.482352364	7.467980734
15	A_52_P302433	Plau	3.130584164	3.044678376	4.164422413
16	A_51_P463003	Tslp	2.160029007	2.965866994	12.90592265
17	A_55_P2088183	Ucn2	2.687236265	2.881792236	858.1670848
18	A_51_P339793	Il1rl1	2.005772593	2.87080388	17.16960283
19	A_51_P271417	Fibcd1	2.711111933	2.798888991	22.88712401
20	A_55_P2027731	Il1rl1	2.687233661	2.65845976	17.52415267
21	A_55_P2404878	Nup188	2.635105891	2.57240736	56.36658962
22	A_52_P608322	Maff	2.956117335	2.528982731	2.799428399

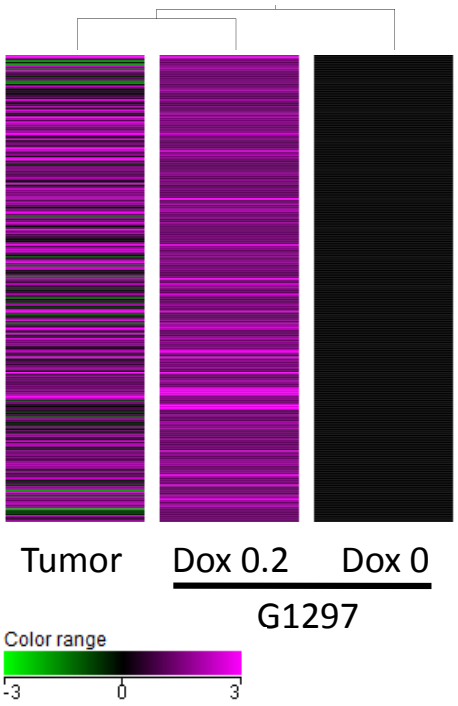
B

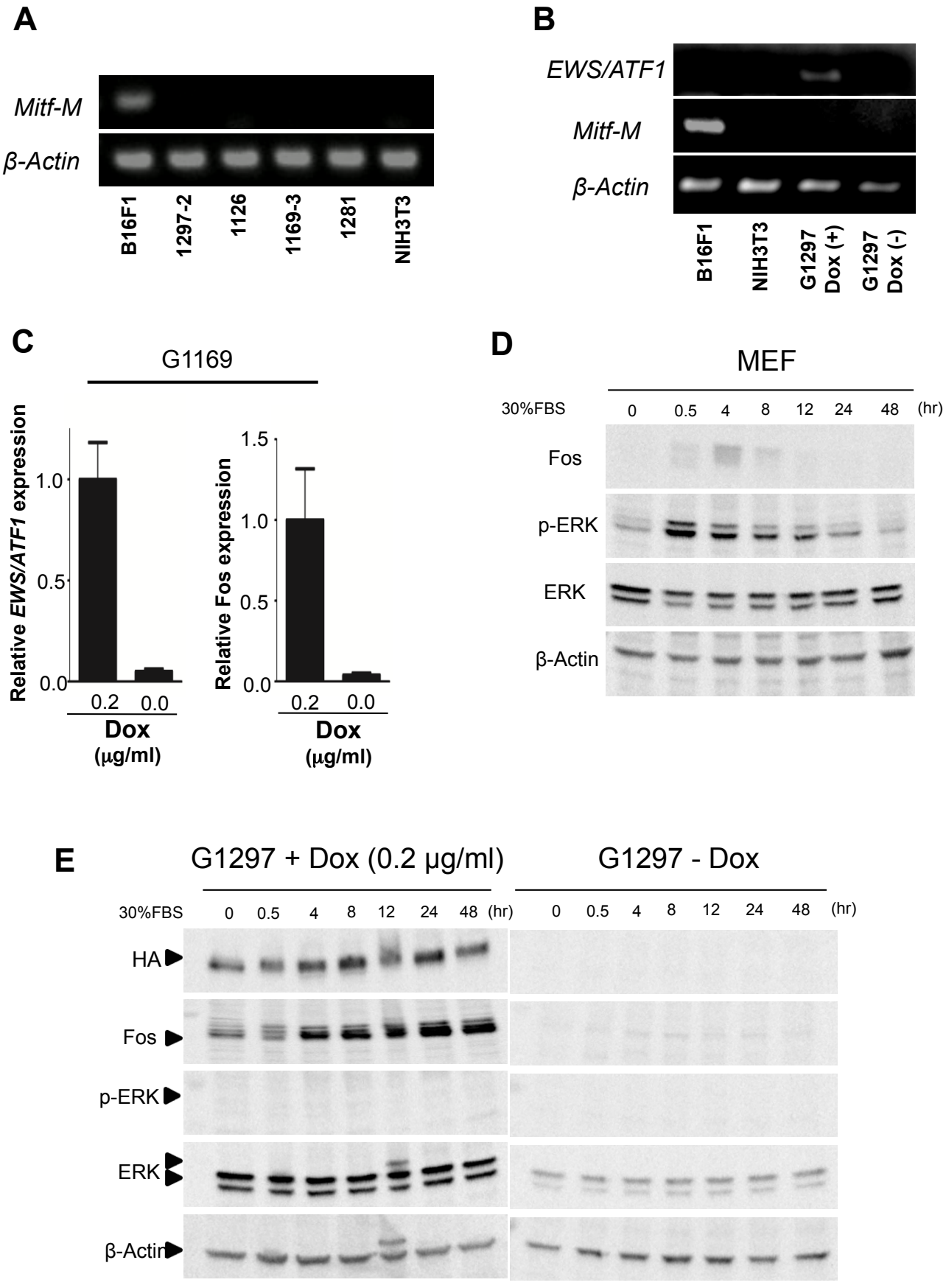
Over-represented TFBS
(transcription factor binding site)

