



Divergent Ewing's sarcoma EWS/ETS fusions confer a common tumorigenic phenotype on NIH3T3 cells

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Ewing's sarcomas express chimeric transcription factors resulting from a fusion of the amino terminus of the EWS gene to the carboxyl terminus of one of five ETS proteins. While the majority of tumors express EWS/FLI1 fusions, some Ewing's tumors contain variant chimeras such as EWS/ETV1 that have divergent ETS DNA-binding domains. In spite of their structural differences, both EWS/ETS fusions up regulate EAT-2, a previously described EWS/FLI1 target gene. In contrast to EWS/FLI1, NIH3T3 cells expressing EWS/ETV1 cannot form colonies in soft agar though coexpression of a dominant negative truncated ETV1 construct attenuates EWS/FLI1 mediated anchorage independent growth. When EWS/ETV1 or EWS/FLI1 expressing NIH3T3 cells are injected into SCID mice, tumors form more often and faster than with NIH-3T3 cells with empty vector controls. The tumorigenic potency of each EWS/ETS fusion is linked to its C-terminal structure, with the FLI1 C-terminus conferring a greater tumorigenic potential than the corresponding ETV1 domain. The resulting EWS/ETV1 and EWS/FLI1 tumors closely resemble each other at both a macroscopic and a microscopic level. These tumors differ greatly from tumors formed by NIH3T3 cells expressing activated RAS. These data indicate that in spite of their structural differences, EWS/ETV1 and EWS/FLI1 promote oncogenesis via similar biologic pathways.

Keywords: EWS/FLI1; EWS/ETV1; chimeric transcription factors; tumorigenesis; Ewing's sarcoma

Introduction

Somatic mutation, and in particular chromosomal translocation, is seen as a causal event in a number of human malignancies (for reviews see Rowley, 1993; Rabbitts, 1994). Many of these translocations juxtapose novel transcriptional activation domains to known DNA-binding domains creating aberrant transcription factors (Denny, 1996; Sorensen and Triche, 1996). These chimeric transcription factors are

thought to aberrantly modulate the expression of genes thereby promoting unregulated cell division and oncogenesis (Braun *et al.*, 1995).

Ewing's sarcoma is associated with a variety of chromosomal translocations all of which fuse the glutamine-rich N-terminus of the EWS gene to the C-terminus of one of several ETS transcription factors (for review see Kovar, 1998). EWS and its family member TLS/FUS, are related to the human TBP-associated factor hTAFII68 (Bertolotti *et al.*, 1996). These proteins normally associate with both the basal transcription factor TFIID and the *PoIII* complex (Bertolotti *et al.*, 1998). In most Ewing's sarcomas, EWS is fused to the carboxyl terminus of either FLI1 or ERG, two very closely related ETS proteins (Delattre *et al.*, 1992, 1994; Sorensen *et al.*, 1994; Giovannini *et al.*, 1994). The resulting EWS/FLI1 fusion demonstrates biochemical and biologic properties that are distinct from those of normal full-length FLI1 (May *et al.*, 1993a,b).

In a minority of cases of Ewing's sarcoma, the amino terminus of EWS is coupled to the ETS transcription factors ETV1/ER81, E1AF/PEA3, or FEV (Jeon *et al.*, 1995; Urano *et al.*, 1996; Kaneko *et al.*, 1996; Peter *et al.*, 1997). The DNA-binding domain of FEV is homologous to that of FLI1, suggesting that EWS/FLI1 and EWS/FEV might function similarly. By contrast, ETV1 and E1AF belong to a distinct structural subclass of ETS proteins; the PEA3 subfamily. Members of this subfamily participate in a number of oncogenic signal transduction pathways and have been implicated in tumor invasion (Janknecht, 1996; de Launoit *et al.*, 1997). While the DNA-binding domains of ETV1 and E1AF are highly homologous to each other (95% amino acid identity), they are structurally divergent from the DNA-binding domains of FLI1, ERG and FEV (60% amino acid identity).

This structural disparity between different ETS family members involved in Ewing's sarcoma chromosomal translocations, suggests that different EWS fusions could have varying biologic potentials. At the same time, in any single tumor specimen, only one type of EWS/ETS fusion is found. Tumors that harbor EWS/ETV1 do not contain other EWS/ETS fusions. This suggests that despite their structural differences, these EWS/ETS fusions may functionally converge on a common oncogenic pathway leading to the phenotypic characteristics of Ewing's sarcoma. To address this question, we have compared EWS/ETV1 with EWS/FLI1 in an NIH3T3 model system.

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Results

EWS/ETV1 and EWS/FLI1 both induce expression of EAT-2 in NIH3T3 cells

To assess possible functional similarity between EWS/ETV1 and EWS/FLI1, the ability for both fusions to modulate expression of a common target gene was determined. Optimally such an indicator gene would be induced only in cells transformed by EWS/ETS genes but not by other stimuli. EAT-2, a gene similar to a recently described family of signal transduction molecules (Sayos *et al.*, 1998), was chosen for this purpose. EAT-2 is up regulated by EWS/FLI1 but not by a panel of unrelated transforming genes including activated RAS, vABL, c-MYC, activated cdc42 and the E2A/PBX fusion gene (Thompson *et al.*, 1996 and data not shown).

