

Figure 2

EWS/ATF1-induced tumors resemble human CCS. *EWS/ATF1* transgenic mice were administered 50 $\mu\text{g}/\text{ml}$ doxycycline in their drinking water for 3 months. (A) *EWS/ATF1* expression caused tumor formation (arrows) in various locations: trunk, head, limbs, and whisker pads. X-ray examination revealed multiple tumors in deep soft tissue. The cut surface of a large tumor on the ventral trunk of an *EWS/ATF1*-inducible mouse revealed a lobulated gray-white mass in the deep soft tissue. Scale bars: 20 mm. (B) Histological analysis of *EWS/ATF1*-induced tumors. Tumors were composed of round or fusiform cells with prominent basophilic nuclei and clear cytoplasm, which were surrounded by fibrous fascicles. HA immunostaining confirmed *EWS/ATF1* expression in the tumor cells. Frequent Ki67-positive cells were present throughout the lesions. Scale bars: 200 μm (H&E, left); 50 μm (H&E, right); 100 μm (HA and Ki67).

The individual tumor cells had a homogeneous appearance. They had round to ovoid vesicular nuclei with prominent basophilic nucleoli and clear or pale-staining cytoplasm (Figure 2B). The majority of the tumor cells expressed *EWS/ATF1* fusion protein in nuclei (Figure 2B and Supplemental Figure 2A). Ki67-positive proliferating cells were observed in about 30%–40% of tumor cells (Figure 2B), indicative of active proliferative activity. The survival curves of *EWS/ATF1*-induced mice were analyzed to evaluate the overall effect of *EWS/ATF1* expression on life span. The transgenic mice treated with doxycycline became moribund within 3–10 months, suggestive of multiple tumor formation in the deep soft tissue, whereas mice without doxycycline treatment survived much longer, and no tumor formation was observed. The median survival time of *EWS/ATF1*-inducible mice treated with doxycycline was 20 weeks (Supplemental Figure 2B).

Previous studies demonstrated that human CCSs express markers for neural crest lineage as well as melanocytic differentiation (8, 9). Therefore, to examine the similarity of mouse *EWS/ATF1*-induced tumors with human CCSs, we performed immunohistochemical analysis for CCS-expressing markers: *EWS/ATF1*-induced tumor cells showed the expression such markers, including S100, Sox10, and Mitf (Figure 3A).

Neural crest-lineage cells are permissive to EWS/ATF1-driven sarcoma development. The cell of origin for CCS remains to be determined. Based on the potential of CCSs for melanocytic differentiation and melanin synthesis, previous studies proposed that CCS may arise from a neural crest progenitor. To determine whether *EWS/ATF1*-induced sarcomas actually arise from neural crest-derived cells, we performed a lineage-tracing experiment in which neural crest-derived cells were tagged by reporter in vivo (32). To label neural crest-derived cells in vivo, we first used transgenic mice containing

Wnt1-Cre and floxed *LacZ* reporter alleles. We further introduced doxycycline-inducible *EWS/ATF1* alleles into the reporter mice to generate compound transgenic mice (Figure 3B). We confirmed that *EWS/ATF1*-induced tumor cells did not express *Wnt1* (Supplemental Figure 3A). Transgenic mice were treated with doxycycline in the drinking water to induce subcutaneous tumors and the developed tumors were then analyzed for the expression of the reporter gene. Importantly, all 14 *EWS/ATF1*-induced tumors were ubiquitously positive for X-gal staining (Figure 3C and Supplemental Figure 3D), which suggests that neural crest-lineage cells are a cell of origin for *EWS/ATF1*-associated sarcomas. We further performed another lineage-tracing experiment using transgenic mice containing *P0-Cre* and floxed *EYFP* reporter alleles (Figure 3D), which have been also widely used to label neural crest-derived cells. Again, we found that all 6 *EWS/ATF1*-induced tumors were positive for EYFP (Figure 3E and Supplemental Figure 4, C and D).

Establishment of tumor cell lines. Tumor samples were obtained from primary tumors of *EWS/ATF1*-induced mice to establish cell lines from *EWS/ATF1*-induced tumors. We established 2 tumor cell lines, G1297 and G1169, from 2 independent mice. These cells grew in the form of an adherent monolayer in the presence of doxycycline (0.2 $\mu\text{g}/\text{ml}$). We cultured the cells up to the fourth passage in medium containing 0.2 $\mu\text{g}/\text{ml}$ doxycycline in order to avoid contamination by fibroblasts. We examined the effect of different concentrations of doxycycline on the growth and morphology of the established cell lines. We confirmed that the expression of *EWS/ATF1* transcript and protein increased in response to doxycycline in a dose-dependent manner in both established cell lines (Supplemental Figure 5, A–C). The growth and morphology of the tumor cells varied in a doxycycline dose-dependent manner: small, round tumor cells grew rapidly at concentrations above 0.1 $\mu\text{g}/\text{ml}$,