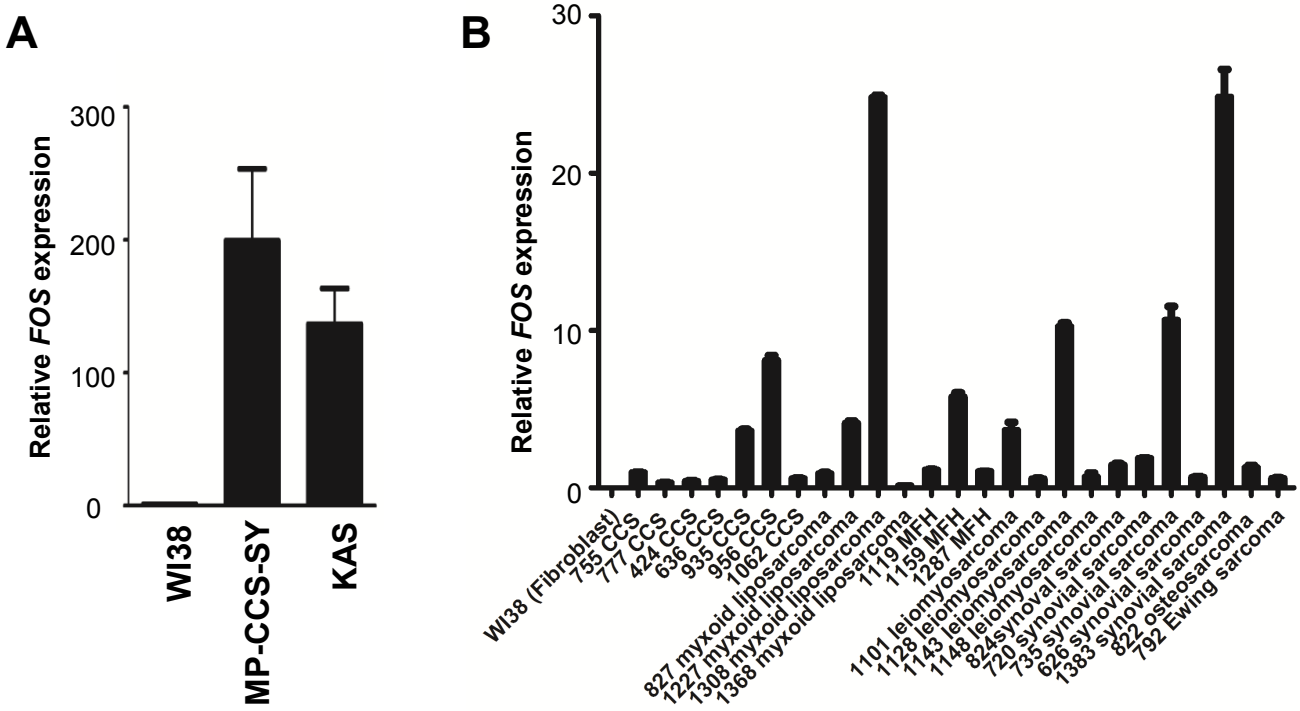
Transcription Factor Binding Site	P	FDR
TF_CREB	0.0081	0.018
ADD_MTF1B	0.0108	0.024
TF_EGR1	0.0185	0.039
TF_TAXCREB1	0.0275	0.057
TF_ZIC	0.0684	0.125
TF_MEISHOX	0.0844	0.148
MIT_009ATF	0.0924	0.159
TF_ATF1	0.0979	0.165
TF_ARNT	0.1001	0.167
TF_TGIF	0.1032	0.171