We had previously shown that on a transcriptional level, EAT-2 was poorly induced by EWS/ETV1. However, these initial studies did not confirm that EWS/ETV1 protein was being adequately expressed. To be able to monitor protein levels on a consistent basis, amino terminal flag-tagged versions of both of EWS/ETV1 and EWS/FLI1 were created. After subcloning into retroviral expression vectors, these constructs were transduced into NIH3T3 cells and stable polyclonal populations were selected. Equivalent levels of EWS/FLI1 and EWS/ETV1 protein were detected in cell lysates from transfectant populations by immunoblot, using an anti-flag monoclonal antibody (Figure 1a). An EWS/FLI1 species of the same molecular weight was also detected using anti-FLI1 antisera, indicating that

the anti-flag reagent was accurately detecting the recombinant EWS proteins (data not shown).

EAT-2 protein levels were assayed in polyclonal NIH3T3 cell lines stably expressing either EWS/ETV1 or EWS/FLI1. EAT-2 protein was clearly detected in cells expressing either epitope tagged EWS/ETS fusion though EAT-2 levels appeared higher in EWS/FLI1 transformed cells (Figure 1b). Up regulation of EAT-2 by EWS/ETV1 is dependent on an intact EWS domain. EAT-2 protein was not detected in NIH3T3 cells expressing (Δ N)ETV1, an N-terminal EWS/ETV1 deletion mutant (data not shown and Figure 2b).

A dominant negative mutant ETV1 inhibits EWS/FLI1 transformation of NIH3T3 cells

Induction of EAT-2 by both EWS/ETS fusions demonstrated functional similarity between these two molecules on a genotypic level. To assess whether both EWS/ETV1 and EWS/FLI1 displayed biologic similarity on a phenotypic level, NIH3T3 populations expressing either fusion were plated in soft agar. In contrast to cells expressing EWS/FLI1, NIH3T3 populations expressing EWS/ETV1 could not form colonies in soft agar even at high plating cell densities and after extended incubation times (Figure 1c).

One possible explanation for these results is that EWS/ETV1 and EWS/FLI1 do not modulate the same repertoire of target genes. There may be a subset of genes necessary for anchorage independent growth that EWS/FLI1 can activate but EWS/ETV1 cannot. However, the observation that both EWS/ETV1 and EWS/FLI1 induce EAT-2 expression indicates that

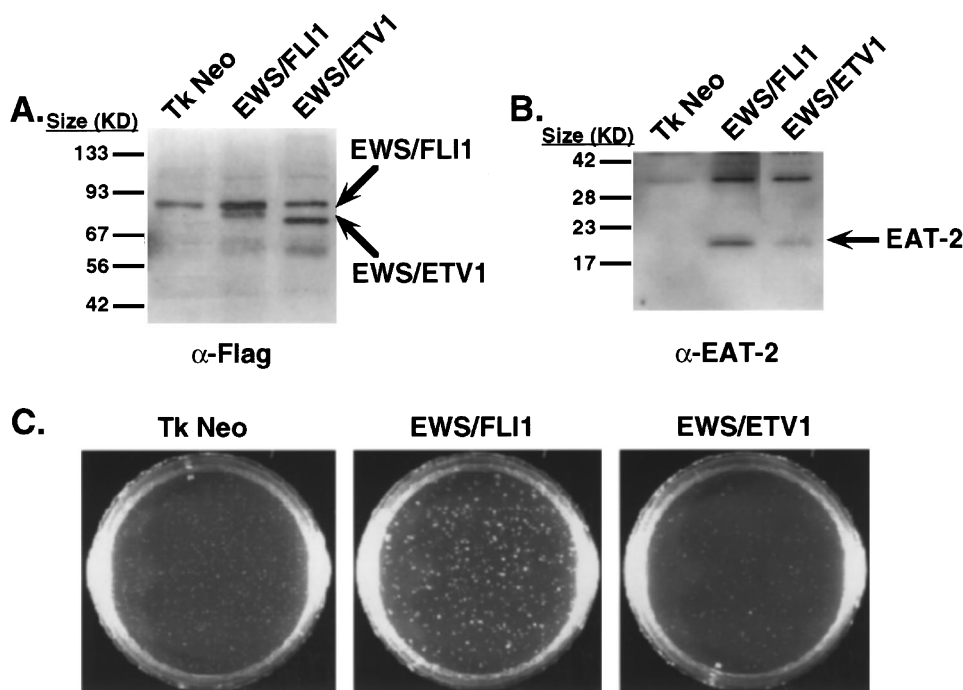


Figure 1 Effect of EWS/ETV1 on target gene expression and anchorage independent growth of NIH3T3 cells. (a) Immunoblot demonstrating equivalent levels of protein expression of flag-tagged EWS/ETV1 and EWS/FLI1. Each EWS/ETS protein was detected using anti-Flag monoclonal antibody. A background band migrating at approximately 84 KD was seen in all NIH3T3 populations grown in tissue culture. (b) Immunoblot showing EAT-2 expression in NIH3T3 cells expressing EWS/ETV1 and EWS/FLI1 but not empty vector (TK Neo). EAT-2 protein was detected using previously described rabbit polyclonal antisera (Thompson *et al.*, 1996 and Materials and methods). (c) Agar assay showing the inability of EWS/ETV1 to promote anchorage independent growth in NIH3T3 cells. Macroscopic colonies are visible in plates containing NIH3T3 cells expressing EWS/FLI1 but not with polyclonal populations expressing EWS/ETV1 or empty vector (TK Neo)

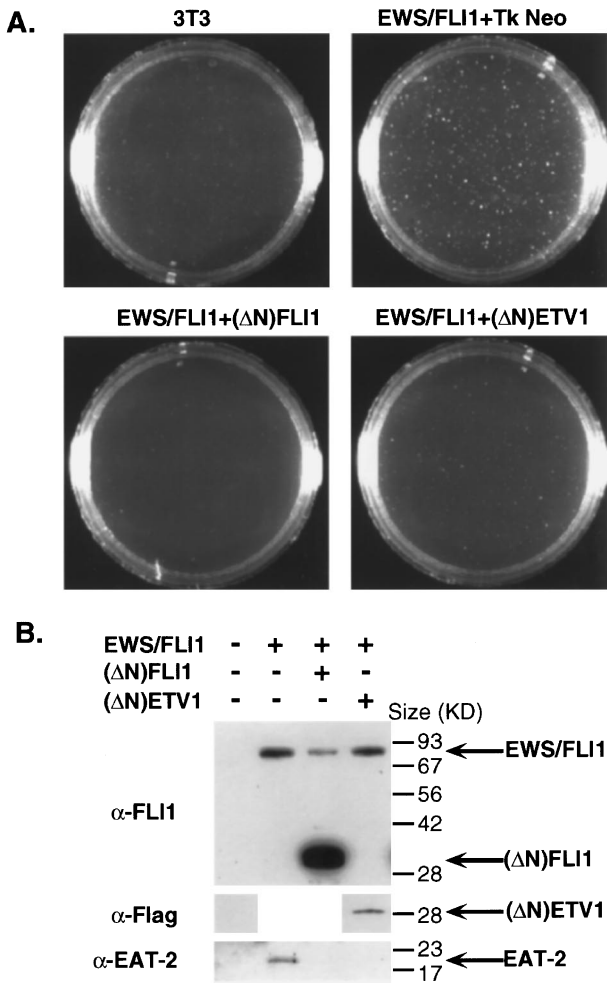


Figure 2 The expression of truncated (ΔN)ETV1 and (ΔN)FLI1 constructs inhibits EWS/FLI1 mediated anchorage independent growth. (a) Soft agar assays with parental NIH3T3 cells or NIH3T3 cells transduced first with EWS/FLI1 then with empty vector (Tk Neo), (ΔN)FLI1 or (ΔN)ETV1. The expression of either (ΔN)ETV1 or (ΔN)FLI1 antagonizes growth in soft agar promoted by EWS/FLI1. (b) Immunoblots showing the expression of EWS/FLI1, (ΔN)FLI1, (ΔN)ETV1 and EAT-2. Anti-FLI1 antibodies demonstrate the expression of both EWS/FLI1 and (ΔN)FLI1 proteins. Anti-flag antibodies detect flag-tagged (ΔN)ETV1 in the line which received this construct. Anti-EAT-2 antibodies reveal that expression of (ΔN)ETV1 or (ΔN)FLI1 reduces the level of activation of EAT-2 by EWS/FLI1

there is some overlap between the EWS/ETV1 and EWS/FLI1 target gene sets. It is possible that expression of some of these shared target genes may also be necessary for anchorage independent growth of NIH3T3 cells. If this is true, inhibitory constructs based on ETV1 would be able to antagonize the transforming effect of EWS/FLI1 in NIH3T3 cells.

To test this hypothesis, a dominant negative mutant of ETV1 was constructed and its ability to inhibit transformation by EWS/FLI1 was assessed. This construct consisted of epitope tagged deletion EWS/ETV1 that contained intact DNA-binding and C-terminal domains but lacked all N-terminal EWS sequences. A similar construct was generated from EWS/FLI1 to serve as a positive control. Similar constructs based on FLI1 had been previously shown to antagonize growth in cells expressing EWS/FLI1 (Kovar *et al.*, 1996). Inhibition experiments were

performed by first transducing EWS/FLI1 into NIH3T3 cells using retroviral constructs lacking an antibiotic resistance marker. These cells were then split and infected with a second retrovirus containing (ΔN)ETV1, (ΔN)FLI1 or empty vector with a neomycin resistance gene. After a short 3 day selection in G418, a portion of these cells were plated in soft agar and the remaining were harvested for protein expression.

Both (ΔN)ETV1 and (ΔN)FLI1 strongly inhibited transformation by EWS/FLI1 (Figure 2a). Inhibition of transformation depended on the stable expression of the dominant negative construct which was documented by immunoblot experiments using either anti-flag or anti-FLI1 serologic reagents (Figure 2b). EAT-2 was used as a marker to assess the effect of (ΔN)ETV1 and (ΔN)FLI1 on EWS/FLI1 signaling. EAT-2 protein levels were undetectable in cells co-expressing EWS/FLI1 and either of the dominant negative constructs (Figure 2b).

EWS/ETV1 and EWS/FLI1 accelerate tumorigenesis of NIH3T3 cells in SCID mice

Though EWS/ETV1 and EWS/FLI1 differ in their ability to transform NIH3T3 cells, our inhibition studies suggest that these molecules do share biochemical similarity. EWS/FLI1 is known to accelerate tumorigenesis of NIH3T3 cells in immune deficient mice (Zinszner *et al.*, 1994). To test whether EWS/ETV1 and EWS/FLI1 may be functionally similar in such an *in vivo* assay, their ability to promote tumorigenesis in inbred Severe Combined Immunodeficiency (SCID) mice was assessed.

Polyclonal NIH3T3 populations expressing EWS/FLI1, EWS/ETV1 or empty vector were generated by retroviral mediated gene transfer. After antibiotic selection, a constant number of cells were injected subcutaneously into SCID mice. Upon the formation of a 2 cm diameter tumor, the mice were sacrificed. Explant cell lines were derived from all tumors. Input and explant cell lines as well as tumor samples were harvested for expression studies.

All mice that received NIH3T3 cells expressing either EWS/ETV1 or EWS/FLI1 formed tumors within the 90 day trial limit (Figure 3). By contrast, approximately half (7/13) of the mice that were injected with cells containing empty vector, formed tumors by day 90. Log rank tests revealed significantly different tumorigenic rates for EWS/FLI1 versus empty vector mice and EWS/ETV1 versus empty vector mice ($P < 0.01$ and $P = 0.014$ respectively). In all cases tumors appeared localized with no apparent signs of gross distant metastasis.

Though all eight mice injected with NIH3T3 cells expressing EWS/ETV1 formed tumors, the time to tumor formation was significantly longer than the mice receiving EWS/FLI1 expressing NIH3T3 cells. Median times to form tumors were 22, 42 and 54 days for EWS/FLI1, EWS/ETV1 and empty vector mice respectively (Table 1). The 95% confidence limits of these three populations did not overlap, suggesting that the observed differences in tumorigenic rates among the EWS/FLI1, EWS/ETV1 and empty vector mice were significant.

Anti-flag or anti-FLI1 immunoblots documented the stable expression of EWS/ETV1 or EWS/FLI1

respectively in both input and explant cell lines (Figure 4). To determine whether the expression of the chimeric genes in these tumors had similar effects on downstream genes, the tumors were also analysed with anti-EAT-2 antibodies. In both EWS/FLI1 and EWS/ETV1 tumors, EAT-2 protein was detected (data not shown).

Potency of EWS/ETV1 and EWS/FLI1 in promoting tumorigenesis is associated with their C-terminal domains

While both EWS/FLI1 and EWS/ETV1 accelerate tumorigenesis of NIH3T3 cells in SCID mice, EWS/

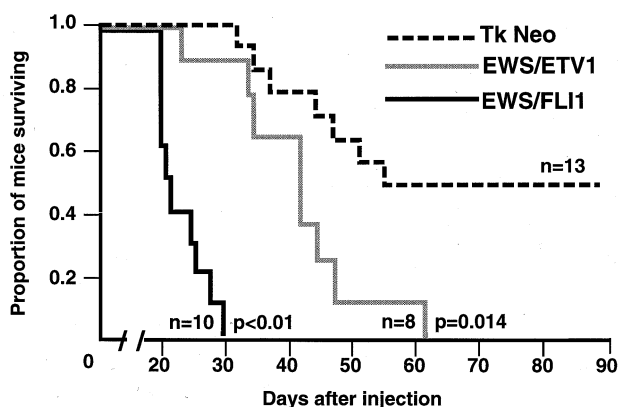


Figure 3 Infection of NIH3T3 cells with EWS/ETV1 or EWS/FLI1 accelerates tumorigenesis in SCID mice. This survival curve shows the per cent of mice that do not have a tumor of 2 cm diameter following a certain number of days after injection of the cells. The median times for cells expressing EWS/ETV1 and EWS/FLI1 differ from that for empty vector (Tk Neo) at *P* values of 0.014 and <0.01 respectively. At the 90th day of these experiments, the remaining Tk Neo mice had no apparent tumors. This graph summarizes at least two independent experiments for each construct involving retroviral transduction of NIH3T3 cells, antibiotic selection and injection into SCID mice. The EWS/FLI1 survival curve represents five independent experiments. Approximately half of the EWS/FLI1 data points were reported previously (May *et al.*, 1997) and were pooled with the present experiments after statistical analyses showed no difference among any of the EWS/FLI1 groups

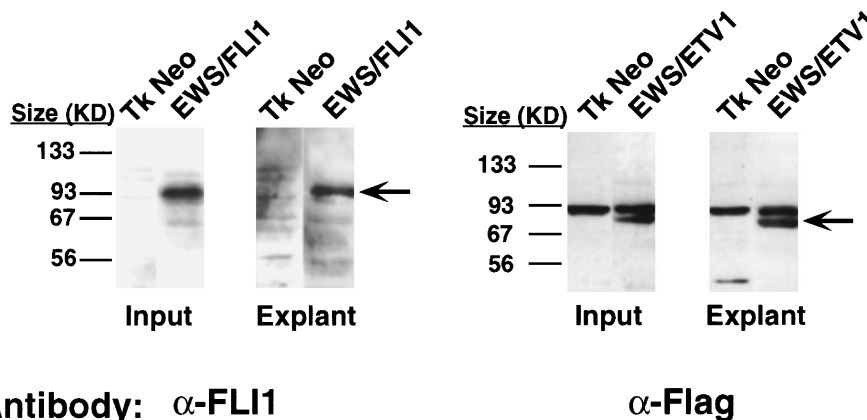
FLI1 was more effective than EWS/ETV1. Cells expressing EWS/FLI1 or EWS/ETV1 form 2 cm tumors in a median time of 22 or 42 days respectively (Table 1). The portions of ETV1 and FLI1 that are fused to EWS consist of two domains: (i) an 86 amino acid region that is both necessary and sufficient for *in vitro* site specific DNA binding and that is 62% identical between FLI1 and ETV1; (ii) C-terminal regions that are highly divergent and variable in length (89 amino acids for FLI1; 60 amino acids for ETV1) whose biochemical function is less clear.

To investigate which domain of FLI1 was responsible for enhanced tumorigenesis, triple chimeric constructs were made that maintain the EWS and DNA binding domains of the two EWS/ETS fusions but switch the C-terminal domains (Figure 5a). Therefore, EWS/FLI1/ETV1 contains EWS, the DNA binding domain of FLI1 and the C-terminal portion of ETV1. EWS/ETV1/FLI1 is the reciprocal that fuses EWS, the ETV1 DNA binding domain and the FLI1 C-terminus. Both constructs were epitope tagged. As with the other EWS/ETS genes, triple chimeras were transduced into NIH3T3 cells by retroviral mediated gene transfer and after antibiotic selection, cells were injected subcutaneously into SCID mice. The time to

Table 1 EWS/ETV1 and EWS/FLI1 accelerate tumorigenesis in NIH3T3 cells

	Median day for 2 cm tumor	95% confidence interval	Fraction with tumor at day 90
Tk Neo	54	46 < y	7/13
EWS/FLI1	22	21 < y < 25	10/10
EWS/ETV1	42	35 < y < 44	8/8

The median day for formation of a 2 cm diameter tumor shifts from 54 days for Tk Neo to 22 and 42 days for EWS/FLI1 and EWS/ETV1 respectively. The 95% confidence interval for Tk Neo is mutually exclusive with those for EWS/ETV1 and EWS/FLI1. Because many of the Tk Neo mice never formed tumors, no upper bound can be placed on the median day. All of the EWS/ETV1 and EWS/FLI1 receiving mice eventually formed 2 cm diameter tumors; six of 13 Tk Neo mice did not have any apparent tumor at the 90th day. A Fisher exact analysis calculates that the fractions of the Tk Neo and EWS/ETV1 mice that eventually formed tumors differ at a *P* value of 0.03



1^o Antibody: α-FLI1

α-Flag

Figure 4 EWS/FLI1 and EWS/ETV1 are expressed in explant lines created from their respective tumors. Immunoblots using anti-FLI1 antibodies show the expression of EWS/FLI1 in both the parental input lines for the tumors and in explanted lines generated from two independent tumors. Similarly, anti-flag antibodies detect flag-tagged EWS/ETV1 in both parental input and tumor explant lines. As before, anti-flag detected a consistent background band in all cell populations. These results are representative of multiple experiments

form 2 cm tumors was monitored and expression of triple chimeras was assessed by immunoblot.

Tumorigenic potency of triple chimeras was associated with the specific C-terminal domain that was expressed (Figure 5b). Cells expressing the EWS/ETV1/FLI1 construct containing the FLI1 C-terminus, formed tumors in a similar time period as those

expressing the full length EWS/FLI1 fusion. Cells expressing the EWS/FLI1/ETV1 fusion containing the ETV1 C-terminus, behaved similarly to EWS/ETV1.

Stable expression of triple chimeric proteins was assayed by immunoblot in input and explant tumor cell lines. EWS/ETV1/FLI1 protein was detected in both cell lines using antisera to the C-terminus of FLI1 (Figure 6). EWS/FLI1/ETV1 was assayed using anti-Flag monoclonal reagent.

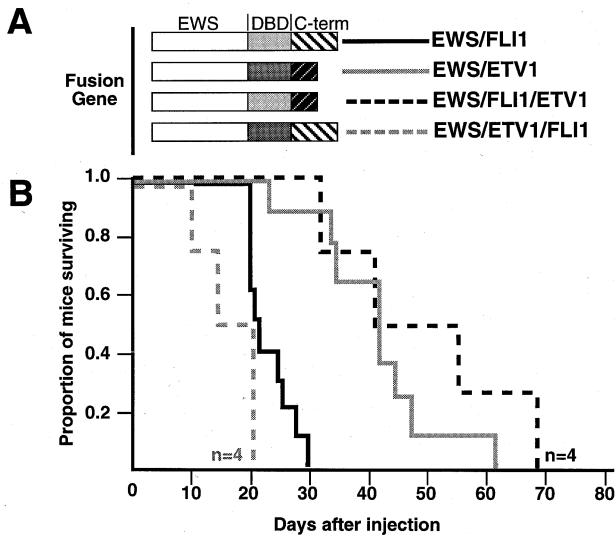


Figure 5 Tumorigenic potency of EWS/ETS triple chimeric constructs. (a) Schematic depicting structures of EWS/FLI1, EWS/ETV1 and triple chimeric fusions. Legend: EWS N-terminus (clear); DNA binding domains FLI1 (light grey) and ETV1 (dark grey); C-termini FLI1 (white stripe) and ETV1 (dark stripe). (b) Switching the C-terminal domain of ETV1 for FLI1 enhances the tumorigenic potential of EWS/ETV1. Mice were injected with equal numbers of NIH3T3 cells expressing EWS/FLI1/ETV1 or EWS/ETV1/FLI1 triple chimeric constructs. Survival curves depict the number of mice without a 2 cm tumor and is in reference to mice injected with EWS/FLI1 or EWS/ETV1 expressing cells from Figure 3. EWS/ETV1/FLI1 mice formed tumors at a rate that approximated EWS/FLI1. By contrast, the survival curve of EWS/FLI1/ETV1 mice was similar to the EWS/ETV1 mice

Tumors formed by NIH3T3 cells expressing either EWS/ETV1 or EWS/FLI1 display a characteristic histologic phenotype

Many dominant acting oncogenes, such as RAS, accelerate the rate of tumor formation but do not greatly alter the fibrosarcoma-like histology seen in tumors derived from NIH3T3 cells (Bernstein and Weinberg, 1985). To investigate potential qualitative effects of different Ewing's sarcoma EWS/ETS chimeras on NIH3T3 derived tumors, gross and microscopic analyses on tumor tissues were performed. Tumors arising from NIH3T3 cells expressing EWS/ETV1 or EWS/FLI1 were compared to those expressing activated RAS, manic fringe (M-FNG), or empty vector. Activated RAS is a well characterized oncogene that transforms NIH3T3 cells and renders them tumorigenic. M-FNG, a gene up regulated by EWS/ETV1 and EWS/FLI1, does not promote growth of NIH3T3 cells in soft agar but does increase their tumorigenic potential (May *et al.*, 1997).

Macroscopically, the EWS/ETV1 and EWS/FLI1 tumors differed from those seen with RAS and M-FNG. The tumors from these chimeric genes were soft and friable with obvious areas of coagulated and liquid hemorrhage. In comparison, RAS, M-FNG and empty vector derived tumors were pale, firm and lacked significant internal necrosis and hemorrhage.

On a microscopic level, tumors from NIH3T3 cells expressing RAS, M-FNG and empty vector resembled fibrosarcomas (Figure 7). These tumors were primarily composed of fascicles of spindle shaped cells. No

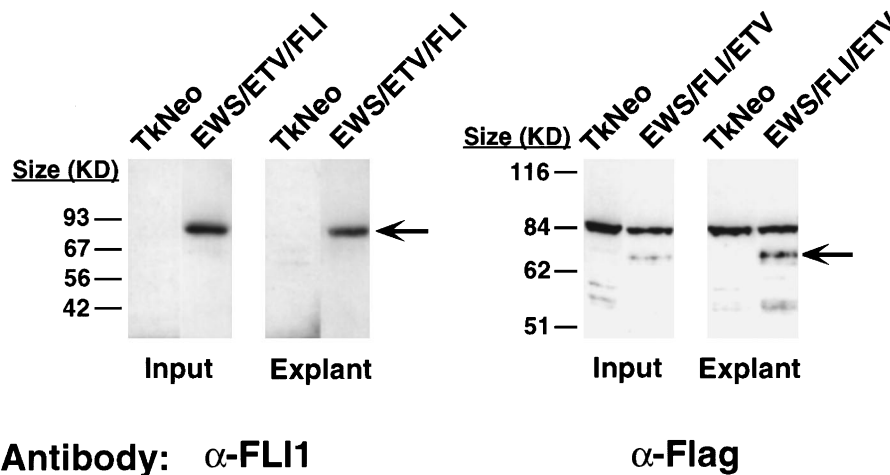


Figure 6 Immunoblots demonstrating stable protein expression of EWS/FLI1/ETV1 and EWS/ETV1/FLI1 in input and explant cell lines. EWS/ETV1/FLI1 protein was detected in both input cell lines and tumor derived explant cell lines using rabbit polyclonal antisera directed towards epitopes at the FLI1 C-terminus. Monoclonal anti-flag antibody was used to detect the EWS/FLI1/ETV1 protein in corresponding input and tumor derived explant cell populations that were infected with this retroviral construct. As seen before, an 84 KD cross reacting background band is seen in all NIH3T3 populations

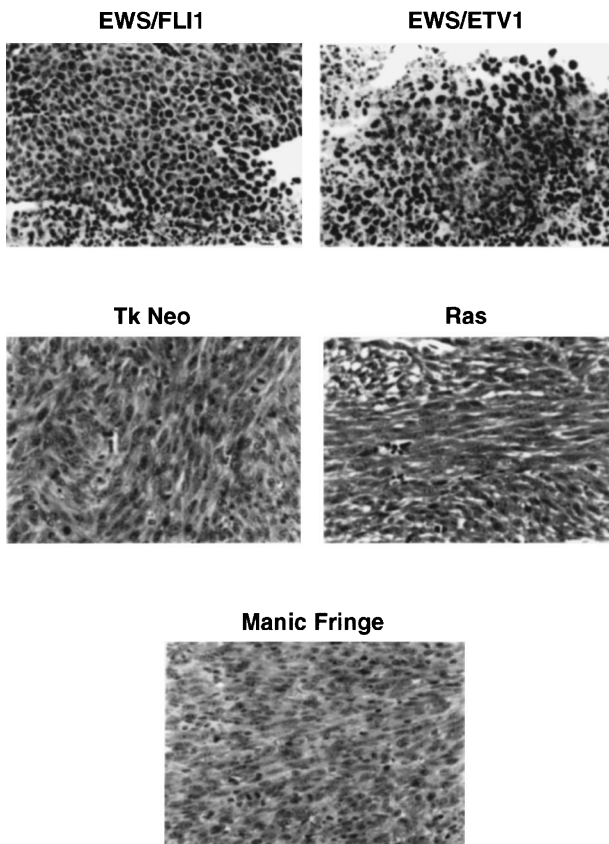


Figure 7 NIH3T3 tumors induced by either EWS/ETV1 or EWS/FLI1 have a characteristic histologic appearance. Hematoxylin and eosin staining reveals necrosis, disorganization, and rounding of cells in tumors expressing EWS/ETV1 or EWS/FLI1. These results are representative of sections examined from eight different EWS/ETV1 and EWS/FLI1 tumors respectively. The same stains demonstrate an organized fascicular pattern of spindle-shaped cells in tumors derived from NIH3T3 cells infected with Tk Neo, activated RAS, or MFNG

cellular necrosis was evident. The EWS/ETV1 and EWS/FLI1 tumors on the other hand, consisted of densely packed sheets of rounded cells with reduced amounts of cytoplasm. There were numerous areas of cellular necrosis. NIH3T3 populations expressing the EWS/FLI1/ETV1 and EWS/ETV1/FLI1 triple chimeras also displayed this same small round cell histologic phenotype (data not shown).

Discussion

In spite of their marked structural differences, EWS/ETV1 and EWS/FLI1 display similar biologic characteristics. The ability of EWS/ETV1 to induce EAT-2 expression and of (Δ N)ETV1 to inhibit transformation by EWS/FLI1, suggests that both EWS/ETS fusions signal through common biologic pathways. In theory the truncated (Δ N)ETV1 mutant competes with full length EWS/FLI1 for binding at specific DNA sites within regulatory regions of genes. A similar strategy has previously been employed for making inhibitors to ETS1, PU.1 and FLI1 (Kovar *et al.*, 1996; Langer *et al.*, 1992; Wasylyk *et al.*, 1994; Conrad *et al.*, 1994). While these constructs have proven to be potent inhibitors, their biologic specificity is probably directly

related to their DNA-binding specificity. Considering that many ETS proteins can bind to the same DNA sites *in vitro*, it is very possible that the inhibitory effect seen with the (Δ N)ETV1 and (Δ N)FLI1 constructs is not only due to competition at FLI1 DNA sites. Though (Δ N)ETV1 expression down regulates EAT-2, a gene specifically modulated by EWS/FLI1, (Δ N)ETV1 may also influence other signal transduction pathways not directly related to EWS/FLI1.

EWS/ETV1 like EWS/FLI1, promotes tumorigenesis, directly demonstrating biologic overlap between these EWS fusion genes. Considering that EWS/ETV1 and EWS/E1AF are highly homologous and that EWS/FLI1, EWS/ERG and EWS/FEV form a second structurally related group, it seems very likely that all of these chimeric genes would be tumorigenic. These results reinforce the notion that all of these EWS/ETS fusion genes play an important role in Ewing's sarcoma oncogenesis. The fact that EWS/ETV1 does not promote anchorage independent growth in soft agar points out that while EWS/FLI1 and EWS/ETV1 may have a similar *in vivo* effect, there are *in vitro* differences between these two fusions. Current studies are in progress to determine whether these biologic differences are reflected in the target gene profiles of EWS/ETV1 and EWS/FLI1.

Not only do EWS/ETV1 and EWS/FLI1 accelerate tumorigenesis but our histologic data suggest that both fusions promote a characteristic microscopic phenotype. This effect differs from other oncogenes and is not simply an effect of tumor growth. NIH3T3 cells expressing activated RAS form solid, spindle cell tumors most consistent with fibrosarcomas. This microscopic appearance is the common phenotype when NIH3T3 cells are rendered tumorigenic by expression of an array of genes including: c-SIS, oncogenic p53 mutants, RhoA, RAC-1, VAV, DBL, OST and ornithine decarboxylase (Berstein and Weinberg, 1985; Krogerman *et al.*, 1997; Cardinali *et al.*, 1997; del Paso *et al.*, 1997; Auvinen *et al.*, 1997). NIH3T3 cells made tumorigenic using organic residue mixtures and basement membrane extracts also resemble fibrosarcomas (Kurzepa *et al.*, 1984; Fridman *et al.*, 1992).

In contrast to all of the above cases, tumors arising from EWS/ETV1 or EWS/FLI1 expressing NIH3T3 cells consist of sheets of small round cells. While rare non-fibrosarcoma phenotypes have also been previously described for NIH3T3 derived tumors, none match the histology seen for EWS/ETV1 and EWS/FLI1 (Rubin, 1984; Lo and Liotta, 1985). Moreover, the histologic appearance of the EWS/EVT1 and EWS/FLI1 NIH3T3 tumors is very reminiscent of that of Ewing's sarcoma. While the small round cell phenotype is always present in Ewing's sarcoma, it can also be found in other pediatric cancers including rhabdomyosarcoma and neuroblastoma. Though this histology has not previously been found in fibroblasts, further analysis is necessary before any firm conclusions can be drawn regarding the relatedness of our murine model system to actual Ewing's tumors.

If multiple ETS genes can promote a specific pattern of tumorigenesis, why is the EWS/FLI1 fusion most commonly found in Ewing's sarcoma? It is very possible that somatic recombination occurs more easily between EWS and FLI1 than with other ETS

partners. Alternatively while many ETS genes when fused to EWS, may acquire some of the biologic attributes needed for oncogenesis, some ETS partners may be more potent than others. In this regard, the fact that EWS/FLI1 accelerates tumorigenesis more effectively than EWS/ETV1, may be significant. This enhanced tumorigenic potential appears to be related to the C-terminal domain of the FLI1. The biochemical function of this domain is unclear though it has been suggested that it encodes an additional transcriptional activation domain (Rao *et al.*, 1993). If this were true, EWS/FLI1 might be able to quantitatively modulate crucial target genes more effectively than EWS/ETV1. Alternatively, the C-terminal domain may facilitate interactions between EWS/ETS proteins and other transcription factors at DNA target sites. Direct physical interactions have been demonstrated that involve the DNA-binding domain and C-terminus of ETS-1 to the FOS/JUN and C/EBP transcription factors (Basuyaux *et al.*, 1997; McNagny *et al.*, 1998).

Though disparate EWS/ETS fusion genes are able to promote a common biologic effect in our model system, they may be distinguishable on a clinical level. Nucleotide sequence analysis of different EWS/FLI1 chimeras from tumor specimens, has documented up to nine possible junction points (Zucman *et al.*, 1993). Recent retrospective studies suggest that specific EWS/FLI1 junctions may have prognostic significance (Zoubek *et al.*, 1996; de Alava *et al.*, 1998; Fletcher, 1998). In this regard, the involvement of particular ETS genes in Ewing's sarcoma EWS fusions may also have an effect on clinical outcome. Considering that NIH3T3 cells expressing EWS/ETV1 are unable to form colonies in soft agar and take a longer time to form tumors in SCID mice, it may be that patients with this variant fusion gene would have a better prognosis. Such a hypothesis requires explicit testing in patient populations.

There is an expanding array of fusion genes with structures consistent with chimeric transcription factors, that are associated with specific human malignancies (Rabbits, 1994). Occasionally multiple variant fusions are found within one tumor type. In these cases, the DNA binding motif is held constant and alternate fusion partners provide other necessary biochemical attributes such as transcriptional activation domains. Such heterogeneity is demonstrated by the many chimeras involving the MLL/ALL1/HRX gene that are found in acute leukemias (for review see Downing and Look, 1996). The variant fusions found in Ewing's sarcoma are unusual in that it is the EWS domain that is held constant while multiple DNA binding domains are interposed. Considering the structural disparity within the ETS gene family, the EWS/ETS set of chimeric fusions can serve as an excellent model system for studying convergent biological pathways in oncogenesis.

Materials and methods

Construction of expression constructs

The flag-tagged EWS/FLI1 and EWS/ETV1 were created by PCR amplification of the parental (non-flag-tagged) construct by the polymerase chain reaction (PCR) using high fidelity Pwo polymerase (Boehringer Mannheim). The flag-tagged

(Δ N)FLI1 was created using the same method above on a version of FLI1 containing only the first six amino acids of EWS as a consensus start site (Δ 22) (May *et al.*, 1993b). The flag-tagged (Δ N)ETV1 was created using the same EWS/ETV1 template and method as above with a 5' primer to the first ETV1 codons found in the EWS/ETV1 fusion (Jeon *et al.*, 1995). Construction of triple chimeras EWS/ETV1/FLI1 and EWS/FLI1/ETV1, was accomplished by a two stage PCR process. For EWS/FLI1/ETV1, EWS/FLI1 was PCR amplified using a chimeric 3' primer containing the 3' FLI1 DNA binding domain fused to the 5' ETV1 C-terminus. A complementary chimeric primer was used to PCR amplify the ETV1 C-terminus. These two PCR products were then fused by amplifying them in the same PCR reaction using vector primers that flanked EWS and ETV1. EWS/ETV/FLI1 was made in reciprocal fashion. The primary structures of all constructs were confirmed by nucleotide sequence analysis.

Retroviral transduction and tissue culture

All EWS/ETS constructs were subcloned into the mammalian expression vector SR α -MSV-TK Neo. These constructs were then cotransfected with $\Psi(-)$ packaging plasmids into 293T cells and high titre viral stocks were collected as conditioned media. Polyclonal NIH3T3 populations expressing EWS/ETS genes were created by infection with replication deficient viral stocks and subsequent selection for neomycin resistance. For dominant negative experiments, cells were first infected with EWS/FLI1 retroviral stocks containing an expression plasmid lacking a neomycin resistance gene. Two days later, the infected cells were split and infected with a secondary construct which contained a neomycin resistance gene. The cells were then selected with G418 for 3–6 days.

Growth of cells in semi-solid media

Following G418 selection, 5000 cells were embedded in soft agar as described by Lugo and Witte (1989). For the doubly infected constructs, G418 was added to all of the layers to allow at least 3 more days of selection. Photographs were taken at days 14–21 following embedding in soft agar; photographs presented together were taken on the same day. Photographs were captured on an Alpha Innotech Corporation imager with an IS-1000 Digital Imaging System and Alpha Imager 2000 software. All images presented were taken at the same exposure and magnification.

Western analysis

Protein lysates were collected within 2 days of the corresponding agar assay or mouse injection by NP-40 lysis and centrifugation to remove nuclei. Source of primary antibodies is as follows: polyclonal anti-FLI1 (Santa Cruz Biotechnology, Inc.); monoclonal anti-Flag (M2) (Sigma); polyclonal anti-EAT-2 antibodies have been previously characterized (Thompson *et al.*, 1996). All secondaries were done with either horseradish peroxidase conjugated goat-anti-mouse or goat-anti-rabbit (Jackson Labs). Immune complexes were detected by chemiluminescence using commercial reagents (Amersham/Pharmacia, Inc.).

Tumorigenesis assays

One million NIH3T3 cells which had been selected for a construct were injected subcutaneously into the nape region of the neck of a CB17 SCID mouse. Mice were monitored until tumors were 2 cm in diameter at which time, the mice were sacrificed, and the tumor was removed by dissection. Small pieces were placed in 10% buffered formalin for later routine paraffin-embedding and hematoxylin and eosin staining. Tumor explant cell lines were created by plating minced/washed tumor tissues in DMEM plus 10% calf serum.

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