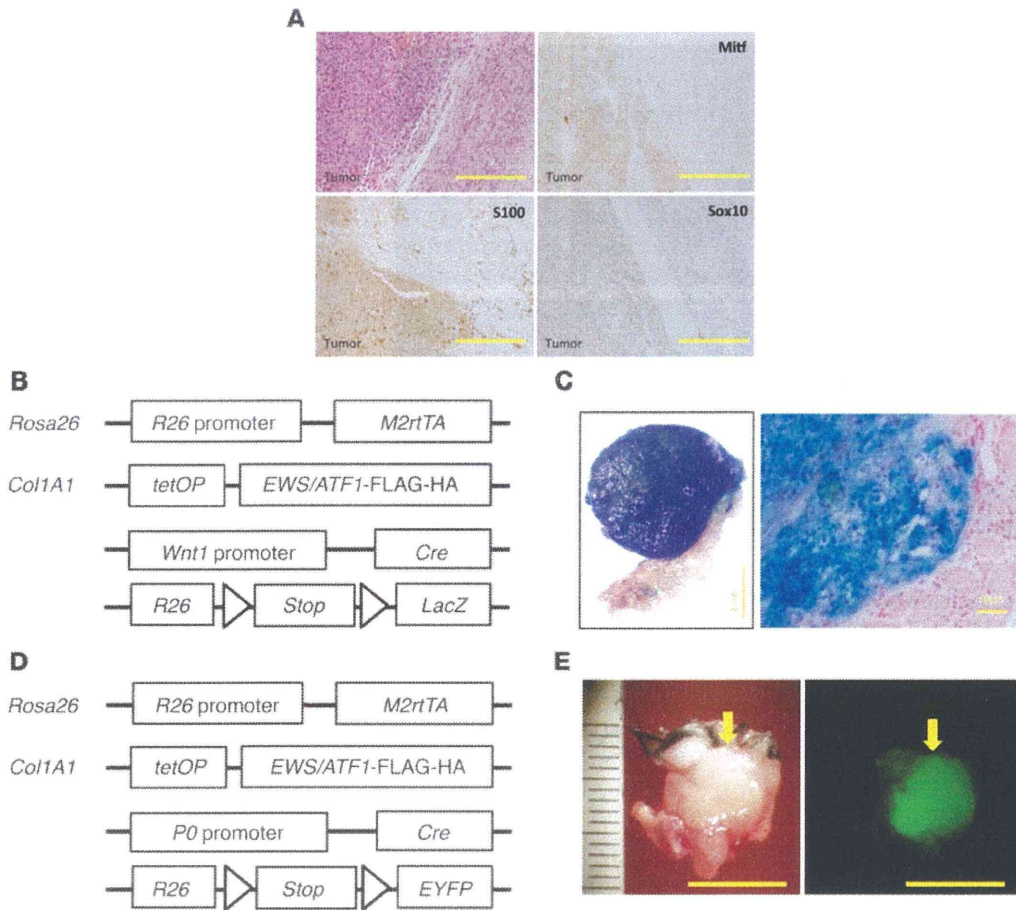


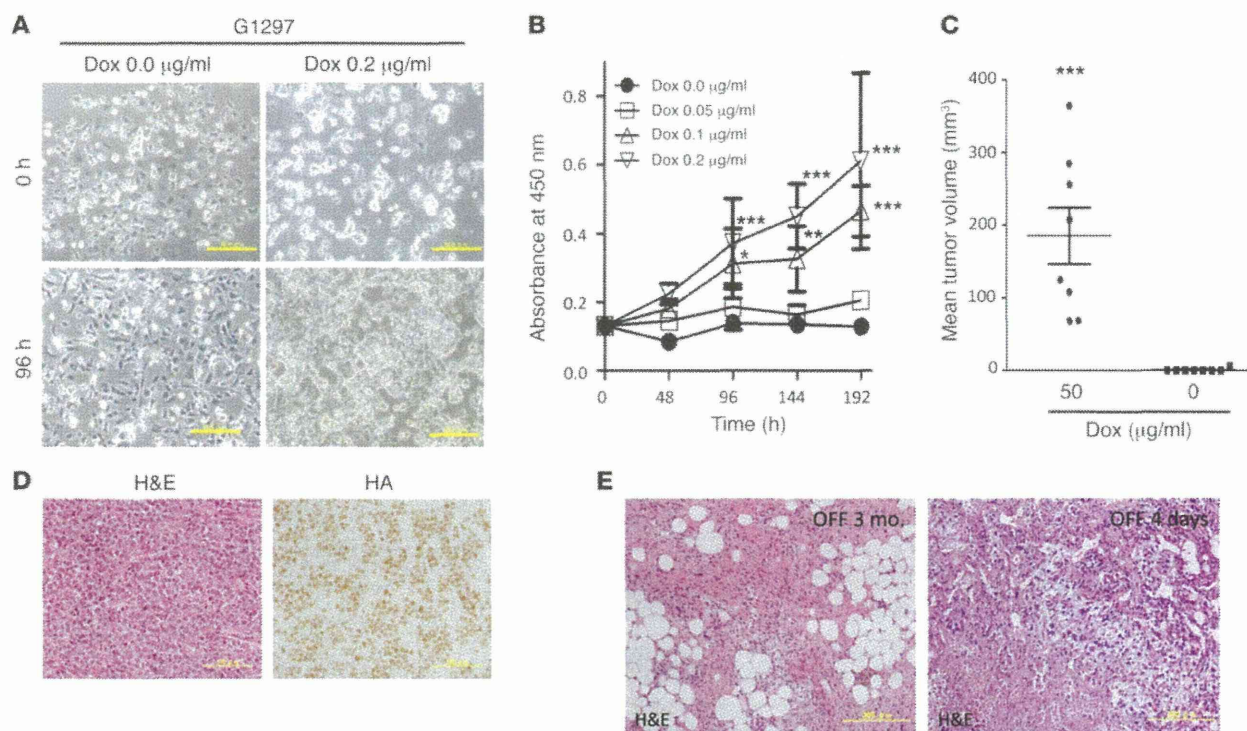
Figure 3

EWS/ATF1-induced tumors arise from neural crest-lineage cells. **(A)** Immunohistochemical analysis for CCS markers. Nuclear staining for S100, Sox10, and Mitf was observed in tumor cells. Sections were counterstained with hematoxylin. Scale bars: 100 μ m. **(B)** Schematic representation of reporter alleles for the lineage-tracing experiment using *Wnt1-Cre* allele. Doxycycline-inducible *EWS/ATF1* alleles were introduced into reporter mice containing the *Wnt1-Cre* and floxed *LacZ* reporter alleles. **(C)** X-gal staining for *EWS/ATF1*-induced tumors with *Wnt1-Cre* and floxed *LacZ* reporter alleles. Positive staining for X-gal indicated that the tumor arose from a neural crest-lineage cell. Histological analysis revealed that neoplastic cells were stained with X-gal. Counterstaining was performed with fast red. Scale bars: 2 mm (left); 50 μ m (right). **(D)** Schematic representation of reporter alleles for the lineage-tracing experiment using *P0-Cre* allele. Doxycycline-inducible *EWS/ATF1* alleles were introduced into reporter mice containing the *P0-Cre* and floxed *EYFP* reporter alleles. **(E)** Representative image of a tumor (arrow) in the trunk of an *EWS/ATF1*-induced mouse with *P0-Cre* and floxed *EYFP* reporter alleles. Fluorescent signals for EYFP expression were detected in the. Scale bars: 10 mm.