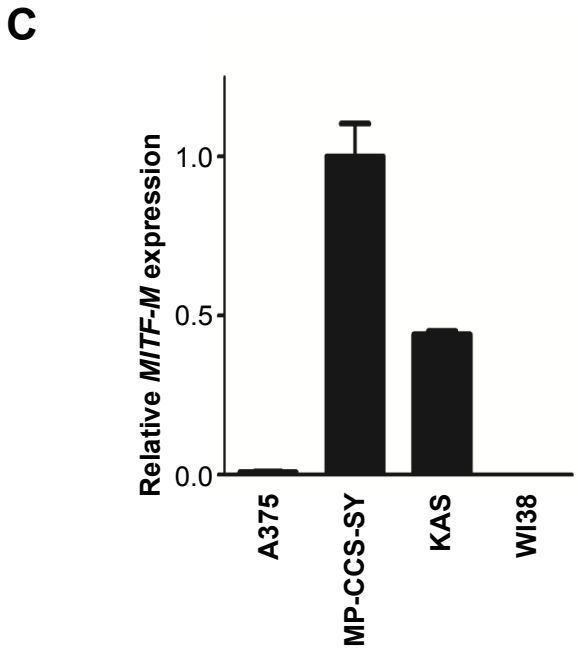
From -1000 bp to +200 bp

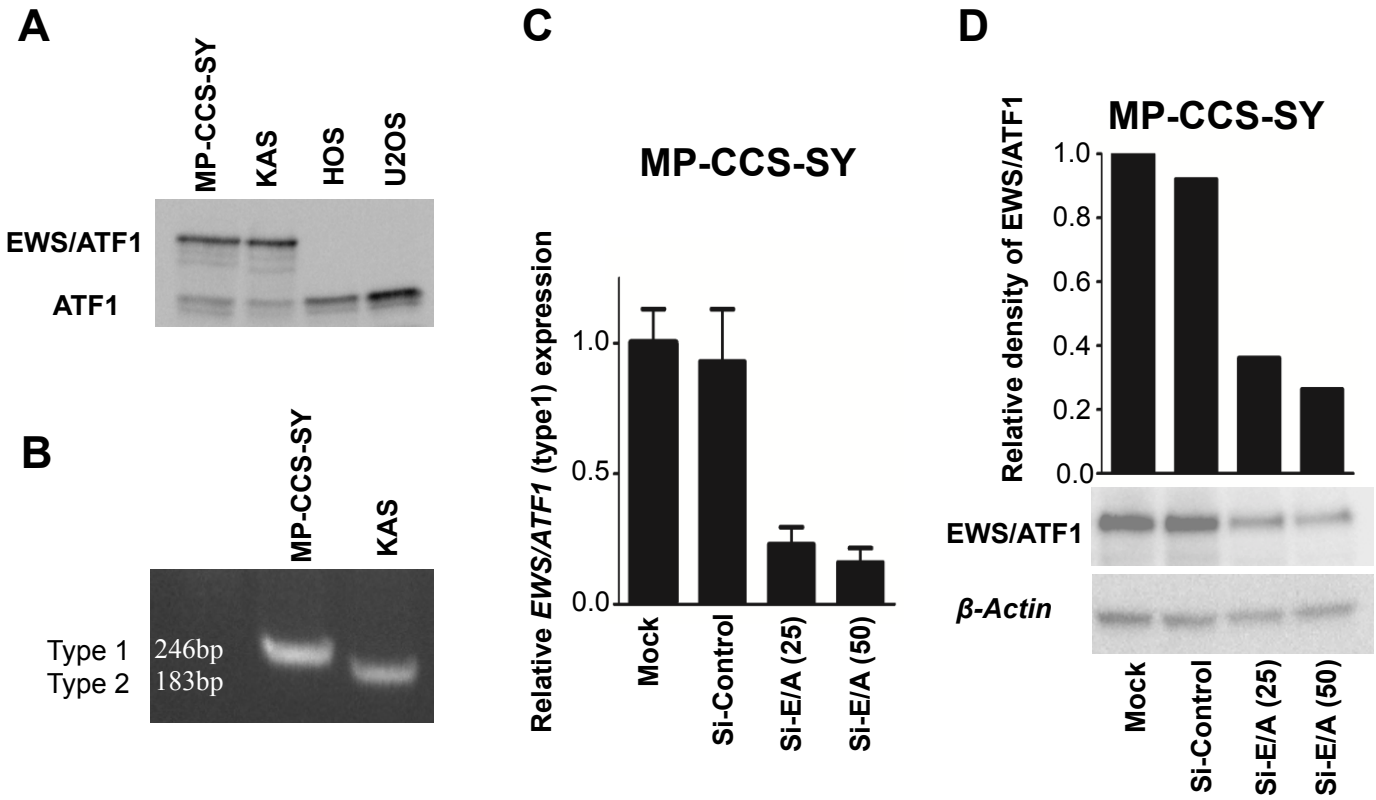
C

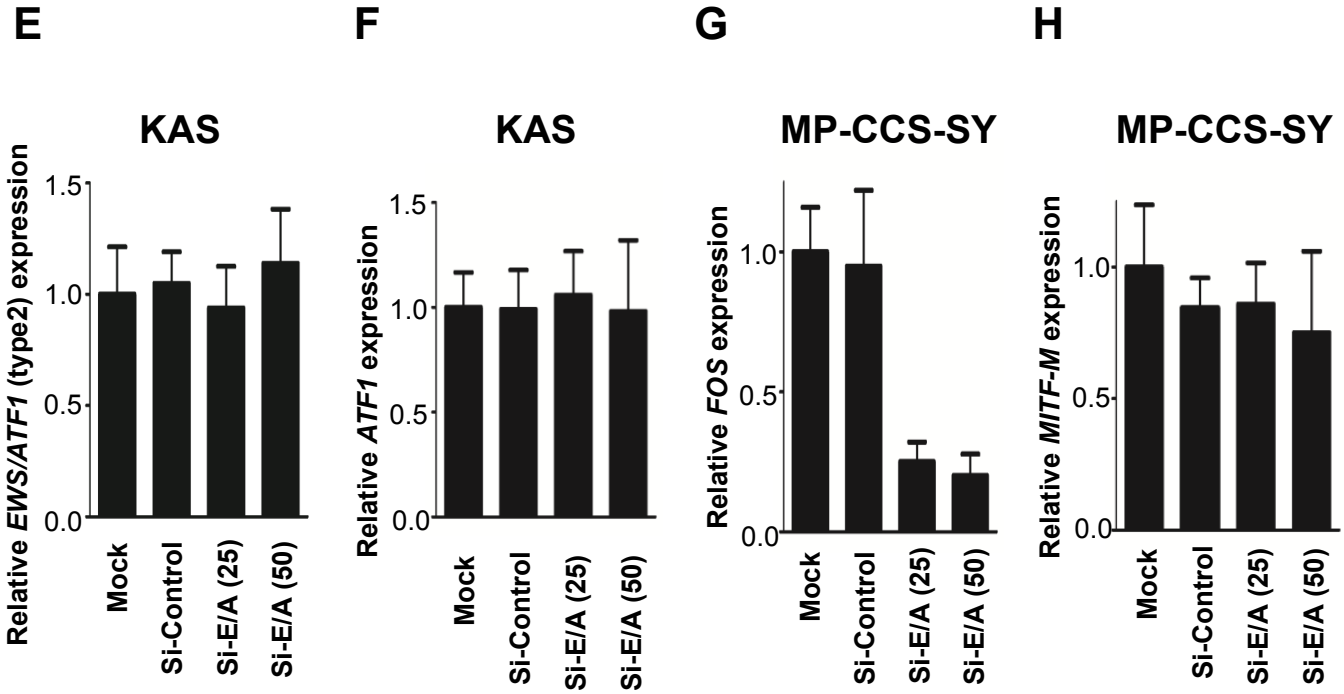


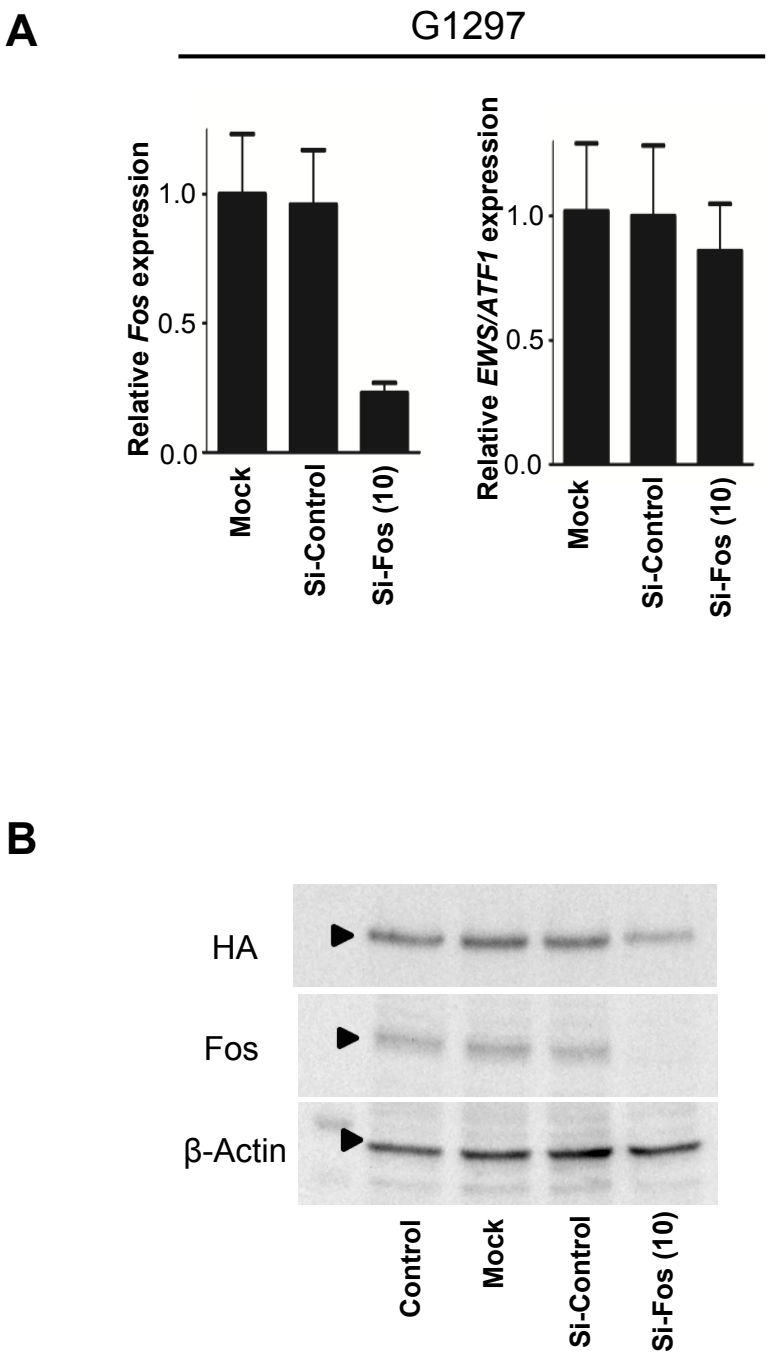

A
Mitf-M
β-Actin
B16F1
1297-2
1126
1169-3
1281
NIH3T3
B
EWS/ATF1
Mitf-M
β-Actin
B16F1
NIH3T3
G1297 Dox (+)
G1297 Dox (-)
C
G1169
Relative EWS/ATF1 expression
1.0
0.5
0.0
0.2
0.0
Dox (μg/ml)
Relative Fos expression
1.5
1.0
0.5
0.0
0.2
0.0
Dox (μg/ml)
D
MEF
30%FBS
0
0.5
4
8
12
24
48
(hr)
Fos
p-ERK
ERK
β-Actin
E
G1297 + Dox (0.2 μg/ml)
G1297 - Dox
30%FBS
0
0.5
4
8
12
24
48
(hr)
HA
Fos
p-ERK
ERK
β-Actin

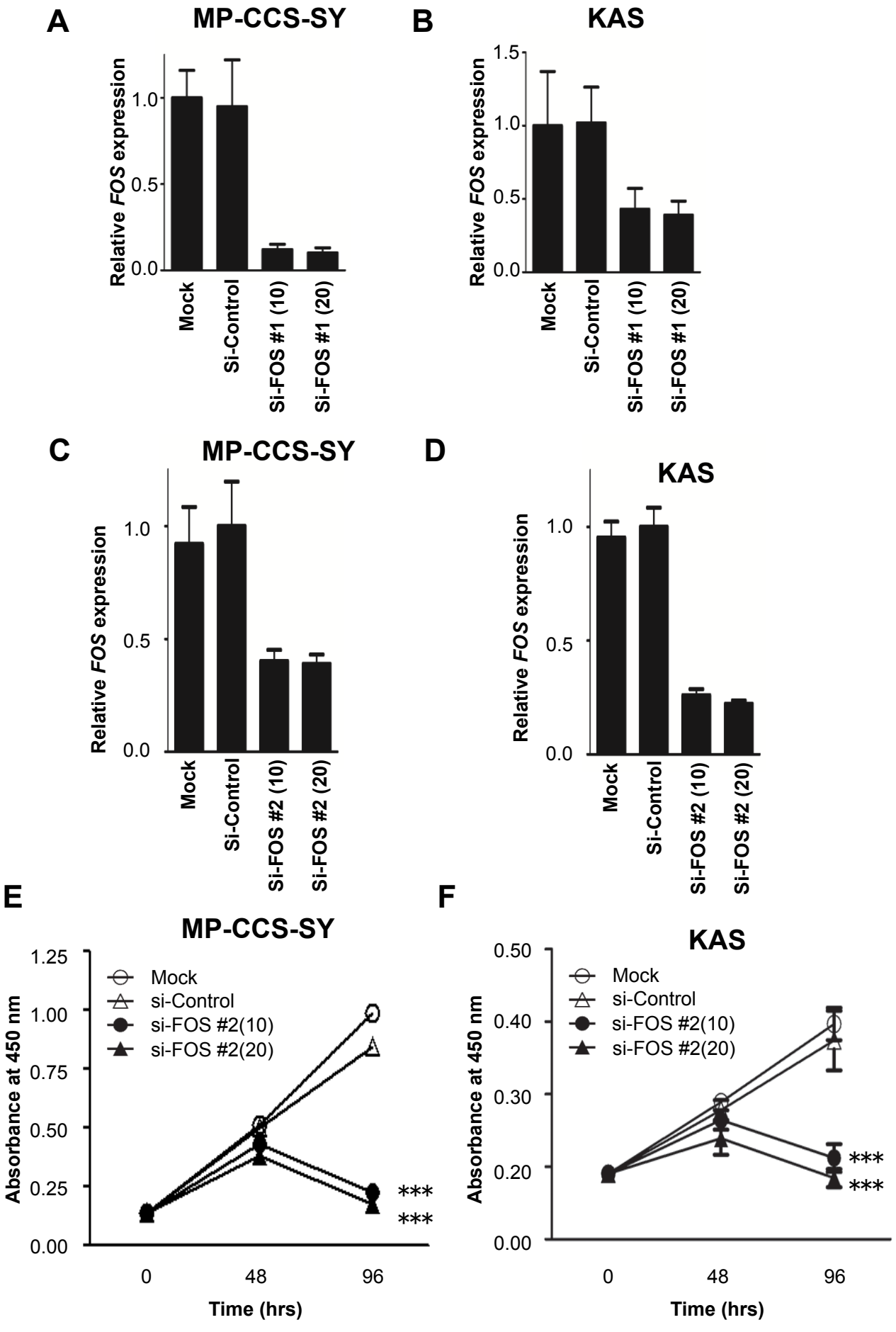

A
Relative FOS expression
300
200
100
0
WI38
MP-CCS-SY
KAS
B
Relative FOS expression
30
20
10
0
WI38 (Fibroblast)
755 CCS
777 CCS
424 CCS
636 CCS
935 CCS
956 CCS
1062 CCS
827 myxoid liposarcoma
1227 myxoid liposarcoma
1308 myxoid liposarcoma
1368 myxoid liposarcoma
1119 MFH
1159 MFH
1287 MFH
1101 leiomyosarcoma
1128 leiomyosarcoma
1143 leiomyosarcoma
1148 leiomyosarcoma
824synovial sarcoma
720 synovial sarcoma
735 synovial sarcoma
626 synovial sarcoma
1383 synovial sarcoma
822 osteosarcoma
792 Ewing sarcoma