whereas dendritic fibroblast-like spindle cells were observed below 0.05 μ g/ml (Figure 4A). Notably, doxycycline withdrawal caused rapid morphological changes, into a fibroblast-like shape, and these tumor cells did not proliferate up to the next passage (Figure 4A). Consistent with these findings, cell viability assay revealed that the number of cells was increased by doxycycline treatment in a dose-dependent manner (Figure 4B). We next examined the effect of *EWS/ATF1* expression on tumorigenesis ability in the subcutaneous tissue of immunocompromised mice. The established cell line G1297 was cultured in medium containing 0.2 μ g/ml doxycycline, and 5.0×10^6 cells were transplanted into the subcutaneous tissue of nude mice. It is important to note that all mice treated with 50 μ g/ml doxycycline in the drinking water developed tumors within 3 weeks, whereas no tumor formation was observed in mice without doxycycline treatment (Figure 4C). Histological analysis revealed that the subcutaneous tumors in nude mice consisted of neoplas-

tic cells that resembled the primary tumor cells in *EWS/ATF1*-induced transgenic mice (Figure 4D). Positive immunoreactivity for HA-Tag was observed in all tumor cells (Figure 4D).

Continuous expression of EWS/ATF1 is required for tumor growth maintenance. To further examine whether continuous expression of *EWS/ATF1* is necessary for the growth of *EWS/ATF1*-induced tumors, we withdrew doxycycline in tumor-bearing *EWS/ATF1* transgenic mice that had been given doxycycline for 3 months. Importantly, doxycycline withdrawal resulted in a rapid reduction of tumor mass in 4 independent mice (7 tumors total). The regressed tumors contained fibrous tissue, but no viable neoplastic cells were observed 3 months after doxycycline withdrawal (Figure 4E), which suggests that *EWS/ATF1*-induced tumor growth depends on continuous *EWS/ATF1* expression. We next examined the histological changes shortly after doxycycline withdrawal in order to investigate the mechanisms of tumor regression.

**Figure 4**

Establishment and analysis of tumor cell lines. G1297 and G1169 cell lines were established from 2 independent *EWS/ATF1*-induced tumors. (A) Morphology of the G1297 line after treatment without or with doxycycline (0 and 0.2 µg/ml, respectively). At concentrations above 0.1 µg/ml, small, round tumor cells grew rapidly, while dendritic fibroblast-like spindle cells were observed; tumor cell growth almost stopped at concentrations less than 0.05 µg/ml. Scale bars: 50 µm. (B) Effect of different levels of *EWS/ATF1* on tumor cell growth. G1297 cells were cultured in different concentrations of doxycycline (0, 0.05, 0.1, and 0.2 µg/ml), and cell viability was determined by WST-8 assay. Data are mean ± SD ($n = 8$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Dox 0.0 µg/ml. (C) Subcutaneous transplantation of 5.0×10^6 G1297 cells in immunocompromised mice resulted in tumor formation in mice treated with 50 µg/ml doxycycline ($n = 8$). Mean tumor volumes ± SEM are shown. *** $P < 0.005$. (D) Representative histology and HA immunostaining of tumors in nude mice. The tumor resembled the original sarcoma with *EWS/ATF1* expression. Scale bars: 100 µm. (E) Doxycycline withdrawal led to rapid tumor regression. At 3 months after doxycycline withdrawal, no viable tumor cells were observed, and tumors were replaced by fibrous tissue. Widespread cell death was observed 4 days after doxycycline withdrawal. Scale bars: 200 µm.

We found widespread cell death within the tumor mass, accompanied by massive infiltration of inflammatory cells, at 4 days after doxycycline withdrawal (Figure 4E), which indicates that neoplastic cells cannot survive *in vivo* without *EWS/ATF1* expression. Taken together, these results clearly indicate that *EWS/ATF1* plays a pivotal role in the proliferation and maintenance of *EWS/ATF1*-induced tumor cells *in vivo*.

Fos is a direct target of *EWS/ATF1*. To determine the downstream targets regulated by *EWS/ATF1*, we next performed gene expression analysis using G1297 cells. First, we confirmed that withdrawal of doxycycline for 96 hours resulted in no detectable expression of *EWS/ATF1* RNA or protein in cultured tumor cells. Next, the tumor cells were exposed again to doxycycline at a concentration of 0.05 or 0.2 µg/ml, and microarray analysis was performed at 3 and 48 hours after doxycycline exposure. Induction of *EWS/ATF1* resulted in altered expression of a number of genes associated with cell growth, such as growth factor genes (*Areg* and *Ereg*), cell cycle regulators (*Cenpa*, *Cna2*, *Ccnb2*, *Cdkn1b*, *Plk1*, and *Aurka*), and a proto-oncogene (*Fos*) at either time point (Supplemental Figure 6A). Although a previous study demonstrated that *MITF-M* is a direct target of *EWS/ATF1* in human CCS cell lines (25), we failed to detect its expression in our *EWS/ATF1*-induced tumor cell lines

and primary tumor samples (Supplemental Figure 7, A and B). Among the transcripts upregulated by *EWS/ATF1*, we focused on the proto-oncogene *Fos*, because this was one of the most highly upregulated genes by *EWS/ATF1* after doxycycline exposure in the microarray analysis (Supplemental Figure 6A). Quantitative real-time RT-PCR (qRT-PCR) confirmed upregulation of both *Fos* and *EWS/ATF1* transgenes in 2 independent tumor cell lines as early as 3 hours after doxycycline exposure (Figure 5A and Supplemental Figure 7C). We also found that the *EWS/ATF1*-induced tumor specimens expressed higher levels of both *Fos* and *EWS/ATF1* transgenes (Figure 5B). Expression of *Fos* is induced by numerous stimuli, which are transmitted through the RAS/Raf/MAP kinase or cAMP-dependent protein kinase pathway (33). In order to investigate the mechanism of *Fos* induction by *EWS/ATF1*, we next examined whether the RAS/Raf/MAP kinase pathway is involved in *EWS/ATF1*-mediated *Fos* activation. In contrast to the rapid and transient induction of *Fos* in MEFs after serum stimulation (Supplemental Figure 7D), expression of *Fos* in the *EWS/ATF1*-expressing tumor cell line was detected even under serum-free conditions, and it gradually increased after serum stimulation (Supplemental Figure 7E). Interestingly, whereas serum-stimulated MEFs revealed immediate phosphorylation of ERK1 and ERK2

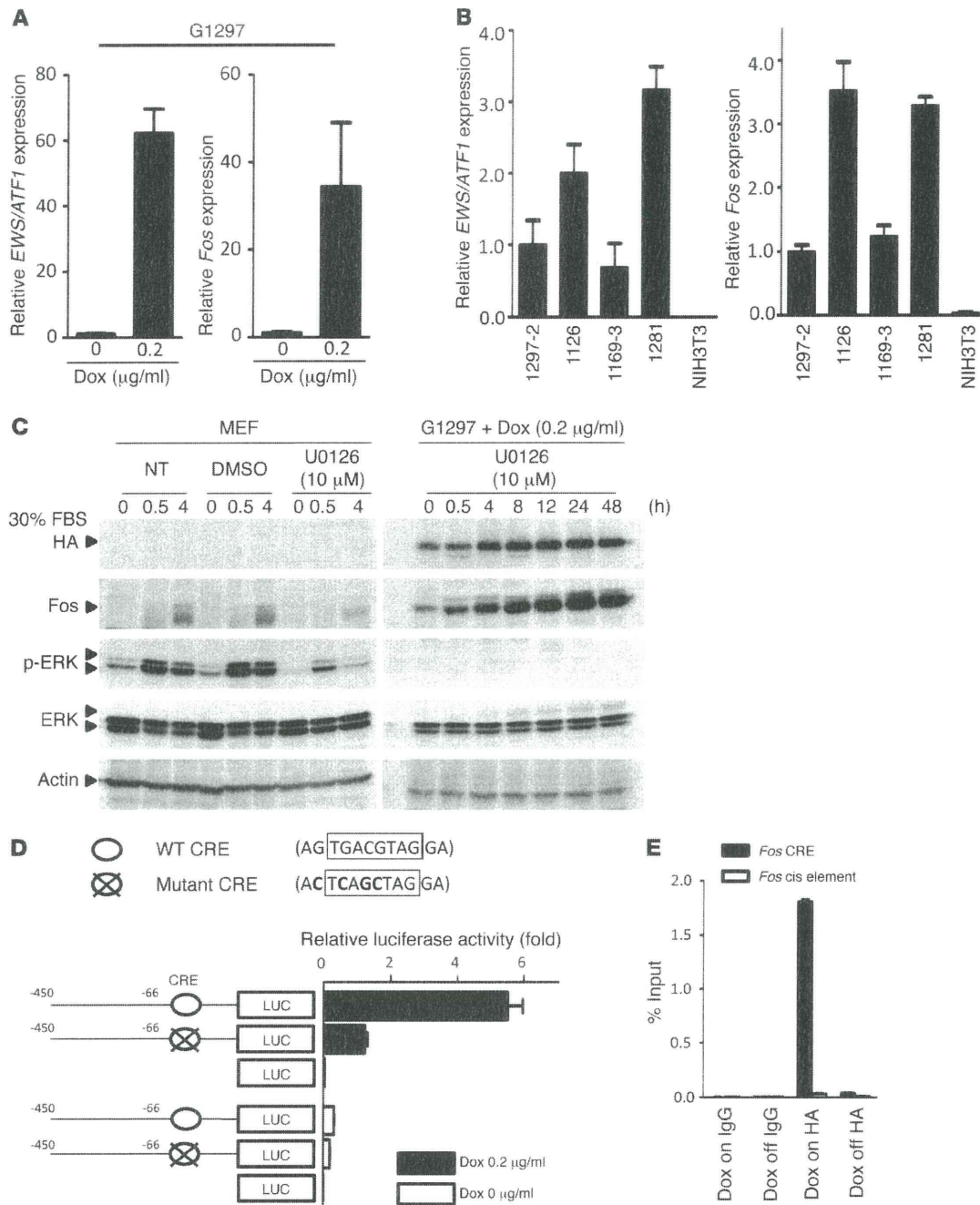
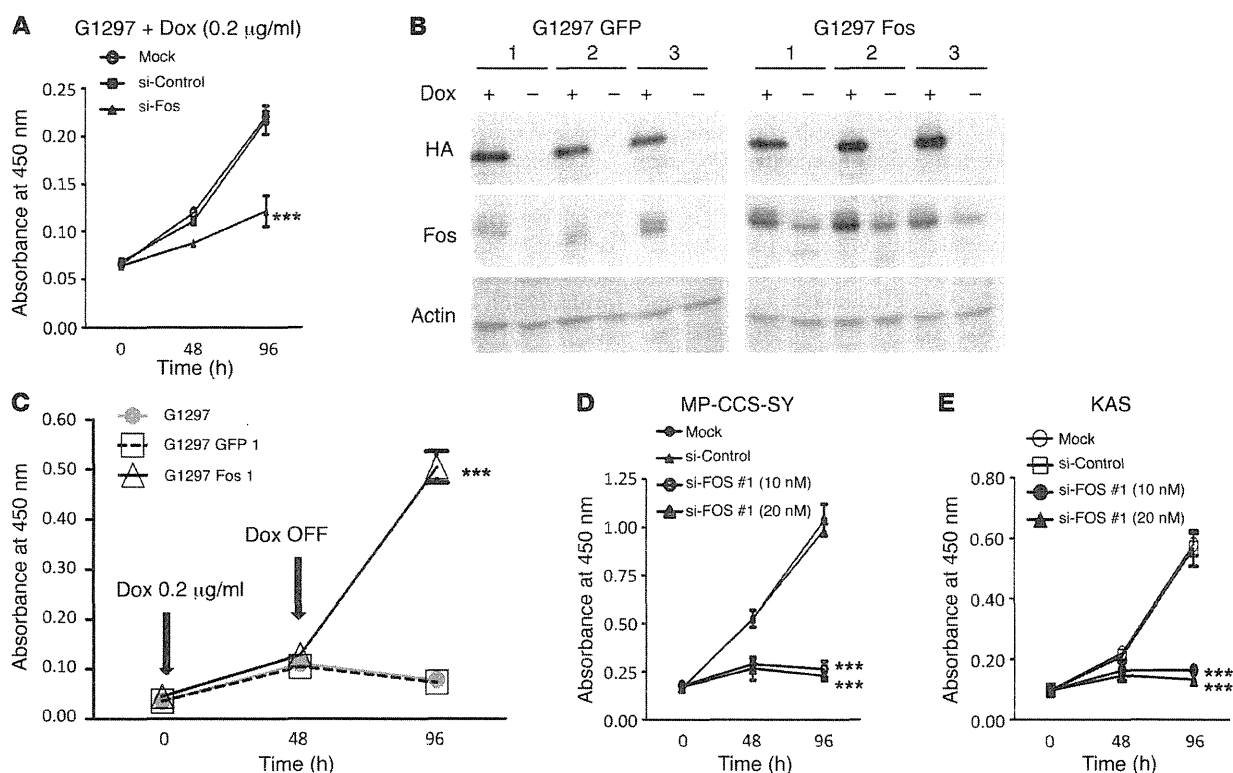