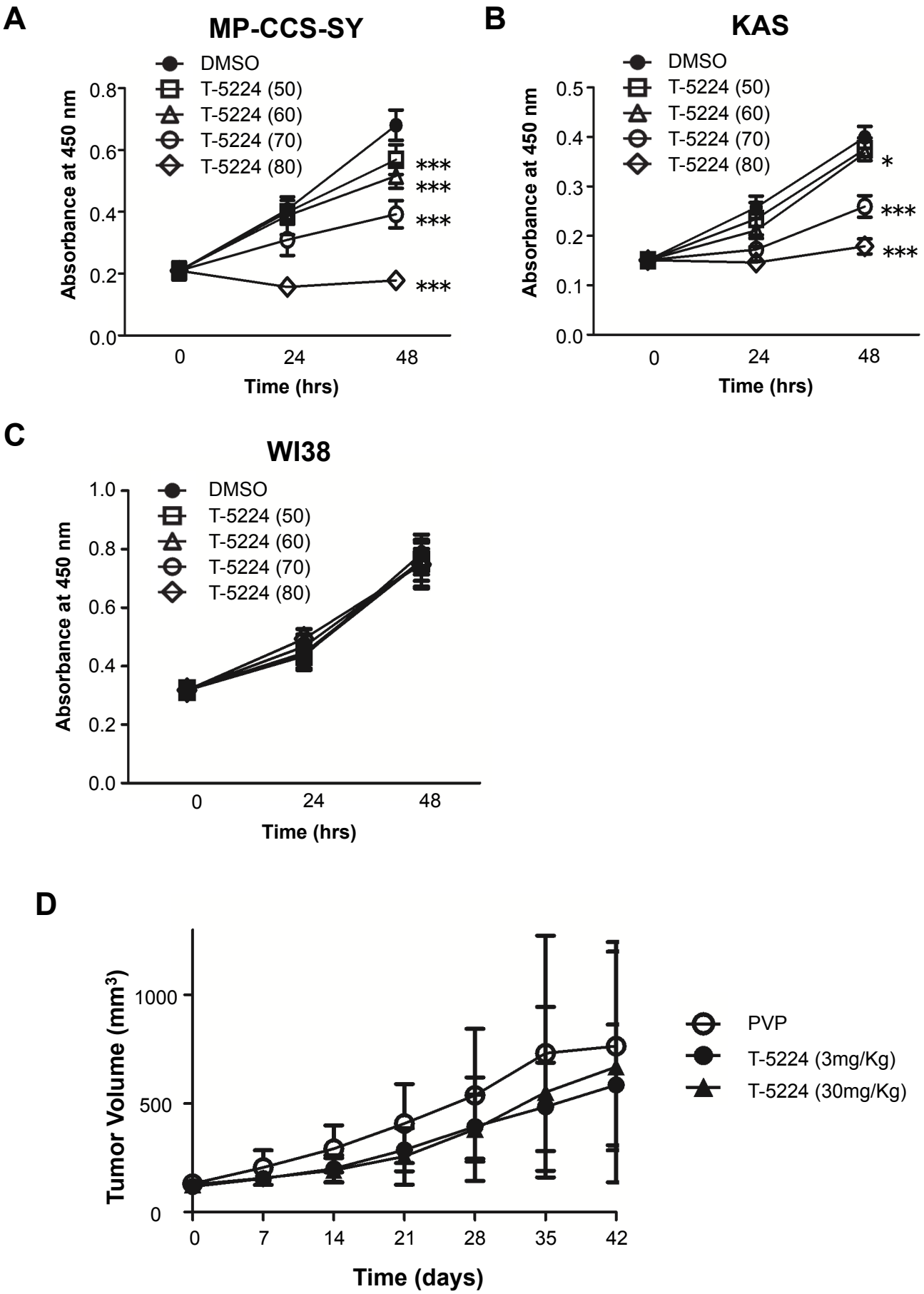

C
Relative MITF-M expression
1.0
0.5
0.0
A375
MP-CCS-SY
KAS
WI38


A
MP-CCS-SY
KAS
HOS
U2OS
EWS/ATF1
ATF1
B
MP-CCS-SY
KAS
Type 1
Type 2
246bp
183bp
C
MP-CCS-SY
Relative EWS/ATF1 (type1) expression
1.0
0.5
0.0
Mock
Si-Control
Si-E/A (25)
Si-E/A (50)
D
MP-CCS-SY
Relative density of EWS/ATF1
1.0
0.8
0.6
0.4
0.2
0.0
EWS/ATF1
β-Actin
Mock
Si-Control
Si-E/A (25)
Si-E/A (50)