Figure 5

Fos is a direct target of EWS/ATF1. (A) Real-time RT-PCR analysis of G1297 cells revealed significant upregulation of both *EWS/ATF1* and *Fos* 3 hours after doxycycline exposure. (B) Relative expression of *EWS/ATF1* and *Fos* in 4 *EWS/ATF1*-induced tumors from 4 independent mice. NIH3T3 cells served as a control. Transcript levels were normalized to β -actin. Data are mean \pm SD ($n = 3$). (C) Fos induction by *EWS/ATF1* was independent of the ERK pathway. Serum-starved MEFs and G1297 cells were stimulated with 30% FBS for the indicated times. Cells were also treated with 10 μ M of the MEK inhibitor U0126. Whereas ERK1/2 inhibition by U0126 decreased Fos in MEFs, U0126 failed to suppress Fos expression in G1297 cells. NT, not treated. (D) Mouse *Fos* promoter–luciferase reporter constructs and pRL-SV40 vector (as an internal control) were cotransfected in G1297 cells treated with or without 0.2 μ g/ml doxycycline. Luciferase activity of each construct was normalized to internal control activity. Data are mean \pm SD ($n = 3$). (E) ChIP-PCR analysis was performed for the *Fos* promoter region containing CRE or the negative control cis element using HA-tag antibody or IgG as nonimmune immunoprecipitation, respectively. EWS/ATF1 was enriched at the CRE element of the *Fos* promoter in G1297 cells after treatment with 0.2 μ g/ml doxycycline. Data (mean \pm SD) were quantified by qRT-PCR and expressed as percent of input DNA.

**Figure 6**

Fos plays a key role in *EWS/ATF1*-induced cell proliferation. (A) Effect of *Fos* knockdown on proliferation of *EWS/ATF1*-induced cells. G1297 cells were treated with siRNA targeting *Fos* (si-Fos; 10 nM), a control siRNA (si-Control; 10 nM), or lipofectamine alone (Mock). 48 and 96 hours later, cell viability was determined by WST-8 assay. Results are mean \pm SD ($n = 4$). *** $P < 0.001$ vs. si-Control and Mock. (B) *EWS/ATF1*-induced tumor cell lines overexpressing *Fos* or *EGFP* (G1297 Fos and G1297 GFP, respectively). pCAG-*Fos*-IZ vector or pCAG-*EGFP*-IZ vector were stably transfected in G1297 cells. Western blot analysis revealed that G1297 Fos cells stably expressed Fos protein even in the absence of doxycycline. (C) Cell proliferation assay for G1297, G1297 GFP, and G1297 Fos cells before and after doxycycline withdrawal. Doxycycline treatment (0.2 µg/ml) was withdrawn for 48 hours. Cell viability was determined by WST-8 assay. *** $P < 0.001$ vs. G1297 and G1297 GFP. (D and E) Effect of *FOS* knockdown on growth of human CCS cell lines. MP-CCS-SY and KAS cells were treated with siRNA#1 targeting *FOS* (si-FOS #1; 10 nM and 20 nM), control siRNA (si-Control; 20 nM), or lipofectamine alone (Mock). 48 and 96 hours later, cell viability was determined by WST-8 assay. Data are mean \pm SD ($n = 4$). *** $P < 0.001$ vs. si-Control and Mock.

(Supplemental Figure 7D), phosphorylation of ERK1/2 was not observed in the *EWS/ATF1*-induced tumor cell line, even after serum stimulation (Supplemental Figure 7E), which suggests that continuous upregulation of Fos in *EWS/ATF1*-induced tumor cells is independent of the RAS/Raf/ERK signaling pathway. We treated *EWS/ATF1*-induced tumor cells with the MEK inhibitor U0126 to block activation of ERK1/2 in order to further confirm the ERK-independent activation of Fos. Although inhibition of ERK1/2 resulted in a substantial decrease of Fos in MEFs, U0126 failed to suppress Fos expression in *EWS/ATF1*-induced tumor cells (Figure 5C). These data indicate that constitutive overexpression of Fos in *EWS/ATF1*-induced tumor cells was mediated by an ERK-independent mechanism.

Previous studies demonstrated an interaction of ATF1 at a CRE in the *Fos* promoter (34, 35), which suggests that *EWS/ATF1* may induce *Fos* expression through interaction with the CRE. Conversely, in the present study, regulatory motif analysis of the upregulated genes by *EWS/ATF1* demonstrated enrichment of CRE near the transcription start site (from -1,000 bp to +200 bp; Supplemental Figure 6B). To evaluate the functional importance of this element in *EWS/ATF1*-mediated activation of *Fos*, we constructed a reporter plasmid con-

taining the mouse *Fos* promoter with wild-type and mutated CRE and examined transcriptional activity by luciferase assay (Figure 5D). We confirmed that induction of *EWS/ATF1* resulted in remarkably increased *Fos* promoter activity with wild-type CRE in G1297 cells. Importantly, luciferase activity of the mutated promoter significantly decreased compared with that of the wild-type promoter. We further examined whether *EWS/ATF1* directly binds to the CRE of the *Fos* promoter. ChIP-PCR analysis revealed that doxycycline-induced *EWS/ATF1* was enriched at the CRE of the *Fos* promoter, but not at the negative control cis element (Figure 5E). Our results indicated that the CRE is crucial for *EWS/ATF1*-mediated transcriptional activity of *Fos* in *EWS/ATF1*-induced tumor cells.

Expression of FOS in human CCS. To investigate whether overexpression of *FOS* is linked to human CCS, we analyzed *FOS* expression in the human CCS cell lines MP-CCS-SY and KAS and in the control lung fibroblast cell line WI38 by qRT-PCR. *FOS* was found to be highly expressed in both human CCS cell lines compared with WI38 (Supplemental Figure 8A). We also found that surgically resected clinical CCS specimens also expressed higher levels of *FOS* than did WI38 (Supplemental Figure 8B), which indicates that human CCS expresses higher levels of *FOS*.



We examined the effect of *EWS/ATF1* knockdown on *FOS* expression in human CCS cell lines to further investigate the association between *EWS/ATF1* expression and increased *FOS* expression in human CCS. Human CCS cell lines MP-CCS-SY and KAS carry the *EWS/ATF1* type 1 and type 2 fusion genes, respectively (Supplemental Figure 9, A and B). We next designed a specific siRNA targeting the breakpoint of the *EWS/ATF1* type 1 fusion gene, which had no effect on the expression of *ATF1* or of the *EWS/ATF1* type 2 fusion gene in KAS (Supplemental Figure 9, E and F). siRNA treatment targeting *EWS/ATF1* type 1 in MP-CCS-SY led to significant downregulation of *FOS* 48 hours after treatment (Supplemental Figure 9G) as well as of *EWS/ATF1* type 1 itself (Supplemental Figure 9, C and D), which indicates that *FOS* is a direct target of *EWS/ATF1* in human CCS. In contrast to *FOS*, we observed a modest reduction of *MITF-M* expression after *EWS/ATF1* knockdown in MP-CCS-SY cells (Supplemental Figure 9H).

FOS could be a promising therapeutic target for human CCS. To examine whether *Fos* overexpression facilitates proliferation of tumor cells expressing *EWS/ATF1*, we knocked down *Fos* in *EWS/ATF1*-induced tumor cells using siRNA. The G1297 cell line was treated with siRNA for *Fos* in the presence of doxycycline. siRNA treatment (10 nM) decreased the expression of *Fos* at the mRNA level by 75% at 24 hours after transfection, although it had no effect on expression of the *EWS/ATF1* transgene compared with the control siRNA (Supplemental Figure 10A). In addition, we confirmed that *Fos* protein levels were also decreased 48 hours after transfection (Supplemental Figure 10B). A WST-8 assay was performed in *EWS/ATF1*-induced tumor cells transfected with the nonfunctional control siRNA or with functional *Fos* siRNA to examine the effect of *Fos* knockdown on the cellular kinetics. The siRNA targeting *Fos* efficiently inhibited cell proliferation of *EWS/ATF1*-induced tumor cells, even in the presence of doxycycline (Figure 6A). In order to further confirm the importance of *Fos* expression for *EWS/ATF1*-induced tumor cell growth, we established *EWS/ATF1*-induced tumor cell lines in which *Fos* is overexpressed (Figure 6B). We found that *Fos*-overexpressed *EWS/ATF1*-inducible cells retained the ability to proliferate for at least 48 hours after doxycycline withdrawal, whereas control cells in which *GFP* is overexpressed stopped their proliferation soon after withdrawal (Figure 6C). We also examined the effect of *FOS* knockdown on cell growth of human CCS cell lines using siRNA targeting *FOS*. Consistent with the results in *EWS/ATF1*-induced tumor cells, siRNA treatment strongly suppressed the growth of CCS cell lines (Figure 6, D and E, and Supplemental Figure 11, A–F). Taken together, these data suggest that *FOS*, a direct target of *EWS/ATF1*, mediates the oncogenic growth of *EWS/ATF1*-related sarcomas and could be a potent therapeutic target for human CCS.

Discussion

The current study revealed that forced expression of the *EWS/ATF1* fusion gene induced sarcoma formation in *EWS/ATF1* transgenic mice. The histology of the tumors in *EWS/ATF1* transgenic mice showed a striking similarity to that of human CCS. In addition, immunohistochemistry demonstrated that *EWS/ATF1*-induced tumor cells express neural crest-associated markers, such as *S100*, *Mitf*, and *Sox10*, which are also expressed in human CCS. Given that the *EWS/ATF1* fusion gene is detected in CCS, our *EWS/ATF1* transgenic mouse is the first mouse model for investigating CCS pathogenesis. Our present results demonstrated that continuous expression of *EWS/ATF1* was required for growth and tumor

formation of *EWS/ATF1*-induced tumor cells. These results indicate that *EWS/ATF1* plays a pivotal role in both development and maintenance of *EWS/ATF1*-associated sarcomas, implying that CCS exhibits oncogene addiction (36) to *EWS/ATF1*, and provide a rationale for targeting *EWS/ATF1* itself to treat CCS.

It is interesting to note that sarcoma formation was observed only in deep soft tissue, although *EWS/ATF1* was induced in a variety of cell types in this experimental system (37, 38). In addition, the cell proliferation rate of MEFs in vitro was reduced by *EWS/ATF1* induction. These results clearly demonstrated that the abnormal proliferation by the forced expression of *EWS/ATF1* requires a specific cell type of origin, accompanied by a specific microenvironment. Consistent with these findings, recent studies of other sarcoma-related genes revealed that introduction of *SYT/SSX*, a synovial sarcoma-related gene, into *Myf5*-positive immature myoblasts specifically resulted in sarcoma formation, whereas its expression in more differentiated cells induced myopathy without tumor induction (39). In addition, introduction of *EWS/FLI1*, a fusion gene detectable in Ewing sarcomas, results in transformation specifically in bone marrow-derived mesenchymal progenitor cells in vitro (40). Taken together, these findings are suggestive of cell type-specific carcinogenesis by expression of sarcoma-related fusion oncogenes.

Our lineage-tracing experiments in vivo suggested that *EWS/ATF1*-associated tumor cells are derived from neural crest-derived cells. This result is consistent with several lines of evidence that CCS often shows melanocytic differentiation and resembles MM. However, our present results do not exclude the possibility that *EWS/ATF1*-induced tumors can arise from non-neural crest-derived cells. In addition, the exact cell type of origin of *EWS/ATF1*-induced tumors remains unclear, since neural crest-lineage progenitors can differentiate into many different cell types, such as neuronal cells, melanocytes, and Schwann cells. Recently, Schwann cell precursors along the peripheral nerve have been shown to be a cellular source of large numbers of melanocytes in the skin during development in mice and chicks (41). Moreover, Schwann cells also retain the potential to differentiate into melanocytes, resulting from a loss of nerve contact (41). Given the finding that *EWS/ATF1*-induced tumor cells expressed markers for melanocytic differentiation, it is possible that the neural crest-derived Schwann cells could be the origin of *EWS/ATF1*-associated sarcomas.

We found that *Fos* was one of the direct targets of *EWS/ATF1* in *EWS/ATF1*-induced tumor cells. *Fos* is an immediate early gene that can be activated by a variety of mitogens and growth factors. The present study showed that *Fos* induction by forced expression of *EWS/ATF1* was independent of the ERK signaling pathway. In contrast, we found that *Fos* upregulation was mediated by a CRE of the *Fos* promoter, accompanied by direct interaction of *EWS/ATF1* with the CRE on the *Fos* promoter. The direct interaction of *EWS/ATF1* at CRE may induce continuous transcriptional activation of *Fos* in *EWS/ATF1*-induced tumor cells. Previous studies have demonstrated a higher expression level of *FOS* to be involved in tumor growth in several cancers (42–44), and overexpression of *Fos* results in osteosarcoma formation in transgenic mice (45, 46). Here we showed that *FOS* was also upregulated in CCS by the *EWS/ATF1* fusion transcript and that the increased *FOS* promoted the growth of *EWS/ATF1*-related sarcomas. Accordingly, blocking the *FOS* pathway might be a promising therapeutic strategy for treating CCS (Supplemental Figure 12).

Methods

Molecular cloning and gene targeting in ES cells. Human *EWS/ATF1*-FLAG-HA was amplified by RT-PCR from the human CCS cell line KAS using primers ACATGGCGTCCACGGATTACAG and CCTAGGCGTAGTC-GGGCACGTCGTAGGGGTATCCTCCAGCGGCCGACTTGTGCATC-GTCGTCCTTGTAGTCTCCTCCAACACTTTTATTGGAATAAAGAT and cloned into pcr2.1-TOPO. Sequence-verified *EWS/ATF1*-FLAG-HA cDNA was subcloned into a unique *EcoRI* site of pBS31 prime (37, 38). KH2 ES cells (obtained from Open Biosystems) were used to insert a single copy of *EWS/ATF1*-FLAG-HA by Flipase (PLP) recombination into the *Col1A1* locus under the control of a minimal CMV tetracycline-inducible promoter using a previously described method (37), and ES cells were selected for hygromycin resistance.

Mouse generation. For blastocyst injections, fertilized zygotes were isolated from the oviducts of day-0.5 pregnant B6D2F1 females and allowed to develop to the blastocyst stage in culture. 7–12 ES cells were injected per blastocyst. The injected blastocysts were transferred into day-2.5 pseudo-pregnant recipient females.

Doxycycline treatment. 6-week-old mice were administered 50 µg/ml doxycycline (Sigma-Aldrich) in their drinking water supplemented with 2 mg/ml sucrose. For cultured cells, doxycycline was used at a concentration of 0.05–0.2 µg/ml.

RNA preparation and RT-PCR. Total RNA was isolated using a RNeasy mini kit (Qiagen). Total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems). qRT-PCR analysis using the fluorescent SYBR green method (Bio-Rad) was performed in accordance with the manufacturer's instructions. The data generated from each reaction were subjected to gene expression analysis using an iCycler iQ Real-Time PCR Detection System (Bio-Rad). See Supplemental Table 1 for specific primer pairs used for amplification. Microarray analysis was performed with SurePrint G3 Mouse GE 8X60K microarray (Agilent Technologies) and Mouse Gene 1.0 ST Array (Affymetrix) according to the manufacturer's instructions. All analyses were performed by Genespring GX (version 12; Agilent Technologies).

Western blot analysis. Western blot analyses were carried out as described previously (47, 48). The following antibodies were used: anti-HA (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti-Fos (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti-ERK1/2 (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti-phospho-ERK1/2 (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti-ATF1 (rabbit IgG, 1:5,000 dilution; EPITOMICS), and anti-β-actin (mouse IgG, 1:5,000 dilution; Calbiochem).

Cell proliferation assay. Cell growth was determined by WST-8 assay using a Cell Counting Kit-8 (Dojindo Laboratories). Absorbance at 450 nm is indicative of the die amount of formazan, which is directly proportional to the number of living cells.

Histological analysis. Normal and tumor tissue samples were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. 4-µm sections were stained with H&E, and serial sections were used for immunohistochemical analyses. Immunostaining was performed using an avidin-biotin immunoperoxidase assay. The primary antibodies used were anti-HA-Tag (1:600 dilution; Cell Signaling), anti-Ki67 (1:250 dilution; Dako), anti-S100 (1:800 dilution; Dako), anti-SOX10 (1:200 dilution; R&D Systems), anti-MITF (1:500 dilution; Exalpa), and anti-GFP (1:1,000 dilution; Abcam).

X-gal staining. Briefly, tumor tissue samples were embedded in OCT compound and frozen. 8-µm cryostat sections were immediately fixed in 0.2% glutaraldehyde for 10 minutes. The sections were stained overnight in an X-gal staining solution, then counterstained with fast red for 3 minutes.

Tumorigenicity studies. 4-week-old male BALB/c athymic mice were obtained from Japan SLC. A total of 5.0×10^6 G1297 cells in 0.1 ml serum-free DMEM was inoculated subcutaneously through a 26-gauge needle

into the posterior flank of each mouse. 3 weeks after inoculation, the tumor diameters were measured with digital calipers, and tumor volume was calculated as $(w^2 \times l)/2$ and expressed in mm³.

siRNA transfection. We performed transient knockdown assays with a siRNA targeting *Fos* (Santa Cruz), *FOS* (Santa Cruz and Dharmacon) or the breakpoint of *EWS/ATF1* type 1 (sense, GCGGUGGAAUGGGAAAAAATT; antisense, AUUUUUCCCAUCCACCGCTT; KOKEN) using Lipofectamine RNAiMAX (Invitrogen). We used nontargeting siRNA (Cosmo Bio Co.) as a control.

Cell lines. MP-CCS-SY and KAS are CCS cell lines carrying *EWS/ATF1* type 1 and type 2, respectively. MP-CCS-SY was established as described previously (49), and KAS was provided by T. Nakamura (Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan; ref. 24). HOS (osteosarcoma), U2OS (osteosarcoma), NIH3T3 (embryonic fibroblast), and WI38 (lung fibroblast) cells were purchased from the American Type Culture Collection. B16-F1 (mouse melanoma) and A375 (human MM) were purchased from the European Collection of Cell Cultures.

Mice. *Wnt1-Cre* mice were provided by S. Iseki (Tokyo Medical and Dental University, Tokyo, Japan; ref. 50), *P0-Cre* mice were provided by K. Yamamura (Kumamoto University, Kumamoto, Japan; ref. 51), and floxed *LacZ* mice were provided by M. Okabe (Osaka University, Suita, Japan; ref. 52). Floxed *EYFP* mice (53) were obtained from Jackson Laboratory.

Construction of the reporter plasmid. To obtain the *Fos* reporter plasmid (pGL3A-1486), the genomic DNA fragment containing –450 to +0 of the 5'-flanking region was amplified by PCR with the primer set 5'-TCTATC-GATAGGTACGAATGTTTCGCTCGCCTTCTC-3' and 5'-ACGCGTAGAGCTCGGGAGTAGTAGGCGCCTCAGC-3' and subcloned into the *KpnI* site of the pGL3 vector (Promega). The *Fos* reporter plasmid with mutant CRE element was generated by PCR-targeted mutagenesis with the primer set 5'-CCAGTTCGCCCCACTCAGCTAGGAAGTCCATCC-3' and 5'-GGATGGACTTCCTAGCTGAGTGGGCGGAAGTGG-3'.

Luciferase assay. Reporter genes were transfected into the G1297 *EWS/ATF1*-induced tumor cell line together with pHRL-SV40 (Promega) using lipofectamine LTX (Invitrogen), and luciferase activity was measured with a luminometer (VERITAS; Promega). Firefly luciferase activities, derived from each reporter construct, were normalized to Renilla luciferase activities from pHRL-SV40.

Stable transfection. To obtain *Fos* expression plasmid (pCAG-Fos-IZ vector), *Fos* cDNA was amplified by RT-PCR from the G1297 cell line with the primer set 5'-ACATGATGTTCTCGGGTTTCAA-3' and 5'-ACTCAGAGGCCAGCAGCGTGG-3' and subcloned into the *ECOR* site of the pCAG-EGFP-IZ vector (provided by H. Niwa, RIKEN, Kobe, Japan). pCAG-EGFP-IZ vector or pCAG-Fos-IZ vector was transfected into the G1297 cell line using Lipofectamine LTX Reagent (Invitrogen) and selected for Zeocine resistance (600 µg/ml).

Patients and tumor tissue collection. Anonymized tumor specimens were obtained by surgical resection or biopsy at Gifu University Hospital or Kyoto University Hospital in accordance with an approved protocol from the Institutional Review Board. Total RNA was isolated using a RNeasy mini kit (Qiagen).

ChIP analysis. A total of 5.0×10^6 *EWS/ATF1*-inducible tumor cells was fixed in 1% formaldehyde for 10 minutes, followed by treatment with 1 ml glycine buffer for 5 minutes. Cells were pelleted, washed, and then resuspended in lysis buffer for 30 minutes. After centrifugation, the pellet was resuspended in NP40 buffer with protease inhibitors (Sigma-Aldrich). Sonication was performed using a XL-2000 (MISONIX), after which the supernatant was used as the input sample for immunoprecipitation experiments. Antibodies used were rabbit HA (Cell Signaling) and rabbit normal IgG (Abcam). Protein G-coated magnetic beads were used to purify specific antibody/DNA complexes. After washes, immunoprecipitated DNA was decrosslinked by elu-



tion buffer at 65°C for 12 hours. To remove protein and RNA, samples were incubated with RNaseA for 1 hour at 37°C and proteinase K treatment for 1 hour at 37°C. Samples were purified by PCR purification kit (Qiagen). The amount of DNA immunoprecipitated with HA-tagged protein was quantified by real-time PCR with primers flanking the *Fos* promoter, including the CRE element (forward, 5'-TCCTACACGCGGAAGGTCTAGG-3'; reverse, 5'-TAGAAGCGCTGTGAATGGATGG-3'). Primers 5'-GCACTAATTAGTC-GCGGTGGTGG-3' (forward) and 5'-CAGGGTCTTAGTGGGATCAAGG-3' (reverse) were used as a negative control.

Accession number. The profiling data cited in Supplemental Figure 6 are available at GEO (accession no. GSE41123).

Statistics. Statistical analyses were carried out using GraphPad Prism (version 5.01; GraphPad Software). Data were analyzed using ANOVA, and *P* values less than 0.05 were considered statistically significant.

Study approval. Animal experiments were approved by the Gifu University Animal Experiment Committee, and the care of the animals was in accordance with institutional guidelines. All clinical samples were approved for analysis by the Ethics Committee at Kyoto University Graduate School and Faculty of Medicine (Kyoto, Japan). Written informed consent was obtained from all patients with cancers analyzed in this study.

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