E
KAS
Relative EWS/ATF1 (type2) expression
1.5
1.0
0.5
0.0
Mock
Si-Control
Si-E/A (25)
Si-E/A (50)
F
KAS
Relative ATF1 expression
1.5
1.0
0.5
0.0
Mock
Si-Control
Si-E/A (25)
Si-E/A (50)
G
MP-CCS-SY
Relative FOS expression
1.0
0.5
0.0
Mock
Si-Control
Si-E/A (25)
Si-E/A (50)
H
MP-CCS-SY
Relative MITF-M expression
1.0
0.5
0.0
Mock
Si-Control
Si-E/A (25)
Si-E/A (50)


A
G1297
Relative Fos expression
1.0
0.5
0.0
Mock
Si-Control
Si-Fos (10)
Relative EWS/ATF1 expression
1.0
0.5
0.0
Mock
Si-Control
Si-Fos (10)
B
HA
Fos
β-Actin
Control
Mock
Si-Control
Si-Fos (10)


A
MP-CCS-SY
Relative FOS expression
1.0
0.5
0.0
Mock
Si-Control
Si-FOS #1 (10)
Si-FOS #1 (20)
B
KAS
Relative FOS expression
1.5
1.0
0.5
0.0
Mock
Si-Control
Si-FOS #1 (10)
Si-FOS #1 (20)
C
MP-CCS-SY
Relative FOS expression
1.0
0.5
0.0
Mock
Si-Control
Si-FOS #2 (10)
Si-FOS #2 (20)
D
KAS
Relative FOS expression
1.0
0.5
0.0
Mock
Si-Control
Si-FOS #2 (10)
Si-FOS #2 (20)
E
MP-CCS-SY
Absorbance at 450 nm
1.25
1.00
0.75
0.50
0.25
0.00
Mock
si-Control
si-FOS #2(10)
si-FOS #2(20)
***
***
0
48
96
Time (hrs)
F
KAS
Absorbance at 450 nm
0.50
0.40
0.30
0.20
0.00
Mock
si-Control
si-FOS #2(10)
si-FOS #2(20)
***
***
0
48
96
Time (hrs)


A
MP-CCS-SY
DMSO
T-5224 (50)
T-5224 (60)
T-5224 (70)
T-5224 (80)
Absorbance at 450 nm
0.0
0.2
0.4
0.6
0.8
0
24
48
Time (hrs)
***
***
***
***
B
KAS
DMSO
T-5224 (50)
T-5224 (60)
T-5224 (70)
T-5224 (80)
Absorbance at 450 nm
0.0
0.1
0.2
0.3
0.4
0.5
0
24
48
Time (hrs)
*
***
***
C
WI38
DMSO
T-5224 (50)
T-5224 (60)
T-5224 (70)
T-5224 (80)
Absorbance at 450 nm
0.0
0.2
0.4
0.6
0.8
1.0
0
24
48
Time (hrs)
D
Tumor Volume (mm³)
0
500
1000
0
7
14
21
28
35
42
Time (days)
PVP
T-5224 (3mg/Kg)
T-5224 (30mg/Kg)

Supplementary Table S1.

Primer pairs used for semi-quantitative RT-PCR and Quantitative real-time PCR

	forward	reverse
EWS/ATF1	GAGGCATGAGCAGAGGTGG	GAAGTCCCTGTACTCCATCTGTG
MITF-M	GGAATTATAGAAAGTAGAGGGA	ACATGGCAAGCTCAGGAC
FOS	GGGGCAAGGTGGAACAGTTAT	CCGCTTGGAGTGTATCAGTCA
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA
Wnt1	GGTTTCTACTACGTTGCTACTGG	GGAATCCGTCAACAGGTTCGT
P0 (Mpz)	TACAGTGACAACGGCACTTTC	GCAGTACCGAATCAGGTAGAAGA
Mitf-M	GCTGGAAATGCTAGAATACAG	TTCCAGGCTGATGATGTCATC
Fos	CGGGTTTCAACGCCGACT	TTGGCACTAGAGACGGACAGA
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT