CHARACTERIZATION OF THE MECHANISMS OF TRANSCRIPTIONAL REGULATION BY EWS/FLI IN EWING SARCOMA

by

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ABSTRACT

Ewing sarcoma is a highly aggressive pediatric malignancy that is characterized by a chromosomal translocation-derived fusion protein, Ewing sarcoma (EWS)/ Friend leukemia insertion (FLI), EWS/FLI. EWS/FLI is an aberrant transcription factor and its downstream targets contribute to oncogenic transformation in Ewing sarcoma. However, the mechanisms of transcriptional regulation by EWS/FLI and the full complement of direct targets of EWS/FLI were previously unknown. The work documented in this dissertation describes a novel basis for EWS/FLI function in gene activation, and also uncovers a mechanism by which EWS/FLI directly represses a subset of critical targets genes in Ewing sarcoma.

Through the identification of direct *in vivo* targets of EWS/FLI, we made an unexpected discovery that EWS/FLI activates some of its critical target genes, including *NR0B1* and *GSTM4*, by binding to microsatellite repeats. These findings suggest a new paradigm for cancer-relevant gene regulation by EWS/FLI, and perhaps other ETS family members.

In addition to the microsatellite repeats, EWS/FLI regulates some of its target genes through the canonical high-affinity consensus E-26 oncogene (ETS) site. We focused on one such target gene, *GLI1*, and uncovered a novel role for GLI1 and its downstream target, KRT17, in coordinating two cancer-relevant functions: oncogenic transformation and cellular adhesion in Ewing sarcoma.

The functional relationship between wild-type EWS and the EWS/FLI fusion protein in Ewing sarcoma was largely unknown. Through global transcription profiling and mechanistic studies we demonstrated that EWS and EWS/FLI coregulate a subset of genes in Ewing sarcoma and that EWS functions as a cofactor of the REST transcription factor to repress neuronal differentiation genes. These data suggest that EWS, and consequently EWS/FLI, have transcriptional repressive roles in Ewing sarcoma.

We next focused on identifying and characterizing the mechanism underlying EWS/FLI-mediated direct transcriptional repression. We demonstrated that EWS/FLI interacts with the nucleosome remodeling and histone deacetylase (NuRD) corepressor complex to repress critical tumor suppressor genes in Ewing sarcoma. These data identify inhibitors of the NuRD complex components as potentially effective therapeutic agents for the treatment of Ewing sarcoma.

Taken together, the work presented in this dissertation advances our molecular understanding of EWS/FLI-mediated gene regulation in Ewing sarcoma.

This work is dedicated to my father, Ramachandran Sankar and my mother, Usha Sankar for being the source of my inspiration.

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CHAPTER 1

INTRODUCTION

Ewing sarcoma: Disease and molecular genetics

Ewing sarcoma accounts for one-third of all primary bone tumors in children and young adults and is the second most common bone-associated malignancy in the pediatric population with a peak incidence at about 15 years of age (1, 2). There are approximately 250 newly diagnosed Ewing sarcoma cases in the United States per year (SEER). It is a highly undifferentiated tumor and histologically has a characteristic small-round blue cell appearance as depicted in Figure 1.1. Ewing sarcoma most frequently arises in the bone, but <10% of tumors arise in soft tissues (3). It is a highly aggressive disease and approximately 25% of patients present with overt metastasis at the time of clinical diagnosis (4). In addition, patients with clinically undetectable metastasis likely have micrometastatic disease, because in the absence of systemic chemotherapy most patients relapse with distant metastatic disease (5, 6). The common sites of metastasis include lung, bone and bone-marrow. The propensity to spread leads to poor prognosis for Ewing sarcoma patients with the five year survival rate for localized disease being ~70%, which drops to ~10% for metastatic disease (7).

Although Ewing sarcoma commonly presents as a bone-associated neoplasm, the cell-of-origin of this tumor is still an area of active debate, since it was first described by

James Ewing in 1921 (8, 9). Several lines of evidence support a neural crest cell of origin for Ewing sarcoma (10). Early studies showed that Ewing sarcomas express cell surface antigens associated with the neuroectodermal lineage (11-13). Later, a gene expression profiling study found that genes expressed in neuronal tissues or during neuronal differentiation are abundantly expressed in Ewing sarcomas (14). Furthermore, ectopic expression of the fusion protein encoded by the Ewing sarcoma gene (EWS) and Friend leukemia insertion gene (FLI), EWS/FLI, in non-Ewing cells including rhabdomyosarcoma, neuroblastoma or human foreskin fibroblast cell lines resulted in an upregulation of genes critical for neural crest development (15-17). Although, these data implicated a role for EWS/FLI in driving the neural phenotype of Ewing sarcoma cells, not much was known about the mechanisms that contributed to the neuronal phenotype and, importantly, the critical factors that prevented full neuronal differentiation of Ewing sarcomas. The work highlighted in Chapter 6 of this dissertation provides new insights in this direction.

In addition to neural crest cells, there is a growing body of recent evidence suggesting that mesenchymal stem cells (MSCs) are the likely progenitor cells of Ewing sarcoma (18-20). Interestingly, it has also been recently suggested that the two proposed cells of origin, neural crest and MSCs, may not be mutually exclusive (21). Since neural-derived MSCs are present in the bone-marrow (22) and, conversely, neural crest stem cells contain some mesenchymal lineage plasticity (23), it is possible that Ewing sarcomas arise from neural-derived MSCs or from neural crest stem cells with mesenchymal features.

In the mid-1980s it was discovered that 85% of Ewing sarcomas harbored a tumor-specific chromosomal translocation, t(11;22)(q22;q12), generating a fusion protein EWS/FLI, from the in-frame fusion of the amino-terminus of EWS (encoded by *EWSR1*), a member of the TET (TLS/EWS/TAF15) family of RNA-binding proteins, and the carboxyl-terminus of FLI (encoded by *FLI1*), a member of the ETS family of transcription factors, as depicted in Figure 1.2 (24, 25). The reciprocal FLI/EWS translocation is not expressed in Ewing sarcoma tumors and the reciprocal translocated chromosome is sometimes lost from these tumors (26, 27). In addition to the gain of function of EWS/FLI, the t(11;22)(q24;q12) translocation causes loss of one allele of the wild-type *EWSR1* and *FLI1* genes (26). The untranslocated wild-type *FLI* allele is not expressed in Ewing sarcomas (27). Therefore, in addition to generation of EWS/FLI the other major consequence of the translocation is the haplo-insufficiency of EWS. Not much was known about the functional consequence of the halo-insufficiency of EWS on Ewing sarcoma oncogenesis. The work described in Chapter 6 of this dissertation has addressed this question. Some insight into the functioning of the EWS/FLI fusion protein also can be gained from knowledge about the wild-type EWS and FLI proteins.

Wild-type EWS

EWS is encoded by the *EWSR1* gene (Ewing's sarcoma rearrangement domain 1), first identified in the context of Ewing sarcoma (25). EWS is a ubiquitously expressed nuclear protein (25, 28). The amino-terminus of EWS included in EWS/FLI is highly unstructured, consists of a repetitive primary sequence with several copies of a degenerate hexapeptide repeat motif (SYGQQS) that resembles the carboxyl-terminal

domain of RNA polymerase II (25), suggesting a potential role for EWS in transcriptional activation. The N-terminal domain of EWS has been shown to interact with the basal transcription factor TFIID, with certain subunits of RNA polymerase II and p300/CBP, providing additional support for the transcriptional activating potential of EWS (29-32). The EWS/FLI fusion protein does not form a stable complex with RNA polymerase II (30) but does interact with the RNA polymerase II subunit RPB7 (29), highlighting a likely conservation in function between wild-type EWS and the EWS/FLI fusion protein in transcriptional activation.

The unstructured nature of the N-terminal EWS domain potentially enables its interaction with several proteins subsequently leading to the pathogenic transcriptional activities of EWS and EWS/FLI (33). As an example, it was demonstrated that wild-type EWS could interact with its oncogenic derivative EWS/FLI to induce mitotic defects and genomic instability and, in doing so, contribute to the transformed phenotype of Ewing sarcoma (34). In support of this are data highlighting a developmental role for EWS in genome surveillance and DNA repair (35, 36). The carboxyl-terminus of EWS, which is not retained in EWS/FLI, is thought to interact with the serine-arginine family of RNA splicing factors as evidenced by the presence of 3 RGG-rich regions (37) (Figure 1.2), suggesting a potential role for wild-type EWS in RNA processing/splicing or export (28).

In addition to the possible roles for EWS in transcriptional activation, DNA-repair, and RNA processing, a recent study demonstrated that EWS could repress the expression of a reporter gene it was tethered to, by inhibition of posttranscriptional gene expression (38). Upon DNA-damage signals, the EWS family member TLS has been shown to be recruited to the *CCND1* gene promoter by a tethered ncRNA to inhibit the

histone acetyl transferase activity of CBP/p300, leading to transcriptional repression of the *CCND1* gene (39). These studies indicate that TET family proteins including EWS may also function in a repressive capacity. Notably, without evidence that EWS binds to double stranded DNA *in vivo*, EWS is more likely to act as a transcriptional cofactor than as a transcription factor.

Since the EWS/FLI fusion protein has been the main focus of study in the field, very little was known about the role of wild-type EWS in Ewing sarcoma, leaving many unanswered questions: Does EWS function as a cofactor to affect transcription of target genes? What are EWS regulated genes, if any, in Ewing sarcoma and does the EWS transcriptional profile contribute significantly to the EWS/FLI transcriptional profile? Does wild-type EWS have a role in oncogenic transformation? The work presented in Chapter 6 provides new insights into the role of wild-type EWS in Ewing sarcoma.

Wild-type FLI

The other portion of the EWS/FLI fusion is contributed by *FLI1*, which encodes the protein FLI. FLI is a member of the ETS family of transcription factors (40). There are 27 known ETS family members in humans, which may be grouped on the basis of similarities in their ETS domains (41). ETS family members play a prominent role in cancer development because genes encoding ETS factors are often involved in chromosomal translocations that generate oncogenic protein derivatives (41). FLI localizes to the nucleus and functions primarily in megakaryocyte, vascular, and neural crest development (42-44). FLI can also function as an oncogene. The murine *Fli* locus was first identified as the predominant Friend murine leukemia virus (F-MuLV) insertion

site, which causes upregulation of the *Fli* gene, and the development of erythroleukemia (45, 46). Exogenous expression of FLI in transforming models like NIH3T3 immortalized murine fibroblasts, however, does not result in oncogenic transformation (47). Therefore, its transforming ability may be limited to the hematopoietic lineage. The amino-terminus of FLI contains a pointed (PNT) domain, which is involved in protein-protein interactions, and functions as a weak transcriptional activation or repression domain (48). However, the PNT domain of FLI is lost in the EWS/FLI fusion protein (refer to Figure 1.2). The carboxyl-terminus of FLI contains a highly conserved ~85 amino acid DNA binding domain (ETS domain) that is both necessary and sufficient for site-specific DNA binding (48). EWS/FLI retains this C-terminal ETS domain of FLI (refer to Figure 1.2).

TET/ETS and non-TET/ETS fusions

EWS/FLI is expressed in ~85% of Ewing sarcoma tumors. In the remaining 10% of cases, translocations resulting in the fusion of EWS [and rarely a related TLS/ETS/TAF15 (TET) family protein] with other ETS family members occur. Thus, EWS/ERG, EWS/ETV1, EWS/ETV4 and EWS/FEV have all been described in Ewing sarcoma, and are all thought to mimic the function of EWS/FLI (25, 49-52). These findings solidified the importance of TET/ETS fusions in the pathogenesis of Ewing sarcoma and have since been used as diagnostic markers for the disease (53).

EWS fusions with non-ETS transcription factor family members have been described in a variety of sarcomas that are distinct from Ewing sarcoma. However, in recent years, a small but increasing number of rare non-TET/ETS rearrangements have been identified in sarcomas called “Ewing’s-like tumors” that anatomically and

histologically resemble certain features of Ewing sarcoma (refer to Chapter 2). These findings have complicated both molecular diagnostics as well as the concept that Ewing sarcoma is strictly a TET/ETS fusion driven malignancy. However, due to lack of a molecular understanding of the non-TET/ETS fusions and their mechanisms of action it is difficult to conclude that these fusions drive the same disease as the TET/ETS fusions.

EWS/FLI functions as a fusion oncoprotein

EWS/ETS proteins are characterized as oncoproteins in Ewing sarcoma. However, introduction of EWS/ETS proteins into different cellular models result in diverse outcomes ranging from the induction of cell cycle arrest or apoptosis to transformation and tumorigenicity, and from blocking differentiation to trans-differentiation (9). Thus, the phenotype driven by EWS/ETS proteins depends on the cellular context. Ectopic expression of EWS/FLI is not sufficient to transform many primary cell types, including primary human fibroblasts, primary mouse fibroblasts and immortalized rat fibroblasts (Rat1 cells), and instead results in cell death or growth arrest (17, 54). Expression of EWS/FLI in more primitive cells including primary neural crest progenitor cells (Ncm1) or tumor cell lines like rhabdomyosarcoma (CTR) cells, results in differentiation defects (9). These studies highlight the importance of a permissive cellular background that is necessary for EWS/FLI function and subsequent cellular transformation.

Ectopic expression of EWS/FLI causes oncogenic transformation of NIH3T3 cells (47, 55, 56), indicating that in this cell type perhaps there are unknown cooperating aberrancies or pathways that permit cellular transformation upon EWS/FLI

overexpression. Subsequent studies within a native Ewing sarcoma cellular context have demonstrated that stable knockdown of EWS/FLI in multiple patient-derived Ewing sarcoma cell lines resulted in loss of oncogenic transformation, assessed by anchorage-independent growth in soft agar *in vitro* and xenograft tumor growth *in vivo* in immunodeficient mice (27, 57). Furthermore, reexpression of an RNAi-resistant EWS/FLI cDNA resulted in restoration of the transformed phenotype (58). Therefore, EWS/FLI or other EWS/ETS fusions are presumably the initiating oncogenic events, and their ongoing expression is required for the transformed phenotype of Ewing sarcoma.

Site directed mutagenesis within the DNA binding domain of the FLI portion, or large deletion mutations within the EWS portion of EWS/FLI, result in a complete loss of biologic activity of the EWS/FLI fusion protein (refer to Chapter 7). Therefore, both the EWS and FLI portions of EWS/FLI, and the DNA binding ability of EWS/FLI are essential for oncogenic transformation (47, 55, 56). The N-terminal EWS domain confers a strong transcription activation domain when fused to heterologous DNA-binding domains of other transcription factors (55). These data suggests that the N-terminal EWS domain that is retained in the EWS/FLI fusion protein functions predominantly as a strong transcriptional activation domain.

Although EWS/FLI binds DNA with the same specificity as wild-type FLI, it acts as a more potent transcriptional activator, owing to the replacement of the weak transcriptional activation domain (from wild-type FLI) by the strong transcriptional activation domain of EWS (47, 56). This concept was supported by a study that found that replacement of the EWS domain in EWS/FLI with other strong transcriptional activation domains could rescue oncogenic transformation in NIH3T3 cells, whereas

replacement with a weak transcriptional activation domain could not (55) (Figure 1.3). These data suggest that EWS/FLI-mediated transcriptional activation drives oncogenic transformation. However, later data from patient-derived Ewing sarcoma cell lines and primary tumors suggested that transcription repression by EWS/FLI is also necessary for oncogenic transformation (27, 57, 59). In fact, as detailed later in Chapter 7, it is the combined effect of EWS/FLI-mediated gene activation and gene repression that causes full oncogenic transformation of Ewing sarcoma cells. Furthermore, EWS/FLI, but not wild-type FLI, transforms NIH3T3 cells, highlighting a gain-of-function of the fusion protein (47). Taken together, these findings indicate that EWS/FLI acts as an aberrant transcription factor, inappropriately regulating the expression of specific repertoires of target genes, thereby orchestrating development of Ewing sarcoma.

DNA binding and regulation by ETS proteins

EWS/FLI retains the ETS-type DNA binding domain of FLI (Figure 1.2). ETS proteins have a characteristic winged helix-loop-helix DNA-binding domain (60, 61). Most ETS family members bind as monomers to sequences of DNA that contain a GGAA (or rarely a GGAT) "core" motif (40, 41, 48); the flanking nucleotides modulate the affinity and specificity of the interaction (62). *In vitro* binding site selection approaches identified a high-affinity consensus ETS binding sequence, ACCGGAAGTG, which acts as a functional binding site for wild-type FLI and for most ETS family members (63). EWS/FLI also binds to this site with high affinity (63).

Genome-wide localization approaches have identified two distinct classes of ETS binding sites *in vivo*: high-affinity (redundant sites), and divergent (specific sites) (64).

The high affinity sites identified were bound by multiple ETS family members *in vivo*, indicating that although there are preferences, the DNA-binding specificity for ETS factors is not stringent. In contrast, the divergent sites consist of partial ETS binding sites overlapping with, or in immediate proximity to, partial binding sites for non-ETS family members. Such binding sites could potentiate gene regulation by cooperative interactions between an ETS protein and another transcription factor. Divergent ETS binding sites have been identified in a number of ETS target genes (65) and DNA binding of ETS proteins is modulated by their interactions with other transcription factors (66, 67). Interestingly, ETS family proteins that participate in Ewing sarcoma, including FLI1, ERG, and ETV1, can cooperatively bind DNA with AP1 (Fos-Jun) proteins (68). Importantly, a truncated mutant form of EWS/FLI that failed to cooperatively bind DNA with AP1 proteins failed to transform NIH3T3 cells (68), highlighting the significance of cooperative DNA binding and transcriptional regulation from weak divergent ETS sites in the pathogenesis of Ewing sarcoma.

The presence of the FLI DNA-binding domain in the EWS/FLI fusion protein suggests that EWS/FLI likely binds similar DNA sequences and targets similar genomic sites as does the wild-type FLI protein. However, little was known about the nature of the binding motifs for EWS/FLI in Ewing sarcoma. Work detailed in Chapter 3 of this dissertation identified, for the first time, *in vivo* binding elements of EWS/FLI in Ewing sarcoma and uncovered novel binding sites for the fusion protein.

EWS/FLI target genes in Ewing sarcoma

While the identification of EWS/FLI was an important step forward, progress in understanding the pathogenesis of Ewing sarcoma has been precluded by the lack of knowledge of the cell-of-origin, and therefore the inability to generate genetic models of the disease (9, 69). EWS/FLI functions as an aberrant transcription factor in Ewing sarcoma which implies that EWS/FLI regulated genes are important for oncogenesis. Early studies made use of NIH3T3 cells, but few of the EWS/FLI "targets" identified in this cell type have been validated as being involved in *bona fide* Ewing sarcoma (70-73). The biggest pitfall of these studies was the use of a heterologous cell type requiring the nonphysiologic overexpression of EWS/FLI and a lack of understanding of the background genetic aberrancies in NIH3T3 cells that aid EWS/FLI-mediated transformation. Indeed, our own work has demonstrated that EWS/FLI expression in NIH3T3 cells induces a gene expression pattern that is quite different from the pattern induced in Ewing sarcoma (58, 74), suggesting that EWS-FLI1 may trigger a generic ETS-mediated gene expression pattern and transformation process in NIH3T3 cells, rather than a Ewing sarcoma-specific process. These data underscored the importance of studying EWS/FLI and the target genes it dysregulates within a native cellular context.

To identify EWS/FLI targets within a native cellular context, our lab developed a strategy to knockdown endogenous EWS/FLI expression in patient-derived Ewing sarcoma cell lines using a retroviral-based stable shRNA approach, without drastically affecting the normal growth rate of cells in tissue culture, followed by gene expression profiling (27). The specificity of the RNAi construct against EWS/FLI was validated by a "rescue" approach re-expressing an RNAi-resistant EWS/FLI cDNA (58). Importantly, as

highlighted previously, stable knockdown of EWS/FLI resulted in loss of oncogenic transformation assessed by *in vitro* and *in vivo* assays and rescue with the RNAi-resistant EWS/FLI cDNA restored the transformed phenotype. Therefore, target genes identified using this approach would potentially identify critical downstream mediators of EWS/FLI function in driving Ewing sarcoma oncogenesis.

Using this approach we and others have identified several thousands of genes regulated by EWS/FLI (27, 57, 59, 75). Importantly, target genes identified within the native cellular context have since been validated as *bone fide* targets, important for various aspects of Ewing sarcoma development. Furthermore, identification of the EWS/FLI transcriptome provided us with target genes that could then be used as tools to understand the mechanisms of regulation by EWS/FLI and to identify EWS/FLI response elements. Specifically, *NR0B1 and GSTM4*, among others, were identified as key upregulated EWS/FLI target genes absolutely essential for oncogenic transformation (27). As detailed in Chapters 3 and 4, we utilized the *NR0B1* and *GSTM4* promoters as candidates to identify and characterize EWS/FLI response elements in Ewing sarcoma.

In contrast to the NIH3T3 transcription profile, the most striking observation from the EWS/FLI transcription profile generated in Ewing sarcoma cells was that there were several fold more downregulated target genes than there were EWS/FLI upregulated targets (76). This was intriguing given that EWS/FLI was predominantly characterized as a transcriptional activator, with the exception of a few reports that suggested that target gene repression by EWS/FLI may be important to the tumorigenic phenotype (47, 55, 75, 77). The repressed gene signature for EWS/FLI was also confirmed by a more recent

RNA-sequencing experiment (refer to Chapter 6). However, the exact mechanism of EWS/FLI-mediated repression in Ewing sarcoma remained unanswered.

Transcription profiling experiments have identified several thousands of EWS/FLI targets including several critical effectors of oncogenic transformation, but the big unanswered questions in the field were: does the EWS/FLI transcription profile contain direct targets in addition to indirect targets? How does the fusion oncoprotein regulate its critical target genes? Therefore, identification of "direct" EWS/FLI targets (defined by EWS/FLI binding to the promoter and/or enhancer elements of target genes) and a detailed characterization of direct binding sites for EWS/FLI at target gene promoters were necessary to better understand the underlying mechanisms of regulation. Overlapping direct EWS/FLI targets with EWS/FLI up or downregulated genes from the transcription profiles would then identify a "core" set of target genes for further focused mechanistic studies. Work detailed in Chapters 3, 4 and 7 have utilized this approach.

Transcriptional activation in Ewing sarcoma

Among the genes activated by EWS/FLI, our lab has previously demonstrated that *NKX2.2* and *NR0B1* are critical upregulated targets necessary for EWS/FLI mediated oncogenic transformation (27, 57). Interestingly, another EWS/FLI upregulated gene initially identified in our microarray-based transcriptional profiling studies was *GLI1* (Glioma associated oncogene homolog 1) (27). GLI1 is a zinc-finger transcription factor and is the principal effector of the Hedgehog signaling pathway (78). Hedgehog signaling is of critical importance during development for proper cellular differentiation, tissue patterning and stem cell maintenance (78, 79). This signaling pathway is well recognized

to contribute to the development and progression of several cancers (80). The canonical pathway for GLI activation requires ligand-dependent inhibition of the receptor Patched (PTCH), which releases inhibition of the signal transducer Smoothened (SMO), allowing for GLI-dependent transcription of genes that mediate cell proliferation and survival.

We demonstrated that GLI1 was transcriptionally activated in a noncanonical fashion in Ewing sarcoma cells, independent of upstream hedgehog pathway components, by direct EWS/FLI binding to a high-affinity ETS consensus site in the *GLI1* promoter. Furthermore, we identified a role for GLI1 in EWS/FLI-mediated oncogenic transformation. While we were working on further characterizing the role of GLI1, several papers were published that highlighted the importance of GLI1 in Ewing sarcoma development. These studies independently validated our findings that EWS/FLI directly activated transcription from the GLI1 promoter (81), and that loss-of-function approaches and pharmacological inhibition of GLI1 significantly decreased oncogenic transformation (81-84). However, the mechanism underlying GLI1-mediated oncogenesis and the critical transcriptional network of genes regulated by GLI1 to achieve this function remained unknown. Work detailed in Chapter 5 of this dissertation addresses these critical questions and provides a mechanistic understanding of GLI1-mediated oncogenic transformation in Ewing sarcoma.

Transcriptional repression in Ewing sarcoma

Transcriptional repression is central to the pathogenesis of hematologic malignancies, including acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). Acute promyelocytic leukemia (APML) is associated with the

chromosomal translocation, t(15;17)(q24;q12) encoding the PML/RARα fusion product. The PML/RARα fusion acts as a transcriptional repressor, due to its interaction with Histone Deacetylases (HDACs) which leads to repression of the retinoic acid response genes (85-87). In contrast, chromosomal fusion-driven solid tumor development has largely been attributed to transcriptional activation. For example, the PAX3/FKRH fusion in alveolar rhabdomysosarcoma, TLS/CHOP in myxoid liposarcoma and the EWS/ATF1 fusion in clear cell sarcoma have been characterized predominantly as transcriptional activators (88-90).

Similarly, evidence from early studies in heterologous cell systems had suggested a role for EWS/FLI in transcriptional activation (47, 55). However, as previously highlighted, gene expression profiling studies in Ewing sarcoma cells displayed a predominant signature of EWS/FLI repressed genes (27). This repression may be attributed, in part, to the upregulation of transcriptional repressors by EWS/FLI. This might suggest an indirect repression model, which would still support the concept that EWS/FLI is predominantly a transcriptional activator.

In support of the indirect repression model, previous studies have shown that EWS/FLI activates the expression of Enhancer of Zeste Homolog 2 (EZH2), the enzymatic subunit of the polycomb PRC2 repressor complex, which methylates histone H3 Lys27, and thereby mediates gene repression. Silencing of EZH2 in Ewing sarcoma cells decreased tumor growth and metastasis, and resulted in a generalized loss of methylation on H3 Lys27 and an increase in H3 acetylation, leading to gene activation (91). Previous work from our lab has identified other EWS/FLI upregulated proteins like NKX2.2 and NR0B1 that function predominantly as transcriptional repressors in Ewing

sarcoma and contribute to a small but significant proportion of the EWS/FLI repressed gene signature (76, 92). NKX2.2 mediates repression in Ewing sarcoma through recruitment of HDAC activity. Consequently, treatment with the HDAC inhibitor vorinostat resulted in a reversal of the NKX2.2-mediated repression of target genes and consequently blocked oncogenic transformation (76). These studies highlight the importance of gene repression in Ewing sarcoma oncogenesis and, in addition, suggest a critical role for HDACs and other epigenetic regulator proteins as effectors of transcriptional repression in Ewing sarcoma.

Histone deacetylases (HDACs)

Gene repression is largely achieved by the combinatorial action of various enzymatic complexes, known as corepressor complexes, which are recruited to DNA by transcription factors and act by modifying histone tails and thereby changing the chromatin architecture at target gene promoters. One of the most important processes that mediates transcriptional repression is deacetylation of histone tails facilitated by HDAC proteins (93). In vertebrates, HDACs contain 11 members (HDAC1-HDAC11), which are divided into three classes: class I (HDAC1-3, HDAC8), class II (HDAC4-HDAC7, HDAC9-10) and class IV (HDAC11). Class III HDACs are the sirtuin family of NAD^+-dependent deacetylases (94-97). Acetylation of histone tails is associated with relaxation of the chromatin structure and increased transcriptional activity. At repressed gene promoters, removal of acetyl groups from histone tails results in a condensed DNA structure, preventing gene expression. Class I HDACs are catalytic subunits of multiprotein corepressor complexes that mediate transcriptional repression. HDAC1 and

HDAC2 are components of the SIN3A corepressor complex (98, 99), the RE1 silencing transcription factor complex (REST) (100-102) and the Nucleosome remodeling and histone deacetylase complex (NuRD) (103-106). HDAC3 is recruited to promoters by association with the NCoR/SMRT repressor complex (93).

NuRD (Nucleosome remodeling and histone deacetylase) complex

The nucleosome remodeling and histone deacetylase complex (NuRD) is an interesting corepressor complex because it combines several enzymatic activities in one large multiprotein complex. The NuRD complex, also known as Mi-2, is about 2MDa in size and is comprised of HDAC1 and HDAC2, and two histone binding proteins, RbAp46 and RbAp48 (107). In addition to HDACs, the NuRD complex also consists of an ATP-dependent nucleosome remodeling activity due to the presence of the Mi-2/CHD family of proteins which have a chromodomain, a DNA helicase/ATPase domain of the SWI/SNF family, and a PHD domain (107). The NuRD complex also contains MTA family proteins, MTA1 or MTA2, first identified as metastasis associated proteins in carcinomas (108). The MTA proteins have a zinc finger and a SANT domain (both are domains that bind DNA). Additionally, the NuRD complex contains the methyl CpG-binding domain protein, MBD3 (109) as well as a lysine-specific histone demethylase, LSD1 (110). It is, however, important to note that some of the components are not exclusive to the NuRD complex and different combinations of these complex members exist in other corepressor protein complexes. Like most classes of chromatin remodeling complexes, the NuRD complex has important roles in transcription, chromatin assembly, cell cycle progression and genomic stability (111). The NuRD complex is evolutionarily

highly conserved and is broadly present in most tissues. The NuRD complex has been shown to associate with oncogenic transcription factors to promote transcriptional repression of downstream targets. As an example, in promyelocytic leukemia, the oncogenic PML/RARα fusion protein recruits the NuRD complex through direct protein-protein interactions to target genes including the tumor suppressor gene retinoic acid receptor β2, to promote gene silencing (112). In cancer, the NuRD complex has been associated with tumor progression or tumor suppression depending on the context (107).

REST (RE1 silencing transcription factor) complex

The RE1 silencing transcription factor, also called Neuron Restrictive Silencing Factor (NRSF) was first discovered as a repressor of neuronal genes containing a 23 bp conserved motif, known as the RE1 repressor element 1 or NRSE (113, 114). REST is critical for embryonic development; perturbations in REST expression or function in developing embryos leads to ectopic expression of neuronal genes in non-neuronal tissues, causing embryonic lethality (115). REST harbors three functional domains: a DNA binding domain containing eight zinc-finger motifs that bind to the NRSE, and two independent repressor domains (116). The amino terminal repressor domain interacts with the mSin3 corepressor that recruits HDACs (117). The carboxyl-terminal repressor domain interacts with the CoREST corepressor that also recruits HDACs. Like the NuRD complex, the REST repressor complex also contains the lysine-specific demethylase, LSD1 (118).

LSD1 (Lysine-specific demethylase)

Histone methylation plays important roles in transcription and epigenetic regulation. LSD1, also referred to as KDM1A, is a flavin-dependent histone demethylase enzyme (119, 120). Identification and characterization of LSD1 demonstrated for the first time that the histone methylation process was a dynamic process. LSD1 functions as part of the NuRD or REST repressor complex, suggesting that DNA binding is essential for stimulating LSD1-mediated nucleosomal demethylation (121). The enzymatic reaction carried out by LSD1 requires the presence of protonated nitrogen on the histone tail to initiate demethylation, therefore limiting it to di-methylated and mono-methylated lysine residues as substrates. In contrast to HDACs, LSD1 functions as both a transcriptional coactivator and a transcriptional corepressor because of its ability to demethylate histone H3 lysine 4 mono or di methyl (H3K4me1/2), a mark of activation, or histone H3 lysine 9 mono or di methyl (H3K9me1/2), a mark of repression. Knockdown or pharmacological inhibition of LSD1 has been shown to reduce proliferation of neuronal progenitor stem cells, suggesting a role for LSD1 in maintaining the balance between self-renewal and differentiation (122). Interestingly, LSD1 has been shown to regulate differentiation of fat and skeletal muscle tissues (123, 124), underscoring the functional importance of LSD1 in tissues of mesenchymal origin (125). LSD1 is highly expressed in sarcomas and, given its function in mesenchymal stem cells, it is likely that LSD1 plays a role in sarcoma pathogenesis (126). In line with this, a recent study demonstrated that Ewing sarcoma primary tumors express high levels of the LSD1 protein (127). LSD1 is also overexpressed in several other cancers and drives their oncogenic growth through

epigenetic mechanisms (128), suggesting that inhibition of LSD1 activity may be a viable and effective therapeutic strategy in multiple cancers.

Dissertation goals

Ewing sarcoma serves as an excellent paradigm for understanding tumorigenesis driven by the expression of chromosomal translocation-derived fusion oncoproteins because of its unique molecular genetics. Most cases of Ewing sarcomas harbor the t(11;22)(q24;q12) translocation encoding the aberrant transcription factor EWS/FLI. Besides the t(11;22), however, these tumors have simple karyotypes with no other demonstrable chromosomal abnormalities. EWS/FLI is therefore considered the central mediator of a hierarchy of transcriptional networks, upregulating and downregulating critical target genes, and leading subsequently to Ewing sarcoma development. The overarching goal of our research is to better understand the biology underlying the pathogenesis of Ewing sarcoma. A mechanistic understanding of how EWS/FLI achieves transcriptional control in Ewing sarcoma, what target genes it directly and indirectly regulates, what DNA motifs EWS/FLI utilizes to achieve this function and what proteins it interacts with will identify key nodes of transcriptional regulation utilized by EWS/FLI to achieve its pathogenic functions. These studies are important to the Ewing sarcoma field as they provide new insights into the mechanistic basis of Ewing sarcoma development and may provide new opportunities for therapeutic intervention. Thus, my thesis work has sought to address several key questions in this context:

1. What are the translocation-based oncogenic fusions that occur in Ewing sarcoma tumors? Chapter 2 is a detailed review article describing the various TET/ETS

fusions that are pathognomonic for the disease. We also discuss rare non-TET/ETS fusions that occur in "Ewing's-like tumors" and highlight important questions that need to be answered to determine the true molecular identities of the "Ewing's-like tumors."

2. What are the direct targets of EWS/FLI and what is the EWS/FLI response element on promoters of activated target genes that are critical for maintenance of oncogenic transformation in Ewing sarcoma? We used genomics approaches to identify direct targets of EWS/FLI in Ewing sarcoma. In addition to the previously characterized high-affinity consensus ETS site, we identified GGAA microsatellites as a novel EWS/FLI response element. Using the promoter of *NR0B1*, an EWS/FLI target gene critical for oncogenesis, we validated binding and transcriptional activation by EWS/FLI from the GGAA microsatellites. Bioinformatics approaches further established the specific association of GGAA-microsatellites with upregulated EWS/FLI target genes. This work is detailed in Chapter 3 of the dissertation.
3. Can we use the presence of GGAA microsatellites as a tool to identify additional EWS/FLI target genes that are critical for Ewing sarcoma development and/or maintenance? Can the presence of GGAA-microsatellites serve as a prognostic marker for Ewing sarcoma? Chapter 4 details our analysis of another GGAA-microsatellite containing EWS/FLI upregulated target gene, *GSTM4*, as an essential gene required for oncogenesis and for chemotherapeutic resistance in Ewing sarcoma. This chapter further establishes a direct correlation between GSTM4 expression levels and survival outcome.

4. What is the role of *GLI1*, a direct upregulated target of EWS/FLI in Ewing sarcoma? What are critical downstream targets of *GLI1* that mediate its function in Ewing sarcoma? Unlike the GGAA microsatellite response element, the *GLI1* promoter harbors the canonical ETS site as the EWS/FLI response element. We demonstrated that GLI1 is necessary for maintenance of oncogenic transformation in Ewing sarcoma. Global transcription profiling studies were performed to identify GLI1 target genes. We identified *KRT17* as a critical GLI1 target gene and characterized a novel role for *KRT17* in coordinating cellular adhesion and oncogenic transformation in Ewing sarcoma by regulating the AKT signaling pathway. These studies are presented in Chapter 5.
5. What is the functional relationship between wild-type EWS and EWS/FLI? Does EWS function as a transcriptional cofactor in Ewing sarcoma? What are EWS target genes in Ewing sarcoma? Does the EWS transcription profile contribute significantly to the EWS/FLI transcription profile? Does EWS play a role in regulating cancer-related phenotypes in Ewing sarcoma? Chapter 6 details our analysis of the EWS and EWS/FLI RNA-sequencing-based transcriptional profiles in Ewing sarcoma. From this overlap, we identified a subset of neuronal genes that are repressed by EWS. The promoters of these neuronal genes harbor the response element for the transcriptional corepressor REST. We demonstrate that REST and EWS cooperate to repress the subset of neuronal genes thereby inhibiting full neuronal differentiation and contributing to the transdifferentiated phenotype of Ewing sarcoma cells. Furthermore, we demonstrate that wild-type

EWS functions as a tumor suppressor and, therefore, haploinsufficiency of EWS drives oncogenic transformation in Ewing sarcoma.

6. Although EWS/FLI has previously been characterized largely as a transcriptional activator, does the significantly large EWS/FLI repressed gene signature contain direct repressed targets? Is transcriptional repression functionally significant to the disease biology? What domains within EWS/FLI are necessary for repression? What corepressor proteins does EWS/FLI interact with to directly repress target genes in Ewing sarcoma? Using genomic approaches we first identified a subset of direct repressed EWS/FLI targets. We validated a subset of these genes and identified two interesting candidate genes with tumor-suppressive properties *in vitro* and *in vivo*. Deletion mapping analysis was used to identify domains within EWS/FLI that are necessary and sufficient for direct repression. Using a candidate approach, we identified a role for the NuRD corepressor complex containing HDACs and LSD1 in EWS/FLI-mediated transcriptional repression. We further identified LSD1 as a novel target for therapeutic intervention in Ewing sarcoma. This work is detailed in Chapter 7.

Taken together, the work presented in this dissertation highlights the identification of direct targets of EWS/FLI, and novel DNA-binding elements for EWS/FLI in Ewing sarcoma. We uncover a novel role for the EWS/FLI up-regulated target gene *GLI1* in Ewing sarcoma oncogenesis. In addition, we unravel a previously unknown role for wild-type EWS in regulating the neural phenotype of Ewing sarcoma. Furthermore, we identify and characterize a less-well understood role for EWS/FLI in direct transcriptional repression of tumor suppressor genes. Finally, through detailed

mechanistic studies we identify HDACs and LSD1 as novel therapeutic targets in Ewing sarcoma.

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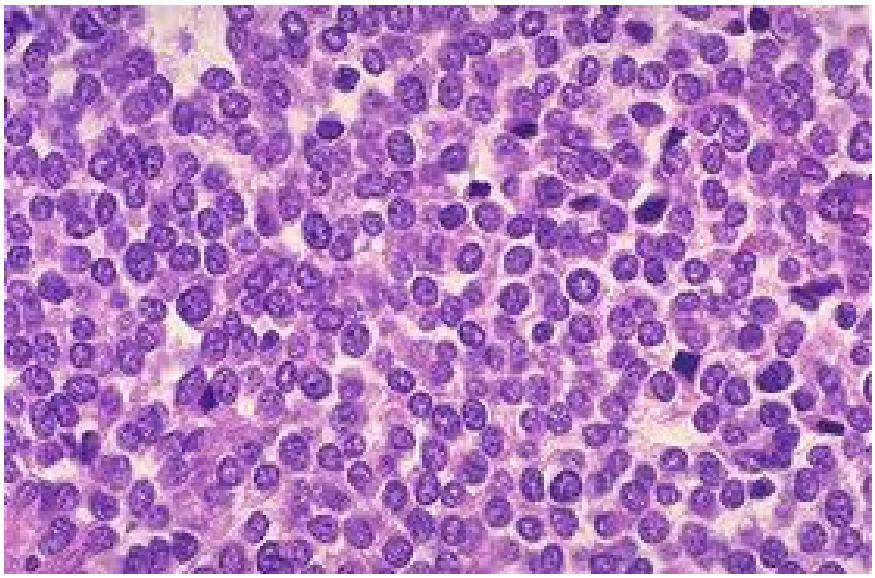

Figure 1.1 Histology of Ewing sarcoma cells. The small round blue cell morphology of Ewing sarcoma tumors. The tumor cells stain blue with hematoxylin and eosin staining. The prevalence of blue staining is because the cells consist predominantly of nucleus, and have little cytoplasm. Figure provided courtesy of Steve Lessnick.

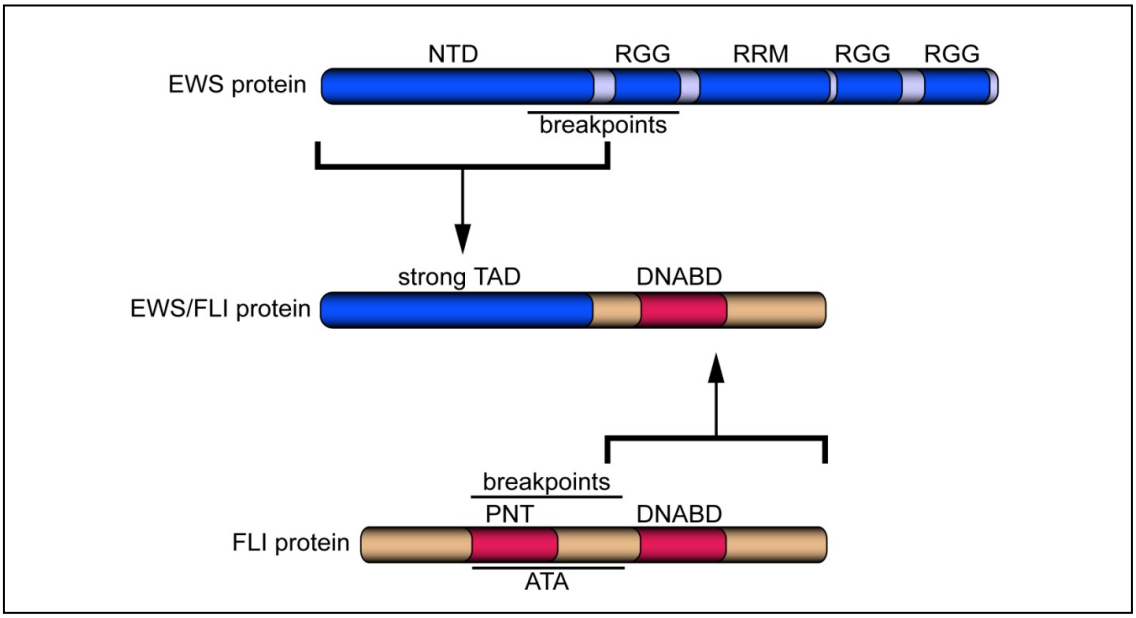

Figure 1.2 Schematic of the EWS/FLI fusion protein. Wild-type EWS protein contains an amino-terminal domain (NTD), an RNA recognition motif (RRM), and three RGG repeat regions. Wild-type FLI protein contains a carboxyl-terminal ETS-type DNA binding domain (DNABD), and an amino-terminal activation domain (ATA) that overlaps a pointed (PNT) domain. The locations of the translocation breakpoints are indicated. EWS/FLI retains the NTD of EWS and the DNABD of FLI. Figure provided courtesy of Steve Lessnick.

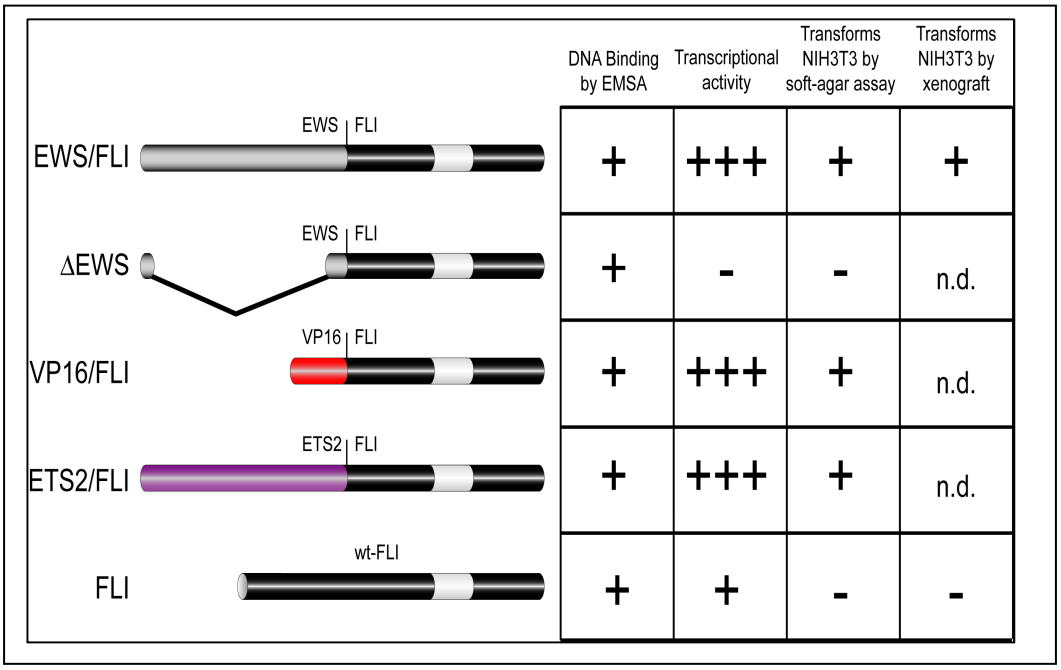

Figure 1.3 Transcriptional activation mutants of EWS/FLI. The DNA binding, transcriptional and transforming abilities of full-length EWS/FLI and various mutants generated by the fusion of the carboxyl-terminal FLI DNA binding domain to heterologous activation domains of other transcription factors. These data have been previously published (47, 55, 56). Data on the xenograft forming abilities of these constructs have also been published (129). Figure provided courtesy of Steve Lessnick.

CHAPTER 2

PROMISCUOUS PARTNERSHIPS IN EWING'S SARCOMA

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REVIEW

Promiscuous partnerships in Ewing's sarcoma

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Ewing's sarcoma is a highly aggressive bone and soft tissue tumor of children and young adults. At the molecular genetic level Ewing's sarcoma is characterized by a balanced reciprocal translocation, t(11;22)(q24;q12), which encodes an oncogenic fusion protein and transcription factor EWS/FLI. This tumor-specific chimeric fusion retains the amino terminus of EWS, a member of the TET (TLS/EWS/TAF15) family of RNA-binding proteins, and the carboxy terminus of FLI, a member of the ETS family of transcription factors. In addition to EWS/FLI, variant translocation fusions belonging to the TET/ETS family have been identified in Ewing's sarcoma. These studies solidified the importance of TET/ETS fusions in the pathogenesis of Ewing's sarcoma and have since been used as diagnostic markers for the disease. EWS fusions with non-ETS transcription factor family members have been described in sarcomas that are clearly distinct from Ewing's sarcoma. However, in recent years there have been reports of rare fusions in "Ewing's-like tumors" that harbor the amino-terminus of EWS fused to the carboxy-terminal DNA or chromatin-interacting domains contributed by non-ETS proteins. This review aims to summarize the growing list of fusion oncogenes that characterize Ewing's sarcoma and Ewing's-like tumors and highlights important questions that need to be answered to further support the existing concept that Ewing's sarcoma is strictly a "TET/ETS" fusion-driven malignancy. Understanding the molecular mechanisms of action of the various different fusion oncogenes will provide better insights into the biology underlying this rare but important solid tumor.

Keywords Ewing's sarcoma, translocations, fusion proteins

Non-random chromosomal translocations are often characteristic features of a number of bone and soft-tissue sarcomas. At the pathophysiologic level, many of these translocations behave as aberrant transcription factor oncogenes that play crucial roles in tumor development by deregulating target gene expression. Ewing's sarcoma is a prototypic example of a solid tumor characterized by the presence of chromosomal translocations (1). Ewing's sarcoma is a highly aggressive primary tumor of the bone with an undifferentiated small round cell phenotype (2,3). The mean age at the time of diagnosis for Ewing's sarcoma is ~15 years, making it the second most common bone tumor in children and adolescents following osteosarcoma (4,5). Ewing's sarcoma typically arises in the bone, but a small portion (less than 15%) of patients present with a primary tumor in soft-tissue, termed extraosseous Ewing's sarcoma (6,7). In addition to bone and soft-tissue Ewing's sarcoma, the Ewing's sarcoma family of tumors also includes Askin's tumors and peripheral primitive neuroectodermal tumors, which harbor the same set of translocation fusions (8–11). For the purpose of this review we shall refer to this entire group simply as "Ewing's sarcoma."

Ewing's sarcoma displays a high propensity to metastasize and the most common sites include lung, bone and bone marrow. About 15-25% of Ewing's sarcoma patients present with metastases at the time of diagnosis (12). It is believed that the vast majority of patients harbor micrometastatic disease, as the relapse rate for surgically-resected Ewing's sarcoma in the absence of systemic chemotherapy is on the order of 90% (13–15). Therefore, the current standard of care for Ewing's sarcoma patients is multimodal treatment, including systemic chemotherapy along with either surgery and/or radiation for control of the primary site of disease. Despite aggressive multimodal treatment the 5-year disease free survival rate for patients drops from 60-70% when the disease is localized to a dismal 10-30% when the disease had metastasized (16,17). The overarching goal in the field is

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to better understand the biology underlying the pathogenesis of Ewing's sarcoma with the hope of developing more efficacious therapy for patients afflicted with this disease. Given the central role of chromosomal translocations in this disease, understanding these translocations is likely to allow for a deeper understanding of the biology of Ewing's sarcoma.

The EWS/FLI fusion in Ewing's sarcoma

Karyotypically, Ewing's sarcoma is a relatively simple neoplasm, harboring the main cytogenetic hallmark t(11;22)(q24;q12) translocation (9,18). Approximately 85% of Ewing's sarcoma tumors harbor this characteristic translocation. The t(11;22) rearrangement creates a fusion between the Ewing's sarcoma breakpoint region 1 gene (*EWSR1*) on chromosome 22 and the Friend leukemia virus integration site 1 gene *(FLI1*) on chromosome 11 (19) (Figure 1A).

The *EWSR1* gene encodes the EWS protein, which is a member of the TET family of proteins (that includes TLS, EWS, and TAF15). Full-length EWS associates with members of the transcriptional machinery, including RNA polymerase II, TFIID and CBP/p300, indicative of a role in transcription activation (20–23). The *FLI1* gene on the other hand encodes for the FLI protein, which normally functions mainly in hematopoietic, vascular and neural-crest development (24–26). FLI is a member of the ETS (E-26 transformation specific) family of transcription factors, which are characterized by a highly-conserved winged helix-loop-helix DNA binding domain known as the ETS domain (27).

The fusion protein EWS/FLI contains the amino-terminus of EWS and the carboxy-terminus of FLI. This in-frame fusion protein acts as an oncogene through its function as an aberrant transcription factor (28–30). The reciprocal FLI/EWS fusion is not expressed in Ewing's sarcoma tumors, and the reciprocal translocated chromosome is sometimes lost from these tumors (9,31).

The amino-terminal domain of the EWS protein, retained in the EWS/FLI fusion product, contains several repeats of the serine-tyrosine-glycine-glutamine rich sequence which resembles transcriptional activation domains seen in other transcription factors. Consequently, when this domain is fused with a heterologous DNA-binding domain the fusion protein functions as a potent transcriptional activator (29,30,32). Subsequent studies have demonstrated that EWS/FLI has a strong repressive capacity at some target genes as well (see below). Understanding the physiologic role of the amino-terminal EWS domain within the context of the wild-type protein has become a growing area of interest since this region is included in similar chromosomal translocations in a variety of different sarcomas (33). The carboxy-terminal domain of FLI, retained in the EWS/FLI fusion product, contains the 85-amino acid ETS DNA binding domain and recognizes purine-rich sequences containing a GGAA/T core motif, similar to other ETS family members (27,34–36).

In addition to encoding the EWS/FLI fusion, the t(11;22) rearrangement has two additional consequences. First, the translocation causes EWS/FLI to be constitutively expressed from the native *EWSR1* promoter (*FLI1* promoter expression is limited to hematopoietic and neural crest lineages, and is non-functional in Ewing's sarcoma cells) (19,29,31,37). The second consequence is that one wild-type copy of *EWSR1*, and one wild-type copy of *FLI1*, are disrupted in the tumor (9). This likely is of no importance for *FLI1* since it is not expressed (31). However, the contribution of haploinsufficiency of *EWSR1* is not well-understood at this time.

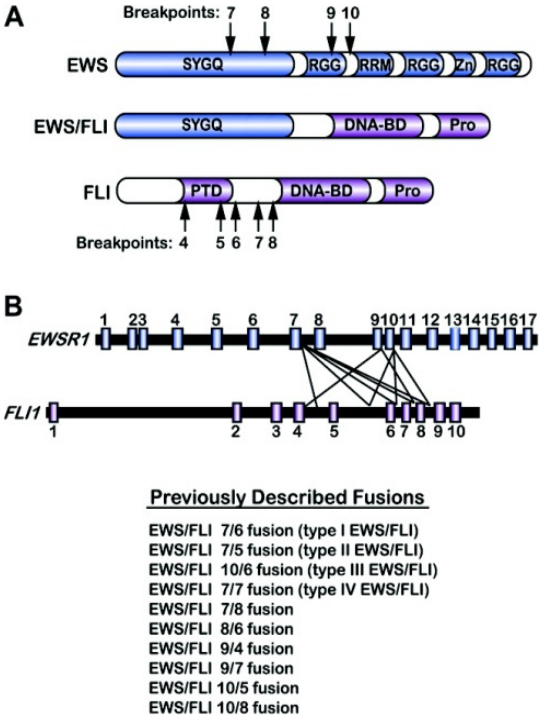

Figure 1 (A) Domain structures of wild-type EWS, wild-type FLI and the EWS/FLI fusion proteins. SYGQ: serine-tyrosine-glycine-glutamine rich transactivation region; RGG: arginine-glycine-glycine rich regions; RRM: RNA-recognition motif; Zn: putative zinc finger; PTD: pointed domain; DNA-BD: DNA binding domain; Pro: proline-rich activation domain. Arrows indicate breakpoints in wild-type EWS and FLI included in commonly observed subtypes of the EWS/FLI fusion protein. (B) Genomic structures of the *EWSR1* and *FLI1* genes. Breakpoints in the *EWSR1* and *FLI1* genes occur in many introns. Following splicing, the exons join together to generate various subtypes of EWS/FLI. Some of the previously described EWS/FLI fusion subtypes are depicted here.

The EWS/FLI fusion protein is known to be a potent oncogene based on its ability to transform NIH3T3-immortalized murine fibroblast cells (28). Furthermore, sustained expression of EWS/FLI is necessary to maintain the oncogenic phenotype of Ewing's sarcoma cells. This has been demonstrated by a number of different studies, where inhibition of endogenous EWS/FLI function or expression in patient-derived Ewing's sarcoma cell lines demonstrated a reduction of oncogenic transformation both *in vitro* and *in vivo* (31,38–44). Structure-function analysis demonstrated that the amino-terminal EWS transactivation domain and the carboxy-terminal ETS-type DNA-binding domain are both required for efficient transformation by the fusion oncogene (28,30). Furthermore, in addition to the amino-terminal transactivation

domain of EWS, the carboxy-terminal proline-rich domain of FLI has been shown to enhance the transcriptional activity of the fusion protein (45). Thus, it is well accepted that EWS/FLI acts as an aberrant transcription factor and that downstream target genes of the fusion protein contribute to the process of oncogenesis in Ewing's sarcoma. It is also possible that potential non-transcriptional functions of the fusion also contribute to its oncogenic activity, but these have not been well-documented or substantiated at this time.

Utilization of RNA-interference (RNAi) based approaches combined with microarray technology has enabled the identification of a large number of target genes dysregulated by EWS/FLI in Ewing's sarcoma (31,42,44,46). Some of the upregulated target genes of EWS/FLI, including *NR0B1*, *NKX2.2* and *GLI1*, have been demonstrated to be critical for the process of EWS/FLI mediated oncogenic transformation (31,44,47,48). Other target genes of EWS/FLI have been implicated in processes that are necessary for sustained tumorigenesis, such as cell proliferation, evasion of apoptosis, drug-resistance, cell cycle control, evasion of growth inhibition, immortalization, angiogenesis, adhesion and maintenance of pluripotency, including *CCND1*, *IGFBP3*, *GSTM4*, *p21*, *TGFBRII*, *hTERT*, *VEGF*, *CAV* and *EZH2* respectively (42,49–56). The growing list of target genes suggests that EWS/FLI modulates a whole network of downstream effector genes to achieve the various hallmarks of oncogenesis. Some of these genes represent direct targets of EWS/FLI, whereas, others are modulated indirectly. The mechanism by which EWS/FLI regulates target genes in Ewing's sarcoma is a growing area of research in the field. Some studies have suggested that EWS/FLI contributes to oncogenesis both in a DNA-binding dependent and independent manner (57,58). However, that the DNA-binding property of EWS/FLI is indispensable to its oncogenic potential has been clearly demonstrated in patient-derived Ewing's sarcoma cells by the inability of DNA-binding mutant versions of EWS/FLI to rescue transformation when endogenous EWS/FLI is down-regulated via an RNAi approach, as well as the lack of any identified Ewing's sarcoma patient tumor sample expressing a DNA-binding deficient form of EWS/FLI (30).

ETS proteins bind to sequences containing a GGAA/T core motif, and flanking sequences further define the binding affinity and specificity for each ETS factor (27). Whole genome localization studies (chromatin immunoprecipitation followed by microarray analysis, or ChIP-chip) in Ewing's sarcoma cell lines revealed that EWS/FLI binds the high affinity ETS-site ACCGGAAGTG, validating previous *in vitro* site selection approaches (27,35,59,60). In addition to regulating some target genes by binding to *bona fide* motifs used by ETS factors, EWS/FLI was also found to bind GGAA-microsatellite repeat sequences in promoters of target genes. Some of these genes (*NR0B1*, *CAV1*, and *GSTM4*) are necessary for EWS/FLI mediated oncogenesis, highlighting the importance of transcriptional regulation via microsatellite repeats in the pathogenesis of Ewing's sarcoma (61). These findings were independently validated using next-generation ChIP-sequencing technology (62). Identification of microsatellite repeats in EWS/FLI-bound chromatin is clearly an example of how advances can be made in unraveling the mechanism of disease pathogenesis using high-throughput genomic approaches (63). From a mechanistic standpoint, studies using multimers of the GGAA core motif have demonstrated that longer GGAA-repeat containing sequences have a higher potential to be activated, possibly by increasing the number of EWS/FLI molecules that bind (64). In addition to the "promoter-proximal" class of GGAA microsatellite response elements, some distant "enhancer regions" have also been reported to harbor EWS/FLI-bound GGAA repeat sequences (62). The mechanism of gene regulation by EWS/FLI bound several hundred kilobases away at these "enhancer microsatellites," however, still needs to be defined.

In addition to its transcriptional activation function, EWS/FLI also represses many downstream target genes in Ewing's sarcoma. Indeed, comprehensive gene expression profiling studies in Ewing's sarcoma cell lines, as well as in primary tumors, suggest that EWS/FLI may repress as many, if not more, genes than it upregulates (31,42,44,46,65). Some repressed targets of EWS/FLI have also been shown to be important contributors to the process of transformation, cell survival and cellular proliferation, further demonstrating the importance of target gene repression in Ewing's sarcoma oncogenesis (42,53,54). In contrast to upregulated target genes, EWS/FLI downregulated target genes do not harbor GGAA-microsatellite response elements in their promoters. The mechanism of repression by EWS/FLI in Ewing's sarcoma is still largely unknown. Understanding this seemingly opposite function of the EWS/FLI transcriptional activator will not only help in furthering our knowledge of EWS/FLI as a molecule, but will also help to identify novel opportunities for therapeutic intervention.

EWS/FLI fusion subtypes

"EWS/FLI" is not a single molecular entity, but rather includes a set of highly related isoforms or subtypes. This diversity is a result of differences in genomic breakpoints in the *EWSR1* and *FLI1* genes. Breakpoints have been observed in a variety of introns in these genes (66–68). In each case, the resultant fusion is really in the introns of the genes, and through typical splicing processes of the transcribed RNA, fusion mRNAs are generated containing 5′ exons derived from *EWSR1* fused to 3′ exons derived from *FLI1* (19,67,68). The nomenclature for such fusions has not been well-defined. However, the most common subtype (originally called a "type I" fusion) consists of *EWSR1* exons 1-7 fused to *FLI1* exons 6-10 (19,67). The second most common subtype (originally called a "type II" fusion) fuses exon 7 of *EWSR1* to exon 5 of *FLI1* (19) (Figure 1B). Many investigators simply refer to the subtypes on the basis of which exons are fused to one-another. Thus, the type I fusion is also called a "7/6" fusion, and the type II fusion is called a "7/5" fusion. Regardless of the details, all the subtypes of EWS/FLI retain the amino-terminal strong transactivation domain of the EWS protein and the carboxy-terminal ETS DNA binding domain contributed by FLI (67). Whether these fine-structure details have functional importance is somewhat unclear. There are data to suggest that the EWS/FLI type 1 fusion has reduced transactivation potential in Ewing's sarcoma cell lines in comparison to the other fusion subtypes and that this may have prognostic significance (69). In support of this notion, two independent retrospective studies reported that Ewing's sarcoma patients with localized tumors harboring type I EWS/FLI had a better overall and event-free

survival in comparison to patients with non-type I translocations (70,71). However, recent studies from large cooperative group trials concluded that fusion subtypes no longer have prognostic significance (72,73). This could be due to the earlier studies demonstrating a statistical anomaly, or alternately, could be due to more intensive therapeutic regimens that have effectively "treated away" any differences in outcome. Regardless of the reason, EWS/FLI fusion subtype is no longer considered a prognostic marker for patients with Ewing's sarcoma.

Other EWS/ETS fusions in Ewing's sarcoma

The ETS family of proteins is comprised of transcription factors that are characterized by the presence of a highly conserved 85 amino acid ETS domain that mediates sequence-specific DNA binding (27). In many cases, ETS proteins function as signal-dependent transcriptional regulators controlling cellular differentiation and proliferation (74,75). Many different members of the ETS family have been shown to be involved in oncogenesis, predominantly, by chromosomal translocations that fuse ETS members to a variety of amino-terminal partners. As mentioned above, ~85% of cases of Ewing's sarcoma have the classic t(11;22) translocation encoding EWS/FLI. Interestingly, in Ewing's sarcoma tumors lacking the EWS/FLI fusion, alternate translocation fusions are present. These alternate translocations result in fusions of the *EWSR1* gene with one of four different *ETS* genes including *ERG* (*ETS*-related gene), *ETV1* (*ETS*-variant gene 1), *ETV4* (*ETS* variant gene 4, also called *E1AF*) or *FEV* (fifth Ewing sarcoma variant) (76–80) (Figure 2). Despite their genetic diversities, the alternate fusions are structurally very similar to EWS/FLI. In each case, the amino-terminal transcriptional activation domain of EWS and an ETS DNA binding domain are retained. These fusions (including EWS/FLI) might be generally referred to as "EWS/ETS fusions." The alternate fusions have not been as extensively studied as EWS/FLI itself. However, the similarity in structure suggests that these chimeric proteins function as aberrant transcription factors as well, and thus contribute to Ewing's sarcoma oncogenesis by deregulating key oncogenic target genes (81–84). Indeed, the few studies that have looked specifically at these alternate fusions support this notion.

The most common of the alternate translocations is the t(21;22)(q22;q12), found in approximately 10% of Ewing's sarcoma tumors. This chromosomal translocation encodes the EWS/ERG fusion protein (67,76). ERG shares 68% overall amino acid identity with FLI and 98% identity within their ETS DNA-binding domains (76,85). Considering the structural similarities of EWS/FLI and EWS/ERG fusions, it is likely that the two proteins function to dysregulate similar target genes in Ewing's sarcoma. Tumors expressing the EWS/ERG fusion lack expression of EWS/FLI, further suggesting that EWS/ERG possess the ability to activate similar oncogenic pathways crucial to Ewing's sarcoma pathogenesis thus generating nearly identical tumors. In fact, a retrospective study comparing 30 EWS/ERG Ewing's sarcoma cases with 106 EWS/FLI cases revealed no significant differences in clinical presentation, age of diagnosis, sex, primary site, metastasis at diagnosis and overall as well as event-free survival. This study further supported the existing notion that EWS/FLI and EWS/ERG fusion proteins function similarly to drive the process of oncogenesis in Ewing's sarcoma (86). Like the EWS/FLI subtypes in Ewing's

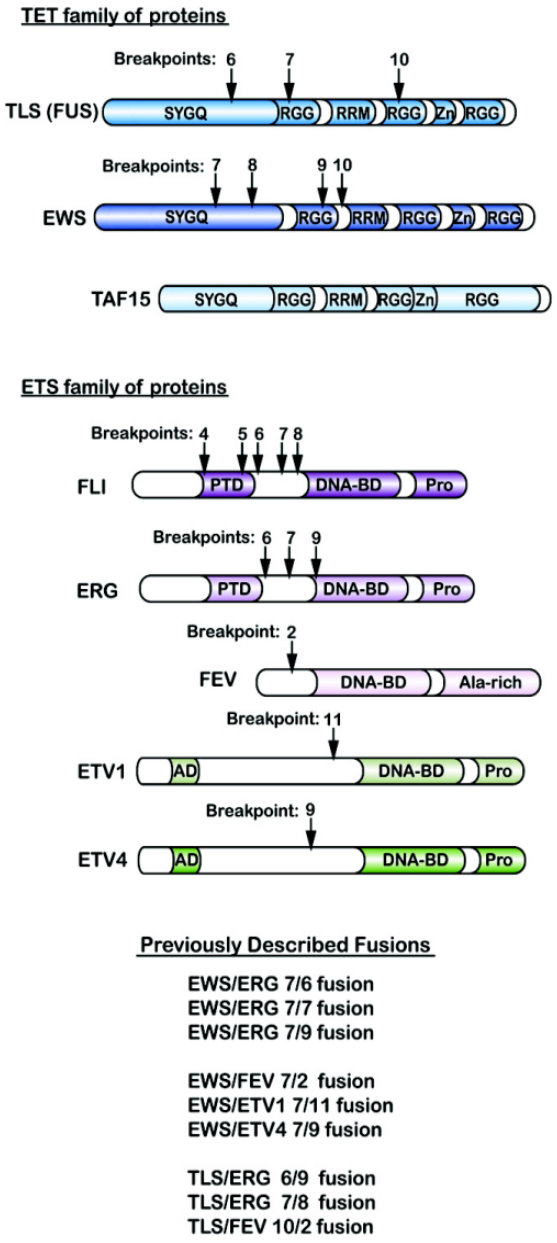

Figure 2 Domain structures of the TET family of RNA binding proteins: TLS (FUS), EWS, TAF15 and the ETS family of transcription factors: FLI, ERG, FEV, ETV1 and ETV4. AD: Activation domain; Ala-rich: Alanine-rich region involved in transcriptional repression. Arrows indicate breakpoints in wild-type EWS, TLS, FLI, ERG, FEV, ETV1 and ETV4 included in TET/ETS fusions in Ewing's sarcoma. Breakpoints occur in the introns of the wild-type genes but following splicing exons are joined to generate various TET/ETS chimeric fusions.

sarcoma, different EWS/ERG fusions have been described in the literature, varying in their breakpoints across different introns in the *EWSR1* and *ERG* genes (Figure 2).

The EWS/ETV1, EWS/ETV4 and EWS/FEV fusions each occur in <1% of Ewing's sarcoma tumors (77–80). FLI, ERG and FEV share 87% identity and 98% similarity in their DNA-binding domains. Therefore, they constitute one subgroup within the 27 known ETS members in the human genome. Based on their structural similarities, one might expect a significant overlap in the repertoire of downstream targets for EWS/FLI, EWS/ERG and EWS/FEV. ETV1 and ETV4 on the other hand, are more closely related to each other and share 96% identity and 100% similarity in their DNA binding domains, thus creating a different subtype in the ETS family. Another ETS protein in this subtype that is highly homologous to ETV1 and ETV4 is ETV5 (also known as ERM) (87). This raises the conceptual possibility that as yet undiscovered cases of Ewing's sarcoma may harbor EWS/ETV5 fusions. However, if EWS/ETV5 fusions do exist they are expected to be extremely rare since more than 99% of Ewing's tumors have already been demonstrated to carry one of the other previously described EWS/ETS fusions.

Although the carboxy-terminal ETS fusion partners have varied tissue-restricted expression patterns, tumor-specific expression of the fusions is accomplished by the strong *EWSR1* gene promoter. This allows for the disruption of the normal gene-expression patterns driven by the full length carboxy-terminal DNA binding counterparts and sets up a new dysregulated gene expression program that drives tumorigenesis. Furthermore, ETS proteins share cooperative DNA binding with AP1 (FOS-JUN) proteins. This cooperative interaction has been established for ETS1-AP1, ERG-AP1 and also for EWS/FLI-AP1, further supporting the idea that various EWS/ETS fusions dysregulate a common target gene pool in Ewing's sarcoma (88–90).

A recurring feature of the various translocation fusions in Ewing's sarcoma is their mutual exclusivity, suggesting that these chimeric proteins can replace each other in the oncogenic pathways leading to Ewing's sarcoma. Whether there are important molecular differences in activity between these alternate fusions is unknown, but some functional differences have been observed. For example, while EWS/FLI, EWS/ERG, and EWS/FEV all efficiently induce oncogenic transformation of NIH3T3 cells (as measured by their ability to grow as colonies in anchorage-independent conditions in soft agar), EWS/ETV1 and EWS/ETV4 were unable to induce this aspect of oncogenic transformation in the same system (84). All five fusions, however, did cause NIH3T3 cells to form tumors when injected into immunodeficient mice. The molecular reasons for these differences are unknown.

Another potential difference between these proteins may be the location of tumors bearing the different fusions. One study found that 11 of 12 cases (92%) of Ewing's sarcomas harboring EWS/FEV, EWS/ETV1, or EWS/ETV4 presented with extraosseous tumors (91). Whether this represents a unique aspect of the tumor biology of the rare EWS/ETS variants, or whether this represents a reporting bias, is unknown at this time. Future research aimed at understanding the impact of different translocation fusions on pathways that impinge on the tumor microenvironment may shed more light on this interesting feature of Ewing's tumors. An alternate hypothesis is that the different fusions occur in different cell types or different developmental stages in the same cell type, due to differences in gene expression, chromatin structure, or other poorly understood characteristics. These differences in origin might then determine if the tumor will be bone-associated or located at extraosseous sites.

TET/ETS fusions in Ewing's sarcoma

To further add to the complexity of Ewing's sarcoma, non-EWS fusions have been identified in rare cases of the disease. As discussed above, EWS is a member of the TET (TLS/EWS/TAF15) family of proteins. Gene fusions have been identified between the TET family member *TLS* (also called *FUS*) and two different ETS family members, *ERG* and *FEV* (Figure 2). TLS/ERG and TLS/FEV fusion proteins are found in <1% of Ewing's sarcoma cases. Conceptually, again, these "TLS/ETS" fusions are likely to functionally recapitulate the EWS/FLI fusion by creating generic "TET/ETS" fusion proteins. Indeed, identification of TLS/ERG and TLS/FEV translocation fusions supports the concept that all possible combinations of TET/ETS fusions might contribute to the development of Ewing's sarcoma (92,93). This further implies that in the years to come Ewing's sarcoma tumors expressing TLS/FLI, TLS/ETV1, and TLS/ETV4 fusions await discovery. One can also imagine the identification of novel TAF15/ETS fusions in Ewing's sarcoma, since members of the TET family of proteins behave in a similar fashion by contributing a strong amino-terminal transactivation domain when fused to members of the ETS family of transcription factors. If such fusions do exist they will be rare. However, they would create a problem for the molecular pathologist, because current diagnostic methodologies used to identify the common fusion proteins (RT-PCR for EWS/FLI and occasionally EWS/ERG, or break-apart FISH probes for *EWSR1*) will not identify these rare variants. Indeed, it is possible that current "diagnostically-challenging" cases of Ewing's sarcoma (that are challenging because an EWS/FLI or EWS/ERG translocation cannot be identified) may have one of the already-described rare variants.

While the molecular mechanisms of EWS/FLI function have been extensively studied, it seems clear that there is a great deal of additional function to understand. Furthermore, it is generally assumed that all of the TET/ETS fusions function in a similar manner, although this has not been rigorously tested, in part because all of the functions of EWS/FLI are not understood. For example, the amino-terminus of EWS functions as a strong transcriptional activation domain (29). It has been demonstrated that in some analyses, the equivalent domain of TLS has weak transcriptional activation function, while in other settings it is equivalent to the EWS domain (30). The molecular basis for this difference is not known. Similarly, the mechanistic basis for transcriptional repression is not understood, and so whether different TET/ETS fusions exhibit similar activity in this respect is not known. Finally, while most investigators believe that the primary function of TET/ETS fusions is to bind DNA and transcriptionally-regulate target genes via interaction with the transcriptional machinery, other modes of action may also contribute to gene regulation. For example, it is possible that TET/ETS fusions also regulate gene expression by interfering with the normal function of TET family members or

ETS family members via a "dominant-negative" mechanism. Thus, EWS/FLI might inhibit wild-type EWS function via protein-protein interactions and block its (poorly understood) normal activity. Similarly, EWS/FLI might bind to some genomic loci and prevent other ETS family members from binding to those sites and thus block their ability to properly regulate target genes.

Regardless of our lack of comprehensive understanding of how TET/ETS fusions function, there is general agreement that fusion of a TET family member to an ETS family member creates a fusion protein that mimics the domain structure of EWS/FLI. Thus, the entire group of fusion proteins is thought to function in a similar fashion, including binding to ETS target sites in the human genome and regulating gene expression through that binding. This is a very satisfying model. However, this working model has been challenged in recent years by the discovery of non-TET/ETS fusions in "Ewing's-like tumors."

Non-TET/ETS fusions in "Ewing's-like tumors"

EWS/NFATc2

In 2009 there was a report on the identification of a new translocation partner of the *EWSR1* gene, *NFATc2* (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 also known as *NFAT1* or *NFATp*), in "Ewing's-like tumors" (94). In all cases identified, an in-frame chimeric fusion between exon 8 of *EWSR1* and exon 3 of the *NFATc2* gene was identified (Figure 3). The EWS/NFATc2 fusion harboring solid tumors were diagnosed in four male patients who were 16, 21, 25 and 39 years of age. Histologically, the tumors stained strongly positive for CD99, a classic marker for Ewing's sarcoma (95) and stained negative for desmin, the latter being a marker for desmoplastic small round cell tumor (DSRCT; a tumor which harbors EWS/WT1 translocations) (Table 1). These features were consistent with these tumors being highly-similar to Ewing's sarcoma.

NFATc2 is a member of the NFAT transcription factor family. The *NFATc2* gene plays a key role in T-cell development and in neuronal development (96) and is normally regulated by calcium signaling (97). Dephosphorylation of the wild-type protein causes functional activation and nuclear translocation, which then results in formation of a complex between NFATc2 and AP1 proteins (FOS and JUN) (98,99). Interestingly, cooperative DNA binding with AP1 proteins is a shared feature of NFAT and ETS proteins, including ETS1, ERG, and EWS/FLI (88–90,98). In the case of the EWS/NFATc2 fusion, the normal tissue-restricted expression pattern of *NFATc2* is overcome by fusion with the constitutively active *EWSR1* gene promoter, as is the case for EWS/ETS fusions.

Intriguingly, the ETS and NFAT gene families recognize sequences that share a core GGAA/T motif, raising the possibility that EWS/ETS and EWS/NFATc2 fusion proteins may be able to bind and regulate a similar pool of target genes, potentially in cooperation with AP1 proteins. Ewing's sarcoma cell lines expressing EWS/FLI demonstrated no detectable expression of NFATc2 but cell lines expressing the EWS/ERG translocation expressed low levels of

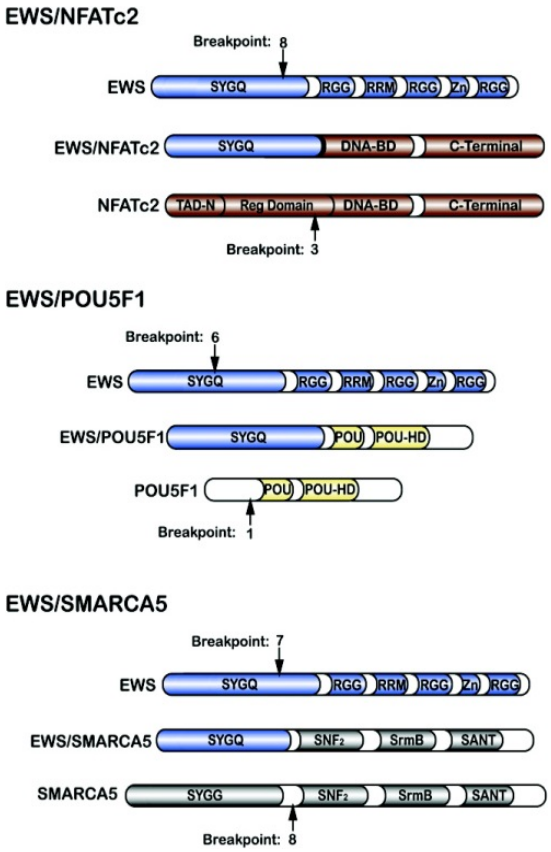

Figure 3 Domain structures of wild-type EWS, NFATc2, POU5F1 and SMARCA5 proteins as well as EWS/NFATc2, EWS/POU5F1 and EWS/SMARCA5 fusion proteins. TAD-N: amino-terminal transactivation domain; Reg domain: Regulatory region containing multiple conserved phosphorylation sites; POU: POU-specific domain; POU HD: POU homeodomain; SNF_2: domain with helicase activity for chromatin unwinding and transcriptional regulation; SrmB: domain found in DNA and RNA helicases, harbors an ATP binding site; SANT: domain involved in DNA-binding. Arrows indicate breakpoints observed in wild-type EWS, NFATc2, POU5F1 and SMARCA5 that are included in the resulting fusion proteins observed in "Ewing's-like" tumors.

NFATc2, suggesting that the EWS/ERG fusion either directly or indirectly may be able to regulate NFATc2 expression in Ewing's sarcoma cells (94). EWS/NFATc2-expressing tumors may represent a variant of Ewing's sarcoma, but additional work is needed to fully validate this concept.

EWS/POU5F1

In addition to the EWS/NFATc2 fusion, fusions between EWS and other transcription factors have been described in "Ewing's-like tumors." For example, in 2005 a t(6;22)(p21;q12) was identified in an undifferentiated bone tumor of the pelvis in a 39 year old woman (100). This translocation created an EWS/POU5F1 chimeric protein through fusion of exon 6 of *EWSR1* with part of exon 1 of *POU5F1* (Figure 3).

Table 1 Clinicopathologic features of non-TET/ETS "Ewing's-like tumors"

Fusion type	No. of patients	Age/Sex	Location of tumor	Immunostaining
EWS/NFATc2	4	16/M 21/M 25/M 39/M	Right femur Right thigh Right femur Right humerus	All 4 tumors were: CD99 + Vimentin + Desmin − NSE − S100 −
EWS/POU5F1	1	39/F	Right pelvic bone	CD99 − Vimentin + S100 + NSE +
EWS/SMARCA5	1	5/F	Lumbosacral spinal canal	CD99 + Vimentin + NSE + SYN + Desmin −
EWS/ZSG	1	16/M	Extraskeletal chest wall	CD99 − Desmin + SYN + Neurofilament −
EWS/SP3	1	16/M	Forehead, lungs and right kidney	CD99 Neurofilament and NSE showed focal staining

Abbreviations: M, Male; F, Female; +, Positive staining; −, Negative staining; NSE, Neuron specific enolase; SYN, Synaptophysin

POU5F1 is a member of the POU homeodomain transcription factor family, and is often referred to by its alternate name OCT4 (101). POU5F1 expression is restricted predominantly to embryonic stem cells and germ cells, and it has clear roles in regulating pluripotency/stemness (102,103). The resulting EWS/POU5F1 fusion protein is thought to function as an aberrant oncogenic transcription factor via its amino-terminal transactivation domain (from EWS) and its carboxy-terminal DNA-binding domain (from POU5F1). *POU5F1* has a tissue restricted expression pattern, but as is the case for EWS/ETS fusions, constitutive expression of the EWS/POU5F1 transcript is driven by the native *EWSR1* promoter.

Histopathologically, tumor cells harboring the EWS/POU5F1 fusion displayed a highly undifferentiated phenotype, stained positive for vimentin and neuron specific enolase (resembling some features of Ewing's sarcoma), but were negative for CD99 staining (100) (Table 1). The predicted structure of the EWS/POU5F1 protein (amino-terminal EWS transactivation domain and carboxy-terminal POU5F1 DNA-binding domain) suggests that it may function as a transcriptional regulator that modulates target genes similar to other EWS chimeric fusion proteins identified in Ewing's sarcoma. Therefore, despite the older age of diagnosis and the negative CD99 staining it is still formally possible that EWS/POU5F1 expressing tumors may represent a variant of Ewing's sarcoma, but further consideration of this issue is needed.

EWS/SMARCA5

The non-TET/ETS fusions found in Ewing's-like tumors have been fusions between EWS and other DNA-binding transcription factors. A recently described translocation in a Ewing's-like tumor, t(4;22)(q31;q12), encodes for a somewhat different type of fusion (104). In this case, the *EWSR1* gene is fused to the chromatin remodeling gene *SMARCA5*. This chimeric fusion is generated by the fusion of exon 7 of *EWSR1* and exon 8 of the *SMARCA5* gene (Figure 3). Similar to many of the rare TET/ETS fusions and the non-TET/ETS fusions described, the location of the EWS/SMARCA5 expressing tumor was extraskeletal. The EWS/SMARCA5 fusion was identified in an extraskeletal tumor in a 5 year old female. Immunohistochemical studies performed on the tumor demonstrated positive staining for CD99, vimentin, synaptophysin, neuron specific enolase and negative staining for desmin, resembling some features of Ewing's tumors (Table 1).

The SMARCA5 protein (also called SNF2H) is the ATPase component of various ATP-dependent chromatin remodeling complexes, including CHRAC, NoRC, RSF, WICH, ACF/BAZ-like and NuRD (105–109). SMARCA5 shares a great deal of similarity at the amino acid level with the ISWI family of chromatin remodelers in *Drosophila*, which function mainly in organized spacing of nucleosomes along DNA, and thereby modulate accessibility of chromatin for transcription factor binding (110).

The EWS/SMARCA5 fusion protein retains the amino-terminal EWS transactivation domain fused with a carboxy-terminal chromatin remodeling domain. One key difference between the previously identified TET/ETS fusions and the EWS/SMARCA5 fusion is the lack of site-specific DNA binding. The assumption here is that EWS/SMARCA5 indirectly causes transcriptional deregulation by altering accessibility of DNA for transcription factor binding. Whether there is specificity to this activity for specific oncogenic gene loci, and whether this specificity is altered by the presence of the EWS domain in the fusion is not known.

EWS/SMARCA5 is an oncoprotein because NIH3T3 cells transduced with the fusion exhibit anchorage independent transforming potential similar to that seen with NIH3T3 cells expressing EWS/FLI (104). While this is an important indicator of oncogenic potential, it appears that NIH3T3 cells expressing EWS/ETS fusions are a poor mimic of Ewing's sarcoma, as the gene expression pattern induced by the fusions in those cells does not recapitulate the gene expression pattern of *bona fide* Ewing's sarcoma (84). This suggests that although NIH3T3 cells are transformed by the EWS/SMARCA5 fusion, the molecular pathways leading to transformation could be different from those used in Ewing's sarcoma.

Considering the structural and functional disparities between EWS/ETS and EWS/SMARCA5, an important underlying question is whether the EWS/SMARCA5 fusion gives rise to Ewing's sarcoma, or does it give rise to a different tumor type that bears some resemblance to Ewing's sarcoma at the histopathological level, but is a completely different tumor at the molecular level?

Of interest to this discussion, atypical teratoid/rhabdoid tumors (AT/RT) are highly malignant tumors that affect children typically in infancy and early childhood. The gene responsible for the initiation of AT/RT is *SMARCB1*, a core component of the SWI/SNF ATP-dependent chromatin-remodeling complex (111,112). Similar to SMARCA5, SMARCB1 functions to displace nucleosomes and in turn regulates transcription by modulating chromatin structure. This regulation has been shown to control proliferation and affect cell cycle progression (113). In AT/RT, loss of the *SMARCB1* gene causes aberrant cell cycle progression, partly via the downregulation of p16INK4a, a tumor suppressor (114). Furthermore, SMARCB1 binds the promoter of Cyclin D1 and regulates its overexpression (113).

SMARCB1 encodes for the protein SMARCB1 (also called SNF5 or INI1). AT/RTs exhibit inactivation of this gene. While the *EWS/SMARCA5* fusion gene is an oncogene, it is at least formally possible that its oncogenic function is dependent on a "dominant-negative" activity that inhibits the function of the remaining free wild-type SMARCA5 as well as protein complexes containing wild-type SMARCA5. Perhaps any inactivation of the ISWI or the SWI/SNF chromatin remodeling complexes can cause tumorigenesis.

EWS/ZSG

Mastrangelo et al. (2000) presented the first report of an intrachromosomal rearrangement by a paracentric inversion of chromosome 22 (22q12) in a tumor that initially was thought to histologically resemble a peripheral primitive neuroectodermal tumor (ie, a Ewing's sarcoma) (115). This inversion results in the fusion of the 5' portion of the *EWSR1* gene to a newly identified zinc finger sarcoma gene (*ZSG*). The EWS/ZSG fusion was identified in an extraskeletal chest wall primary tumor in a 16 year old male patient.

ZSG encodes for a novel Cys_2-His_2 motif containing zinc-finger protein. This zinc finger protein shares a high level of similarity with the human myc-associated zinc finger protein (MAZ), a transcription factor that binds the *c-MYC* promoter and regulates its transcription (116), suggesting that ZSG may also function as a transcription factor. The translocation produces a chimeric fusion protein as a result of an in-frame fusion of *EWSR1* exon 8 with part of exon 1 of *ZSG*, due to the creation of an acceptor splice site within exon 1 of *ZSG* (Figure 4). As has been documented for the EWS/FLI fusion, expression of the reciprocal ZSG/EWS fusion rearrangement could not be detected. Additional analysis of the tumor from which this fusion was identified revealed a rearrangement of the second *ZSG* allele (in addition to the primary translocation), leading to complete loss of wild-type ZSG expression. This suggests that wild-type *ZSG* might function as a tumor suppressor gene. The EWS/ZSG expressing primary tumor was localized to the chest wall, similar to the Askin's tumor variant of Ewing's sarcoma. Immunophenotyping showed positive staining for desmin, a feature more typical of DSRCT and negative staining for CD99 protein (a classic hallmark of Ewing's sarcoma) (Table 1).

Structurally, the EWS/ZSG fusion protein retains the carboxy-terminal DNA-binding domain of the wild-type zinc finger protein but lacks the amino-terminal POZ domain which typically functions as a transcriptional repression domain. This is an interesting finding in light of another zinc finger protein involved in transcriptional repression, WT1, which is fused to EWS in DSRCT (117). Hence, the chimeric products EWS/WT1 and EWS/ZSG share similarities of being zinc finger proteins, lacking amino-terminal repression domains as a result of the translocations, and each occurs in tumors that lack CD99 expression. Thus, both of these fusions may convert zinc-finger transcriptional repressive proteins into transcriptional activators.

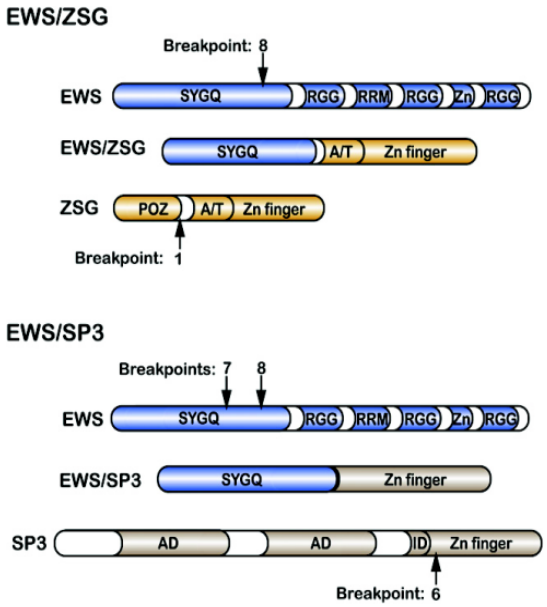

Figure 4 Domain structures of wild-type EWS and Zinc-finger proteins, ZSG and SP3. The resultant EWS/ZSG and EWS/SP3 chimeric fusions are non-TET/ETS fusions in "Ewing's-like" tumors. A/T: A-T hook DNA binding motif, a minor groove tether; Zn finger: Cys_2-His_2 zinc fingers; ID: Inhibitory domain. Arrows indicate breakpoints observed in wild-type EWS, ZSG and SP3 included in the EWS/ZSG and EWS/SP3 fusions in "Ewing's-like" tumors.

One important question is what disease do tumors harboring EWS/ZSG represent? Is this a variant of DSRCT? Is this a rare variant of Ewing's sarcoma? Is this an entirely new entity?

EWS/SP3

In 2007, one sarcoma case was reported with a fusion of the *EWSR1* gene with *SP3*, a gene of the Sp zinc-finger family. The EWS/SP3 fusion was identified in a 16 year old Caucasian male who presented with disseminated disease of the forehead, bones, right kidney and lungs. The tumor expressed an in-frame fusion of *EWSR1* exon 7 with exon 6 of *SP3* (Figure 4) (91). A second longer chimeric EWS/SP3 fusion was also detected in the tumor, generated by the fusion of *EWSR1* exon 8 and *SP3* exon 6 with a 31 nucleotide sequence insertion to restore the reading frame. The longer EWS/SP3 "8/6" fusion transcript, however, was less abundant in comparison to the shorter EWS/SP3 "7/6" fusion transcript. Immunophenotyping showed weak focal staining for CD99, neuron specific enolase and neurofilament (Table 1). Similar to the rare EWS/ETS fusions EWS/ETV1, EWS/ETV4 and EWS/FEV, the EWS/SP3 expressing tumor was found in an extraskeletal primary site. The wild-type SP3 protein possesses an inhibitory domain (ID) which is lost in the EWS/SP3 fusion protein. Loss of a repression domain in the context of the translocation fusion protein is a feature that EWS/SP3 shares with EWS/ZSG and EWS/WT1. This finding further highlights the importance of transactivating functions driven by aberrant fusion oncogenes in sarcomas and suggests that the EWS/zinc-finger fusion proteins participate in tumorigenesis through transcriptional deregulation. As was the question with the EWS/ZSG fusion discussed above, the question here again is whether EWS/SP3 fusions give rise to variant-small round cell tumor (SRCTs) or do they cause Ewing's-like tumors that share phenotypic resemblances, but are molecularly distinct, from Ewing's sarcoma?

Cell-of-origin and cellular context in Ewing's sarcoma

One important aspect that is crucial to understanding the biology of Ewing's sarcoma is the identification and characterization of the cell-of-origin for the disease. Currently there is no resolution to this issue. On the one hand, there is a growing body of work suggestive of a mesenchymal stem or progenitor cell as the precursor cell type for Ewing's sarcoma (118–122). On the other hand, several observations are consistent with a neural crest cell-of-origin (123–125). Interestingly, a new idea that has been recently put forth encompassing both the above mentioned cell types suggests that Ewing's sarcoma may arise from a neural crest stem cell exhibiting mesenchymal features or from a mesenchymal stem cell that is neural derived (126,127).

Given the enigma surrounding the cell-of-origin of Ewing's sarcoma, the cellular context most appropriate to study EWS/FLI and other variant translocation fusions also remains unresolved. Expression of EWS/FLI in a variety of different cell types including primary human fibroblasts, mouse embryonic fibroblasts, immortalized rat fibroblasts, neural crest progenitor cells and rhabdomyosarcoma cells results in growth arrest (33,128). Although NIH3T3 cells display a transformed phenotype when transduced with EWS/FLI, the gene expression pattern of these cells is markedly different from that found in the *bona fide* disease (84).

The importance of cellular context is further highlighted by a number of recent attempts to generate a mouse model for Ewing's sarcoma. Since constitutive expression of EWS/FLI in mice leads to embryonic lethality, one attempt at making a Ewing's mouse was based on a strategy in which EWS/FLI was knocked-in at the Rosa-26 locus. EWS/FLI expression was then targeted to bone marrow progenitor cells by crossing to mice expressing an *Mx1-cre* driver (129). However, these mice did not develop sarcomas and developed myeloid/erythroid leukemias instead, likely due to the preferential expression of EWS/FLI in hematopoietic precursors driven by the *Mx1-cre* recombinase. In another study, expression of EWS/ERG in mice driven by *Rag-1 cre* resulted in the generation of T-cell lymphomas likely due to the fact that the *Rag-1 cre* recombinase is preferentially expressed in lymphocytes (130,131). Although it was surprising that EWS/FLI and EWS/ERG expression gave rise to leukemias and lymphomas but not sarcomas, it is important to note that TLS/ERG, a related TET/ETS chromosomal translocation, is a recurrent genetic abnormality associated with poor prognosis in human acute myeloid leukemia (AML), secondary AML associated with myelodysplastic syndrome (MDS), and chronic myeloid leukemia (CML) (132–134). Furthermore, there are rare reports that support the findings that expression of EWS/FLI and other EWS/ETS fusion proteins occur in isolated cases of leukemias and biphenotypic sarcomas exhibiting features of myogenic and neural differentiation (135,136). This suggests that EWS/FLI and other TET/ETS fusions can trigger oncogenic transformation in cell types different from precursors that gives rise to Ewing's sarcoma.

In an attempt to avoid expression of EWS/FLI in the hematopoietic compartment, conditional expression of EWS/FLI in the primitive mesenchyme of the early limb bud (mesoderm-derived tissues) was achieved by crossing with the *Prx-1 cre* driver (137). This model also did not give rise to sarcomas spontaneously and did so only when the *Tp53* gene was simultaneously mutated. The tumors that were generated were described as "undifferentiated sarcomas." The authors did not specifically claim that these were true Ewing's sarcomas. This model further underscores the significance of cellular context and brings up an important concept that additional mutations may be required to cooperate with EWS/FLI in order to give rise to Ewing's sarcoma. This concept is further supported by the inability of human mesenchymal stem cells expressing EWS/FLI to form tumors when injected into immunodeficient mice, likely due to the lack of critical cooperating mutations required for transformation (121). Hence, the tumors that arise due to the expression of an EWS/ETS translocation fusion, whether it is Ewing's sarcoma, a variant Ewing's-like tumor, or a leukemia, depends on the cellular background in which the translocation occurs and its interplay with cooperating mutations.

One highly speculative hypothesis is that differences in gene regulation by EWS/FLI via GGAA-microsatellites is the primary reason why no organisms except for humans (with the exception of a possible single case in a camel (138))

have ever been reported to develop Ewing's sarcoma. For example, NR0B1 is required for the oncogenic phenotype of Ewing's sarcoma. The human *NR0B1* promoter harbors a GGAA microsatellite that is critical for upregulation of this gene by EWS/FLI. In contrast, the mouse *Nr0b1* promoter lacks the GGAA-microsatellite, and cannot be induced by EWS/FLI in murine NIH3T3 cells (59). This suggests that even if the EWS/FLI fusion were expressed in mice via genetic engineering it would be unable to upregulate critical microsatellite-containing target genes like *NR0B1, CAV1,* and *GSTM4*. Without upregulation of these critical targets, Ewing's sarcomas could not form.

Potential mechanisms of chromosomal translocations in Ewing's sarcoma

There is relatively little known about the mechanism of generation of TET/ETS (and non-TET/ETS) chromosomal translocations in Ewing's and Ewing's-like tumors, but a few hypotheses have been advanced since the discovery of EWS/FLI nearly 20 years ago. Homologous recombination at site-specific sequences has been suggested as the potential mechanism of chromosomal translocations in human hematological malignancies such as lymphoid neoplasms (139). In contrast, analysis of 113 interchromosomal junctions of the t(11;22) translocation from 77 Ewing's sarcoma tumors and cell lines demonstrated that generation of the EWS/FLI fusion does not rely on site-specific recombination because translocations were initiated independently on each chromosome (11 and 22) in regions that lacked homology. This study suggested that the generation of chromosomal translocations in Ewing's sarcoma may be mediated by a mechanism of illegitimate recombination that initiates the translocation event independently on each chromosome before interchromosomal joining (66).

Another emerging concept in the field is related to nuclear architecture and chromosomal positioning. This hypothesis is based on the idea that actively transcribed genes are found in euchromatin, and these regions tend to cluster together near the center of the nucleus (140). Given their close proximity in three-dimensional nuclear space, occasional spontaneous DNA breakage may lead to fusion of non-homologous chromosomes (potentially because of regions of micro-homology or because of illegitimate recombination) resulting in chromosomal translocation (141). In support of this hypothesis, it has been shown that the derivative chromosomes (11 and 22) involved in the translocation exhibit shifted positions in Ewing's sarcoma cell nuclei in comparison to the native non-aberrant *EWSR1* and *FLI1* loci (142).

A newer hypothesis is that DNA strand breaks occur specifically at sites of active transcription. It has been shown that topoisomerase IIβ (TOP2B) is recruited to sites of active transcription by nuclear hormone receptors (143,144). TOP2B enables transcription by relieving topological strain on the DNA by cleaving and re-annealing double stranded DNA. Interestingly, it was recently suggested that the generation of the TMPRSS2-ERG translocation in prostate cancer is mediated by androgen signaling induced androgen receptor-TOP2B activity (144). The details of transcriptional regulation of *EWSR1* and *FLI1* in a "precursor" Ewing's sarcoma cell are unknown. However, given the peak incidence of Ewing's sarcoma during puberty, with its associated elevated hormonal signaling, it is tempting to speculate that a similar nuclear hormone receptor-TOP2B double strand break mechanism also mediates translocation development in Ewing's sarcoma.

Is it time to revisit the concept that TET/ETS fusions are pathognomonic for Ewing's sarcoma?

The initial discovery of EWS/FLI and the demonstration that this fusion functions as an aberrant transcription factor to mediate oncogenesis in Ewing's sarcoma provided a simple model for Ewing's sarcoma tumorigenesis: EWS/FLI sits at the top of a transcriptional hierarchy to dysregulate a set of target genes that together mediate tumor formation. The discovery of the EWS/ERG fusion, with its highly conserved domain structure to EWS/FLI, provided additional support for this hypothesis. Indeed, a number of downstream gene targets of EWS/FLI have been identified that participate in the oncogenic phenotype of Ewing's sarcoma. Identification of additional examples of EWS/ETS and TLS/ETS fusions further supported this notion. Indeed, many investigators have suggested that Ewing's sarcoma could be molecularly defined by the presence of a TET/ETS fusion. While the vast majority of Ewing's sarcomas would harbor EWS/FLI fusions, the ongoing identification of other TET/ETS fusions provided some support that "non-EWS/FLI containing Ewing's sarcomas" simply had one of the rare TET/ETS variants instead.

This straightforward hypothesis, however, now has to be revisited. The "alternate" EWS-based fusions, including EWS/NFATc2, EWS/POU5F1, EWS/SMARCA5, EWS/ZSG, and EWS/SP3, are unlikely to bind and regulate exactly the same set of target genes as EWS/FLI and the other TET/ETS fusions. Furthermore, it is unclear whether tumors harboring these alternate fusions are similar enough to Ewing's sarcoma to be considered the same tumor type. At least EWS/ZSG and EWS/SP3-containing tumors have some similarity to DSRCTs. The others, though, have more similarity to Ewing's sarcoma, at least on a histological basis.

If we assume that these alternate fusions arise in tumors that are similar to Ewing's sarcoma, we can reason that there are at least three different hypotheses to explain such findings. These hypotheses are not necessarily mutually exclusive.

Hypothesis 1: The "coincidence hypothesis"

This hypothesis states that it is simply coincidence that the non-TET/ETS containing tumors are histologically similar to Ewing's sarcoma, but they have no significant similarities to that disease in their underlying molecular biology. In this scenario, the non-TET/ETS fusions are indeed oncogenic, but they regulate a different set of target genes than the TET/ETS fusions do. These fusions could occur in the same (currently unknown) precursor cell that is also the cell-of-origin for Ewing's sarcoma, and so may have histological similarities because of a similar cell-of-origin. Alternately, they may arise in distinct cell types, but cause a similar cell morphologic and

marker pattern to Ewing's sarcoma by chance alone. One conceptual possibility is that the various TET/ETS and non-TET/ETS fusions give rise to sarcomas that bear phenotypic resemblance but may occur in different cell types.

An alternate version of this hypothesis is that the non-TET/ETS fusions are simply "passenger" mutations. Although EWS/FLI has been formally shown to function as a "driver" mutation (for example, through model systems that "knock-down" EWS/FLI expression in patient-derived Ewing's sarcoma cells), a similar level of analysis has not been performed for the other fusions. Thus, although unlikely, it is possible that there are yet-to-be-discovered "driver" mutations in the Ewing's-like tumors that mediate oncogenesis. Similarities between these tumors and EWS/ETS-containing tumors would then simply be coincidental as described above.

Hypothesis 2: The "common gene-target hypothesis"

This hypothesis states that although the TET/ETS and the non-TET/ETS fusions have vastly different DNA binding domains (or in the case of EWS/SMARCA5, a chromatin-remodeling ATPase domain), they would have significant overlap in target genes that are dysregulated. The common overlap might include a small group of "core" regulators of the Ewing's sarcoma oncogenic program. For example, these might include genes such as *NR0B1*, *NKX2.2*, and *GLI1*, which have been shown to be absolutely essential for oncogenic transformation in Ewing's sarcoma (31,44,48). Alternately, the common overlap might include a broader array of EWS/FLI target genes that contribute to various other aspects of tumor growth and progression, such as *IGFBP3*, *GSTM4*, *CDKN1A*, *TGFBRII*, *VEGF*, and *CAV1* (42,52–55). Some of these genes represent direct targets of EWS/FLI, while others are indirectly regulated by the fusion. The non-TET/ETS fusions may also regulate these target genes via other transcription regulatory sites (ie, non-ETS binding sites), or alternately, the non-TET/ETS fusions may regulate one or more ETS-family transcription factors that subsequently regulate transcription of some/many/all EWS/FLI target genes. This would effectively recapitulate the oncogenic transcriptional program of Ewing's sarcoma.

An alternate form of this hypothesis is that some of the requisite transcriptional patterns might be normally expressed by the tumor cell-of-origin. Thus, the non-TET/ETS fusions might modulate the expression of a limited set of genes, while other required genes might be "contributed" by the tumor cell-of-origin. Some required genes might even be activated or repressed through somatic mutations in the tumor. Other mechanisms to recapitulate the TET/ETS gene expression pattern could also be envisioned, such as contribution by unique tumor microenvironments, specific signaling milieu, etc.

Hypothesis 3: The "non-gene expression hypothesis"

This hypothesis would suggest that although gene regulatory function is likely to be important in oncogenesis by both TET/ETS and non-TET/ETS fusions, there may be a significant contribution by non-DNA binding-dependent activities of the fusion proteins. As noted earlier, there is some evidence that EWS/FLI may have DNA binding independent function (57,58). While the molecular details of these alternate functions are not known, they could be related to a dominant-negative function of EWS/FLI blocking the normal function of wild-type EWS. Non-TET/ETS fusions still contain the amino-terminus of EWS, and so if that domain is important for non-DNA binding functions, it could contribute to the oncogenic activity in a similar manner in the non-TET/ETS fusions. Similarly, it is at least formally possible that EWS-based fusion proteins participate in signaling cascades required for oncogenesis. The non-TET/ETS fusions may also be able to participate productively in such signaling pathways and thus contribute to "Ewing's-like" oncogenesis.

Should we care if non-TET/ETS-containing tumors are Ewing's sarcoma?

Ewing's sarcoma itself is a rare tumor, with approximately 250 new cases occurring in the United States each year (145). If each of the rare TET/ETS and non-TET/ETS fusion variants occurs in 1% of cases or less, these will contribute to a very small portion, and a very small total number of tumors. Are these an important set of tumors to understand?

We would suggest that these are indeed important tumors to understand. In the first place, patients will develop tumors harboring these rare fusion variants. If they turn out to be similar or identical to Ewing's sarcoma in their response to therapies, then it is important to make a definitive diagnosis so that physicians may provide the best possible treatments for these patients. In contrast, if these tumors do not respond in a manner similar to Ewing's sarcoma, then we are providing ineffective therapies for these patients by simply lumping them together with other Ewing's sarcoma patients. Indeed, this is a major consideration for all rare tumors, where the likelihood of gathering adequate numbers of patients to perform well-powered clinical trials is slim at best.

In the second place, we believe that understanding these tumors may provide unique opportunities to understand the specifics of Ewing's sarcoma development. For example, if the non-TET/ETS tumors harbor specific mutations that inactivate important tumor suppressors, such observations may allow researchers to determine whether these tumor suppressors are inhibited by a TET/ETS fusion in Ewing's sarcoma. Indeed, one significant problem in understanding the oncogenic program mediated by EWS/FLI and other TET/ETS fusions is that there are hundreds to thousands of genes dysregulated by the fusion. Understanding which of these are important to oncogenesis is a Herculean task. Rare non-TET/ETS fusions may be extremely helpful in this understanding.

In the third place, even if such rare non-TET/ETS Ewing's-like tumors do not provide a better understanding of Ewing's sarcoma itself, they may provide unique insights into the underlying mechanisms of tumorigenesis. Such an understanding may have general applicability, or at least applicability to some other types of tumors. Furthermore, understanding rare tumor types has often allowed for a deeper understanding of basic cell biology and molecular processes.

Summary and conclusions

Ewing's sarcoma is an enigmatic cancer driven by chromosomal translocation derived fusion oncogenes. TET/ETS proteins are undoubtedly the central mediators in the pathogenesis of Ewing's sarcoma. In particular EWS/FLI, the most common gene rearrangement in Ewing's sarcoma, is widely used as a molecular diagnostic marker for the disease. However, recent identification of an increasing number of similar TET/ETS as well as non-TET/ETS rearrangements has further complicated molecular diagnostics for Ewing's sarcoma. Large scale genomic approaches followed by detailed molecular and functional studies could be performed to dissect out the shared, as well as the divergent, mechanisms driven by the rare non-TET/ETS fusions. Such studies will help in characterizing the nature of the non-TET/ETS fusion harboring tumors and in deciphering if these tumors are in fact Ewing's sarcoma. The knowledge gained may help shed more light on the mechanisms that drive the pathogenesis of Ewing's sarcoma and may translate into new targeted therapies for patients afflicted with this aggressive disease. Ultimately, the improved molecular insights may advance our understanding of other cancers that are driven by the dysregulation of the TET and ETS family of proteins.

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CHAPTER 3

MICROSATTELITES AS EWS/FLI RESPONSE ELEMENTS

IN EWING'S SARCOMA

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Microsatellites as EWS/FLI response elements in Ewing's sarcoma

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The ETS gene family is frequently involved in chromosome translocations that cause human cancer, including prostate cancer, leukemia, and sarcoma. However, the mechanisms by which oncogenic ETS proteins, which are DNA-binding transcription factors, target genes necessary for tumorigenesis is not well understood. Ewing's sarcoma serves as a paradigm for the entire class of ETS-associated tumors because nearly all cases harbor recurrent chromosomal translocations involving ETS genes. The most common translocation in Ewing's sarcoma encodes the EWS/FLI oncogenic transcription factor. We used whole genome localization (ChIP-chip) to identify target genes that are directly bound by EWS/FLI. Analysis of the promoters of these genes demonstrated a significant over-representation of highly repetitive GGAA-containing elements (microsatellites). In a parallel approach, we found that EWS/FLI uses GGAA microsatellites to regulate the expression of some of its target genes including *NR0B1*, a gene required for Ewing's sarcoma oncogenesis. The microsatellite in the *NR0B1* promoter bound EWS/FLI *in vitro* and *in vivo* and was both necessary and sufficient to confer EWS/FLI regulation to a reporter gene. Genome wide computational studies demonstrated that GGAA microsatellites were enriched close to EWS/FLI-up-regulated genes but not down-regulated genes. Mechanistic studies demonstrated that the ability of EWS/FLI to bind DNA and modulate gene expression through these repetitive elements depended on the number of consecutive GGAA motifs. These findings illustrate an unprecedented route to specificity for ETS proteins and use of microsatellites in tumorigenesis.

ChIP-chip | transcription | gene regulation | ETS | NR0B1

ETS proteins are extremely important in human tumor development. The first ETS gene, *v-ets*, was initially identified as part of the E26 avian erythroblastosis virus and corresponded to the human *ETS1* protooncogene (1, 2). Based on the presence of a DNA-binding ETS domain, 27 unique human ETS family members have been identified (3). ETS family members are frequently dysregulated and/or mutated in human cancers through chromosomal rearrangements. Indeed, the observation that ≈70% of prostate cancers harbor translocations between ETS genes (*ERG*, *ETV1*, or *ETV4*) and androgen-responsive genes indicates that ETS gene rearrangements may be the most common chromosomal abnormalities in human cancer (4, 5).

Most members of the ETS family bind to DNA sequences containing a GGAA (or in some cases, GGAT) core motif, with sequences flanking the GGAA core contributing to the affinity and specificity of the interaction (3, 6, 7). Because most cell lines examined express multiple ETS family members simultaneously, and because ETS factors in many cases are not functionally redundant, there are likely mechanisms to allow for gene-specific regulation by different ETS proteins (8). Indeed, recent whole genome localization studies have supported this concept by demonstrating that *in vivo* ETS-binding sites may be grouped into two classes (9): (*i*) high-affinity binding sites found close to transcription start sites and (*ii*) lower-affinity binding sites found in close proximity to low affinity binding sites for other transcription factors that allow for cooperative DNA binding.

Ewing's sarcoma was the first tumor in which ETS family members were shown to be involved in chromosomal translocations and serves as a paradigm for ETS-driven cancers (10). Ewing's sarcoma is a highly malignant solid tumor of children and young adults that usually harbors a recurrent chromosomal translocation, t(11;22)(q24;q12), that encodes the EWS/FLI fusion oncoprotein (10). The oncoprotein consists of a transcriptional activation domain from EWS, joined, in frame, to a region of the ETS transcription factor FLI harboring a DNA-binding domain (10–12). EWS/FLI functions as an aberrant transcription factor that regulates genes involved in the tumorigenic phenotype of Ewing's sarcoma (11–15).

EWS/FLI plays a critical role in establishing and maintaining the tumorigenic phenotype of Ewing's sarcoma cells (13, 15–18). Thus, EWS/FLI regulates its downstream target genes nonredundantly with other coexpressed ETS factors in Ewing's sarcoma (13, 15, 18). EWS/FLI gene expression signatures include genes that are important for Ewing's sarcoma oncogenesis, such as *NR0B1* (13). EWS/FLI up-regulates *NR0B1* in Ewing's sarcoma cells, and this up-regulation is required for their transformed phenotype (13). Whether *NR0B1* is regulated by EWS/FLI directly, or through other intermediary proteins, is unknown. Indeed, the transcriptional response elements that EWS/FLI uses to regulate its target genes are largely unknown.

One difficulty in the study of EWS/FLI is that the human cell of origin of Ewing's sarcoma is not currently known. Thus, some studies of EWS/FLI function have relied on heterologous cell types as model systems, but results from these systems may not be applicable to the human disease (19, 20). We recently developed a system that allows for the study of EWS/FLI in a relevant model system: in Ewing's sarcoma itself (13, 15, 17, 20). To understand the mechanisms by which EWS/FLI regulates its target genes in Ewing's sarcoma itself, we undertook two parallel approaches, including a genome wide analysis of fusion protein-binding sites in patient-derived Ewing's sarcoma cells and a directed analysis of EWS/FLI-regulated promoters. We found that EWS/FLI uses GGAA-containing microsatellites to regulate some of its target genes, including its key oncogenic target *NR0B1*. This demonstrates a new role for microsatellites in human cancer and suggests a unique mechanism for ETS transcription factor regulation of target genes.

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Results

EWS/FLI functions as an aberrant ETS-type transcription factor in Ewing's sarcoma to regulate genes involved in tumorigenesis (11). To identify genes that are direct EWS/FLI targets, we performed genome wide localization studies ("ChIP-chip") of the endogenous fusion protein in patient-derived A673 Ewing's sarcoma cells. Because wild-type FLI protein is not expressed in these cells, the anti-FLI antibody used for these studies only immunoprecipitates EWS/FLI in this context (15). Agilent 244k promoter microarrays were used, which interrogate ≈17,000 human promoters from −5.5 kb to + 2.5 kb relative to the transcriptional start site. Approximately 900 genes were identified that were directly bound by EWS/FLI [supporting information (SI) Dataset S1 (XLS)]. These included previously identified direct targets, such as *TGFBR2*, *CAV1*, and *IGFBP3* (18, 21, 22).

Of importance, the gene whose promoter was most highly enriched in the ChIP-chip dataset was *NR0B1*. *NR0B1* is regulated by EWS/FLI in Ewing's sarcoma cells and is absolutely required for the oncogenic phenotype of Ewing's sarcoma (13). This earlier study was not able to determine whether *NR0B1* was a direct or indirect target of EWS/FLI. The ChIP-chip data demonstrate that *NR0B1* is bound by EWS/FLI and suggest that it may be regulated directly by the fusion oncoprotein. Inspection of the promoter did not reveal any sequences matching the previously identified *in vitro* high-affinity ETS-binding site, which also binds FLI and EWS-FLI with high affinity (ACCGGAAG/aT/c; data not shown; ref. 23). We, therefore, sought to understand the regulation of this key target to understand how EWS/FLI might function in the absence of a high-affinity binding site.

We cloned ≈1.6 kb of the *NR0B1* promoter upstream of the luciferase cDNA and used this reporter construct to test for EWS/FLI responsiveness. Knock down of endogenous EWS/FLI (using the EF-2-RNAi retroviral construct) (15) showed that this promoter fragment was responsive to the fusion protein (Fig. 1*A*). This promoter fragment was also EWS/FLI-responsive in a heterologous cell type (293EBNA; Fig. 1*B*). These data support the notion that *NR0B1* is a direct EWS/FLI target gene.

To identify the EWS/FLI response element in the *NR0B1* promoter, a series of deletion constructs were tested in the luciferase reporter assay. We found that EWS/FLI responsiveness was contained within the −1.6 to −1.1 kb promoter region (Fig. 1*B*). This 500-bp region was also sufficient to confer EWS/FLI responsiveness to a minimal promoter derived from SV40 (data not shown).

Whereas the 500-bp *NR0B1* promoter region did not contain high-affinity ETS-binding elements (ACCGGAAG/aT/c) (23), it did contain a 102-bp microsatellite consisting of 25 GGAA repeats (as well as two single base insertions; Fig. 1*C*). Because ETS family members, including EWS/FLI, bind with high affinity to consensus sequences containing a GGAA core element (9), we hypothesized that the GGAA microsatellite might represent a previously unrecognized *in vivo* EWS/FLI-binding element. We cloned the 102-bp GGAA microsatellite into a luciferase reporter construct that contained a minimal promoter derived from SV40. The microsatellite was sufficient to mediate EWS/FLI responsiveness in heterologous 293EBNA cells (Fig. 1*D*). Thus, the GGAA microsatellite is the EWS/FLI response element in the *NR0B1* promoter.

In a parallel approach to identify potential EWS/FLI binding and response elements, we used an unbiased computational approach [multiple Em for motif elicitation (MEME)] (24) to identify sequence motifs that were enriched near the most highly EWS/FLI-bound DNA fragments identified in the ChIP-chip experiment. The MEME analysis identified a sequence (ACCGGAAGTG; E value = 1.4×10^{-40}) that perfectly matched the

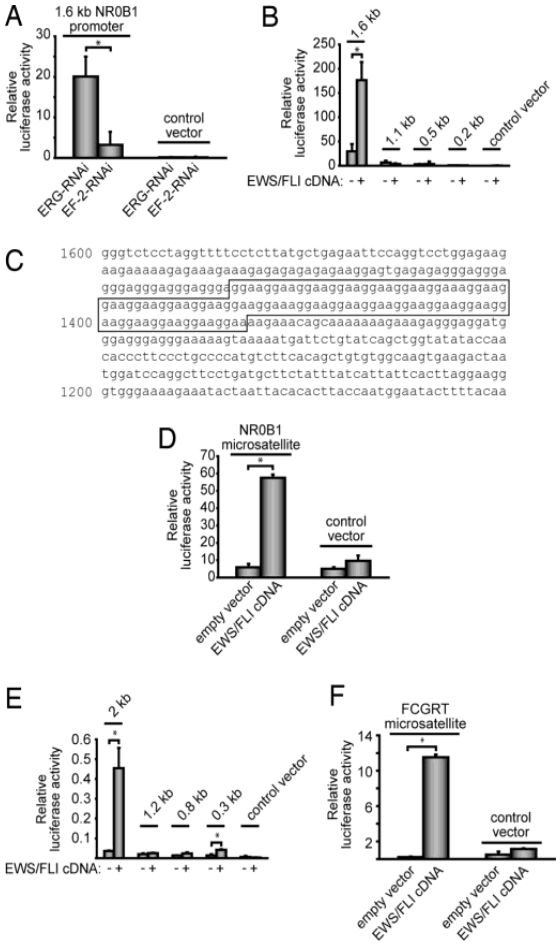

Fig. 1. The GGAA microsatellites in the *NR0B1* and *FCGRT* promoters are the EWS/FLI response elements. (*A*) TC71 Ewing's sarcoma cells cotransfected with a 1.6-kb *NR0B1* promoter luciferase vector and either EF-2-RNAi (targeting EWS/FLI) or ERG-RNAi (negative control). The "control vector" does not contain *NR0B1* promoter elements. The error bars the figure indicate SDs, and asterisks indicate $P < 0.05$. (*B*) 293EBNA cells cotransfected with the indicated *NR0B1* promoter luciferase vectors (containing the indicated amount of promoter sequence upstream of the transcriptional start site) and an EWS/FLI (or empty control) cDNA expression vector. The control vector does not contain *NR0B1* promoter elements. (*C*) *NR0B1* promoter with GGAA microsatellite indicated. The microsatellite contains 25 GGAA repeats. (*D*) Luciferase assays in 293EBNA cells with the full 102-bp *NR0B1* microsatellite upstream of a minimal promoter element. The control vector does not contain the microsatellite, but does contain the minimal promoter element. (*E* and *F*) Luciferase reporter assays by using 293EBNA cells and either the indicated *FCGRT* promoter deletion constructs or the isolated *FCGRT* GGAA microsatellite upstream of a minimal promoter, respectively. Of note, the *FCGRT* microsatellite is present at approximately −1.6 kb upstream of the transcriptional start site.

previously described high-affinity ETS-binding element (23). However, the most highly enriched sequence identified by MEME was a repetitive GGAA motif (that corresponded to the microsatellite sequence in the *NR0B1* promoter; GGAAGGAAGGAAGGAA; E value = 1×10^{-173}). The GGAA-repetitive sequence element was identified in 12 of the 134 top EWS/FLI-bound gene promoters (9%) that were used for this analysis. There was virtually no overlap between promoter elements that harbored GGAA microsatellites vs. those that contained the high-affinity ETS-binding elements (data not shown).

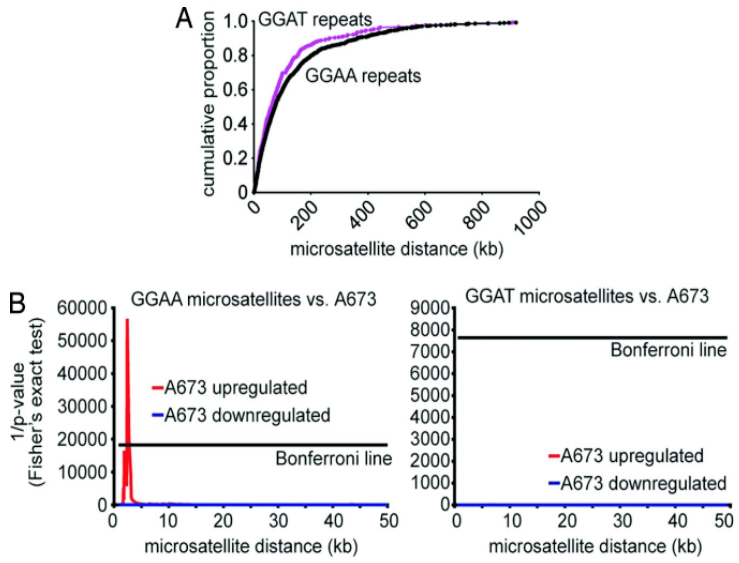

Fig. 3. Enrichment of GGAA microsatellites in the promoters of EWS/FLI-up-regulated genes. (*A*) Cumulative portion of microsatellites (GGAA or GGAT) plotted as a function of distance between the microsatellites and the closest 5′ gene edge. (*B*) Correlation between EWS/FLI-up- and -down-regulated genes (in red and blue, respectively) and microsatellite distance analyzed by Fisher's exact test in the A673 Ewing's sarcoma cell line. Significant correlations cross over the "Bonferroni line" (see *Methods*). N.B., only up-regulated genes vs. GGAA microsatellites can be seen at the scales used.

fragments bearing five, six, or seven repeats. Similar results were obtained with highly purified, recombinant FLI-derived protein (data not shown). These *in vitro* binding studies demonstrated direct binding and strongly suggested that *in vivo* occupancy detected by ChIP experiments was attributable to sequence-specific DNA binding between EWS/FLI and the microsatellite repeats and did not require other cellular proteins. Interestingly, nuclear extract containing 3xFLAG-FLI also showed a similar binding pattern with these variable repeats oligonucleotides (Fig. S1*A*).

Promoter fragments with the same set of synthetic GGAA repeats were tested for transcriptional activity in the context of the minimal SV40 promoter. Transcriptional activity required at least five consecutive GGAA repeats and exhibited increased activity with six and seven repeats (Fig. 4*C*). Similar results were observed with DNA probes representing the endogenous *NR0B1* microsatellite (data not shown). Wild-type FLI, on the other hand, was not able to regulate reporter gene activity via the *NR0B1* microsatellite (Fig. S1*B*). Taken together, although both EWS/FLI and FLI are capable of binding GGAA microsatellites, only EWS/FLI is able to transcriptionally activate via these elements.

Discussion

Our findings demonstrate that EWS/FLI uses GGAA-containing microsatellites as specific response elements for a subset of fusion protein-up-regulated genes. The use of microsatellites as cancer-relevant genetic elements has not been previously demonstrated. Up-regulation of the EWS/FLI target gene *NR0B1* has been shown previously to be necessary for the transformed phenotype of patient-derived Ewing's sarcoma cells (13). The present report demonstrates that the regulation of *NR0B1* by EWS/FLI depends on the GGAA microsatellite in the *NR0B1* promoter. Similarly, a second EWS/FLI-regulated gene, *CAV1*, which also contains a GGAA microsatellite in its promoter, has also been shown to be involved in the tumorigenic phenotype of Ewing's sarcoma (22). Although the cancer-relevant protein p53 has been shown to use a microsatellite as a response element at one of its target genes, *PIG3*, no functional role for PIG3 has been defined in tumorigenesis (26). Thus, the data presented in this report suggest a new role for microsatellites in human cancer development.

Experimental analysis of DNA probes and promoter fragments with variable numbers of repeats indicated that at least four to five consecutive GGAA motifs are required for DNA binding and gene activation and that the efficiency of these processes increased with increasing numbers of repeats. These results suggest interactions between multiple binding events. We speculate that protein–protein interactions may mediate cooperative DNA binding or that the presence of multiple sites affects the local effective concentration of active protein. Both phenomena could explain the use of suboptimal binding sequences within the GGAA-repetitive elements. The relatively high ChIP signal from the *in vivo* occupancy study and direct ChIP experiment is consistent with a mechanism that enhances the binding affinity of EWS/FLI to the microsatellites. Whereas eukaryotic promoters are often characterized by multiple transcription factor binding sites in close proximity, these findings indicate that the GGAA repetitive elements have emergent properties and do not simply represent a collection of independent binding sites.

In addition to the length-dependent interaction between EWS/FLI and GGAA microsatellites, other features may contribute to the selection of certain microsatellites as EWS/FLI-binding targets. Indeed, only ≈30% of GGAA microsatellites that could be detected by the Agilent promoter microarray used in our studies were bound by EWS/FLI (S.C.H., K.G., and S.S., unpublished observations). Furthermore, we have been unable to detect binding of endogenous wild-type FLI to GGAA microsatellites in Jurkat T cells (K.G., unpublished observation), nor have we observed enrichment of GGAA microsatellite binding in previously published ChIP-chip data of three other ETS transcription factors, ETS1, ELF, and GABPα (P.C.H., unpublished observation; ref. 9). Additional features that may contribute to ETS protein binding to microsatellites *in vivo* include local chromatin structure, nucleosome positioning, and

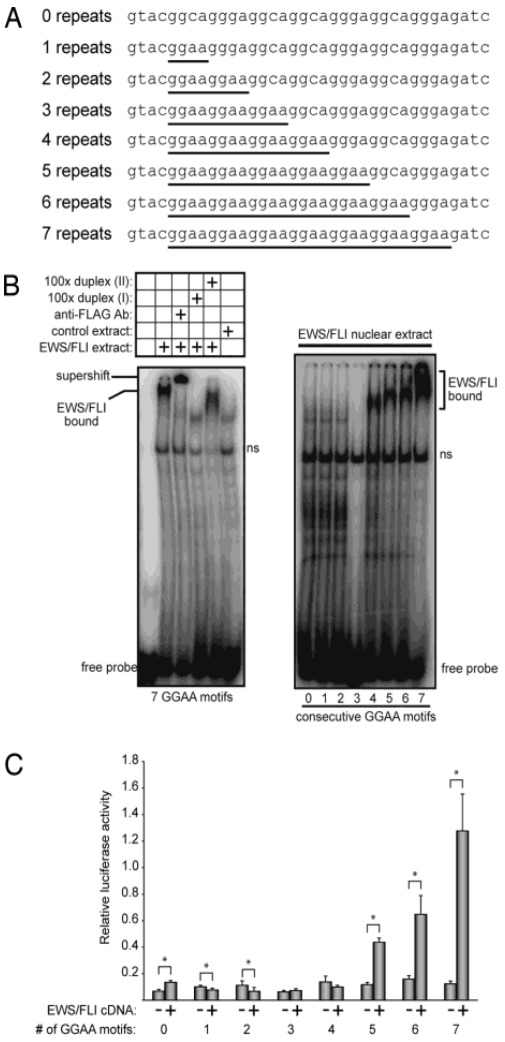

Fig. 4. Ability of EWS/FLI to bind and activate via GGAA repetitive regions depends on the number of consecutive GGAA motifs. (*A*) Sequences of the oligonucleotides used for these analyses. The GGAA repeats are underlined. (*B*) *Left* shows an EMSA with a DNA duplex containing seven consecutive GGAA motifs. A specific EWS/FLI band is present when 3xFLAG-EWS/FLI from nuclear extracts is included. This specific band is supershifted with anti-FLAG antibody and competed with DNA duplex (I) containing a high-affinity ETS site but is not competed with DNA duplex (II) containing a PU.1 site that does not bind EWS/FLI (14). A control extract that does not contain EWS/FLI produces only nonspecific binding (indicated by "ns"). *Right* shows EMSA with DNA duplexes containing the indicated number of consecutive GGAA motifs and 3xFLAG-EWS/FLI. The positions of specific EWS/FLI-bound complexes are indicated. (*C*) Luciferase assays in 293EBNA cells with 36-bp sequence containing the indicated number of consecutive GGAA motifs (as indicated in *A*) upstream of a minimal promoter. The error bars indicate SDs, and asterisks indicate $P < 0.05$.

the presence of other bound proteins at these sites, all of which may modulate the accessibility of the microsatellite for ETS protein binding. Minor sequence variations could also play a role in binding affinity to these sites. Additionally, although EWS/FLI can bind to microsatellite sequences *in vitro* without the assistance of other proteins (K.G., unpublished observations), it is possible that other proteins are required for binding *in vivo*. We demonstrated that both EWS/FLI and wild-type FLI bind to GGAA microsatellites with similar efficiency *in vitro*, but only EWS/FLI is capable of activating a microsatellite-containing reporter construct (Fig. S1). This suggests that activation of genes through these microsatellites, but not microsatellite binding, is a neomorphic function of the EWS/FLI fusion protein. Whereas these data suggest that the use of microsatellites by EWS/FLI may be a unique function of the fusion protein in Ewing's sarcoma, additional work will be required to fully address this question.

One interesting implication of this work is that differences in microsatellites (e.g., size, sequence, or location) could result in differences in Ewing's sarcoma susceptibility. Ewing's sarcoma is ≈10 times more prevalent in Caucasian populations than in African American populations (27). It may be that some microsatellites that regulate cancer-relevant EWS/FLI target genes are polymorphic between populations. Five microsatellites (including the *NR0B1* microsatellite) were analyzed for length polymorphisms in genomic DNA from groups of individuals of defined ethnic backgrounds (CEPH reference panel) (28). Whereas we did not detect any significant length polymorphism differences between African and European populations in this small series (W.S.W. and L.B.J., unpublished observations), a more comprehensive analysis is needed to fully evaluate this hypothesis.

Ewing's sarcoma has only been observed in humans. Thus, no other organism develops Ewing's sarcoma spontaneously, nor have genetically engineered mouse models of Ewing's sarcoma been reported. We speculate that at least part of this difference in susceptibility is related to differences in GGAA microsatellites between organisms. For example, there is no GGAA-containing microsatellite in the *Mus musculus Nr0b1* promoter, even though there are thousands of such microsatellites in the murine genome (data not shown). Murine Nr0b1 is not induced by EWS/FLI in NIH 3T3 cells (M.K., unpublished observation). Similarly, the GGAA microsatellites found in the EWS/FLI target genes *FCGRT* and *CAV1* are also absent in the murine orthologs (data not shown). This suggests that even if the EWS/FLI fusion were expressed in mice, either spontaneously or via genetic engineering, it would not up-regulate critical genes required for oncogenic transformation, and thus tumors would not form (13, 22). This has important implications for the development of a genetically engineered mouse model of Ewing's sarcoma and may explain why Ewing's sarcoma development is limited to humans.

Methods

DNA Cloning. The 3xFLAG-EWS/FLI MSCV-hygro retroviral expression construct and the EF-2-RNAi retroviral construct have been described previously (15, 19). 3xFLAG-FLI was prepared by using standard molecular biology approaches and was cloned into the MSCV-neo retroviral plasmid. Full-length and 5′ deleted *NR0B1* and *FCGRT* promoters were cloned into the pGL3-Basic luciferase reporter vector (Promega Corporation). Constructs without promoter elements (e.g., the 500 bp *NR0B1* microsatellite region, isolated microsatellites, and constructs containing varying numbers of GGAA motifs) were cloned into the pGL3-Promoter vector (Promega Corporation).

Cell Culture. TC71, A673, and 293EBNA cells were cultured as described previously (13, 15, 29).

Luciferase Assays. 293EBNA or TC71 cells were transfected with each firefly reporter, *Renilla* plasmid, and cDNA or RNAi plasmids. Firefly luciferase activity was normalized to *Renilla* luciferase activity to control for transfection efficiency. Two-tailed Student's *t* tests were used for statistical comparisons.

EMSA. Nuclear extracts were prepared from 293EBNA cells transfected with 3xFLAG-EWS/FLI, 3xFLAG-FLI, or empty vector control expression plasmids. Twenty milligrams of nuclear extract protein, 5 nM [^{32}P]-labeled probes, and 1× Gel Shift Binding Buffer (Promega Corporation) were used in each reaction. DNA duplex (I) (500 nM) (containing a high-affinity EWS/FLI-binding site, called "ETS2 probe" in ref. 12) and 500 nM DNA duplex (II) (bearing a variant

To test whether other genes use their GGAA microsatellites as EWS/FLI response elements, we analyzed the *FCGRT* promoter. *FCGRT* was also identified as an EWS/FLI-bound target in the ChIP-chip analysis and contains a GGAA microsatellite at approximately −1.6 kb relative to its transcriptional start site. A 2-kb region of the *FCGRT* promoter was EWS/FLI-responsive in luciferase reporter assays (Fig. 1*E*). Deletion analysis of the promoter demonstrated that the GGAA microsatellite containing region was necessary for EWS/FLI responsiveness (Fig. 1*E*). Furthermore, the GGAA microsatellite was sufficient to confer EWS/FLI responsiveness to a reporter containing a minimal SV40 promoter (Fig. 1*F*). These data provide an independent confirmation of the role of GGAA microsatellites as EWS/FLI response elements.

To further validate *in vivo* occupancy of GGAA microsatellites by EWS/FLI, directed ChIP experiments were performed at six microsatellite-containing genes: *NR0B1*, *FCGRT*, *CAV1*, *CACNB2*, *FEZF1*, and *KIAA1797*. Each of these genes contain GGAA microsatellites within 5 kb of their transcriptional start sites. We found that EWS/FLI bound to each of these GGAA microsatellite-containing promoters *in vivo* but not to control *TP53* or *RPS26* promoters, neither of which contain GGAA microsatellites (Fig. 2). None of the six microsatellite-containing promoters was significantly occupied by two other members of the ETS family, ETS1 or ELK1 (Fig. 2). Thus, binding of these promoters appears to be specific to EWS/FLI.

To determine whether EWS/FLI regulation through microsatellites is a generalized phenomenon in Ewing's sarcoma, we asked whether there was a correlation between the presence of a GGAA-containing microsatellite and EWS/FLI responsiveness. All 2,577 GGAA microsatellite-containing genes in the human genome were mapped, and the distances between the microsatellites and the transcriptional start sites were determined. As a control, all 942 GGAT microsatellite-containing genes were similarly identified and mapped. The distribution of GGAA and GGAT microsatellites relative to adjacent genes was similar (Fig. 3*A*). Genes were rank-ordered by distance between the microsatellite and the transcriptional start site. Fisher's exact test was then performed reiteratively to determine whether there was an over-representation of EWS/FLI-up- or -down-regulated genes at each rank position. The very conservative Bonferroni correction was applied to control for multiple hypothesis testing. A dataset derived from A673 cells showed significant enrichment of EWS/FLI-up-regulated genes within 5 kb of GGAA-containing microsatellites (Fig. 3*B*). EWS/FLI-down-regulated genes were not enriched, and there was no enrichment of EWS/FLI-regulated genes when compared to GGAT-containing microsatellites (Fig. 3*B*). A second independent EWS/FLI dataset derived from two other Ewing's sarcoma cell lines (TC71 and EWS502) showed a similar pattern of enrichment of EWS/FLI-regulated genes close to GGAA, but not GGAT, microsatellites (data not shown).

A series of controls that included either randomly sampled gene sets or published "cancer gene neighborhood" gene sets [group C4 from the Molecular Signature Database (MsigDB version 2, January 2007 release; http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html)] were tested in the same analyses. The A673 and TC71/EWS502 Ewing's sarcoma datasets were significantly enriched over the randomly sampled gene sets (P = 0.0005 and P = 0.0001, respectively) and were also significantly enriched as compared to the "cancer gene neighborhood" gene sets (P = 0.005 and P = 0.007, respectively). These data strongly suggest that the use of GGAA microsatellites as EWS/FLI response elements for gene up-regulation is not limited to *NR0B1* and *FCGRT* but is more widespread.

The *in vivo* occupancy and direct ChIP experiments suggested direct binding between the ETS domain of EWS/FLI and the microsatellite repeat. However, the GGAA tandem repeats are spaced too closely for each to be used as a binding site for an ETS domain. Site size requirement experiments indicate that an ETS domain requires at least 15 bp of DNA duplex, although only 9–10 bp show sequence preference (25). Furthermore, the GGAA flanks surrounding the GGAA core of a microsatellite/repetitive element do not create a sequence similar to the selected consensus site for FLI (23). To evaluate the binding properties of the repetitive elements, we performed electrophoretic mobility shift assays with DNA probes bearing variable number of repeats (Fig. 4*A*). Initially, nuclear extract from cells expressing recombinant 3xFLAG-EWS/FLI were used. EWS/FLI-specific DNA complexes were detected as confirmed by supershifts with anti-FLAG antibodies and competition experiments (Fig. 4*B*). A minimum of four repeats was necessary to detect binding (Fig. 4*B*). This result indicated that the 9- to 10-bp sequence centered within three repeats, GAAGGAAGGA, does not create a strong binding site and suggested that the affinity of EWS/FLI for the microsatellite might be enhanced by multiple binding events possible with additional repeats. Indeed, the mobility of the shifted complex was reduced with added number of repeats suggesting multiple binding events on the

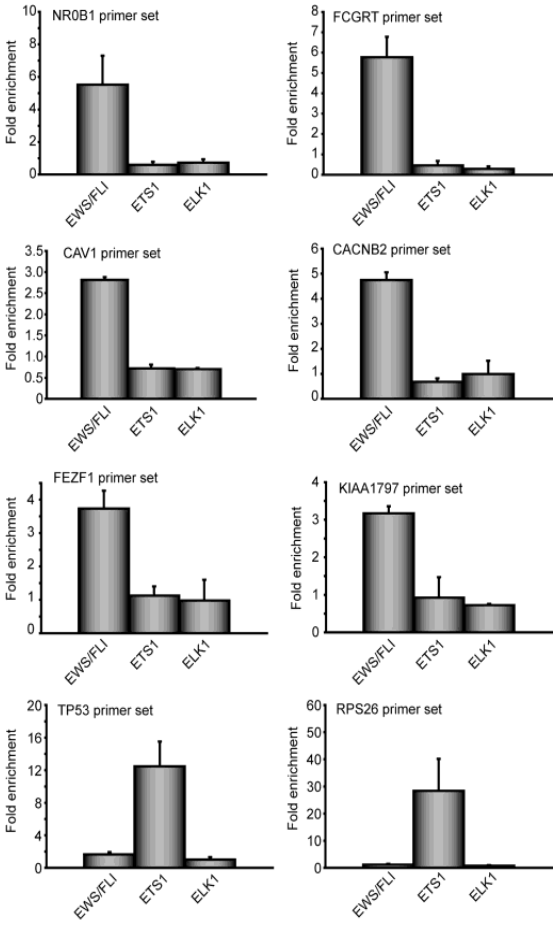

Fig. 2. EWS/FLI occupies GGAA microsatellite containing promoters *in vivo*. Chromatin immunoprecipitation of the indicated promoters from A673 Ewing's sarcoma cells by using antibodies against FLI (which recognizes EWS/FLI), ETS1, or ELK1. Data are plotted as fold enrichment for each region compared to the average enrichment of two negative control genes. The error bars indicate SEMs of two to five independent experiments.

ETS motif, which binds the ETS protein PU.1, but not EWS/FLI, called "PU.1 probe" in ref. 12) were used as specific and nonspecific unlabeled competitors for protein binding, respectively.

ChIP and Whole Genome Localization Studies (ChIP-chip). ChIP from A673 cells was performed as previously described (9), by using anti-ETS1, anti-ELK1, or anti-FLI-1 antibodies (sc-350, sc-355, or sc-356, respectively; Santa Cruz Biotechnology, Inc.). Quantitative PCR was performed with *NR0B1*, *FCGRT*, *CAV1*, *CACNB2*, *FEZF1* (*LOC389549*), *KIAA1797* (*hsa-mir-491*), *RPS26*, or *TP53* primers and with *ALB* and *BCL2L1* primers (as normalization controls). See Table S1 for sequences. For ChIP-chip, anti-FLI immunoprecipitated genomic DNA samples from A673 cells (two independent biological replicates) were processed and hybridized to Agilent 244k promoter microarrays, as described (9). These microarrays interrogate ≈17-kb human promoters from −5.5 to +2.5 kb relative to the transcriptional start site. Initial analysis of the datasets was performed by using the Agilent ChIP Analytics software (version 1.3.1) to average both replicates as previously described (9).

MEME. After processing the ChIP-chip data via the Agilent ChIP Analytics software, the most highly enriched DNA probe for each gene was identified. In some cases, the software package identified two enriched segments, suggesting that there were two separate EWS/FLI-binding sites in that gene. In each case, the genomic DNA sequence for the region surrounding the most enriched probe(s) (including the adjacent proximal and distal probes) were downloaded from the University of California at Santa Cruz Genome Browser (http://genome.ucsc.edu) by using the May 2004 Human genome assembly. Because of input data size limitations of the web-based MEME application (http://meme.sdsc.edu/meme/meme.html; version 3.5.7), only 60 kb of sequence could be used as input data (24). This corresponded to sequences from the 134 most highly enriched promoter fragments. The data were analyzed with the following parameters: any number of repetitions, minimum width of eight bases, maximum width of 16 bases, and identify two motifs.

Genome Wide *in Silico* Analysis. For the purposes of this study, microsatellites were defined as sequence elements that contained at least 20 GGAA (or GGAT) motifs in a window of 120 bp and were identified from *Homo sapiens* genome data (Ensembl version 35), and the distance between the closest microsatellite "edge" and the transcriptional start site of the two closest genes in either direction was determined. There were 2,577 GGAA microsatellite-containing genes, and 942 GGAT microsatellite-containing genes identified.

For A673 cells, previously published "stable knockdown" data consisting of 320 EWS/FLI-up-regulated and 1,151 EWS/FLI-down-regulated genes were used (15). For TC71 and EWS502, the 1,610 EWS/FLI-up-regulated and 436 EWS/FLI-down-regulated gene sets derived from a similar stable knockdown experiment were used (13). ProbeIDs were mapped to their Ensembl identifiers by using the HG-U133A.na21.annot.csv and HG-U133A_2.annot.csv (September 2005 release) annotation files from Affymetrix. Probes without associated Ensembl identifiers were masked from further analysis.

For the Fisher's exact test analyses, the microsatellite-neighboring genes were rank-ordered based on distance between the microsatellite and the gene. Fisher's exact test was performed at each position of the rank-ordered list by using the following two-by-two table: genes with microsatellites at or closer than a particular distance (where the distance increases with each iteration of the analysis) vs. those greater than that particular distance and genes that are EWS/FLI-regulated vs. those that are not. EWS/FLI-up- or -down-regulated genes from A673 cells or TC71/EWS502 cells were considered in separate analyses (13, 15). The total number of genes analyzed was the intersection between genes containing microsatellites that were also present on the U133A microarray (Affymetrix). The Bonferroni correction was calculated by dividing 0.05 by the total number of genes in each analysis.

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Supporting Information

Gangwal *et al.* 10.1073/pnas.0801073105

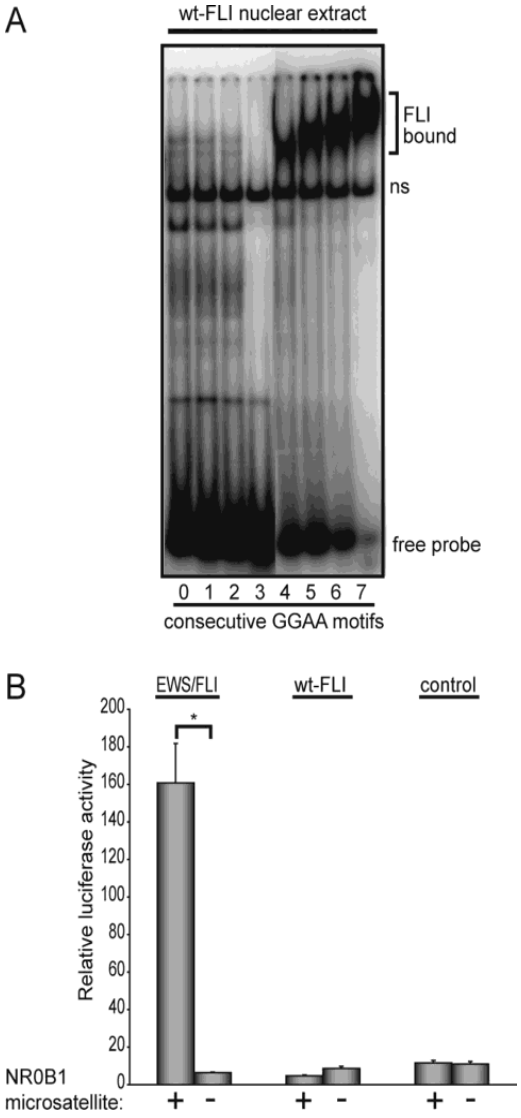

Fig. S1. Wild-type FLI binds, but does not activate, via GGAA-repetitive regions. (*A*) EMSA with DNA duplexes containing the indicated number of consecutive GGAA motifs and 3xFLAG-FLI. The positions of specific FLI-bound complexes are indicated. Nonspecific binding is indicated by "ns". (*B*) Luciferase assays in 293EBNA cells with a reporter construct containing the NR0B1 GGAA-containing microsatellite upstream of a minimal promoter (+) or an "empty" reporter construct that does not contain the microsatellite (−). Cells were transfected with either 3xFLAG-EWS/FLI, 3xFLAG-FLI or an empty vector control. Error bars indicate SDs, and asterisks indicate $P < 0.05$. Of note, 3xFLAG-FLI was expressed at similar levels to 3xFLAG-EWS/FLI, as determined by Western blot (data not shown).

Table S1. Primer sets used for real-time PCR validation of ChIP enrichments

Locus	Forward primer	Reverse primer
NR0B1 primer set	GATTCTGTATCAGCTGGTATATACC	GCATCAGGAAGCCTGGATCC
FCGRT primer set	GAAAGCAGATGAATGGTTGCCAGG	CTGTCACCTCTATCCGAGTTCC
CAV1 primer set	GGTTCAAGAGTACATGTGCAGG	GGGAGTAGGCTTTGTAGCTGG
CACNB2 primer set	GCAAGACTGTAAGCCCCATGG	CTAGGACACATTCAGAATCTGGC
FEZF1 primer set	GTGCGGCTAACCTGTTCTACG	GGTCTGGCTCCTGTGTGC
KIAA1797 primer set	GTAGGTGACCTAGAGGGCC	GAAGGCAGGTGGTAAAGCAGG
RPS26 primer set	CAGCAGAAATGCTGAATGTAAAGG	CATGAGATCCCTACGCGGAC
TP53 primer set	GCCTATATCAGTGCTGGGTAG	ACCTCTTCTGCATCTCATTCTC
ALB primer set	GGTATGCCTGGCCCAAGTACTC	CTCCTTATCGTCAGCCTTGC
BCL2L1 primer set	CACTCCCAGTCCAAATGTCC	GAGGCCATAAACAGCTCTGG

Other Supporting Information File

Dataset S1 (PDF)

Additional supporting information can be found:
http://www.pnas.org/content/105/29/10149/suppl/DCSupplemental

CHAPTER 4

GSTM4 IS A MICROSATELLITE-CONTAINING EWS/FLI TARGET INVOLVED IN EWING'S SARCOMA ONCOGENESIS AND THERAPEUTIC RESISTANCE

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SHORT COMMUNICATION

GSTM4 is a microsatellite-containing EWS/FLI target involved in Ewing's sarcoma oncogenesis and therapeutic resistance

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Ewing's sarcoma is a malignant bone-associated tumor of children and young adults. Most cases of Ewing's sarcoma express the EWS/FLI fusion protein. EWS/FLI functions as an aberrant ETS-type transcription factor and serves as the master regulator of Ewing's sarcoma-transformed phenotype. We recently showed that EWS/FLI regulates one of its key targets, *NR0B1*, through a GGAA-microsatellite in its promoter. Whether other critical EWS/FLI targets are also regulated by GGAA-microsatellites was unknown. In this study, we combined transcriptional analysis, whole genome localization data, and RNA interference knockdown to identify glutathione *S*-transferase M4 (*GSTM4*) as a critical EWS/FLI target gene in Ewing's sarcoma. We found that EWS/FLI directly binds the *GSTM4* promoter, and regulates *GSTM4* expression through a GGAA-microsatellite in its promoter. Reduction of GSTM4 levels caused a loss of oncogenic transformation. Furthermore, reduction of GSTM4 resulted in an increased sensitivity of Ewing's sarcoma cells to chemotherapeutic agents, suggesting a role for this protein in drug resistance. Consistent with this hypothesis, patients with Ewing's sarcoma whose tumors had higher levels of GSTM4 expression had worse outcomes than those with lower expression levels. These data show that GSTM4 contributes to the cancerous behavior of Ewing's sarcoma and define a wider role for GGAA-microsatellites in EWS/FLI function than previously appreciated. These data also suggest a novel therapeutic resistance mechanism, in which the central oncogenic abnormality directly regulates a resistance gene.
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Keywords: Ewing's sarcoma; EWS/FLI; GSTM4; transformation

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Most cases of Ewing's sarcoma harbor recurrent chromosomal translocations, the most common of which encodes the EWS/FLI fusion oncoprotein (Delattre *et al.*, 1992). EWS/FLI requires both its strong transcriptional activation domain (derived from EWS) and its ETS-type DNA-binding domain (derived from FLI) for oncogenic function (May *et al.*, 1993a, b). A variety of studies have identified a large number of EWS/FLI-regulated genes (Prieur *et al.*, 2004; Smith *et al.*, 2006). However, only a handful of these genes have been shown to participate in Ewing's sarcoma oncogenesis. It seems likely that other EWS/FLI-regulated genes will be involved in oncogenic transformation or other cancer-relevant phenotypes, such as invasion, migration, angiogenesis or drug resistance. One of the challenges, however, for identifying which EWS/FLI targets participate in cancer-relevant phenotypes is the complexity of assays required to assess many of these phenotypes, which precludes the development of high-throughput screening approaches. An alternate approach, then, is to limit the number of gene products to be assessed in a lower-throughput screening assays, for example, by comparing microarray profiles across multiple different experiments (Kinsey *et al.*, 2006; Hancock and Lessnick, 2008), or by identifying which genes are 'direct' EWS/FLI targets (Gangwal *et al.*, 2008).

We recently showed that EWS/FLI uses a GGAA-microsatellite to regulate *NR0B1*, a gene that is required for oncogenic transformation in Ewing's sarcoma cells (Kinsey *et al.*, 2006; Gangwal *et al.*, 2008). During the course of these studies, we identified thousands of GGAA-microsatellite-containing genes, suggesting that at least a portion of these might be regulated by direct binding of EWS/FLI to their promoters. We further hypothesized that other GGAA-microsatellite-containing genes might also be required for oncogenic transformation, or other cancer-relevant phenotypes (Gangwal and Lessnick, 2008).

To evaluate this hypothesis, we now compare three data sets we had previously generated to identify genes that might be directly regulated by EWS/FLI though GGAA-microsatellites. These data sets included (i) the transcriptional profile of EWS/FLI in Ewing's sarcoma cells (produced by comparing patient-derived Ewing's

sarcoma cells with retroviral-mediated RNA interference (RNAi) constructs targeting EWS/FLI with those harboring control constructs; Kinsey *et al*., 2006; Smith *et al*., 2006), (ii) genome-wide localization data of EWS/FLI in Ewing's sarcoma cells (produced by 'ChIP-chip' experiments; Gangwal *et al*., 2008) and (iii) comprehensive mapping data of GGAA-microsatellites in the human genome (produced computationally; Gangwal *et al*., 2008). Through these cross-platform comparisons, we identified genes that have all of the characteristics we sought. These genes include previously identified EWS/FLI targets such as *NR0B1*, *FCGRT* and *CAV1* (Tirado *et al*., 2006; Gangwal *et al*., 2008). In addition, we identified glutathione *S*-transferase mu 4 (*GSTM4*) as a potential new EWS/FLI target gene in Ewing's sarcoma.

Glutathione *S*-transferases (GST) are detoxification enzymes which inactivate a variety of endogenous and exogenous reactive compounds by conjugation to glutathione. At present, eight distinct classes (alpha, kappa, mu, omega, sigma, theta, pi and zeta) of soluble and six membrane-bound GSTs have been identified. GSTM4 belongs to the mu class of soluble forms. GSTM4 has a high level of amino-acid sequence identity, but distinct physiochemical properties and tissue distributions, as compared with other GSTMs (Comstock *et al*., 1993, 1994). GSTM4 does not show comparable activity with the standard GST substrate and its specific substrates have yet to be identified. *GST* genes are known to be highly polymorphic to accommodate an increasing number of foreign compounds (Hayes and Strange, 2000). This polymorphism can change an individual's susceptibility to disease and responsiveness to therapeutic drugs. For instance, a T2517C polymorphism in *GSTM4* was shown to associate with an increased risk of developing lung cancer (Liloglou *et al*., 2002). The mechanistic basis for this association is currently unknown.

To evaluate the role of EWS/FLI in regulating *GSTM4*, we first extracted the *GSTM4* data from our transcriptional profiling studies (Kinsey *et al*., 2006; Smith *et al*., 2006). In these studies, two different retroviral RNAi constructs targeting the 3′-UTR of endogenous EWS/FLI (EF-2-RNAi and EF-4-RNAi) were introduced into patient-derived Ewing's sarcoma

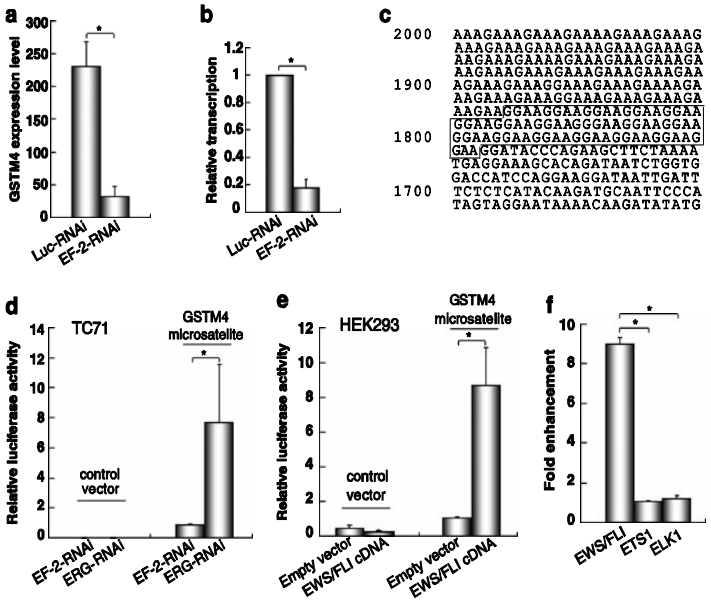

Figure 1 *GSTM4* is regulated by EWS/FLI binding to a GGAA-microsatellite in its promoter in Ewing's sarcoma. (**a**) GSTM4 transcriptional profiling data extracted from Smith *et al*., 2006. EF-2-RNAi is a retroviral construct targeting EWS/FLI, while luc-RNAi is a negative control construct. In this and subsequent panels, columns indicate the mean of triplicate samples; error bars indicate the s.d.; asterisks indicate $P<0.05$. (**b**) Quantitative reverse transcription–PCR results for GSTM4 RNA levels from A673 Ewing's sarcoma cells infected with the indicated retroviral RNAi constructs and selected in puromycin (primer sequences available upon request). (**c**) Partial sequence of GSTM4 promoter with GGAA-microsatellite indicated. (**d**) Luciferase assays using a pGL3-promoter luciferase vector (Promega Corporation, Madison, WI, USA) containing the GSTM4 GGAA-microsatellite, or an empty control vector, co-transfected into TC71 Ewing's sarcoma cells with the EWS/FLI RNAi construct (EF-2-RNAi), or a negative control construct (ERG-RNAi). Luciferase activities were determined as previously described (Gangwal *et al*., 2008). (**e**) Luciferase assays using a pGL3-promoter luciferase vector containing the GSTM4 GGAA-microsatellite, or an empty control vector, co-transfected into HEK293 cells with an EWS/FLI cDNA expression vector, or an empty negative control construct (Gangwal *et al*., 2008). (**f**) Chromatin immunoprecipitation of the GGAA-microsatellite-containing region of the *GSTM4* promoter from A673 cells was carried out by using antibodies against FLI (which recognizes EWS/FLI), ETS1 or ELK1 (see Gangwal *et al*., 2008 for experimental details; primer sequences available upon request). Data are plotted as the fold enrichment with respect to the average enrichment of two negative controls. The error bars indicate the s.e. of the mean of two to five independent experiments.

cell lines A673, TC71 and EWS502, and were compared with a similar RNAi construct targeting the luciferase gene (luc-RNAi) as a negative control (which is not present in the cells used). We found that knock down of EWS/FLI resulted in the reduced levels of GSTM4 mRNA levels in Ewing's sarcoma cell lines (Figure 1a, data not shown). This result was confirmed using quantitative reverse transcription–PCR, in which knock down of EWS/FLI resulted in an approximately 80% decrease in GSTM4 expression (Figure 1b). Unfortunately, we were unable to assess protein levels as the currently available antibodies were inadequate for western blot experiments.

Direct inspection of the *GSTM4* promoter revealed the presence of a GGAA-microsatellite, consisting of 18 GGAA repeats (Figure 1c). We have previously shown that a GGAA-microsatellite regulates the *NR0B1* gene in Ewing's sarcoma cells, and that EWS/FLI is capable

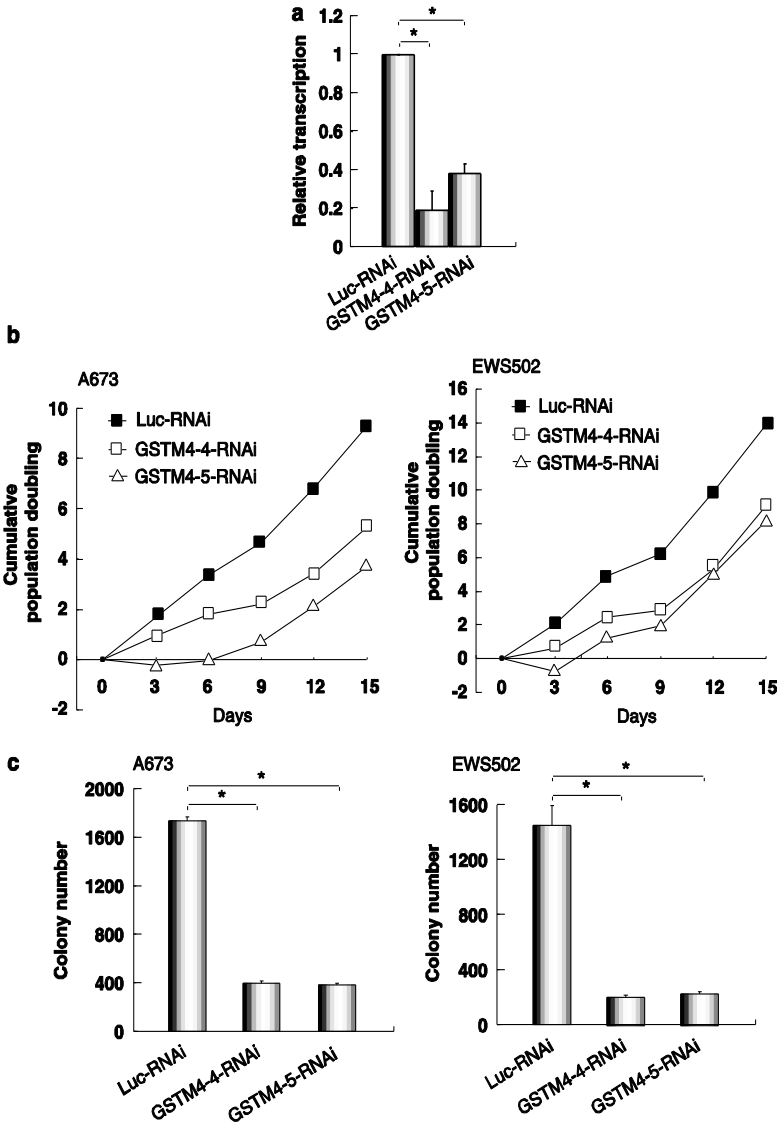

Figure 2 GSTM4 is necessary for oncogenic transformation of Ewing's sarcoma cells. (**a**) Quantitative reverse transcription–PCR results for GSTM4 RNA levels from A673 Ewing's sarcoma cells infected with the indicated retroviral RNAi constructs targeting GSTM4 (GSTM4-4-RNAi and GSTM4-5-RNAi) or a negative control construct (luc-RNAi) and selected in puromycin (primer sequences available upon request). Columns indicate the mean of triplicate samples; error bars indicate the s.d.; asterisks indicate $P<0.05$. (**b**) Growth assays (3T5 assays; Lessnick *et al*., 2002) of Ewing's sarcoma cells (A673 or EWS502) infected with the indicated retroviral RNAi constructs and selected in puromycin. (**c**) Soft agar assays (Kinsey *et al*., 2006) using the indicated Ewing's sarcoma cell lines infected with the indicated retroviral RNAi constructs and selected in puromycin. Columns indicate the mean of duplicate samples; error bars indicate the s.d.; asterisks indicate $P<0.05$.

of binding GGAA-microsatellites *in vitro* as well as *in vivo* (Gangwal and Lessnick, 2008; Gangwal *et al.*, 2008). To determine whether the GGAA-microsatellite in the *GSTM4* promoter is an EWS/FLI-response element, we cloned this element upstream of a minimal promoter and a luciferase reporter cDNA. This construct was EWS/FLI responsive in TC71 Ewing's sarcoma cells, as shown by relatively high levels of luciferase activity when co-transfected with an ERG-RNAi negative control vector, and an ~85% reduction in luciferase reporter activity when EWS/FLI was knocked down with an EWS/FLI RNAi construct (EF-2-RNAi; Figure 1d). In complementary experiments, we found that co-transfection of the luciferase reporter with an EWS/FLI cDNA in HEK293 cells resulted in an ninefold stimulation as compared with co-transfections with an empty vector negative control construct (Figure 1e).

To determine whether EWS/FLI occupied the *GSTM4* promoter *in vivo*, we carried out directed chromatin immunoprecipitation experiments (ChIP) using antibodies and conditions we previously validated (Gangwal *et al.*, 2008). We found that immunoprecipitation of EWS/FLI pulled down the *GSTM4* microsatellite region, whereas immunoprecipitation of two other ETS family members, ETS1 and ELK1, did not (Figure 1f). Taken together, these data support our hypothesis that *GSTM4* is upregulated in Ewing's sarcoma cells by direct binding of EWS/FLI to the GGAA-microsatellite in the *GSTM4* promoter.

We next sought to determine whether GSTM4 has a functional role in Ewing's sarcoma. First, we investigated whether GSTM4 is required for maintaining oncogenic phenotype of Ewing's sarcoma through a loss-of-function approach. We designed small hairpin RNAs against the 3′-UTR of GSTM4 and cloned these into retroviral constructs (called GSTM4-4-RNAi and GSTM4-5-RNAi). Polyclonal-infected and -selected populations of A673 Ewing's sarcoma cells showed an efficient reduction of endogenous GSTM4 mRNA levels as assessed by quantitative reverse transcription–PCR, as compared with cells infected with the negative control luc-RNAi retrovirus (Figure 2a). We found that cells in which GSTM4 was knocked down grew slightly more slowly than control cells in tissue culture (Figure 2b). Importantly, decreased GSTM4 expression resulted in a significant reduction in oncogenic transformation, as shown by diminished anchorage-independent growth in soft agar experiments (Figure 2c). The fact that two different retroviral RNAi constructs showed nearly identical phenotypes in two different patient-derived Ewing's sarcoma cell lines suggested that these results are specific to reduced GSTM4 expression, and were not 'off-target' effects. These results show that ongoing GSTM4 expression is required for the transformed phenotype of Ewing's sarcoma.

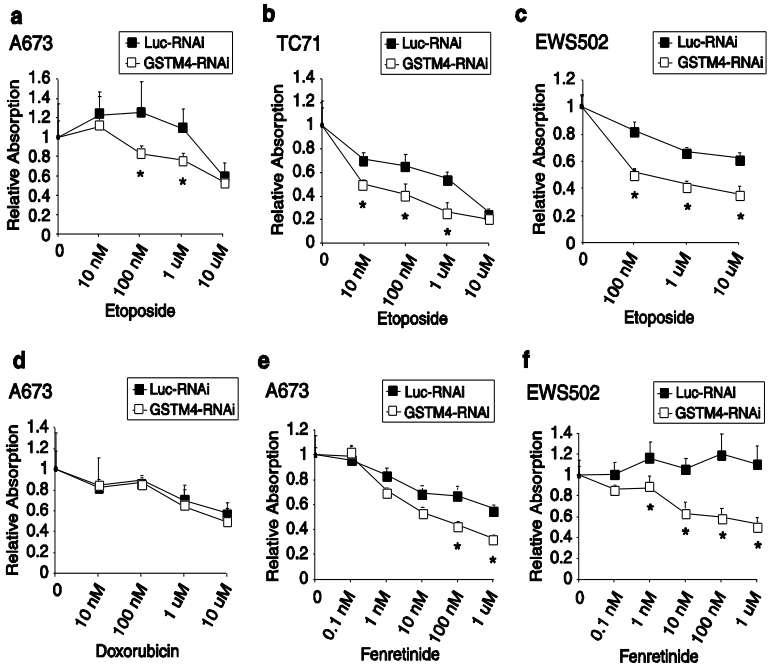

Figure 3 Depletion of GSTM4 increases sensitivity of Ewing's sarcoma cells to etoposide (**a–c**) or fenretinide (**e**, **f**), but not doxorubicin (**d**). The indicated Ewing's sarcoma cells were seeded at a density of 2×10^4 cells/well in 24-well plate on day 0. On day 1, cells were incubated with varying concentrations of the indicated drugs for 3 days before MTT cell proliferation assays (Cayman Chemical Company, Ann Arbor, MI, USA) were performed according to the manufacturer's instructions. Error bars indicate the s.d. of triplicate samples in a representative experiment. The same trend was seen in three independent biological replicates. Asterisks indicate $P<0.05$.

To determine whether GSTM4 is required for oncogenic transformation in other tumor types, we depleted GSTM4 from U2OS osteosarcoma cells and found that neither cell growth nor colony formation in soft agar was changed (data not shown). Furthermore, knockdown of GSTM4 in RH28 rhabdomyosarcoma cells did not alter cell growth in tissue culture, but as these cells did not form colonies in soft agar at baseline, the effects of GSTM4 loss on oncogenic transformation could not be assessed (data not shown). These data indicate that a role for GSTM4 in proliferation and oncogenic transformation is likely specific to Ewing's sarcoma.

Therapeutic approaches to Ewing's sarcoma consist of either surgery and/or radiation therapy to the primary site of disease, along with intensive systemic chemotherapy to eradicate micrometastatic disease. We reasoned that because GST enzymes detoxify various reactive compounds, including therapeutic drugs, GSTM4 might contribute to the resistance profile of Ewing's sarcoma to chemotherapeutic agents. To test this possibility, we prepared Ewing's sarcoma cells with either wild-type (luc-RNAi) or reduced levels (GSTM4-4-RNAi) of GSTM4, and treated them with increasing dosages of chemotherapeutic drugs that are currently used to treat patients with Ewing's sarcoma, including etoposide, doxorubicin and melphalan, and tested cell survival using an MTT assay. We found that knockdown of GSTM4 resulted in an increase in sensitivity to etoposide as compared with luc-RNAi-infected cells in three different patient-derived Ewing's sarcoma cell lines (A673, TC71 and EWS502; Figure 3a–c). This effect was specific for etoposide among the initial agents tested, as there were no differences in sensitivity to doxorubicin or melphalan following GSTM4 knockdown (Figure 3d, data not shown).

The synthetic retinoid derivative fenretinide (4-hydroxy(phenyl) retinamide) has been reported to induce high levels of cell death in Ewing's sarcoma cell lines *in vitro* and to delay growth of Ewing's sarcoma xenografts *in vivo* (Magwere *et al*., 2008; Myatt and Burchill, 2008). Interestingly, it was recently reported that depletion of glutathione by pretreatment with l-buthionine (*S*,*R*) sulphoximine-sensitized Ewing's sarcoma cells to the toxic effects of fenretinide (Magwere *et al*., 2008). This suggested that fenretinide toxicity is modulated by glutathione-dependent functions in Ewing's sarcoma cells. On the basis of these observations, we next investigated whether the function of a glutathione-dependent enzyme (GSTM4) would alter the sensitivity of Ewing's sarcoma cells for fenretinide. We found that Ewing's sarcoma cells with reduced GSTM4 levels (through the GSTM4-4-RNAi construct) were more sensitive to fenretinide than cells containing a control RNAi construct (luc-RNAi; Figures 3e and f). Because fenretinide is known to generate reactive oxygen species in Ewing's sarcoma cells (Magwere *et al*, 2008), we tested whether GSTM4 depletion in A673 cells caused alterations in reactive oxygen species production using a cell permeable dye (5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester). We found that there were no significant changes in reactive oxygen species production following reduction of GSTM4 levels with the GSTM4-4-RNAi construct (data not shown). Taken together, the drug-sensitivity data show that the reduction of GSTM4 levels confer increased sensitivity to some cytotoxic agents in Ewing's sarcoma, and further suggest that inhibition of GSTM4 activity might be used in combination with chemotherapy to increase therapeutic responses in Ewing's sarcoma.

Because GSTM4 modulates Ewing's sarcoma oncogenic transformation and therapeutic resistance, we

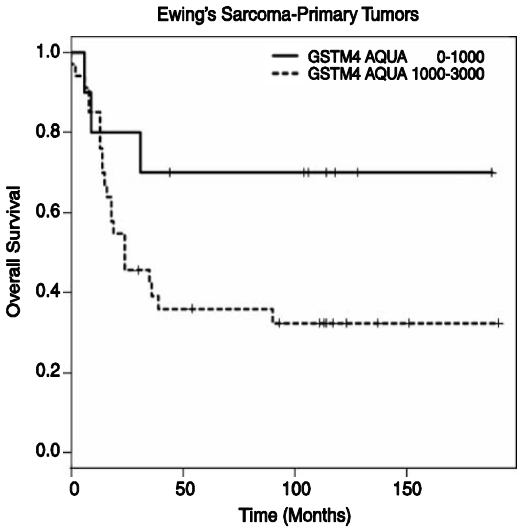

Figure 4 GSTM4 expression in tumors correlates with overall survival in Ewing's sarcoma patients. Analysis was performed following the Institutional Review Board approval. Forty four cases of morphologically confirmed Ewing's sarcoma pretreatment primary tumor samples (from the University of Michigan Medical Center Department of Pathology) included on a tissue microarray (TMA) were analysed by double immunofluorescence staining for both CD99 (which marks Ewing's sarcoma tumor cells; Abcam, Cambridge, MA, USA, Ab-27271, rabbit polyclonal antibody, 1:100) and GSTM4 (Abcam, ab-49484, mouse monoclonal antibody, 1:80). The AQUA system was used for the automated image acquisition and analysis. Briefly, images of each TMA core were captured with an Olympus BX51 microscope at different extinction/emission wavelengths. Within each TMA spot, the area of tumor was distinguished from stromal and necrotic areas by creating a tumor-specific mask from the CD99-staining pattern, which was visualized using the Alexa Fluor 555 signal. Within the masked region, the fluorescence pixel intensity of the GSTM4 protein/antibody complex was obtained from the Cy5 signal and reported as pixel intensity from 0 to 3000. In cases where multiple primary pretreatment tumor samples were present, the average of the values from each sample was used. Patients were stratified into two groups, those with relatively low-level GSTM4 expression (0–1000; $n=10$), and those with relatively high-level GSTM4 expression (1000–3000; $n=34$). These data were plotted with stratified Kaplan–Meier curves using the outcome data from the University of Michigan. Cox proportional hazards models were fit using the counting process formulation of Andersen and Gill, 1982. Goodness of fit was tested using the method presented in Grambsch and Therneau, 1994. All statistical analysis was performed using R 2.8.0 statistical software (Copyright 2008, The R Foundation for Statistical Computing, Vienna, Austria). The 'survival' package in R was used for the survival analysis.

hypothesized that patients whose tumors exhibited higher levels of GSTM4 expression would have a worse outcome than patients whose tumors expressed lower levels of the protein. To test this hypothesis, we stained tissue microarrays containing Ewing's sarcoma tumor specimens for GSTM4 protein, using immunohistochemistry, and read out the results in a semiquantitative manner using an AQUA system (HistoRx, New Haven, CT, USA) (Camp *et al.*, 2008). As anticipated by our hypothesis, we found that patients with higher GSTM4 levels in their primary tumors had lower overall survival rates, although this result did not reach statistical significance due to a small sample size ($P=0.054$ by likelihood ratio test; Figure 4). These data support the notion that higher GSTM4 levels are correlated with therapeutic resistance and worse outcome in Ewing's sarcoma.

The data in this report show that *GSTM4* is a direct target gene of the EWS/FLI oncoprotein and is required for oncogenic transformation and therapeutic drug resistance in Ewing's sarcoma cells. These findings show the utility of combining transcriptional profiling, ChIP-chip and computational promoter analyses in the identification of target genes that are involved in oncogenesis and other cancer-relevant phenotypes, such as drug resistance. Indeed, our observation that GSTM4 has a role in resistance to therapeutic agents in this disease suggests a new paradigm for drug resistance: that key oncogenic events involved in tumorigenesis may directly regulate drug resistance programs, in addition to their more widely recognized role in promoting oncogenic transformation. This has important implications for the design of molecularly targeted agents. We would predict that agents targeting EWS/FLI (such as cytarabine or RNAi-based approaches; Dubois *et al.*, 2008; Hu-Lieskovan *et al.*, 2005; Kinsey *et al.*, 2006; Owen *et al.*, 2008; Stegmaier *et al.*, 2007) might be most effective when combined with standard cytotoxic agents. In this way, the loss of the key oncoprotein will reduce both oncogenic transformation and drug resistance, thereby allowing the tumor to be more effectively treated with standard chemotherapies. Conversely, inhibitors of GSTM4, if developed, may also be combined with standard chemotherapeutics to provide more efficacy in tumor kill by these agents.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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CHAPTER 5

A NOVEL ROLE FOR KERATIN 17 IN COORDINATING ONCOGENIC TRANSFORMATION AND CELLULAR ADHESION IN EWING SARCOMA

In review process at Molecular and Cellular Biology as:

Sankar S, Tanner JM, Bell R, Chaturvedi A, Beckerle MC and Lessnick SL. (2013) A novel role for Keratin 17 in coordinating oncogenic transformation and cellular adhesion in Ewing sarcoma.

Abstract

Oncogenic transformation in Ewing sarcoma is caused by EWS/FLI, an aberrant transcription factor fusion oncogene. Glioma-associated oncogene homolog 1 (*GLI1*) is a critical target gene activated by EWS/FLI, but the mechanism by which GLI1 contributes to the transformed phenotype of Ewing sarcoma was unknown. In this work we identify Keratin 17 (*KRT17*) as a direct downstream target gene upregulated by GLI1. We demonstrate that KRT17 regulates cellular adhesion by activating AKT/PKB (Protein Kinase B) signaling. In addition, KRT17 is necessary for oncogenic transformation in Ewing sarcoma and accounts for much of GLI1-mediated transformation function but via a mechanism independent of AKT signaling. Taken together, our data reveal previously unknown molecular functions for a cytoplasmic intermediate filament protein KRT17 in coordinating EWS/FLI and GLI1 mediated oncogenic transformation and cellular adhesion in Ewing sarcoma.

Introduction

Ewing sarcoma is a highly aggressive bone and soft tissue associated malignancy that affects children and young adults (1). The vast majority of these tumors are characterized by a t(11;22)(q24;q12) chromosomal translocation, that generates a fusion oncogene, *EWS/FLI* (2). Persistent expression of EWS/FLI is necessary for maintenance of the transformed phenotype in Ewing sarcoma (3-5). Previous studies demonstrate that Ewing sarcoma tumors have a relatively low frequency of mutations in known oncogenes and tumor suppressors, supporting the concept that EWS/FLI is largely responsible for oncogenic transformation (6, 7). EWS/FLI functions as an aberrant transcription factor

and dysregulates the expression of myriad target genes (8-10). Over the years, several critical EWS/FLI target genes have been identified that are all necessary for maintenance of oncogenic transformation in Ewing sarcoma; however, no target gene alone has proven to be sufficient for EWS/FLI mediated oncogenic transformation (3, 4). These findings highlight the unique biology of Ewing sarcoma and its sole reliance on a single oncogenic transcription factor, EWS/FLI, as the central regulator of a hierarchy of transcriptional networks.

Hedgehog signaling is of critical importance during development in regulating tissue patterning and stem cell maintenance (11, 12). This signaling pathway is inappropriately activated in a diversity of cancers (13-22). GLI1 is a zinc-finger transcription factor and is the principal effector of the Hedgehog signaling pathway (11). Previous microarray studies and a recent RNA-sequencing (RNA-seq) experiment have identified *GLI1* as an EWS/FLI upregulated target gene in Ewing sarcoma (3, 10), (Sankar *et al*., submitted). EWS/FLI has been shown to bind and directly activate transcription from the GLI1 promoter (23). Furthermore, loss-of-function approaches and pharmacological inhibition have demonstrated that GLI1 is necessary for EWS/FLI mediated oncogenic transformation (23-25). These studies highlight the importance of GLI1 in Ewing sarcoma development.

However, the mechanism underlying GLI1 mediated oncogenesis in Ewing sarcoma and the critical transcriptional network of genes regulated by GLI1 to achieve this function were unknown. Here, we sought to define the mechanistic role of GLI1 in Ewing sarcoma, and in doing so, identified a unique target gene, *KRT17* that has novel

functions in coordinating parallel functions of cellular adhesion and oncogenic transformation.

Materials and methods

Ethics statement

Patient tumor specimens were used in a deidentified way, and were therefore deemed "non-human subject research" by the University of Utah Institutional Review Board via protocol IRB_00035414. Animal experiments were performed following approval from the University of Utah Institutional Animal Care and Use Committee.

Constructs and retroviruses

The Luciferase-RNAi (Luc-RNAi), EWS/FLI-RNAi (EF-2-RNAi), 3x-FLAG EWS/FLI and 3x-FLAG NKX2.2 cDNA have been described previously (3, 10, 26). The GLI1 and KRT17 shRNAs were designed to target the cDNA and 3'UTR, respectively, and cloned into the pMKO.1 retroviral vector. 3x-FLAG GLI1, 3x-FLAG KRT17 and 3x-FLAG S44A KRT17 cDNAs were generated and subcloned into the Murine Stem Cell Virus (MSCV) retroviral vector (Clontech). One kilobase KRT17 promoter including the 5'UTR was cloned into the pGL3 basic vector (Promega), immediately upstream of the luciferase reporter gene. The constitutively active (myristoylated) AKT in the MSCV retroviral vector has been described previously (27).

Cell culture

Ewing sarcoma cell lines (A673, TC-71, TC-32, SK-N-MC and EWS502) and HEK293 EBNA cells were infected with retrovirus, and polyclonal populations were grown in the appropriate selection media, as previously described (4, 28). Growth assays (3T5) were performed as previously described (28).

Soft agar and methylcellulose assays

Soft agar assays were performed as described previously (28). Methylcellulose assays were performed by plating $1x10^5$ cells in 2% methylcellulose mixed with an equal volume of appropriate growth media as described previously (29).

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted using an RNAeasy kit (Qiagen). Total RNA from cells was then amplified and detected using SYBR green fluorescence for quantitative analysis. Normalized fold enrichment was calculated by determining the fold-change of each condition relative to the control (either Luc-RNAi or Luc-RNAi re-expressing an empty vector). The data in each condition was then normalized to internal housekeeping control genes GAPDH and RPL19. Primer sequences used to amplify target genes by qRT-PCR are provided in Supplemental Data (Table S5.1).

Luciferase reporter assays

A one kilobase promoter region including the 5'UTR of *KRT17* was cloned into the pGL3 basic vector (Promega) immediately upstream of the luciferase reporter gene.

Luciferase reporter assays were performed in HEK293 EBNA as previously described (30).

Xenograft and intratibial injection assays

A673 cells infected and selected with a control ERG-RNAi or KRT17-RNAi were injected into the flanks of nude mice at 1×10^6 cells per flank or 2.5×10^5 cells into the tibia of NOD/SCID mice. For the xenograft tumor assay, four mice were injected subcutaneously with control knockdown cells and five mice were injected subcutaneously with KRT17 knockdown cells. Both flanks of each mouse was injected subcutaneously, therefore, eight and ten tumors were measured for the two groups, respectively. For the intratibial tumor assay, five mice were each injected in the right tibia; therefore, five tumors were measured per group. Tumors were measured using digital calipers and three-dimensional tumor volumes were calculated using the equation (Length x Width x Depth)/2. The mice in each group were sacrificed once their tumors reached a size limit of 2 cm for the subcutaneous injection model and 1.5 cm for the intratibial injection model. The data from both assays are plotted as Kaplan-Meier survival curves using GraphPad Prism.

Antibodies and reagents

The following antibodies were used for immunodetection: M2-anti-FLAG (HRP; Sigma A8592), anti-FLI-1 (Santa-Cruz sc-356X), antiα-Tubulin (Calbiochem CP06), anti-KRT17 (Abcam ab-53707) and anti-phospho AKT (S473) (Cell Signaling # 9271S), anti-AKT (pan) (Cell Signaling # 4691S), anti-GLI1 (Cell Signaling # 2643S). The

isozyme selective AKT inhibitor (Akti1/2) was obtained from Millipore Cat. No.124017. The inhibitor was used at a final concentration of 2 μM. At this concentration it inhibits all three forms of AKT (AKT1, AKT2 and AKT3). Cells were treated with the inhibitor for twenty four hours before they were used for experiments.

Adhesion and migration assays

Ewing sarcoma cells infected and selected with different constructs were seeded at 5 x10^5 cells per well in a non-ECM coated 24-well plate. Cells were allowed to adhere for two hours at 37ºC and then were processed as previously described (31). Cells that adhered were stained with Toluidine Blue and O.D. was measured at 620nm as previously described. Cell migration was measured using the Boyden Chamber haptotactic cell migration assay as previously described (31).

Immunofluorescence assays

Sterile coverslips were coated with 10 μg/ml fibronectin in 12-well plates overnight at 4ºC. 75 x 10^3 cells/well were seeded, allowed to adhere for twenty four hours, fixed with 3.7% formaldehyde as described previously (31). Cells were stained with anti-paxillin antibody (1:100) for one hour at 37ºC and then with AlexaFluor secondary antibody (1:200) and AlexaFluor-phalloidin and were imaged using a Zeiss Axioskop2 mot plus microscope with a 40x objective as previously described (31).

RNA sequencing analysis, GSEA and Venn overlaps

RNA from A673 cells stably infected and selected for expression of a control Luc-RNAi or the KRT17-RNAi was extracted using the RNAeasy kit (Qiagen) with an on-column DNAse digestion protocol. Libraries for deep-sequencing were prepared according to the manufacturer's instructions (Illumina) and sequenced on an Illumina Hi-Seq with 50 cycles of single end reads. Sequences were aligned to the human genome build hg19. Raw sequence reads can be found in the NCBI SRA #121863. The USeq analysis package was used to identify differentially expressed genes. Significance parameters were set to a two-fold or four-fold change in expression and an FDR of 0.1 (10%) or 1.0×10^{-10}.

Overlaps between the different gene sets were performed using the VennMaster program. Statistical significance of the overlaps was determined using Chi square analysis. Gene set enrichment analysis (GSEA) was performed using GSEA v2.0.10 program. Functional annotation analysis was performed by Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis. Identification of potential direct GLI1 target genes was performed by Find Individual Motif Occurrences (FIMO) analysis.

Results

GLI1 is a downstream target of EWS/FLI and is necessary for oncogenic transformation

Previous studies using loss of function approaches have identified GLI1 as an upregulated target of EWS/FLI (24, 25). To further demonstrate that GLI1 is specifically

regulated by EWS/FLI, we used a retroviral-based stable knockdown/rescue approach in A673 cells (a patient-derived Ewing sarcoma cell line). We found that reduction of EWS/FLI resulted in a significant reduction in GLI1 expression, which was restored by reexpression of an RNAi-resistant EWS/FLI cDNA (Figure 5.1A, S5.1A). This result demonstrated that GLI1 is specifically upregulated by EWS/FLI and is not an off-target or other nonspecific RNAi effect. EWS/FLI did not regulate GLI2 or GLI3 (Figure S5.1B). GLI1 is not the sole downstream effector of EWS/FLI-mediated oncogenic transformation because GLI1 expression (Figure 5.1B) failed to rescue oncogenic transformation following knockdown of EWS/FLI (Figure 5.1C).

To test the necessity of GLI1 in Ewing sarcoma oncogenesis, we performed GLI1 knockdown/rescue experiments (Figure 5.1D, S5.1C). In comparison to a control knockdown (Luc-RNAi) GLI1 knockdown did not affect monolayer growth of cells in tissue culture but significantly reduced colony growth in soft agar (Figures 5.1E, 5.1F). This is not an "off-target" effect because reexpression of GLI1 rescued the loss of transformation induced by GLI1 knockdown (Figure 5.1D-5.1F). These results demonstrated that GLI1 is necessary for maintenance of oncogenic transformation in Ewing sarcoma cells.

GLI1 has been shown to transcriptionally activate NKX2.2 (25). NKX2.2 is a critical target of EWS/FLI that is necessary for oncogenic transformation in Ewing sarcoma (3). We therefore asked if NKX2.2 could rescue GLI1-knockdown mediated loss of transformation. Interestingly, we found that NKX2.2 (Figure S5.1D) was unable to rescue the loss of transformation mediated by GLI1-knockdown (Figure 5.1F), indicating

that other GLI1 target genes are necessary for full oncogenic transformation in Ewing sarcoma.

Determining the transcriptional signature of GLI1 in Ewing sarcoma

We next sought to identify the full-complement of genes regulated by GLI1 in Ewing sarcoma. We performed an RNA-seq experiment in A673 cells comparing genome-wide transcripts from cells expressing a control or GLI1-RNAi constructs (Figure 5.2A, Table S5.2). Venn master analysis was used to generate overlaps of the up-regulated and downregulated genesets obtained from the GLI1 RNA-seq and the EWS/FLI RNA-seq (Sankar *et al*., submitted). Of the 1796 genes upregulated by EWS/FLI, 327 genes were also upregulated by GLI1 ($p=3.19 \times 10^{-162}$; Figure 5.2B), and of the 2227 genes repressed by EWS/FLI, 319 genes were also repressed by GLI1 ($p=1.01 \times 10^{-170}$; Figure 5.2B), demonstrating that GLI1 contributes significantly to the EWS/FLI transcriptional profile in Ewing sarcoma cells. Using very stringent cutoffs of a four-fold change and an FDR of 1.0×10^{-10} we limited the list to 86 genes upregulated and 55 genes downregulated by GLI1 (Table S5.2). We used this stringent set of genes to perform Gene Set Enrichment Analysis (GSEA) against EWS/FLI-regulated genes to better determine the relationship between the EWS/FLI and GLI1 transcriptional profiles. We found that the GLI1 upregulated genes clustered strongly with the most highly upregulated EWS/FLI genes (NES=2.0; $p<0.001$) and vice-versa (NES=-1.8; $p<0.001$) (Figure 5.2C), indicating that GLI1-regulated genes make up a significant portion of the EWS/FLI transcriptional signature. We performed similar analyses using microarray datasets generated from TC71 and EWS502 Ewing sarcoma cells and again found

significant correlations between GLI1 and EWS/FLI in gene regulation (Figure S5.2A and S5.2B). We also validated a subset of GLI1-regulated genes identified in the RNA-seq data by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (Figure S5.2C).

To gain further insight into the functional significance of the differentially expressed genes from the GLI1 RNA-seq, we used the functional annotation tools from the Database for Annotation, Visualization and Integrated Discovery (DAVID). We found that the most significant classes among the GLI1 upregulated genes corresponded to neuronal development and cell cycle regulation (Figure 5.2D), which is consistent with the well-studied role of GLI1 in neuronal development (32) and its ability to transcriptionally regulate cell cycle proteins (33). Interestingly, neuronal features have previously been noted in Ewing sarcoma (34, 35), and thus the RNA-seq data suggests that GLI1 and its downstream target genes may contribute to the neuronal phenotype of Ewing sarcoma. Among the downregulated geneset, the most significant classes were related to signaling and membrane activity (Figure 5.2D).

Identification of KRT17 as a direct downstream target of GLI1

To further investigate the role of GLI1 target genes identified from the RNA-seq analysis, we focused on *KRT17,* which is the second most upregulated GLI1 target gene (Figure 5.2A) and is also regulated by EWS/FLI (Figure 5.2B, 5.2C, S5.2A, S5.2B). qRT-PCR analysis demonstrated that both GLI1 and EWS/FLI upregulate KRT17 in multiple Ewing sarcoma cell lines (Figure 5.3A, 5.3B, and 5.3C). Western blot analysis revealed that the KRT17 protein is expressed at detectable levels in all Ewing sarcoma

cell lines tested, albeit, at varying levels (Figure 5.3D), and KRT17 RNA is expressed in five independent Ewing sarcoma primary tumors (Figure 5.3E). These results demonstrate that KRT17 is an up-regulated target of GLI1 in Ewing sarcoma.

The GLI1 RNA-seq analysis does not distinguish direct from indirect targets. GLI1 is a well-studied transcription factor, and previous work has identified and characterized a conserved 10-base pair motif as the preferred binding site (GACCACCCAC/A) for GLI1 on target gene promoters (36, 37). To predict potential direct targets of GLI1, we used Find Individual Motif Occurrences (FIMO) (38), by combining a previously published weighted matrix for binding affinity and a weighted matrix for activation potential of GLI1 at the 10-base pair motif, to search for genes in our RNA-seq dataset that had a significant match (p-value cut-off of 1.0×10^{-5}) to the known GLI1 binding motif. We identified 23 potential direct upregulated and 12 direct downregulated targets of GLI1 (Figure S5.3). Interestingly, *KRT17* was one of the potential direct targets of GLI1 (Figure S5.3, Table 1). Luciferase reporter assays demonstrated a dose-dependent increase in luciferase activity from a 1Kb KRT17 promoter region with increasing concentrations of the GLI1 cDNA (Figure 5.3F). These data indicate that KRT17 is likely a direct upregulated target of GLI1.

KRT17 is necessary for oncogenic transformation in vitro and in vivo

KRT17 is a cytoplasmic intermediate filament protein (39) that is overexpressed in several cancers (40-46). High KRT17 expression correlates with poor prognosis in breast, pancreatic and gastric adenocarcinomas (47-49). In basal cell carcinomas, which are associated with aberrant hedgehog signaling, KRT17 promotes tumor growth by

modulating the immune response (40). However, it is unknown whether KRT17 plays a more direct role in oncogenic transformation.

To determine if KRT17 is involved in oncogenic transformation in Ewing sarcoma, we performed knockdown/rescue of KRT17 in A673, EWS502 and SK-N-MC Ewing sarcoma cells. We found that knockdown of KRT17 had no effect on cell growth in tissue culture, but significantly reduced colony formation in soft agar (Figure 5.4A, 5.4B, 5.4C, 5.4D, S5.4A, and S5.4B). Furthermore, reexpression of KRT17 in knockdown cells restored their ability to form colonies in soft agar, demonstrating a specific function of KRT17 in maintaining the transformed phenotype of Ewing sarcoma cells (Figure 5.4D, 5.4E). Importantly, qRT-PCR analysis of endogenous KRT17 transcript levels demonstrated that the KRT17 knockdown was maintained even in the KRT17 cDNA rescue samples (Figure S5.4D), suggesting that rescue of oncogenic transformation was not merely due to loss of the KRT17-RNAi. KRT17 knockdown had no effect on oncogenic transformation in the non-Ewing sarcoma cell line HEK293 EBNA (human embryonic kidney cells) (Figure S5.4C), suggesting that KRT17 is specifically required for oncogenic transformation in Ewing sarcoma.

We next used two *in vivo* tumor models, a subcutaneous model and an orthotopic intratibial model, to evaluate the role of KRT17 in tumor growth *in vivo*. We noted a significant improvement in overall survival of immunocompromised mice injected with KRT17 knockdown A673 cells when compared to those with control (ERG) knockdown cells in both *in vivo* models (Figure 5.4F). In the tumors that did form in mice injected with KRT17 knockdown cells, we noted that the knockdown effect was lost in tumors that grew actively, while the slow growing (indolent) tumors from the opposite flanks of

those mice still maintained the KRT17 knockdown (Figure 5.4G), indicating that KRT17 is necessary for aggressive tumor growth *in vivo*.

To evaluate if KRT17 was a critical target gene downstream of GLI1, we performed anchorage-independent colony forming assays with A673 cells following control or GLI1 knockdown and re-expressing an empty vector, GLI1 or KRT17 cDNA constructs (Figure 5.4H). Surprisingly, expression of the KRT17 cDNA rescued GLI1 knockdown mediated loss of transformation (Figure 5.4I). In cells harboring the GLI1-RNAi and re-expressing the KRT17 cDNA, maintenance of GLI1 knockdown and lack of rescue of GLI1 target genes was demonstrated by qRT-PCR analysis (Figure S5.4E, S5.4F), indicating that rescue of oncogenic transformation was not due to reexpression of GLI1 when KRT17 is expressed. Taken together these results demonstrate that KRT17 is necessary for maintaining the transformed phenotype of Ewing sarcoma cells both *in vitro* and *in vivo,* and that *KRT17* is a critical target gene downstream of GLI1 that contributes significantly to GLI1 mediated maintenance of oncogenic transformation in Ewing sarcoma.

KRT17 mediated activation of AKT signaling is necessary and sufficient to regulate cellular adhesion in Ewing sarcoma

KRT17 is known to regulate protein synthesis and epithelial cell growth by inducing phosphorylation and activation of the AKT protein (50). We therefore asked whether KRT17 regulated AKT phosphorylation downstream of GLI1, and if this genetic interaction was necessary for KRT17 function in Ewing sarcoma. GLI1 knockdown significantly reduced AKT phosphorylation levels in A673 cells, and this effect was

rescued by GLI1 or KRT17 reexpression (Figure 5.5A), but not KRT17 S44A (Figure 5.5B), a previously described mutant that fails to induce phosphorylation of AKT (50, 51), demonstrating that KRT17 is the critical mediator of AKT phosphorylation downstream of GLI1.

To characterize the functional significance of KRT17 mediated activation of AKT signaling, we performed immunofluorescence studies on A673 cells expressing reduced levels of KRT17. Interestingly, we noted a significant decrease in staining for Paxillin protein, a marker of focal adhesions, in the cells expressing reduced KRT17 levels in comparison to control cells (Figure 5.5C). As a control, EWS/FLI knockdown cells expressed higher levels of Paxillin (Figure 5.5C) as noted previously (31). To test if KRT17 is directly involved in regulating cellular adhesion in Ewing sarcoma cells, we performed cellular adhesion assays with KRT17 knockdown cells re-expressing wild-type KRT17 or the S44A mutant. Interestingly, wild-type KRT17, but not the S44A mutant, rescued basal levels of cellular adhesion in Ewing sarcoma cells (Figure 5.5D).

To directly test the contribution of active AKT signaling in cellular adhesion mediated by KRT17, we took two complementary approaches: (i) a genetic approach by expressing a constitutively active form of AKT (myristoylated AKT) (27), and (ii) a pharmacological approach using a selective AKT inhibitor (Akti1/2; Millipore). We found that expression of the constitutively active AKT following knockdown of endogenous KRT17 (Figure 5.5E) phenocopied KRT17 mediated cellular adhesion (Figure 5.5F). We also found that selective inhibition of AKT by the pharmacological inhibitor significantly decreased the basal levels of cellular adhesion in Ewing sarcoma cells, similar to levels achieved with KRT17 knockdown (Figure 5.5G). These data

clearly define the genetic and functional interaction between GLI1, KRT17 and active AKT signaling in regulating cellular adhesion in Ewing sarcoma cells.

KRT17 mediated oncogenic transformation is independent of the AKT pathway

AKT signaling is frequently activated in cancer (52). We therefore asked whether AKT phosphorylation was necessary for KRT17 mediated oncogenic transformation in Ewing sarcoma. Interestingly, the KRT17 S44A mutant that failed to phosphorylate AKT (Figure 5.5B) retained the ability to rescue KRT17 knockdown mediated loss of transformation to an extent comparable to wild-type KRT17 (Figure 5.6A), suggesting that oncogenic transformation by KRT17 is independent of the AKT pathway.

To directly test the contribution of active AKT signaling in Ewing sarcoma oncogenesis, we used the constitutively active form of AKT (myristoylated AKT) (27), and the selective AKT inhibitor (Akti1/2; Millipore). The constitutively active form of AKT failed to rescue the loss of oncogenic transformation following KRT17 knockdown, even though high levels of AKT phosphorylation were achieved (Figure 5.6B, 5.5E). Pharmacological inhibition of AKT phosphorylation also had no effect on oncogenic transformation of Ewing sarcoma cells (Figure 5.6C). Maintenance of AKT inhibition in the anchorage-independent environment was ensured by assessing the phosphorylation status of AKT in the colonies that did form (Figure 5.6D). These results suggest that AKT signaling is completely dispensable to the anchorage-independent colony-forming phenotype of Ewing sarcoma cells. Taken together, our data highlight a central role for

KRT17 downstream of GLI1 in coordinating two important, but independent, phenotypes of cancer cells, oncogenic transformation and cellular adhesion.

Discussion

In this work we identified KRT17 as an upregulated target of EWS/FLI and GLI1 in Ewing sarcoma. Furthermore, we unraveled novel functional roles for KRT17 in regulating oncogenic transformation and cellular adhesion in Ewing sarcoma: KRT17 induces AKT signaling to mediate cellular adhesion, while KRT17 modulates oncogenic transformation (as measured by colony formation in anchorage-independent conditions and by xenograft tumor formation) independent of AKT signaling. To our knowledge, this is the first demonstration of such a coordinating function for an intermediate-filament protein for these cancer-relevant phenotypes.

Hyperactive AKT signaling is characteristic of several cancers (52). Interestingly, oncogenic transformation mediated by KRT17 is independent of the AKT signaling pathway in Ewing sarcoma. Consequently, inhibiting the AKT signaling pathway had no impact on growth or oncogenic transformation of Ewing sarcoma cells. These observations suggest that cooperating molecules or pathways necessary for AKT to mediate oncogenic transformation in other cancers may be absent in Ewing sarcoma cells. Indeed, polymerization of KRT17 with KRT5/6α/6β is required to form stable cytoskeletal structures (39), and mutations in KRT17 or its partners KRT5, 6α or 6β result in human genetic diseases. We inspected our global transcriptional profiling datasets and found very low, if any, expression for KRT5, 6α or 6β in Ewing sarcoma cells, suggesting that KRT17 functions in a novel capacity to regulate oncogenic

transformation. This also indicates that in addition to regulating AKT signaling, KRT17 might impinge on multiple critical growth factor signaling pathways in the context of Ewing sarcoma cells - all of which together contribute to the transformed phenotype. Further studies are ongoing to identify the precise mechanism by which KRT17 regulates oncogenic transformation in Ewing sarcoma.

Importantly, we demonstrate in this report that KRT17-mediated AKT phosphorylation is necessary and sufficient for regulating cellular adhesion. There is a growing body of evidence indicating that alterations in the adhesion properties of cells play a pivotal role in the development and progression of cancer (53). Expression of EWS/FLI has profound effects on adhesion and cytoskeletal architecture of Ewing sarcoma cells (31). In support of this are transcriptional profiling data for EWS/FLI in Ewing sarcoma cells that reveal significant downregulation of adhesion and cytoskeletal proteins, suggesting that Ewing sarcoma cells have low basal levels of cellular adhesion (10). In fact cellular adhesion is dramatically increased upon EWS/FLI knockdown in Ewing sarcoma cells (31). Ewing sarcoma is a highly metastatic tumor and in the absence of chemotherapy, the vast majority of patients die from metastatic disease, suggesting that most patients have micrometastases at presentation (54, 55). In support of this, circulating tumor cells can be identified in Ewing sarcoma patients (56). These observations suggest that, in contrast to epithelial cancers, which are thought to follow a multistep process for metastasis, a mesenchymal tumor like Ewing sarcoma may display metastatic dissemination of tumor cells early in the disease process (31). The ability of Ewing sarcoma tumor cells to readily disseminate clearly highlights the importance of regulating adhesion levels in these tumors. Although EWS/FLI largely inhibits cellular

adhesion proteins likely to promote metastatic dissemination in Ewing sarcoma, these tumor cells still need to maintain low basal levels of adhesion to be able to form tumors, and to adhere to and colonize secondary sites of metastasis. Our data suggests that KRT17 is one of the critical cytoskeletal proteins, downstream of EWS/FLI and GLI1 that is necessary to maintain basal levels of cellular adhesion in Ewing sarcoma by activating the AKT signaling pathway. Interestingly, the AKT signaling pathway has been previously shown to activate Focal Adhesion Kinase (FAK)-dependent adhesion in cancer (57) further supporting our finding that AKT signaling regulates cellular adhesion in Ewing sarcoma.

Our data in this study suggest that AKT-signaling uncouples KRT17-mediated cellular adhesion and oncogenic transformation in Ewing sarcoma. A similar uncoupling of cellular adhesion and oncogenic transformation has previously been noted in activated Src kinase signaling. Src kinase expression/activity is frequently increased in various cancers where it affects oncogenic transformation by activating RAS, PI3K and STAT signaling pathways (58). Activated mutants of Src play a role in oncogenic transformation and affect morphological changes including cellular adhesion (59). Interestingly it has been shown that integrin α5β3 signaling regulates Src-kinase mediated oncogenic transformation but this interaction does not affect Src-mediated cellular adhesion (60). Our data suggest that signaling downstream of KRT17 may occur through multiple independent pathways, one of which is AKT signaling, that is necessary for cellular adhesion but dispensable for oncogenic transformation.

Based on our findings, we would hypothesize that inhibiting the AKT signaling pathway alone would be an ineffective therapy for Ewing sarcoma patients. In support of

this are findings that Insulin-like Growth Factor 1 Receptor (IGF1R) antagonists that have shown efficacy in Phase I/II clinical trials for the treatment of Ewing sarcoma patients (61, 62) inhibit not only the PI3K-AKT signaling but also the RAS-MAPK and JAK-STAT pathways. Therefore, inhibiting multiple crucial signaling pathways may be necessary to inhibit growth and transformation of Ewing sarcoma cells. Also, targeting pathways downstream of IGF1R, with MEK/MAPK inhibitors (PD98059 and U0126) and the PI3K inhibitor (LY294002) decreases Ewing sarcoma cell survival and increases sensitivity to doxorubicin (63). Interestingly, blocking AKT activation alone did not have any effect on survival or proliferation of Ewing sarcoma cells (Sankar, unpublished observations). Our results demonstrate that active AKT signalling is not required for proliferation or oncogenic transformation in Ewing sarcoma.

In conclusion, we have defined a new pathway downstream of GLI1 in Ewing sarcoma that highlights the central role of KRT17 in coordinating both oncogenic transformation and cellular adhesion in Ewing sarcoma. Future work will be required to identify the critical factors and pathways downstream of KRT17 that affect oncogenic transformation. These studies will be key to a better understanding of the biology of Ewing sarcoma, and may lead to more effective targeted therapies for patients with this devastating disease.

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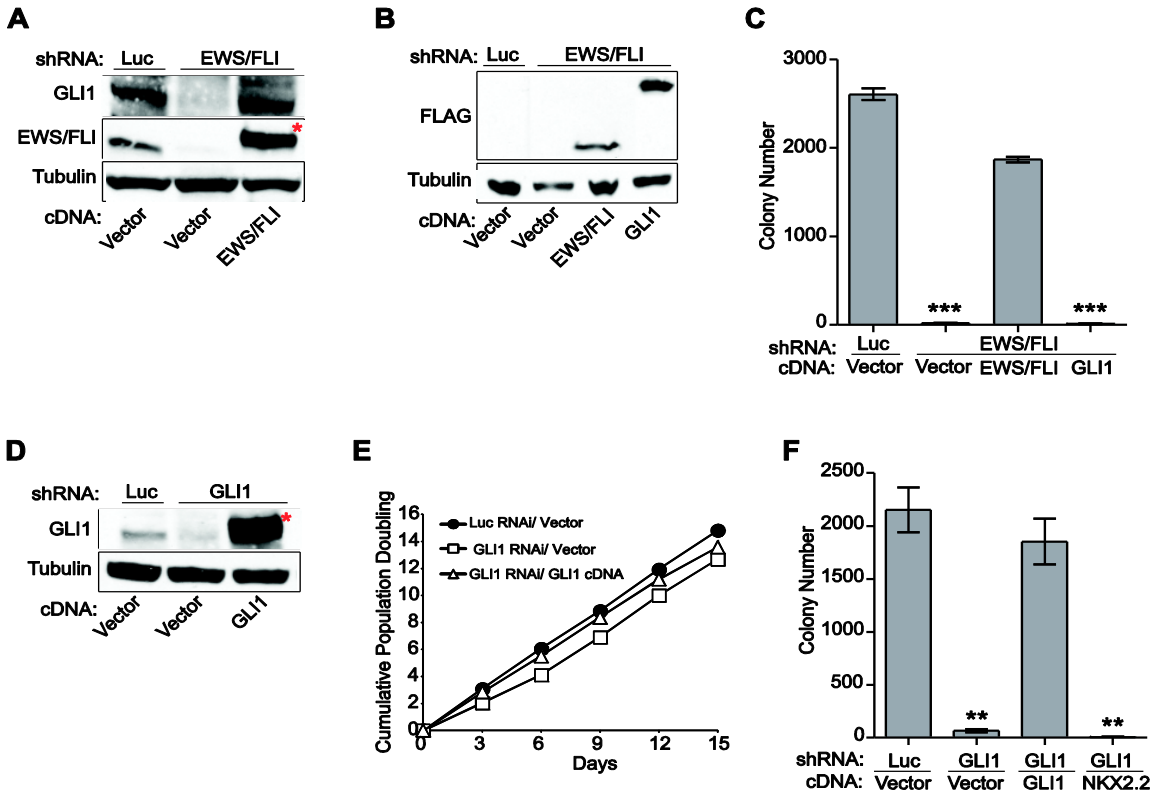


Figure 5.1 GLI1 is upregulated by EWS/FLI and is necessary for oncogenic transformation in Ewing sarcoma cells. (A) Western blot analysis to demonstrate EWS/FLI mediated activation of GLI1. GLI1 and EWS/FLI levels were assessed in A673 cells infected with a control shRNA (Luc) or an shRNA targeting EWS/FLI followed by rescue with an empty vector or an RNAi-resistant EWS/FLI cDNA using anti-GLI1 and anti-FLI antibodies. Tubulin was used as a loading control. (*) indicates the 3x-FLAG tagged EWS/FLI cDNA that runs slightly higher than endogenous EWS/FLI. (B) Western blot analysis to demonstrate expression of the RNAi-resistant 3x-FLAG tagged EWS/FLI cDNA or 3x-FLAG tagged GLI1 cDNA constructs using an anti-FLAG antibody in A673 cells expressing a control shRNA (Luc) or an EWS/FLI shRNA. Tubulin was used as the loading control. (C) Quantification of colonies formed by A673 cells described in (B). Error bars indicate SD of duplicate assays. P-values were determined using a student's t-test comparing all conditions to the control knockdown/empty vector condition (*** for $p \leq 0.001$). (D) Western blot analysis of GLI1 levels in A673 cells infected with a control shRNA (Luc) or an shRNA targeting GLI1 followed by rescue with an empty vector or an RNAi-resistant GLI1 cDNA. Tubulin was used as a loading control. (*) indicates the 3x-FLAG tagged GLI1 cDNA that runs slightly higher than endogenous GLI1. (E) Growth assays (3T5) for A673 cells described in (D). Student's t-test showed no significant difference in growth curves. (F) Quantification of colonies formed in soft agar by A673 cells expressing a control shRNA (Luc) or a GLI1 shRNA, re-expressing an empty vector or an RNAi-resistant GLI1 or NKX2.2 cDNA constructs. Error bars indicate SD of duplicate assays. P-values were determined using a student's t-test comparing all conditions to the control knockdown/empty vector condition (** for $p \leq 0.01$).

Figure 5.2 GLI1 regulates a significant portion of the EWS/FLI transcription profile in Ewing sarcoma cells. (A) Heat map representation of the rank-ordered expression profiling data from the GLI1 RNA-seq. Genes were ranked by mean deviation of the log transformed FPKM (Fragments Per Kilobase per Million mapped reads). Each row represents a different gene. The top 15 upregulated (left) and downregulated (right) genes from the GLI1 RNA-seq are shown. (B) Venn diagram representations of the overlap between the EWS/FLI and the GLI1 transcription profiles, both generated by RNA-seq in A673 cells. The Chi square-determined p-values are indicated. (C) Gene set enrichment analysis (GSEA) using the EWS/FLI regulated genes in A673 cells (RNA-seq) as the rank-ordered dataset and the 86 Gli1 upregulated and 55 GLI1-downregulated genesets (RNA-seq). The positions of the 86 and 55 GLI1 genes are indicated as black vertical lines in the center portion of the panel. The normalized enrichment scores (NES) and p-values are shown. (D) Top ten categories identified by DAVID functional analysis of the GLI1 up and downregulated genesets. The log transformed enrichment scores for each category are indicated on the x-axis.

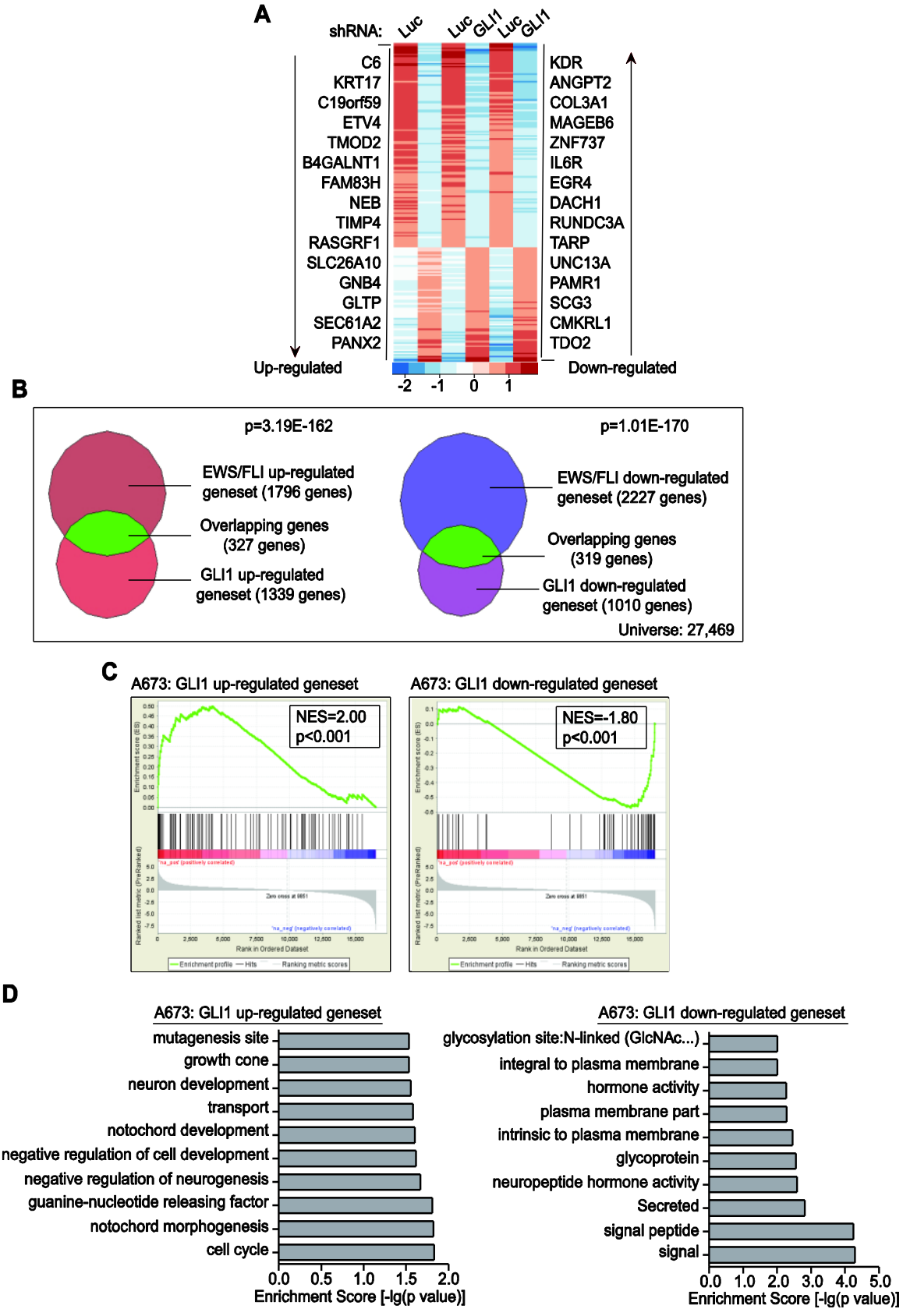

A
shRNA:
Luc
Luc
GLI1
Luc
GLI1
C6
KRT17
C19orf59
ETV4
TMOD2
B4GALNT1
FAM83H
NEB
TIMP4
RASGRF1
SLC26A10
GNB4
GLTP
SEC61A2
PANX2
KDR
ANGPT2
COL3A1
MAGEB6
ZNF737
IL6R
EGR4
DACH1
RUNDC3A
TARP
UNC13A
PAMR1
SCG3
CMKRL1
TDO2
Up-regulated
Down-regulated
-2
-1
0
1
B
p=3.19E-162
EWS/FLI up-regulated geneset (1796 genes)
Overlapping genes (327 genes)
GLI1 up-regulated geneset (1339 genes)
p=1.01E-170
EWS/FLI down-regulated geneset (2227 genes)
Overlapping genes (319 genes)
GLI1 down-regulated geneset (1010 genes)
Universe: 27,469
C
A673: GLI1 up-regulated geneset
NES=2.00
p<0.001
A673: GLI1 down-regulated geneset
NES=-1.80
p<0.001
D
A673: GLI1 up-regulated geneset
mutagenesis site
growth cone
neuron development
transport
notochord development
negative regulation of cell development
negative regulation of neurogenesis
guanine-nucleotide releasing factor
notochord morphogenesis
cell cycle
0.0
0.5
1.0
1.5
2.0
Enrichment Score [-lg(p value)]
A673: GLI1 down-regulated geneset
glycosylation site:N-linked (GlcNAc...)
integral to plasma membrane
hormone activity
plasma membrane part
intrinsic to plasma membrane
glycoprotein
neuropeptide hormone activity
Secreted
signal peptide
signal
0.0
1.0
2.0
3.0
4.0
5.0
Enrichment Score [-lg(p value)]

Figure 5.3 KRT17 is regulated by GLI1 in multiple Ewing sarcoma cell lines. (A) Validation of KRT17 being an EWS/FLI and GLI1 target gene. qRT-PCR analysis of KRT17 in A673 cells infected with a control shRNA (Luc), an EWS/FLI shRNA or a GLI1 shRNA, followed by rescue with an empty vector, an RNAi-resistant EWS/FLI cDNA or a GLI1 cDNA construct. Error bars indicate SD. P-values were determined using a student's t-test comparing all conditions to the control knockdown/empty vector condition (** for $p \leq 0.01$; *** for $p \leq 0.001$). (B) Western blot analysis of cells described in (A) using KRT17, EWS/FLI and GLI1 antibodies. Tubulin was used as the loading control. (*) indicates the 3x-FLAG tagged EWS/FLI and GLI1 cDNAs. (C) qRT-PCR validation of KRT17 being a GLI1 target gene in multiple patient-derived Ewing sarcoma cell lines (TC71, TC32, SK-N-MC and EWS502). Cells were infected with a control shRNA (Luc) or a GLI1 shRNA. GLI1 and KRT17 mRNA levels were analyzed. Error bars indicate SD. P-values were determined using a student's t-test comparing all conditions to the control knockdown (Luc-shRNA) (** for $p \leq 0.01$; *** for $p \leq 0.001$). (D) Western blot analysis of KRT17 expression in multiple patient-derived Ewing sarcoma cell lines (A673, TC71, TC32, SKNMC, SKES1 and EWS502). Tubulin was used as the loading control. (E) RT-PCR analysis of KRT17 transcript levels in five independent Ewing sarcoma patient tumor samples compared to KRT17 transcript levels in A673 cells infected with a control shRNA (Luc) or a KRT17 shRNA as well as a water negative control. (F) Luciferase reporter assay in HEK293 EBNA cells cotransfected with a 1 Kb. KRT17 promoter region upstream of luciferase or a control vector (that does not contain the KRT17 promoter) and an empty vector or increasing concentrations of the GLI1 cDNA. Relative luciferase activity is the ratio of firefly luciferase activity to *Renilla* luciferase activity (to control for transfection efficiency). Error bars indicate SD. P-values were determined using a student's t-test comparing all GLI1 cDNA transfected conditions to the vector transfected condition (** for $p \leq 0.01$).

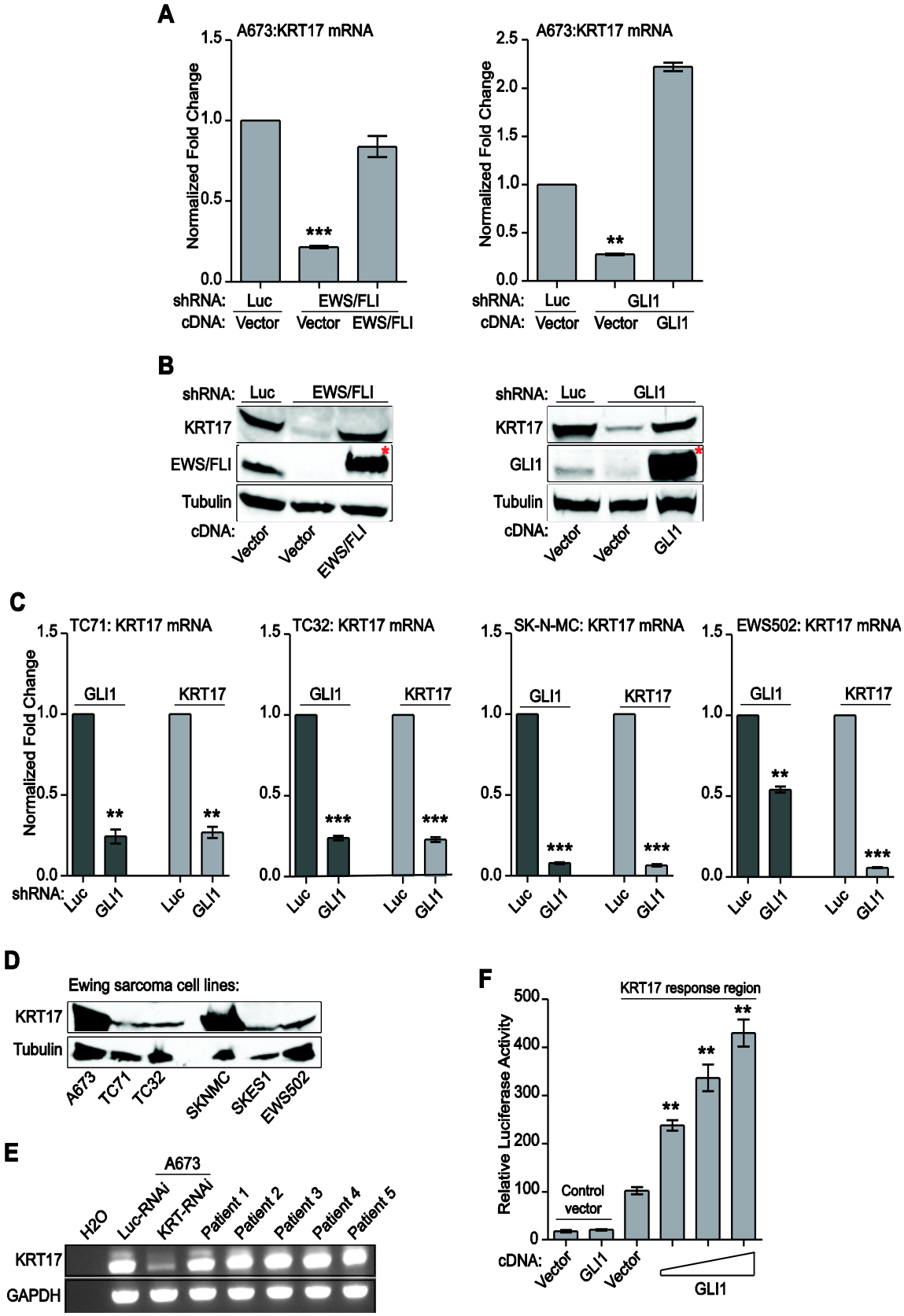

A
A673:KRT17 mRNA
Normalized Fold Change
1.5
1.0
0.5
0.0
***
shRNA: Luc EWS/FLI
cDNA: Vector Vector EWS/FLI
A673:KRT17 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
**
shRNA: Luc GLI1
cDNA: Vector Vector GLI1
B
shRNA: Luc EWS/FLI
KRT17
EWS/FLI
Tubulin
cDNA: Vector Vector EWS/FLI
shRNA: Luc GLI1
KRT17
GLI1
Tubulin
cDNA: Vector Vector GLI1
C
TC71: KRT17 mRNA
TC32: KRT17 mRNA
SK-N-MC: KRT17 mRNA
EWS502: KRT17 mRNA
Normalized Fold Change
GLI1
KRT17
shRNA: Luc GLI1
D
Ewing sarcoma cell lines:
KRT17
Tubulin
A673 TC71 TC32 SKNMC SKES1 EWS502
E
A673
H2O Luc-RNAi KRT-RNAi Patient 1 Patient 2 Patient 3 Patient 4 Patient 5
KRT17
GAPDH
F
KRT17 response region
Relative Luciferase Activity
500
400
300
200
100
0
Control vector
cDNA: Vector GLI1 Vector GLI1

Figure 5.4 KRT17 is necessary for GLI1 mediated oncogenesis in Ewing sarcoma. (A) shRNA knock-down of KRT17 in A673 cells infected with a control shRNA (Luc) or two different shRNA constructs targeting KRT17, measured by qRT-PCR. Error bars indicate SD. P-values were determined using a student's t-test comparing all conditions to the control knockdown (Luc-shRNA) (** for $p \leq 0.01$; *** for $p \leq 0.001$). (B) Western blot analysis of KRT17 in cells described in (A). Tubulin was used as the loading control. (C) Growth assays (3T5) for A673 cells described in (A). Student's t-test showed no significant difference in growth curves. (D) Quantification of colonies formed in methylcellulose by A673 cells expressing a control shRNA (Luc) or two different KRT17 shRNAs, re-expressing an empty vector or an RNAi-resistant KRT17 cDNA construct. Error bars indicate SD of duplicate assays. P-values were determined using a student's t-test comparing all conditions to the control knockdown/empty vector condition (** for $p \leq 0.01$). (E) Western blot analysis of cells described in (D). The KRT17 blot demonstrates maintenance of KRT17 knockdown in cells infected with two independent KRT17 shRNAs and re-expressing an empty vector control, compared to control cells. The FLAG blot demonstrates expression of the RNAi-resistant KRT17 cDNA construct. (F) Survival curves for immunodeficient mice subject to subcutaneous or intratibial injections with A673 cells expressing a control shRNA (ERG) or a KRT17 shRNA. Five mice were used per condition. For the subcutaneous model, both flanks of each mouse was injected subcutaneously. In the control condition one mouse died due to the anesthesia and was censored from the analysis. Therefore 8 and 10 tumors were measured for the control knockdown and KRT17 knockdown groups, respectively. For the intratibial model, the right tibia of each mouse was injected, and therefore, 5 tumors were measured for each group. The mice in each group in the subcutaneous model were sacrificed once their tumors reached a size limit of 2 cubic cm. The mice in each group in the intratibial model were sacrificed once their tumors reached a size limit of 1.5 cubic cm. Percent survival was plotted for both models as Kaplan-Meier survival curves using GraphPad Prism. The log-rank (Mantel-Cox Test) determined p-values using GraphPad Prism are indicated. (G) Western blot analysis of control (ERG) shRNA or KRT17 shRNA expressing tumors from the subcutaneous injection model described in (F). KRT17 levels in the tumors were compared to levels in the parental A673 cells expressing either the control shRNA or KRT17 shRNA, used to inject mice. Tubulin was used as the loading control. (H) Western blot analysis of A673 cells expressing a control shRNA (Luc) or a GLI1 shRNA and re-expressing an empty vector, 3x-FLAG tagged GLI1 or 3x-FLAG tagged KRT17 cDNA constructs using a FLAG antibody. Tubulin was used as the loading control. (I) Quantification of colonies formed in methylcellulose by A673 cells described in (H). Error bars indicate SD of duplicate assays. The P-value was determined using a student's t-test comparing the GLI1 knockdown/empty vector condition to the control knockdown/empty vector condition (*** for $p \leq 0.001$).

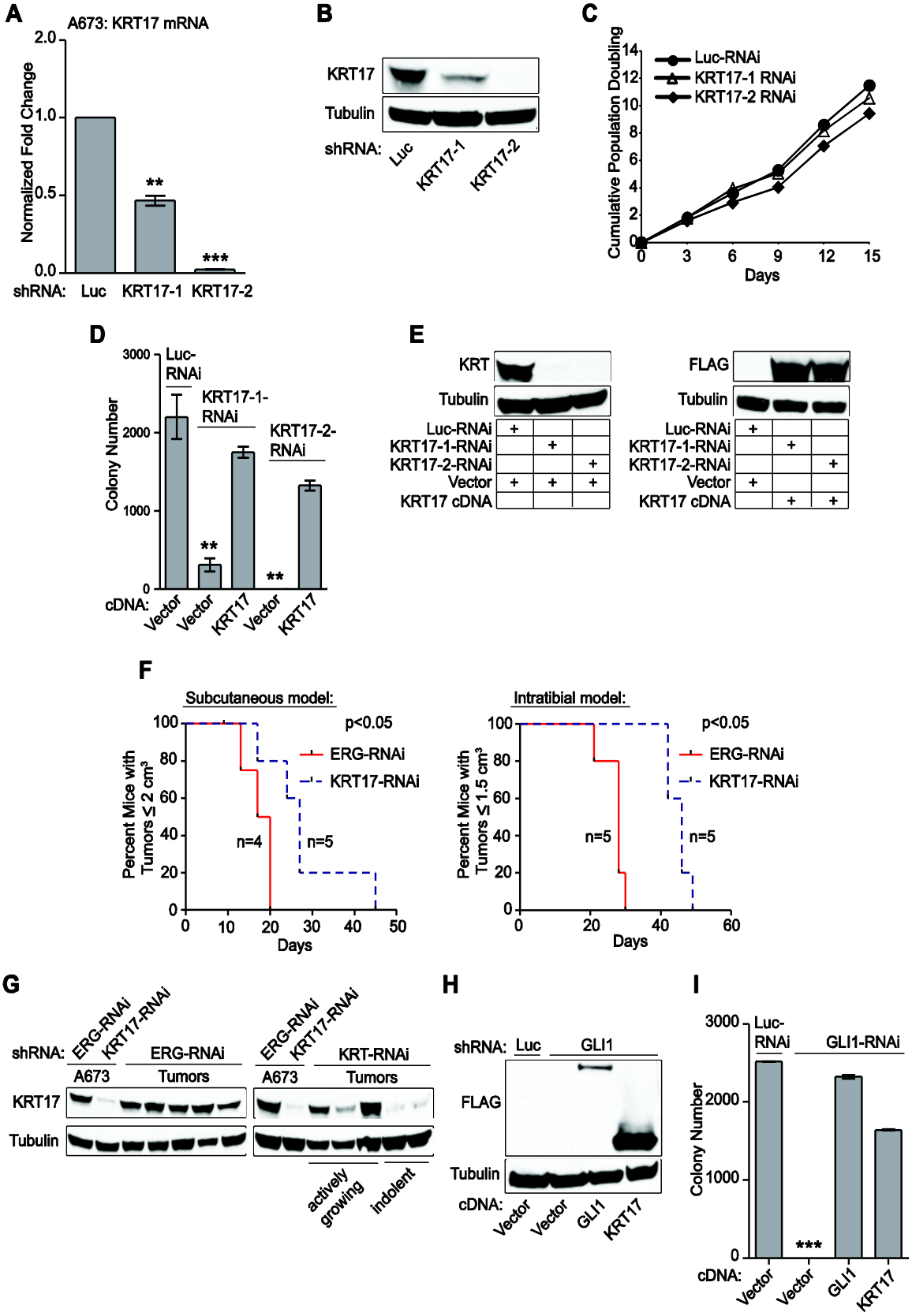
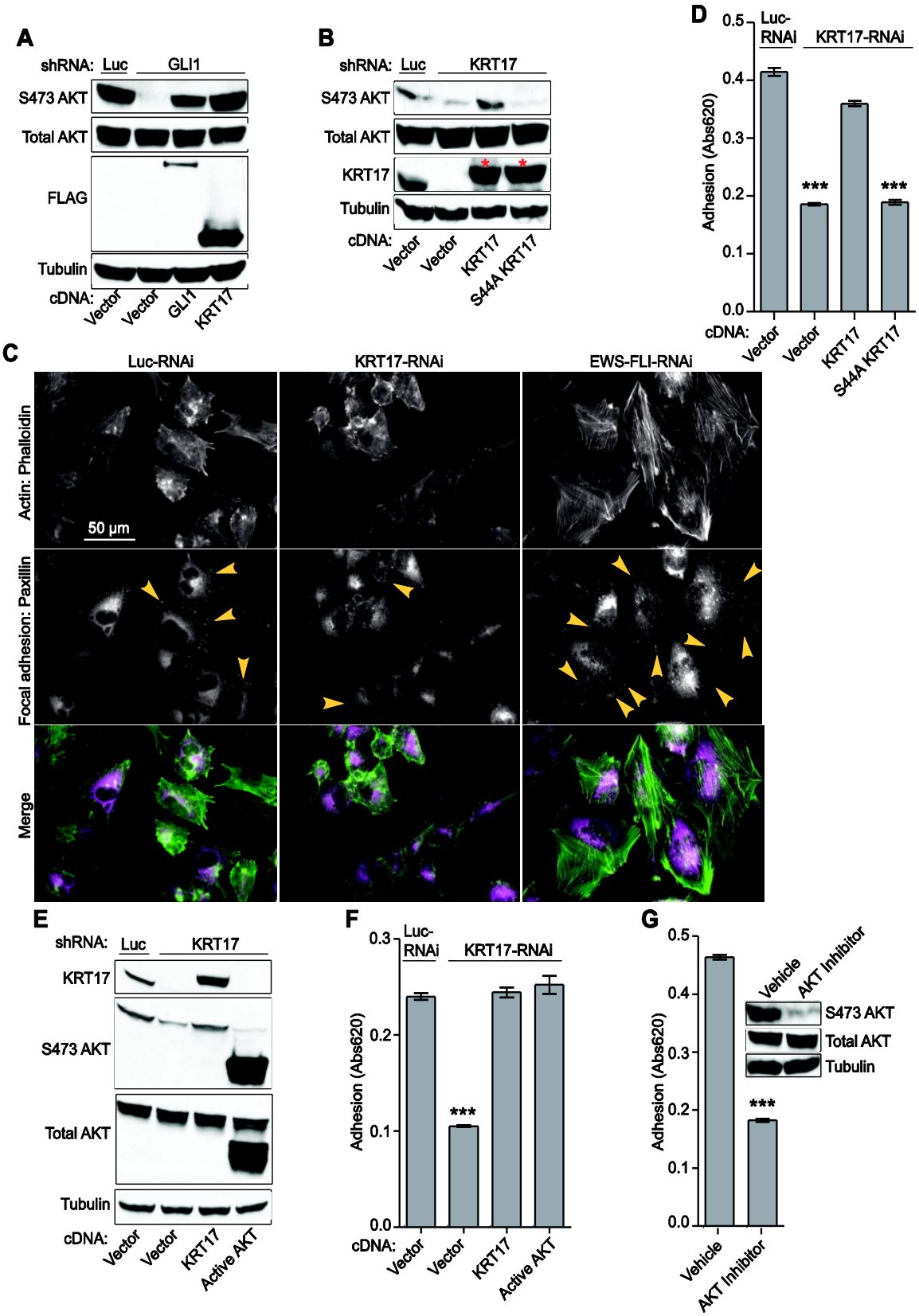

A
A673: KRT17 mRNA
Normalized Fold Change
2.0
1.0
0.5
0.0
**
***
shRNA: Luc KRT17-1 KRT17-2
B
KRT17
Tubulin
shRNA: Luc KRT17-1 KRT17-2
C
Cumulative Population Doubling
Luc-RNAi
KRT17-1 RNAi
KRT17-2 RNAi
Days
D
Colony Number
Luc-RNAi
KRT17-1-RNAi
KRT17-2-RNAi
cDNA: Vector Vector KRT17 Vector KRT17
E
KRT
Tubulin
FLAG
Luc-RNAi
KRT17-1-RNAi
KRT17-2-RNAi
Vector
KRT17 cDNA
F
Subcutaneous model:
Intratibial model:
p<0.05
ERG-RNAi
KRT17-RNAi
Percent Mice with Tumors ≤ 2 cm³
Percent Mice with Tumors ≤ 1.5 cm³
n=4
n=5
Days
G
shRNA: ERG-RNAi KRT17-RNAi ERG-RNAi ERG-RNAi KRT17-RNAi KRT-RNAi
A673 Tumors A673 Tumors
KRT17
Tubulin
actively growing
indolent
H
shRNA: Luc GLI1
FLAG
Tubulin
cDNA: Vector Vector GLI1 KRT17
I
Colony Number
Luc-RNAi
GLI1-RNAi
***
cDNA: Vector Vector GLI1 KRT17

Figure 5.5 KRT17 is necessary and sufficient for AKT phosphorylation mediated cellular adhesion in Ewing sarcoma cells. (A) Western blot analysis of A673 cells infected with a control shRNA (Luc) or the GLI1 shRNA and re-expressing an empty vector, GLI1 or KRT17 cDNA constructs. The protein lysate from these cells were probed with phosphorylated-AKT (S473), total AKT and FLAG antibodies. Tubulin was used as a loading control. (B) Western blot analysis of A673 cells infected with a control shRNA (Luc) or a KRT17 shRNA and re-expressing an empty vector, KRT17 wild-type or an S44A mutant KRT17 cDNA construct. The protein lysate from these cells were probed with phosphorylated-AKT (S473), total AKT and KRT17 antibodies. (*) indicates 3x-FLAG tagged KRT17 and 3x-FLAG tagged S44A KRT17 cDNA constructs, which run slightly higher than endogenous KRT17. Tubulin was used as a loading control. (C) Immunofluorescence images of A673 cells infected with a control shRNA (Luc), KRT17 shRNA or an EWS/FLI shRNA stained for focal adhesions (paxillin antibody) and for actin filaments (phalloidin). Arrow heads indicate paxillin-rich focal adhesions. (D) Adhesion assay with A673 cells infected with a control shRNA (Luc) or a KRT17 shRNA, re-expressing an empty vector, KRT17 wild-type or S44A mutant KRT17 cDNA constructs. Error bars indicate SD. P-values were determined using a student's t-test comparing all conditions to the control knockdown/empty vector condition (*** for $p \leq 0.001$). (E) Western blot analysis of A673 cells infected with a control shRNA (Luc) or a KRT17 shRNA and re-expressing an empty vector, KRT17 cDNA or a constitutively active (myristoylated) form of AKT. The protein lysate from these cells were probed with KRT17, phosphorylated-AKT (S473) and total AKT antibodies. Tubulin was used as a loading control. (F) Adhesion assay with A673 cells described in (E). Error bars indicate SD. The P-value was determined using a student's t-test comparing the KRT17 knockdown/empty vector condition to the control knockdown/empty vector condition (*** for $p \leq 0.001$). (G) Adhesion assay with A673 cells treated with the selective AKT inhibitor or vehicle control for 24 hours. Error bars indicate SD. The P-value was determined using a student's t-test comparing the inhibitor treated condition to the vehicle treated condition (*** for $p \leq 0.001$).


A
shRNA: Luc GLI1
S473 AKT
Total AKT
FLAG
Tubulin
cDNA: Vector Vector GLI1 KRT17
B
shRNA: Luc KRT17
S473 AKT
Total AKT
KRT17
Tubulin
cDNA: Vector Vector KRT17 S44A KRT17
D
Luc-RNAi
KRT17-RNAi
Adhesion (Abs620)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
cDNA: Vector Vector KRT17 S44A KRT17
C
Luc-RNAi
KRT17-RNAi
EWS-FLI-RNAi
Actin: Phalloidin
50 μm
Focal adhesion: Paxillin
Merge
E
shRNA: Luc KRT17
KRT17
S473 AKT
Total AKT
Tubulin
cDNA: Vector Vector KRT17 Active AKT
F
Luc-RNAi
KRT17-RNAi
Adhesion (Abs620)
0.3
0.2
0.1
0.0
***
cDNA: Vector Vector KRT17 Active AKT
G
Vehicle
AKT Inhibitor
S473 AKT
Total AKT
Tubulin
Adhesion (Abs620)
0.5
0.4
0.3
0.2
0.1
0.0
***
Vehicle
AKT Inhibitor

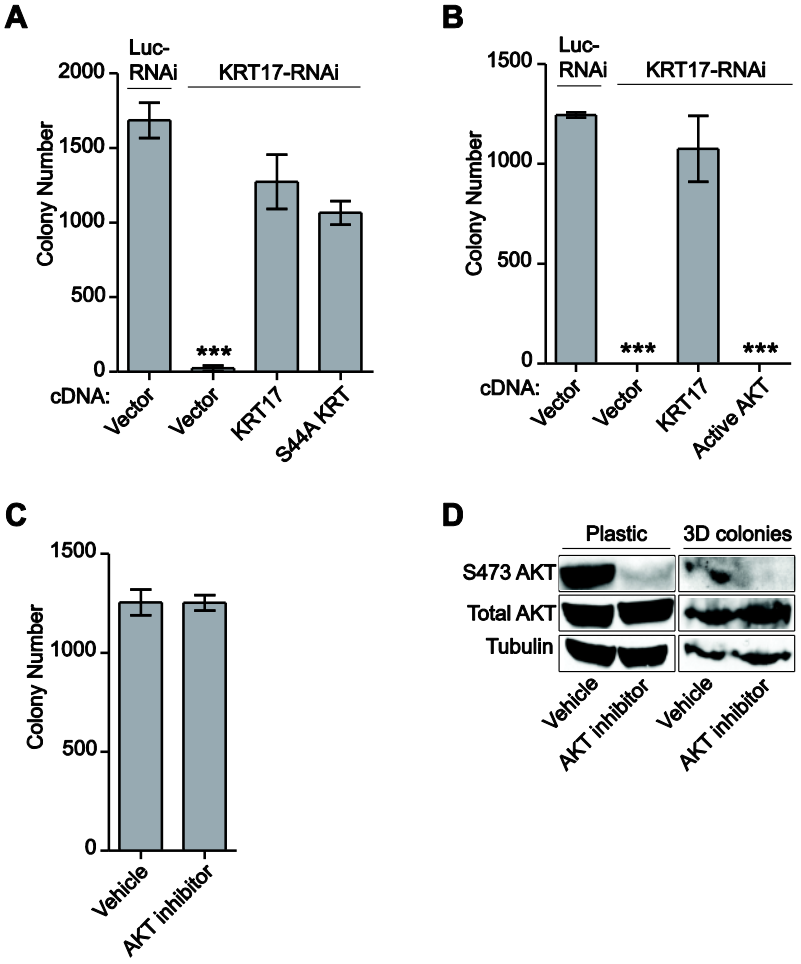


Figure 5.6 KRT17 mediated oncogenic transformation is independent of AKT signaling. (A) Quantification of colonies formed in methylcellulose by A673 cells infected with a control shRNA (Luc) or a KRT17 shRNA and re-expressing an empty vector, KRT17 wild-type or the S44A mutant KRT17 cDNA construct. Error bars indicate SD of duplicate assays. The P-value was determined using a student's t-test comparing the KRT17 knockdown condition rescued with an empty vector to the control knockdown condition rescued with an empty vector (*** for $p \leq 0.001$). (B) Quantification of colonies formed in methylcellulose by A673 cells infected with a control shRNA (Luc) or a KRT17 shRNA and re-expressing an empty vector, KRT17 cDNA or a constitutively active (myristoylated) form of AKT. Error bars indicate SD of duplicate assays. P-values were determined using a student's t-test comparing all conditions to the control knockdown/empty vector condition (*** for $p \leq 0.001$). (C) Quantification of colonies formed in methylcellulose by A673 cells treated with a selective AKT inhibitor or a vehicle control for 24 hours. Error bars indicate SD of duplicate assays. (D) Western blot analysis of A673 cells described in (C). Protein lysates from treated cells and from 3D colonies at the end of the anchorage-independent colony forming assay, were probed with phosphorylated-AKT (S473) and total AKT antibodies. Tubulin was used as the loading control. The total amount of protein obtained from the 3D colonies was much less that that achieved from cells grown and treated on plastic.

CHAPTER 6

EWS AND RE1 SILENCING TRANSCRIPTION FACTOR INHIBIT NEURONAL PHENOTYPE DEVELOPMENT AND ONCOGENIC TRANSFORMATION IN EWING SARCOMA

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Original Article

EWS and RE1-Silencing Transcription Factor Inhibit Neuronal Phenotype Development and Oncogenic Transformation in Ewing Sarcoma

Genes & Cancer
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Submitted 14-Feb-2013; accepted 10-Apr-2013

Abstract

The gene encoding EWS (*EWSR1*) is involved in various chromosomal translocations that cause the production of oncoproteins responsible for multiple cancers including Ewing sarcoma, myxoid liposarcoma, soft tissue clear cell sarcoma, and desmoplastic small round cell sarcoma. It is well known that EWS fuses to FLI to create EWS/FLI, which is the abnormal transcription factor that drives tumor development in Ewing sarcoma. However, the role of wild-type EWS in Ewing sarcoma pathogenesis remains unclear. In the current study, we identified EWS-regulated genes and cellular processes through RNA interference combined with RNA sequencing and functional annotation analyses. Interestingly, we found that EWS and EWS/FLI co-regulate a significant cluster of genes, indicating an interplay between the 2 proteins in regulating cellular functions. We found that among the EWS–down-regulated genes are a subset of neuronal genes that contain binding sites for the RE1-silencing transcription factor (REST or neuron-restrictive silencer factor [NRSF]), neuron-restrictive silencer element (NRSE), suggesting a cooperative interaction between REST and EWS in gene regulation. Co-immunoprecipitation analysis demonstrated that EWS interacts directly with REST. Genome-wide binding analysis showed that EWS binds chromatin at or near NRSE. Furthermore, functional studies revealed that both EWS and REST inhibit neuronal phenotype development and oncogenic transformation in Ewing sarcoma cells. Our data implicate an important role of EWS in the development of Ewing sarcoma phenotype and highlight a potential value in modulating EWS function in the treatment of Ewing sarcoma and other EWS translocation–based cancers.

Keywords

EWS, REST, oncogenic transformation, neuronal phenotype, Ewing sarcoma

Introduction

Ewing sarcoma is a translocation-based pediatric bone and soft tissue tumor. In most Ewing sarcoma cases, translocation causes a fusion between the *EWSR1* gene (encoding EWS) and the *FLI1* gene (encoding FLI) and gives rise to the fusion protein EWS/FLI. It is well known that EWS/FLI functions as an aberrant transcription factor to deregulate the expression of target genes and promote tumor development.[1-3] In addition to the gain of function of EWS/FLI, translocation also results in the loss of 1 *EWSR1* allele. In fact, a case of Ewing sarcoma with both copies of *EWSR1* translocated, and therefore no wild-type EWS expression, has been reported,[4] suggesting that EWS is dispensable for tumor growth. However, EWS function is disrupted or insufficient in several EWS translocation–based cancers, indicating that EWS may contribute to the suppression of cancer-related phenotypes. So far, little is known about the role of EWS, if any, in regulating cancer-related phenotypes.

In general, EWS is an RNA-binding protein and has been implicated in transcription regulation and RNA

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[7]Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
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(Email: wen.luo@hci.utah.edu).

processing.[5] EWS interacts with RNA polymerase II and the TFIID transcription preinitiation complex. EWS co-transcriptionally binds to its target mRNA and regulates the alternative splicing or exon skipping of genes involved in DNA repair and related signaling upon cellular stress.[6,7] In addition, EWS binds to noncoding RNA and inhibits the histone acetyltransferase (HAT) activity of CBP/p300 on a repressed gene target, *CCND1*, upon radiation.[8] Recently, EWS was found to be associated with RNA granules under genotoxic stress.[9,10] Most of the above known EWS functions are characterized under conditions of cellular stress in non–Ewing sarcoma settings. Notably in Ewing sarcoma, EWS co-exists with EWS/FLI, which has been shown to bind EWS and interfere with EWS-mediated transcription regulation and splicing in a dominant-negative manner.[11-13] However, neither the function of EWS nor the relationship between EWS and EWS/FLI in Ewing sarcoma is well defined.

Interesting neuronal features have been identified in Ewing sarcoma cells and tumors, such as the presence of Homer-Wright rosettes, neural processes, neurosecretory granules, and neural immunohistochemical markers.[14,15] It has been suggested that the neural phenotype of Ewing sarcoma may be a consequence of the translocation and resultant expression of EWS/FLI because the introduction of EWS/FLI into NIH3T3 or rhabdomyosarcoma RD cells induces the features of neural differentiation.[16,17] However, Ewing sarcoma and peripheral primitive neuroectodermal tumor (pPNET), which contain the same t(11;22)(q24;q12) translocation[18-20] and represent the same disease, exhibit varying levels of neural differentiation. This suggests that other modulators may exist to regulate the neuronal phenotype in Ewing sarcoma.

RE1-silencing transcription factor (REST or neuron-restrictive silencer factor [NRSF]) is a transcription repressor that has diverse functions in a context-dependent manner through interactions with distinct co-factors.[21-23] REST was originally found to repress neuronal gene expression in non-neuronal cells.[21] It is now widely recognized that REST also plays a role in tumorigenesis.[24-27] REST was identified as a tumor suppressor in an RNA interference (RNAi)–based genetic screen in epithelial cells using an *in vitro* breast cancer precursor model.[24] Later studies revealed that REST is frequently deleted in colon and small cell lung cancers,[24,25] supporting a role for this transcription repressor as a tumor suppressor. In breast cancer, a nonfunctional, truncated splice variant of REST was identified in some tumor subtypes, and expression of this truncated variant of REST was shown to correlate with poor prognosis.[28] Interestingly, these REST-deficient tumors acquire certain neuronal phenotypes such as the expression of neuronal genes that are normally not expressed outside the nervous system.[24,25]

In this article, we sought to characterize the function of EWS in Ewing sarcoma and found that EWS contributes to cancer phenotypes in that EWS cooperates with REST to repress neuronal phenotype development and EWS and REST inhibit oncogenic transformation in Ewing sarcoma cells.

Results

Identification of EWS-regulated genes and cellular processes in Ewing sarcoma. To characterize the function of EWS in Ewing sarcoma, we silenced EWS in A673 Ewing sarcoma cells (Fig. 1A) and performed high-throughput sequencing of RNA (RNA-seq) from control (luciferase) or EWS knockdown cells to identify EWS-regulated genes. Sequencing reads were mapped to Ensembl annotations (www.ensembl.org), and expression levels of genes based on the Ensembl annotation are shown in Supplementary File S1. Genes were ranked by the mean ± standard deviation of log-transformed FPKM (fragments per kilobase per million mapped reads) and shown as a heat map in Figure 1B. To gain insight into the functional significance of the differentially expressed genes, we performed DAVID functional annotation analysis (david.abcc.ncifcrf.gov) of 99 genes that pass the filter of a 5% false discovery rate (FDR) and $\log_2$ ratio >1 or <–1. We found that these genes are associated with diverse functions, including those that have previously been indicated for EWS in non–Ewing sarcoma cells, such as a response to various cellular stresses, as well as previously unidentified functions including cell signaling, secretion, blood vessel development, and neuronal-related processes (Fig. 1C and Suppl. File S2). A subset of EWS–up-regulated and –down-regulated genes was randomly selected and validated by qRT-PCR) (Fig. 1D and 1E).

EWS-regulated genes are differentially regulated by EWS/FLI. Because EWS/FLI has previously been shown to interfere with EWS functions,[12,13] we next sought to determine the relationship between EWS and EWS/FLI in regulating cellular processes in Ewing sarcoma. We performed RNA-seq following EWS/FLI silencing and compared the EWS/FLI-regulated transcriptional profile (Suppl. File S3) with that of EWS. We used VennMaster analysis (informatik.uni-ulm.de/ni/staff/HKestler/vennm) to identify genes commonly regulated by EWS and EWS/FLI. Fifty-three of the 99 EWS-regulated genes were found to also be regulated by EWS/FLI (P = 3.86345E-30) (Fig. 2A). Of these 53 EWS and EWS/FLI commonly regulated genes, 14 genes were up-regulated by EWS and down-regulated by EWS/FLI (P = 4.30758E-9), and 13 genes were down-regulated by EWS and up-regulated by EWS/FLI (P = 4.62419E-8), suggesting that the 2 proteins inversely regulate a significant subset of target genes (Fig. 2B). Interestingly, we also identified 7 genes that were up-regulated (P = 0.00897) and 19 genes that were down-regulated (P = 4.19203E-14) by both EWS and EWS/FLI (Fig. 2B), indicating that the 2 proteins also regulate a significant subset of genes in the same

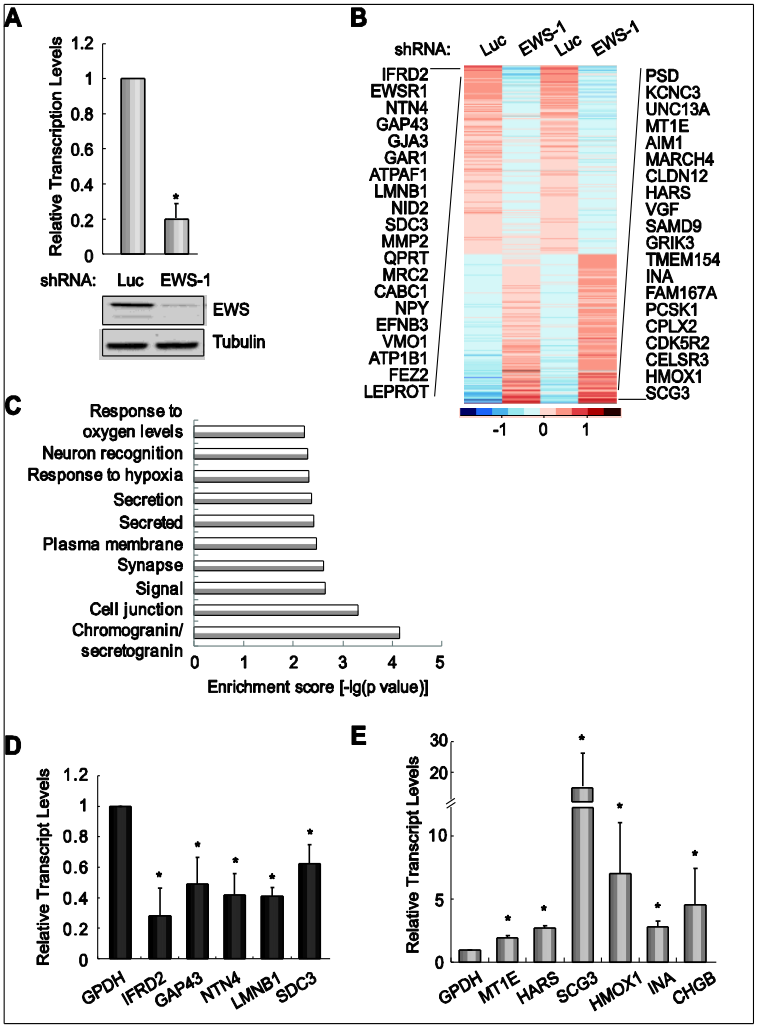

Figure 1. Identification of EWS-regulated genes and functions in Ewing sarcoma. (**A**) EWS knockdown by shRNA. qRT-PCR analysis shows that the EWS transcript level decreased about 80% by EWS knockdown. Normalized fold change was calculated by determining the fold change of the EWS-RNAi condition relative to the control Luc-RNAi condition, with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. Columns indicate the mean of 3 independent replicate experiments, error bars indicate the standard deviation, and asterisks indicate $P < 0.05$. EWS and tubulin (loading control) protein levels after control or EWS shRNA treatment are shown in the bottom panel. (**B**) Expression profiles for all detected and rank-ordered Ensembl genes are represented as a heat map. The FPKM values were mean-centered and normalized, with each row representing a different gene. The top 20 genes that either increase (left) or decrease (right) with increased EWS are shown. (**C**) Top 10 categories identified by DAVID functional annotation analysis of EWS-regulated genes. (**D, E**) RT-PCR validation of randomly selected EWS–up-regulated (**D**) or –down-regulated (**E**) genes. Normalized fold change was calculated by determining the fold change of the EWS-RNAi condition relative to the control Luc-RNAi condition, with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. Columns indicate the mean of 3 independent replicate experiments, error bars indicate the standard deviation, and asterisks indicate $P < 0.05$.

manner. We next performed Gene Set Enrichment Analysis (GSEA) using the EWS/FLI-regulated genes as the ranked list and the EWS–up-regulated or –down-regulated targets as the gene sets and applied a ! ^{2} test[29] to establish enrichment of the EWS-regulated genes for both the EWS/FLI–up-regulated and –down-regulated genes. We found that the EWS–up-regulated genes cluster significantly (P " 0.001) with both the EWS/FLI–up-regulated and –down-regulated genes and *vice versa* (Fig. 2C), suggesting a correlated regulation of genes by EWS and EWS/FLI in either the same or opposite directions. Notably, the correlation seemed to be stronger for the EWS–down-regulated genes as compared to the EWS–up-regulated genes. These results suggest that EWS and EWS/FLI differentially regulate genes and cellular processes in Ewing sarcoma.

EWS inhibits a subset of REST target neuronal genes in Ewing sarcoma cells. Because Ewing sarcoma was found to display some neuronal features, we focused our study on the EWS-regulated genes in the category of neuronal-related processes. We noted a set of neuronal genes including *chromogranin A* (*CHGA*), *cholinergic receptor nicotinic beta 2* (*CHRNB2*), *pleckstrin and Sec7 domain containing* (*PSD*), *secretogranin III* (*SCG3*), *synaptotagmin IV* (*SYT4*), and *VGF nerve growth factor inducible* (*VGF*). These 6 genes are involved in different aspects of neuronal functions[30-35] and are all down-regulated by EWS. Interestingly, elevated CHGA is an indicator for pancreas and prostate cancers.[36] Detection of SCG3 and VGF transcripts is a prognostic biomarker for small cell lung cancer and large cell neuroendocrine carcinoma,[37,38] respectively. More importantly, a putative REST response element (neuron-restrictive silencer element [NRSE]) is found in the regulatory regions such as the promoter or 5#untranslated region (5#-UTR) of each of these genes (Suppl. File S4). Most of these genes are known to be repressed by REST as well.[39-41]

To test if REST is involved in the repression of these neuronal genes in Ewing sarcoma, and to validate the RNA-seq data that EWS down-regulates the same subset of neuronal genes, we performed shRNA- and siRNA-mediated silencing of EWS or REST and analyzed

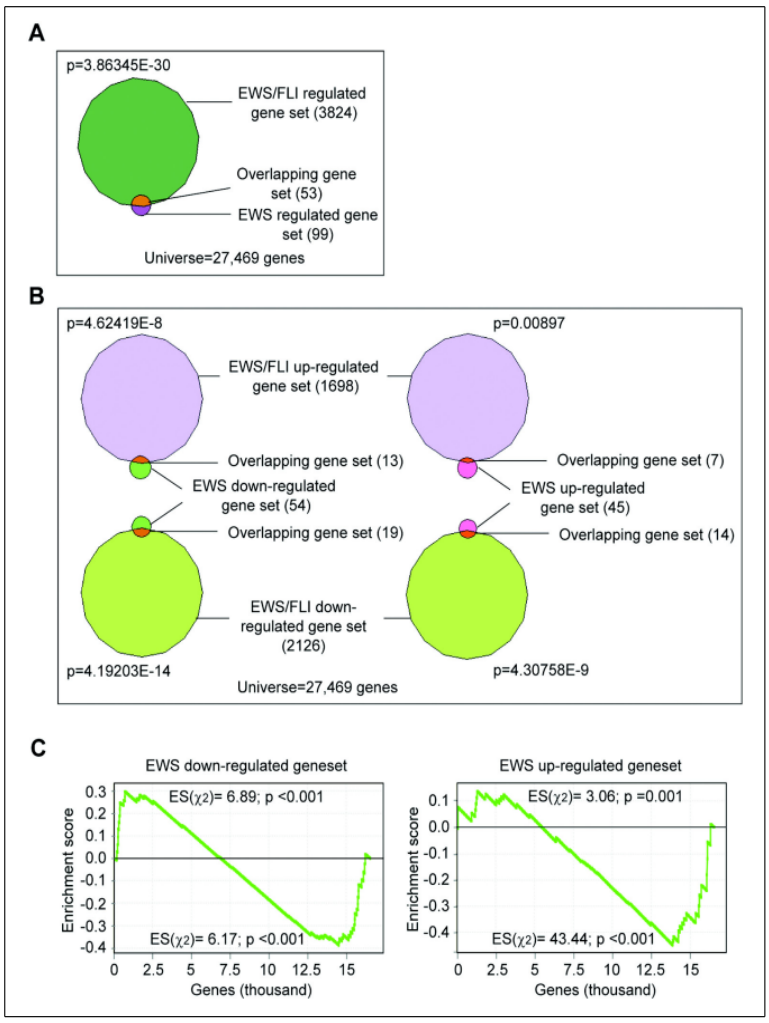

Figure 2. EWS/FLI differentially regulates EWS-regulated genes. **(A)** Venn diagram shows a significant overlap of the EWS- and EWS/FLI-regulated gene sets. **(B)** Venn diagrams showing significant overlapping gene sets between the EWS–up-regulated or –down-regulated and the EWS/FLI–up-regulated or –down-regulated gene sets. The *P* value was calculated by χ^2 analysis. **(C)** GSEA using EWS/FLI-regulated genes in A673 Ewing sarcoma cells as the rank-ordered data set and the EWS–up-regulated or –down-regulated targets as the gene set. The χ^2 test–derived enrichment scores and *P* values are shown for each end of the GSEA curve.

the expression of these genes by qRT-PCR (Fig. 3A and Suppl. Fig. S1). Stable knockdown of EWS or REST by shRNA significantly increased the expression of each of these neuronal genes in both A673 and TC71 cells (Fig. 3A and Suppl. Fig. S1A). Transient knockdown of EWS or REST using siRNA (Suppl. Fig. S1B) generated the same pattern of increased expression for each of these neuronal genes (Suppl. Fig. S1C). These findings demonstrate the repression of a subset of neuronal genes by REST in Ewing sarcoma, confirm the EWS RNA-seq results, and suggest a direct connection between EWS and REST in the regulation of neuronal gene expression.

Since knockdown of EWS increased the expression of the neuronal genes of interest, we next asked whether the overexpression of EWS would decrease the expression of these genes. There are 5 known alternative splice variants for EWS. We identified at least 3 variants during the process of cloning the EWS cDNA from A673 cells. Two variants, V2 and V3 (NM_005243.3 and NM_001163285.1), have previously been reported, and they differ only by 3 nucleotides in exon 11. The third variant, V6 (BankIt1574452 EWS JX977847), has not been identified previously. Variant V6 contains an alternative exon 9 (9b), which is 105 bases shorter than exon 9 (9a) that is found in V2 and V3 (Fig. 3B, upper panel). Following the enforced expression of each EWS isoform in A673 cells, we observed an overall repression of the neuronal genes tested to various extents (Fig. 3B, lower panel). It should be noted that the shRNA used to knock down EWS targets all 3 EWS variants and that EWS knockdown consistently increased the expression of the neuronal genes of interest (Fig. 3A). Taken together, these findings demonstrate that EWS indeed represses the set of neuronal genes tested in Ewing sarcoma cells.

EWS physically interacts with REST and binds to NRSE sites in the genome. Next, we examined the mechanism by which EWS and REST co-regulate these neuronal genes. One possibility is that EWS and REST regulate one another. However, knockdown of REST failed to change EWS levels and *vice versa* (Fig. 3A). EWS has previouisly been shown to regulate transcription by binding to proteins in the transcriptional machinery or to other transcription factors.[42,43] REST has also been shown to require co-factor interactions to mediate the repression of its target genes.[8,44] Therefore, we hypothesized that EWS and REST interact with each other to regulate the subset of neuronal genes. To test this hypothesis, we first asked whether EWS and REST physically interact in Ewing sarcoma cells. We immunoprecipitated endogenous EWS proteins from A673 cell lysates and tested for the presence of REST in the

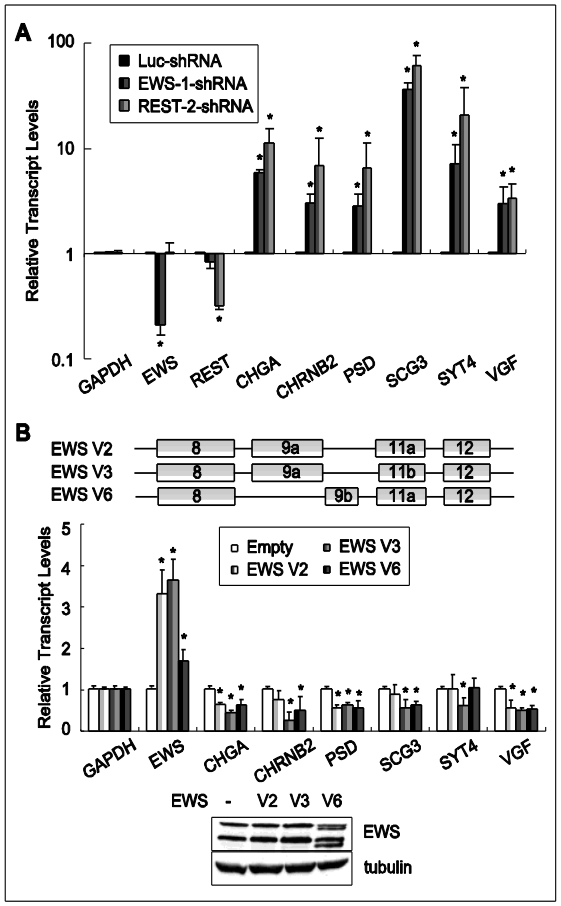

Figure 3. EWS and REST repress neural gene expression in Ewing sarcoma cells. (**A**) qRT-PCR analysis showing that EWS and REST down-regulate the transcription of all 6 neural genes. Normalized fold enrichment was calculated by determining the fold change of each condition relative to the control Luc-RNAi condition, with the data in each condition normalized to *GAPDH*. Columns indicate the mean of the 3 independent replicate experiments, error bars indicate the standard deviation, and asterisks indicate $P < 0.05$. (**B**) Overexpression of EWS represses the neuronal genes of interest. Schematic representation of 3 EWS splice variants found in Ewing sarcoma (upper panel). Expression of indicated neuronal genes with the overexpression of each EWS variant was investigated by RT-PCR. Normalized fold enrichment was calculated by determining the fold change of each condition relative to the empty vector condition, with the data in each condition normalized to *GAPDH*. Columns indicate the mean of 3 independent replicate experiments, error bars indicate the standard deviation, and asterisks indicate $P < .05$. EWS protein expression was examined by Western blotting, shown in the lower panel.

immunoprecipitates and *vice versa*. We found that EWS and REST co-immunoprecipitate in the reciprocal pull-downs (Fig. 4A). Domain mapping was then carried out by overexpressing flag-tagged full-length EWS, full-length EWS/FLI, which contains only the N-terminal portion of EWS, or 22 (a deletion mutant of EWS/FLI in which almost the entire EWS portion is deleted) (Fig. 4B) in HEK293 cells. After performing co-immunoprecipitation (co-IP), we found that full-length EWS and EWS/FLI, but not 22, were able to interact with REST (Fig. 4B). This indicates that the N-terminal domain of EWS is required for REST binding.

We next reasoned that if EWS and REST regulate these neuronal genes by mutual interaction, they would closely bind to chromatin at the set of neuronal genes. To examine whether EWS contacts chromatin at the NRSE sites present in the set of neuronal genes or at NRSE sites in the genome in general, we expressed epitope-tagged EWS in Ewing sarcoma EWS502 cells in which endogenous EWS/FLI had been silenced. We performed ChIP-seq analysis[45] and observed EWS signals at regions 100 to 200 bp from the predicted NRSE sites at the neuronal genes of interest (Fig. 4C). We also observed EWS signals at other regions of some genes, which is consistent with previous findings that EWS regulates transcription and RNA splicing. We then examined EWS signals at computationally predicted NRSE sites (TTCAGCACCA/T/GC/ANGGACAGC /AG/AC/GC, N = 3-9).[40] We found that the EWS signal was enriched and centered around NRSE sites (Fig. 4D, left panel) and that the signal intensity was even greater (Fig. 4D, right panel) when the analysis was limited to algorithmically selected EWS peaks (Zero-Inflated Negative Binomial Algorithm [ZINBA][46]). As a validation of the signal-based enrichment of EWS at NRSE, we performed a permutation analysis of EWS binding at NRSE sites (Fig. 4E) and observed a significant association between EWS and NRSE. We also observed this relationship with the highly conserved insulator element CTCF but not the binding sites of 2 neuronal transcription factors SOX2 or PAX6 (Fig. 4E). These results suggest that EWS preferentially binds chromatin at or near NRSE sites in Ewing sarcoma cells. Taken together, our data indicate that EWS and REST interact with each other and bind chromatin at or near NRSE sites to repress the expression of the target neuronal genes.

EWS and REST inhibit the neuronal phenotype and tumorigenesis in Ewing sarcoma cells. Next, we sought to determine the biological function of the co-regulation of neuronal genes by EWS and REST. Because the depletion of EWS increases expression of the neuronal genes, we reasoned that EWS knockdown may induce a neuronal phenotype in Ewing sarcoma cells. We therefore examined the protein levels of 3 neuronal markers, ! -III tubulin (TUBB3), neurofilament heavy polypeptide (NEFH), and microtubule-associated protein 2 (MAP2), following the knockdown of EWS. These 3 proteins have been previously used to assess neural differentiation in Ewing sarcoma cells.[17,47] We found that silencing of EWS in both A673 and TC71 cells resulted in an increased expression level of all 3 neuronal markers

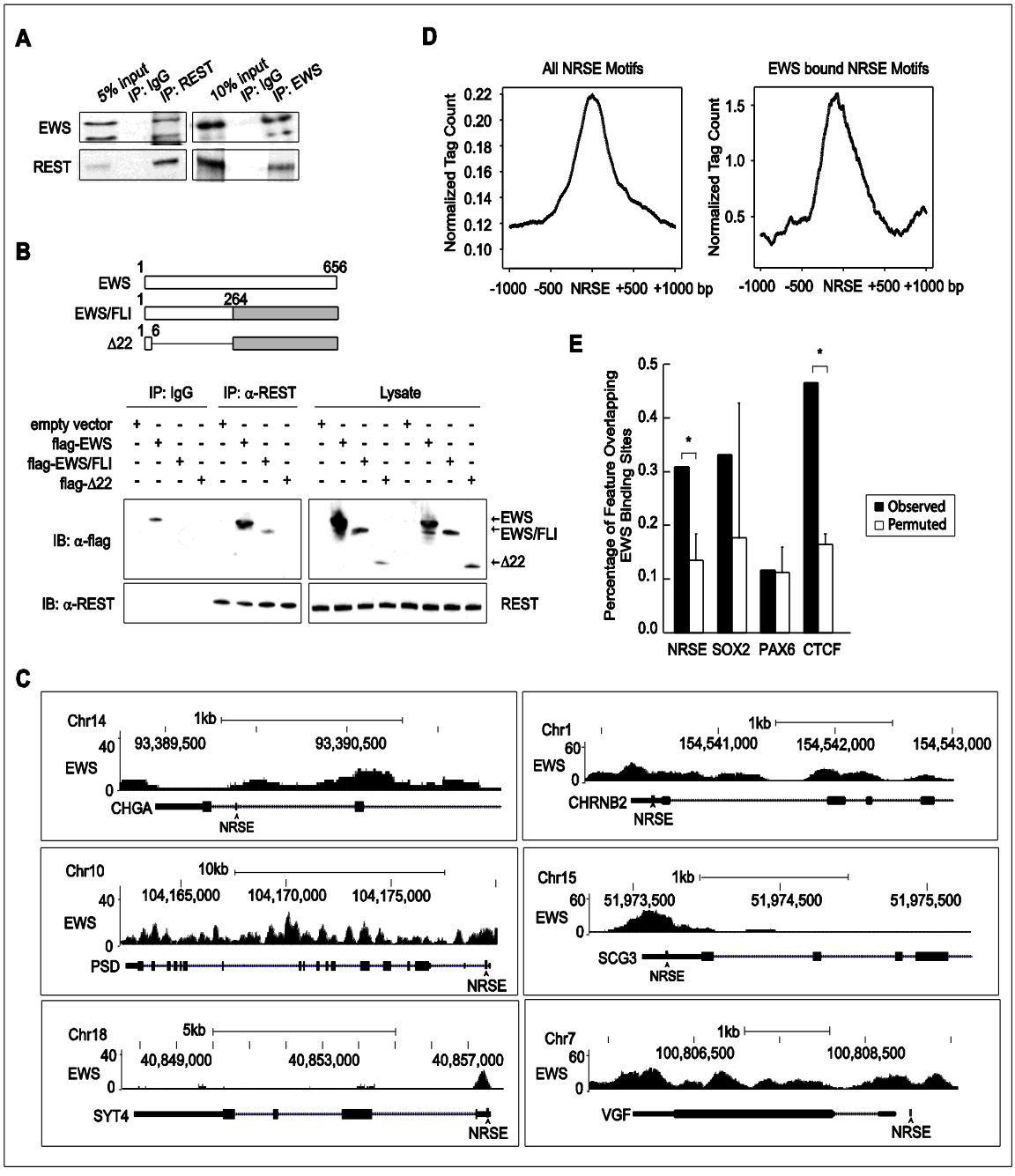

Figure 4. EWS physically interacts with REST and binds to chromatin regions at or near NRSE sites. (**A**) Reciprocal co-IP of EWS and REST. (**B**) The N-terminus of EWS or EWS/FLI is responsible for binding to REST. Schematic representation of constructs used in the analysis is shown in the top panel. The number equals the numerical value of amino acid residues of the EWS protein. HEK293 cells were transfected with flag-tagged full-length EWS, full-length EWS/FLI, or EWS/FLI ! 22. Cell lysates were subject to immunoprecipitation with anti-REST antibody for endogenous REST. Normal mouse IgG was used as a negative control. Co-immunoprecipitates of flag-tagged proteins were detected by the anti-flag antibody. All co-IP experiments were performed 3 times. (**C**) EWS binds to the REST-repressed neural genes at or near their NRSE sites. ChIP-seq analyses were carried out as described previously.[45] UCSC Genome Browser screenshots of the EWS ChIP-seq signal at indicated genes are shown. NRSE sites are indicated by arrows. (**D**) Mean EWS ChIP signal across all NRSE elements ±1 kb (left panel) and EWS-bound NRSE elements ±1 kb (right panel). Tags have been normalized for sequencing depth. (**E**) Overlap of EWS-binding sites across NRSE, SOX2, PAX6, and CTCF sites (dark gray bars). For comparison, overlap was also performed using randomly permuted EWS peaks (light gray bars). Asterisks indicate $P < 0.001$.

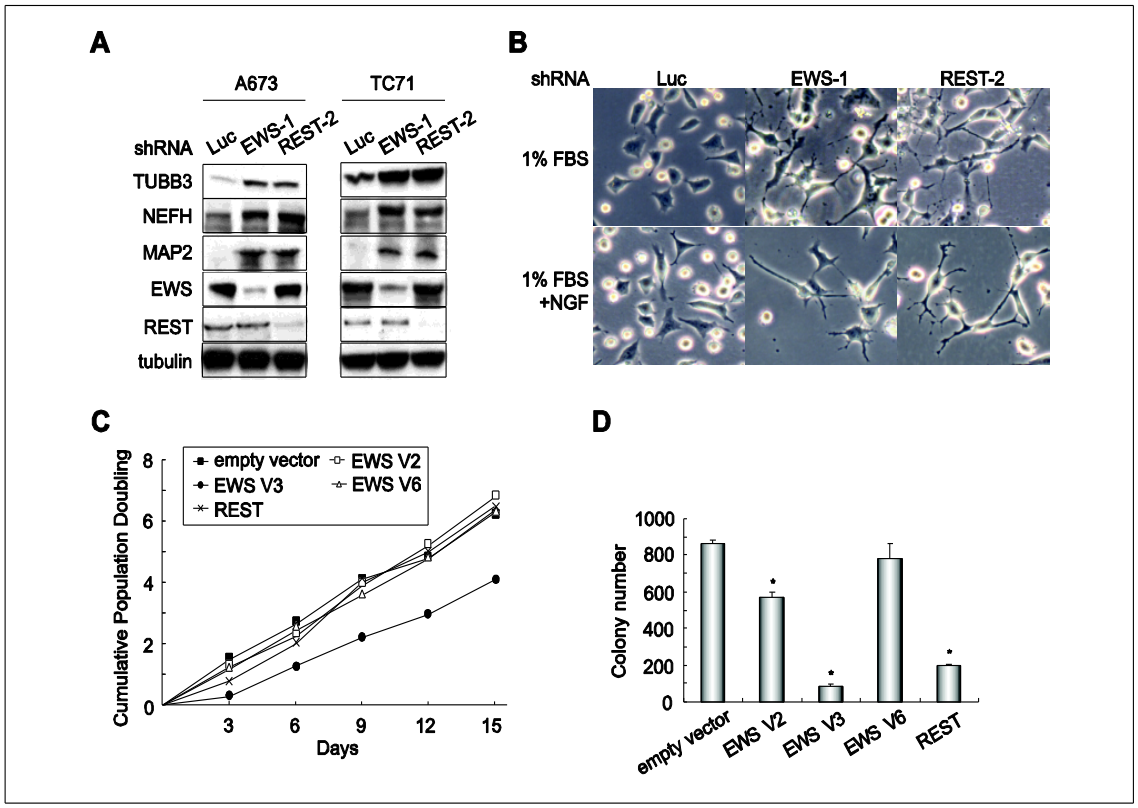

Figure 5. EWS and REST repress the neuronal phenotype and oncogenic transformation in Ewing sarcoma cells. (**A**) EWS or REST knockdown increases the expression of neuronal markers TUBB3, NEFH, and MAP2 in A673 and TC71 cells. (**B**) EWS or REST but not control knockdown cells display a neuronal phenotype when grown in media with 1% FBS and/or 20 ng/mL of nerve growth factor. (**C**) Growth curves of A673 cells with the overexpression of EWS V2, V3, or V6 or REST. (**D**) Soft agar assay with A673 cells overexpressing each of the 3 EWS splice isoforms or REST. Asterisks indicate $P < 0.05$.

(Fig. 5A). REST knockdown also caused an increase in expression of the 3 neuronal markers, which is consistent with its role in inhibiting neural differentiation. Furthermore, we observed a significant morphological change of EWS or REST knockdown cells, as demonstrated by the increase in neurite outgrowth when the cells were exposed to low-serum medium and/or nerve growth factor (20 ng/mL) (Fig. 5B). In contrast, the control knockdown cells displayed only minor morphological changes when maintained under the same conditions of neuronal differentiation. These results suggest that both EWS and REST mediate repression of the neuronal phenotype in Ewing sarcoma.

Given that EWS is translocated in multiple cancers, and that REST has been implicated as a tumor suppressor in lung, breast, and colon cancers, we next tested whether EWS and REST inhibit tumorigenesis in Ewing sarcoma. We silenced EWS or REST by shRNA in A673 and TC71 cells and found that the cells grew similar in tissue culture and formed a similar number of colonies in soft agar as the control knockdown cells (Suppl. Fig. S2A-D). Because Ewing sarcoma cells are highly transformed at baseline, we reasoned that a decrease of the EWS or REST expression level may not result in a significant increase in colony formation. Therefore, we next enforced the expression of each of the 3 EWS isoforms or REST in A673 and TC71 cells. We also observed a similar growth rate of these cells in tissue culture compared to control cells (Fig. 5C and Suppl. Fig. S2E). However, expression of EWS V2 or V3 isoforms or REST resulted in a significant reduction in oncogenic transformation, as shown by diminished anchorage-independent growth in soft agar (Fig. 5D and Suppl. Fig. S2F). Interestingly, EWS V6 overexpression failed to inhibit colony formation in soft agar, suggesting that exon 9 in the V2 and V3 isoforms is necessary for EWS-mediated inhibition of anchorage-independent growth. These results indicate that EWS and REST inhibit the maintanence of oncogenic transformation in Ewing sarcoma.

Discussion

In the current study, we identified EWS-regulated genes and cellular processes in Ewing sarcoma by combining

RNAi with RNA-seq and DAVID functional annotation analysis (Fig. 1). Interestingly, we found that the wild-type EWS and the fusion oncoprotein EWS/FLI have significantly overlapping profiles of transcriptional regulation and that they have opposite effects on some genes and similar effects on others (Fig. 2). Several previous reports have shown that EWS/FLI interferes with EWS functions in splicing, transcription, and maintanence of genomic stability[12,48] via its interaction with EWS. However, functional regulation in the same manner by EWS and EWS/FLI has not previously been reported. Given the domain structure of the 2 proteins (Fig. 4B), it is tempting to speculate that the similar regulation of genes by EWS and EWS/FLI may be mediated by their identical N-terminal domains, which has been shown to display transcriptional activation as well as repression activity[49] and is able to bind to proteins in the transcriptional initiation complex.[5]

We focused on a subset of EWS-regulated neuronal genes in this report and found that EWS cooperates with REST to repress the neuronal phenotype (Figs. 3-5). Interestingly, in addition to Ewing sarcoma, 2 other EWS translocation–based tumors, desmoplastic small round cell tumor and extraskeletal myxoid chondrosarcoma, also show neuronal features such as the expression of neuron-specific enolase.[50,51] This supports the notion that EWS represses the neuronal phenotype, and therefore, loss of EWS leads to acquiring neuronal features in these cancers. Further studies are required to fully understand the mechanism by which EWS and REST cooperatively regulate these neuronal genes. One candidate mechanism is epigenetic regulation of these genes by EWS and REST. EWS has previously been shown to inhibit the HAT activity of CBP/p300 via its interaction with noncoding RNA.[8] Interestingly, REST/coREST/LSD1 and the PRC2 complex (Polycomb Repressive Complex 2), 2 histone-modifying complexes, were found to simultaneously tether to the long, noncoding RNA, HOTAIR, to mediate coupled histone H3K27 methylation and K4 demethylation.[52]

In addition to EWS and REST, EWS/FLI was also found to regulate the neuronal phenotype development in Ewing sarcoma. A previous report suggested that EWS/FLI induces neuronal features by up-regulation of an array of genes important for neural crest development, such as *EGR2*, *MSX1*, *CITED2*, *c-Myc*, *ID2*, *Cadherin 11*, *RUNX3*, and *Rho* family members.[17] Although EWS and EWS/FLI may regulate the neuronal phenotype via different pathways, their opposite effects may explain how Ewing sarcoma family tumors exhibit varying levels of neural differentiation. Increased relative EWS/FLI levels would result in tumors with a more neuronal phenotype such as pPNET, whereas higher EWS expression would cause a reduced neuronal phenotype as observed for Ewing sarcoma.

Another important finding of this article is that both EWS and REST inhibit oncogenic transformation in Ewing sarcoma. EWS has not previously been implicated in tumor suppression except that it has been shown to control cell proliferation via posttranscriptional regulation of the Akt substrate PRAS40.[53] Dysfunction of REST is evident in several cancers and is achieved through diverse mechanisms. In prostate cancer, loss of REST results in the derepression of IB1/JIP1 (Islet-Brain1/c-Jun amino-terminal kinase interacting protein 1) to prevent JNK activation and apoptosis.[54] Impaired REST function in the breast cancer model stimulates the phosphorylation of Akt and leads to increased PI3-kinase signaling.[24] REST activity has also been shown to be affected by the changes in the availability of REST/co-factor complexes.[55] Based on our data, it is possible that the haploinsufficency of EWS in Ewing sarcoma decreases the abundance of the REST/EWS complex and abolishes REST activity on downstream effectors, leading eventually to tumorigenesis. Since EWS and EWS/FLI have been shown to interact, it is possible that EWS exerts a dominant-negative effect on EWS/FLI and therefore decreases colony formation in soft agar. The underlying mechanism of EWS- and/or REST-mediated inhibition of oncogenic transformation is under investigation.

Our findings that EWS and REST play roles in both repressing neuronal differentiation and inhibiting oncogenic transformation raise an interesting question of whether it is rational to see a neuronal phenotype in rapidly proliferating cancer cells. Indeed, both colon and breast cancers with REST deletion can display some neuroendocrine features.[26] Also, it is well documented that many neuroendocrine genes are aberrantly expressed in small cell lung cancer.[25] It has been postulated that when REST activity is lost in the precursor cells of these tumors, some neuronal genes are expressed outside their normal context. If the loss of REST activity is incomplete, such as the decrease in REST/co-factor abundance due to the haploinsufficiency of the co-factor, cancer cells may more closely resemble poised neural progenitor cells than mature neurons, remaining in the cell cycle but allowing the expression of some REST target genes. This transdifferentiation phase is very close to what we observe in Ewing sarcoma in which a highly undifferentiated phenotype coexists with neuronal features.

As indicated in our functional annotation analysis, EWS is also involved in the regulation of genes in other aspects of tumor development, such as angiogenesis and invasion. This suggests a more complex and broader impact of EWS on cancer development. A thorough study on EWS and its downstream effectors will benefit patients with Ewing sarcoma and other EWS translocation–based cancers.

Materials and Methods

Cell lines. Ewing sarcoma cell lines A673, TC71, and EWS502 were purchased from American Type Culture

Collection (ATCC, Manassas, VA) and grown as previously described.[56] Growth curve and soft agar colony formation assays were performed as previously described.[2]

Constructs. To clone the 3xFlag EWS construct, full-length EWS was amplified by PCR using the cDNA library from A673 cells. EWS was then fused in-frame with the 3xFlag tag and cloned into the pQCXIN vector. Constructs for the 3 EWS isoforms were generated by the amplification of individual EWS cDNA for each isoform, followed by ligation into the pMSCV-neo vector. The pMSCV-hygro 3xFlag EWS/FLI and mutant 22 have been previously described.[57]

RNAi. A human EWS-specific 19-mer oligonucleotide, 5!-gactctgacaacagtgcaa-3!, that maps to nucleotides 1083 to 1102 of the *EWS* gene was inserted into the pMKO.1-puro vector for stable knockdown of EWS. The nucleotide for REST knockdown is 5!-gaaactttgaacaaggttt-3!. Vectors for control (Luc-RNAi) knockdown and production of retrovirus-expressing shRNA have previously been described.[1] siRNA for transient knockdown of EWS or REST was purchased from Dharmacon (Thermo Scientific, Rockford, IL).

RT-PCR. RT-PCR was performed using the iScript SYBR green RT-PCR kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Primer sequences are available in Supplementary File S5.

RNA-seq and data analysis. Total RNA was extracted from Ewing sarcoma cells and treated with DNase using the Qiagen RNeasy kit (Germantown, MD). mRNA was enriched by oligo-dT magnetic beads and was used to construct Illumina (San Diego, CA) sequencing libraries. The libraries were single-end sequenced on Illumina Genome Analyzer IIx for 36 cycles (EWS) or HiSeq 2000 for 50 cycles (EWS/FLI). Reads were mapped to the hg19 genome build with Casava (Illumina) for EWS or Novoalign (Novocraft, Petaling Jaya, Malaysia) for EWS/FLI. The RNA-seq analysis was carried out using USeq (useq.sourceforge.net) versions 8.1.5 for EWS and 8.3.9 for EWS/FLI. Sorted, mapped files were converted to PointData representation with the USeq Eland Parser application for EWS and SAM Parser for EWS/FLI (useq.sourceforge.net). The knockdowns were compared with control using either Defined Region Scan Seqs (EWS) or Overdispersed Region Scan Seqs (EWS/FLI) applications with default parameters and a gene reference file derived from a merger of Ensembl and RefSeq (using a USeq Merge UCSC Gene table). Differential expression was defined as EWS: FDR " 0.05 and $|\log_2$ fold change$|$ # 1.3 and EWS/FLI: FDR " 1e-20 and $|\log_2$ fold change$|$ # 2.

GSEA and $\$^2$ testing. EWS gene sets, both the up-regulated and down-regulated, were divided according to their corresponding log fold change in the EWS/FLI RNA-seq experiment. Gene set enrichment was determined using GSEA.[58] To quantitatively establish enrichment of the EWS-regulated genes in the EWS/FLI–up-regulated and –down-regulated genes, we applied a $\$^2$ test as described previously[29] and calculated the enrichment score separately for EWS/FLI–up-regulated and –down-regulated genes.

ChIP-seq and data analysis. ChIP-seq analysis was performed as described previously.[45] NRSE, SOX2, PAX6, and CTCF motif locations were derived from MotifMap (motifmap.ics.uci.edu).[59] EWS peaks were permuted 1,000 times over the mappable genome (hg19) and assayed for overlap with the NRSE, SOX2, PAX6, and CTCF sites.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were carried out as described previously.[2] Anti-TUBB3 (MAB1195) and anti-NEFH (AF3108) were from R&D Systems (Minneapolis, MN), anti-MAP2 (Ab32454) was from Abcam (Cambridge, MA), and anti-REST (sc-374611), anti-EWS (sc-48404), and normal mouse IgG (sc-2025) were from Santa Cruz Biotechnology (Santa Cruz, CA).

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Note

The EWS and EWS/FLI RNA-seq raw data can be accessed from the Sequence Read Archive (SRA) database (accession numbers PRJNA174537 and SAMN01163407 for EWS and SRA059239 for EWS/FLI). The EWS V6 sequence is deposited in GenBank (BankIt1574452 EWS JX977847).

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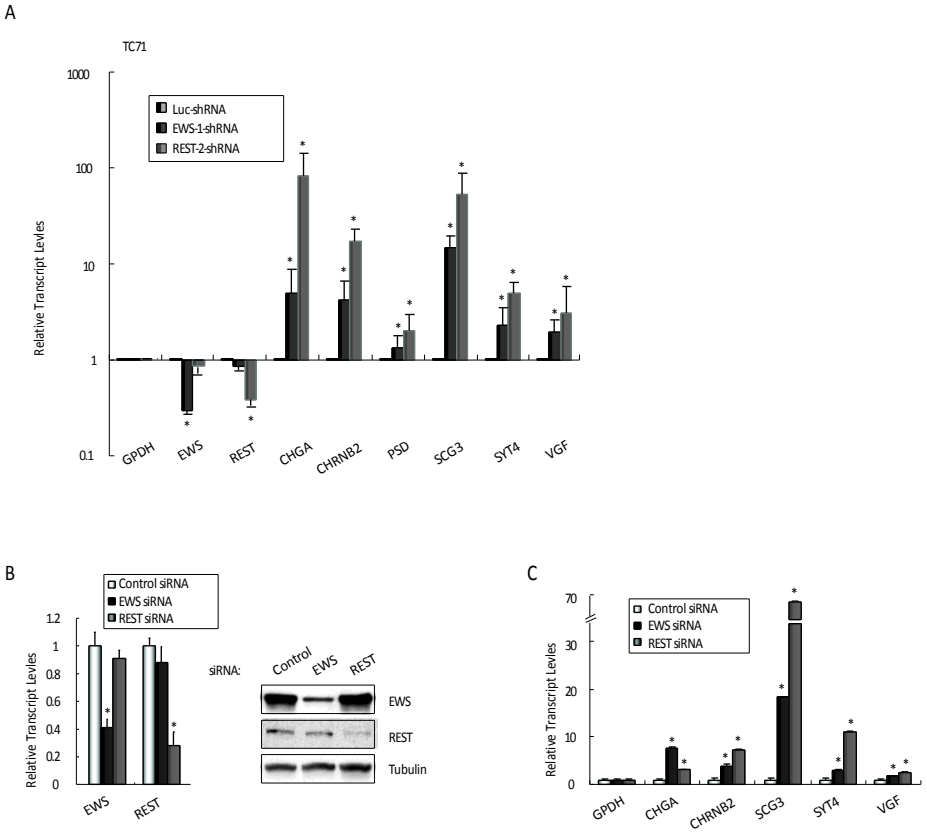
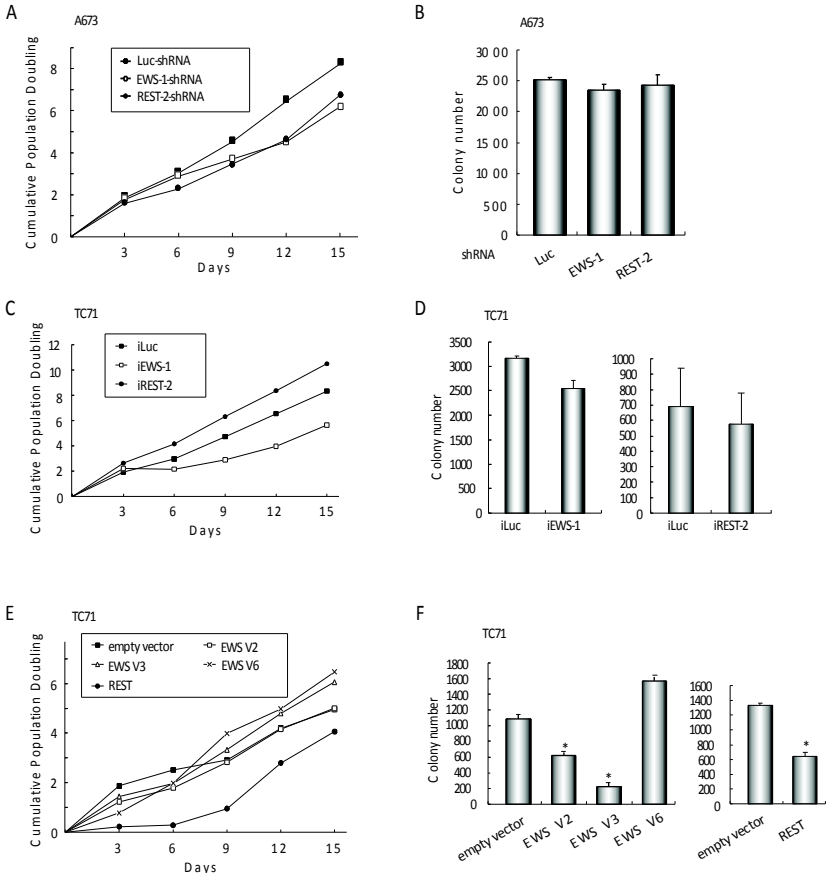
Supplementary Information



Supplementary Figure S6.1 Validation of EWS and REST coregulated genes. (A) Short-hairpin RNA (shRNA) mediated knockdown of EWS or REST in TC71 cells results in increased expression of tested neuronal genes. (B) Transient knockdown of EWS or REST by siRNA. (C) The expression of indicated neuronal genes were increased upon EWS or REST knockdown by siRNAs.



Supplementary Figure S6.2 Functional analysis of EWS and REST. (A) Growth curves of A673 cells with control or EWS or REST knockdown. (B) Anchorage-independent growth analyses of A673 cells with control or EWS or REST knockdown. (C) Tissue culture growth of TC71 cells with reduced control or EWS or REST level. D, Soft agar analyses of TC71 cells with reduced control or EWS or REST level. E, TC71 cells with enforced expression of EWS isoforms or REST grow similarly in tissue culture. F, Increased expression of EWS V2 or V3 or REST but not EWS V6 in TC71 cells decrease the number of colonies formed in soft agar.

CHAPTER 7

MECHANISM AND RELEVANCE OF EWS/FLI-MEDIATED TRANSCRIPTIONAL REPRESSION IN EWING SARCOMA

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ORIGINAL ARTICLE

Mechanism and relevance of EWS/FLI-mediated transcriptional repression in Ewing sarcoma

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Ewing sarcoma provides an important model for transcription-factor-mediated oncogenic transformation because of its reliance on the ETS-type fusion oncoprotein EWS/FLI. EWS/FLI functions as a transcriptional activator and transcriptional activation is required for its oncogenic activity. Here, we demonstrate that a previously less-well characterized transcriptional repressive function of the EWS/FLI fusion is also required for the transformed phenotype of Ewing sarcoma. Through comparison of EWS/FLI transcriptional profiling and genome-wide localization data, we define the complement of EWS/FLI direct downregulated target genes. We demonstrate that LOX is a previously undescribed EWS/FLI-repressed target that inhibits the transformed phenotype of Ewing sarcoma cells. Mechanistic studies demonstrate that the NuRD co-repressor complex interacts with EWS/FLI, and that its associated histone deacetylase and LSD1 activities contribute to the repressive function. Taken together, these data reveal a previously unknown molecular function for EWS/FLI, demonstrate a more highly coordinated oncogenic transcriptional hierarchy mediated by EWS/FLI than previously suspected, and implicate a new paradigm for therapeutic intervention aimed at controlling NuRD activity in Ewing sarcoma tumors.

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Keywords: Ewing sarcoma; EWS/FLI; NuRD; transcription; repression

INTRODUCTION

Ewing sarcoma provides an attractive model to understand the role of transcriptional regulation in tumor development because of its reliance on a single oncogenic transcription factor, EWS/FLI, created by the t(11;22)(q24;q12) chromosomal translocation.[1] Although the function of wild-type EWS is uncertain, the portion of EWS contained in the fusion contributes a strong transcriptional activation domain.[2] FLI is an ETS-family transcription factor, and contributes an ETS-type DNA-binding domain to the fusion.[3]

Early studies suggested that EWS/FLI functions as a transcriptional activator to mediate oncogenic transformation. In support of this model were data demonstrating that the EWS portion of the fusion functions as a strong activation domain relative to the amino-terminal portion of FLI lost in the fusion protein,[2] that EWS/FLI binds the RNA polymerase II core subunit, hsRBP7[4] and co-activators CBP/p300,[5] and that replacement of the EWS portion of the fusion with strong transcriptional activation domains (such as the VP16 transcriptional activation domain) resulted in heterologous fusions that retained full oncogenic activity in NIH3T3 murine fibroblasts.[6] Thus, in the NIH3T3 model, EWS/FLI functions mainly as a transcriptional activator, and transcriptional activation was thought to be solely required for its oncogenic function.[2,6]

Subsequent analyses with patient-derived Ewing sarcoma cell lines suggested a possible role for transcriptional repression by EWS/FLI in oncogenic transformation. For example, gene expression profiling after modulation of EWS/FLI levels in Ewing sarcoma cells revealed that many more genes were downregulated by EWS/FLI than upregulated.[7,8] A part of this downregulated signature is due to upregulation of transcriptional repressors, such as NKX2.2 and NR0B1, and is therefore indirectly regulated by EWS/FLI.[9,10] However, other studies suggested that some targets, such as *TGFBR2* and *IGFBP3*, may be directly-repressed by EWS/FLI.[8,11] These data suggested that the dogmatic model of EWS/FLI functioning as a transcriptional activator may be incomplete. However, the full complement of directly-repressed EWS/FLI target genes, the importance of transcriptional repression for the oncogenic function of EWS/FLI, and the mechanistic basis of this repression, remain unknown. We therefore performed experiments to address these questions.

RESULTS

Identification of directly-downregulated targets of EWS/FLI

To identify the complement of genes downregulated through direct binding of EWS/FLI, we compared previously published EWS/FLI gene expression data and genome-wide EWS/FLI chromatin immunoprecipitation on microarray (ChIP-Chip) data[9,12] and found 100 genes that overlapped between the two data sets as potential EWS/FLI direct-downregulated target genes (Figure 1a; Supplementary Table S1). We validated a subset of these genes with quantitative reverse-transcriptase polymerase chain reaction (qRT–PCR) following knockdown of EWS/FLI using the EF-2-RNAi construct (Supplementary Figure S1A). This gene set included Lysyl Oxidase (*LOX*), which has been shown to function as a tumor suppressor in a number of cancers, and had not been previously studied in Ewing sarcoma. We therefore analyzed this gene in greater detail, and used transforming growth factor β receptor II (*TGFBR2*) as a positive control as it had been previously

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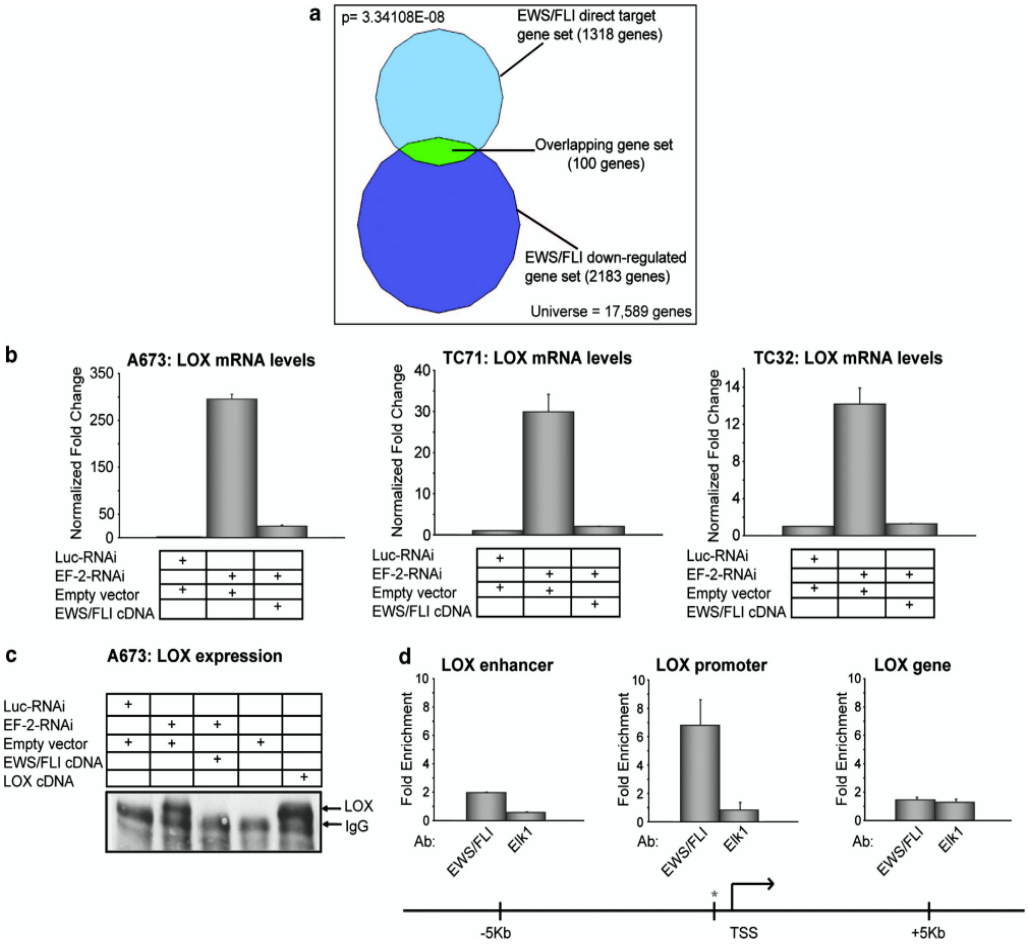

Figure 1. Identification of direct downregulated targets of EWS/FLI. (**a**) Venn diagram representation of the overlap between EWS/FLI downregulated genes (by transcriptional profiling) and EWS/FLI direct targets (by ChIP-chip) in A673 Ewing sarcoma cells. The χ^2-determined *P*-value is indicated. (**b**) qRT–PCR validation of EWS/FLI-mediated transcriptional repression of *LOX* in A673, TC71 and TC32 Ewing sarcoma cells following retroviral knockdown of endogenous EWS/FLI (EF-2-RNAi, versus the negative-control Luc-RNAi) and rescue with an RNAi-resistant EWS/FLI cDNA (versus an empty vector control). Error bars indicate standard deviations. Normalized fold enrichment was calculated by determining the fold-change of each condition relative to the control Luc-RNAi condition, with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. (**c**) Immunoprecipitation-western blot analysis of LOX protein from A673 cells expressing the indicated RNAi constructs (Luc-RNAi negative-control versus EWS/FLI knockdown with EF-2-RNAi), and the indicated expression vectors (empty vector negative control, EWS/FLI cDNA resistant to the RNAi construct or a LOX cDNA). The same antibody was used to perform the immunoprecipitation and the western blotting. The IgG band refers to the heavy chain. (**d**) ChIP of EWS/FLI at the *LOX* locus using antibodies against FLI (which recognizes only EWS/FLI) or ELK1 (negative control). The ChIP experiments were performed in A673 Ewing sarcoma cells which express EWS/FLI but do not have any detectable expression of wild-type FLI,[7] and so the anti-FLI antibody used only detects the fusion protein. Thus, there is no competition between the two transcription factors for the same binding sites. The red asterisk indicates the ChIP-Chip identified EWS/FLI binding site at the *LOX* promoter, and the transcriptional start site (TSS) is indicated. The level of enrichment for EWS/FLI or ELK1 are plotted as fold enrichment compared with the average enrichment of EWS/FLI or ELK1 at two negative control housekeeping genes *ALB* and *BCL2L1* used as normalization controls. Elk1 immunoprecipitation is used as a negative control for the ChIP experiment. Enrichment of EWS/FLI or Elk1 at regions 5 kb upstream and downstream of the ChIP-Chip identified binding site were used as negative controls to further demonstrate binding specificity for EWS/FLI at the *LOX* promoter. The error bars indicate standard error of the means of five independent experiments.

identified as a direct downregulated target of EWS/FLI.[11] Downregulation of *LOX* and *TGFBR2* were not off-target RNAi effects because the gene expression changes mediated by EWS/FLI knockdown were reversed by re-expression of an RNAi-resistant EWS/FLI cDNA in multiple Ewing sarcoma cell lines (Figure 1b; Supplementary Figure S1B). Changes in LOX mRNA levels were well correlated with LOX protein levels (Figure 1c).

To determine if repression of *LOX* and *TGFBR2* are directly mediated by EWS/FLI, we performed chromatin immunoprecipitation experiments, and found that EWS/FLI bound both the *LOX* and *TGFBR2* promoters *in vivo* (Figure 1d; Supplementary Figure S1C). These data suggest that *LOX* is a directly-downregulated target of EWS/FLI in Ewing sarcoma and provide independent validation of *TGFBR2* as an appropriate control gene for our study.

LOX functions as a tumor suppressor in Ewing sarcoma

Since *LOX* was directly bound and downregulated by EWS/FLI, we asked whether it functions as a tumor suppressor in Ewing sarcoma. Forced expression of LOX (or TGFBR2) impaired colony growth in soft agar and *in vivo* xenograft tumor formation without affecting monolayer growth (Figures 2a–c; Supplementary Figures S2A–E). In the tumors that did form, LOX or TGFBR2 were still expressed, indicating that the tumors may have adapted to overcome the suppressive effect of each protein (Supplementary Figure S2F).

If *LOX* and *TGFBR2* are indeed important EWS/FLI-repressed target genes, then their expression should be relatively low in primary Ewing sarcoma tumor samples. To test this, we analyzed the expression pattern of *LOX* and *TGFBR2* in a published microarray data set.[13] This data set includes 37 Ewing sarcoma samples consisting of 27 primary tumors and 10 cell lines. Analysis of this data set revealed low levels of expression of both *LOX* and *TGFBR2* in nearly all of the primary tumor samples analyzed (Figure 2d; Supplementary Figure S2G). We also evaluated a second published data set containing 59 Ewing sarcoma primary tumors and five different Ewing sarcoma cell lines (in which endogenous EWS/FLI was knocked-down using an RNAi approach), compared with mesenchymal progenitor cells as the reference tissue (a suggested cell-of-origin of Ewing sarcoma). This data set also demonstrated low levels of *LOX* and *TGFBR2* in primary Ewing sarcoma tumors (Figure 2e).[14] These data sustain the assertion that repression of *LOX* and *TGFBR2* is important for the development of Ewing sarcoma.

Mapping of the EWS/FLI transcriptional repressive domains

We next sought to identify the key domains required for transcriptional repression mediated by EWS/FLI by knocking-down endogenous EWS/FLI and re-introducing mutant forms of the protein (Supplementary Figures S3A–E), followed by evaluation of endogenous *LOX* and *TGFBR2* expression. We found that the DNA-binding mutant R2L2[15] failed to repress *LOX* or *TGFBR2*, but the mutant Δ89-C, lacking the carboxyl-terminal 89 amino acids of EWS/FLI[16] retained full repressive capability at these loci (Figures 3a and b; Supplementary Figures S3A–C). In contrast, the Δ22 mutant,[17] lacking nearly all of the EWS portion of EWS/FLI, did not repress *LOX* or *TGFBR2* when introduced in place of full-length EWS/FLI (Figures 3a and b; Supplementary Figures S3A–C). This indicates that the repressive activity is localized to the EWS portion of EWS/FLI and that DNA binding is required for transcriptional repression at these loci.

We then used a previously described panel of deletion mutants to further refine the location of the repression domain within EWS/FLI (Figure 3b; Supplementary Figures S3C–E).[6] Mutants 2, 3 and 9 retained full transcriptional repressive activity, but mutants 10 and 11 failed to repress target genes (Figures 3c and d). All deletion mutants tested were appropriately localized to the nucleus and bound DNA *in vitro* (Supplementary Figure S3F and data not shown).

These findings suggest the presence of two general regions in the EWS portion of EWS/FLI that mediate repression: an amino-terminal region (amino acids 1–82), and a distal region (amino acids 118–264). This underlying organization was reminiscent of the organization previously found for transcriptional activation in EWS/FLI.[6] Consistent with this observation, EWS/FLI mutants that retained transcriptional repressive function also retained the ability to activate the critical EWS/FLI upregulated target genes *NKX2.2* and *NR0B1*, while mutants deficient in transcriptional repression also failed to transactivate these genes (Figures 3e and f). Importantly, the mutants that mediate both transcriptional repression and activation also rescue oncogenic transformation of Ewing sarcoma cells following EWS/FLI knockdown, while those that are inactive in repression and activation do not (Figure 3g).

We next wanted to test the contribution of transcriptional repression to EWS/FLI-mediated oncogenesis directly. However, we were unable to identify mutants that separate transcriptional repression and activation functions in the fusion protein. As an alternate approach, we replaced EWS/FLI with a previously described engineered construct, VP16/FLI, that contains two copies of the strong transcriptional activation domain of the VP16 protein fused to the carboxyl-terminal FLI protein[6] (Supplementary Figure S4A). This construct was previously shown to effectively transactivate a reporter construct and induce oncogenic transformation of NIH3T3 cells.[6] VP16/FLI rescued activation of *NKX2.2* and *NR0B1* nearly as well as EWS/FLI itself (Figure 4a), but was unable to transcriptionally repress *LOX* or *TGFBR2* (Figure 4b). Thus, VP16/FLI functions as a transcriptional activator in Ewing sarcoma cells.

We found that Ewing sarcoma cells expressing VP16/FLI (in lieu of EWS/FLI) could not form *in vitro* colonies under anchorage-independent conditions, and that *in vivo* xenograft formation was also reduced, as compared with Ewing sarcoma cells expressing EWS/FLI (Figures 4c and d; Supplementary Figure S4B). Thus, transcriptional activation without transcriptional repression, in the context of a FLI-based DNA-binding domain, is insufficient to rescue oncogenic transformation of Ewing sarcoma cells. Thus, transcriptional repression is required for the oncogenic function of EWS/FLI.[6,18–21]

Transcriptional repression by EWS/FLI requires HDAC activity

Given the importance of EWS/FLI-mediated transcriptional repression in Ewing sarcoma oncogenesis, we next sought to determine the mechanistic basis for this function. We previously demonstrated that two critical EWS/FLI target genes, *NKX2.2* and *NR0B1*, act as transcriptional repressors in Ewing sarcoma.[9,10] Neither of these are required for repression of *LOX* or *TGFBR2* as shown by knockdown experiments (Supplementary Figure S5A).

Histone deacetylases (HDACs) play an important role in transcriptional repression mediated by NKX2.2 in Ewing sarcoma.[9] To test if HDACs are also involved in EWS/FLI-mediated transcriptional repression, we used gene set enrichment analysis (GSEA) to compare the transcriptional profile of A673 cells treated with the HDAC inhibitor vorinostat[9] to a gene set consisting of the 100 EWS/FLI-bound and downregulated genes (Figure 1a; Supplementary Table S1). We found that these 100 genes clustered strongly with those genes upregulated by vorinostat (NES = 2.08; $P < 0.001$; Figure 5a), demonstrating that the HDAC inhibitor reverses the EWS/FLI-mediated direct transcriptional repressive signature in Ewing sarcoma cells. Interestingly, *LOX* was most correlated with derepression by vorinostat. These data strongly suggest that HDACs are involved in direct transcriptional repression of *LOX* and *TGFBR2* (and other genes) by EWS/FLI in Ewing sarcoma.

Quantitative RT–PCR analysis demonstrated a dose-dependent increase in the expression of *LOX* and *TGFBR2* following vorinostat treatment (Figure 5b). This effect was absolutely dependent on the expression of EWS/FLI, as the effect was lost following knockdown of EWS/FLI (Figures 5c and d), and neither *LOX* nor *TGFBR2* was upregulated by vorinostat in HEK 293 cells (human embryonic kidney cells that do not express EWS/FLI; Supplementary Figure S5B). Taken together, these data demonstrate that full transcriptional repression by EWS/FLI requires HDAC activity.

These data also suggested a model in which EWS/FLI interacts with one or more HDACs (either directly, or through binding to an HDAC-containing complex) to mediate transcriptional repression. To test this model, we performed co-immunoprecipitation experiments in A673 cells expressing a 3 × -FLAG tagged version of EWS/FLI in place of the wild-type fusion. We found that EWS/FLI co-immunoprecipitates with HDAC2 and HDAC3, but not HDAC1,

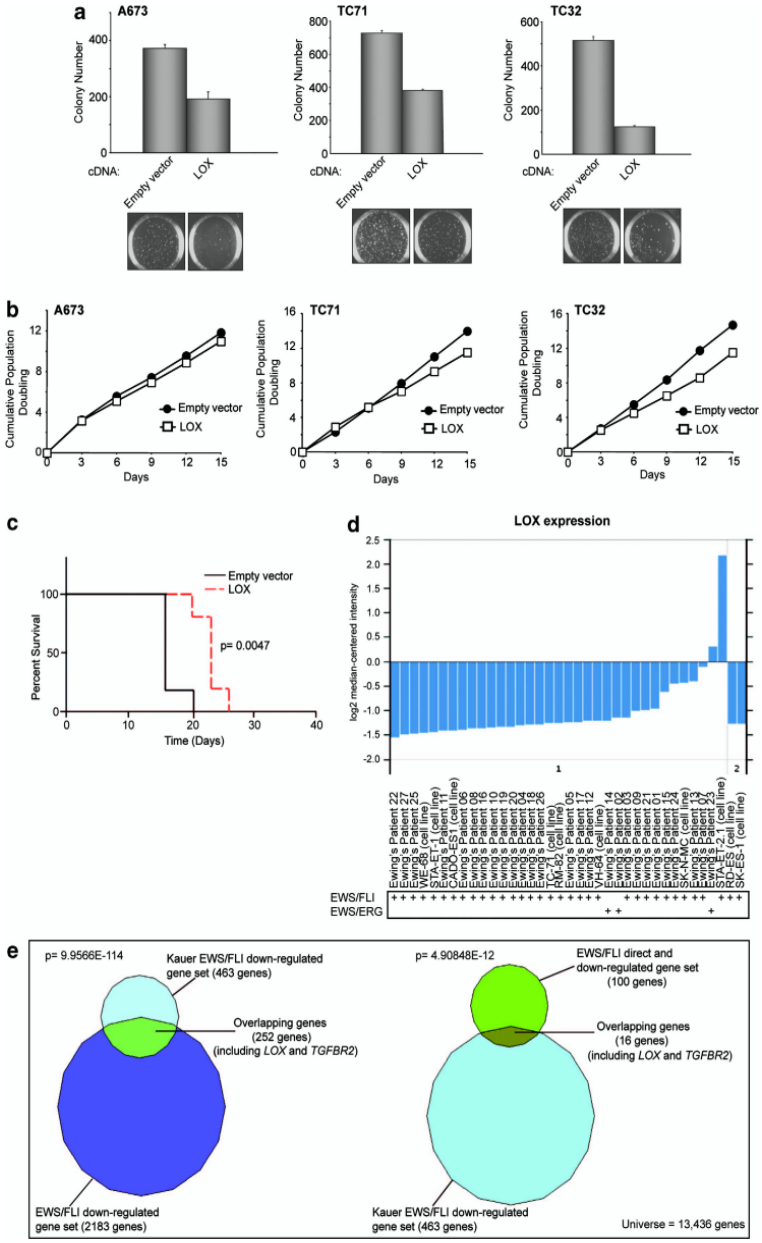

Figure 2. LOX functions as a tumor suppressor in Ewing sarcoma. (**a**) Quantification of colonies formed in soft agar by A673, TC71 and TC32 cells expressing a 3 × -FLAG LOX cDNA construct in comparison to an empty vector control. Error bars indicate standard deviations of duplicate assays. Representative images of soft agar colonies are included. (**b**) Growth assays (3T5) for A673, TC71 and TC32 cells described in (**a**). (**c**) Survival curves for immunodeficient mice injected with TC32 cells expressing 3 × -FLAG LOX or an empty vector construct. Five mice underwent bilateral subcutaneous injection for each condition, and each animal was sacrificed once one of their tumors reached a 2-cm endpoint. Percent survival was plotted as a Kaplan–Meier survival curve using GraphPad Prism. The log-rank (Mantel–Cox test) *P*-value is indicated. (**d**) Graphical representation of *LOX* expression levels in 27 primary Ewing sarcoma patient-derived tumors and 10 Ewing sarcoma cell lines in the Schaefer *et al.* data set. The EWS/FLI or EWS/ERG translocation fusion status of each sample is indicated. The reason for the higher levels of *LOX* expression observed in the STA-ET-2.1 cell line is uncertain, but possibilities include that this cell line may harbor a mutant *LOX* allele, or perhaps has activated an adaptive 'bypass' pathway that allows for growth in the presence of high levels of *LOX* expression (such as the effect seen in our own xenograft experiments with Ewing sarcoma cells expressing the LOX cDNA (**c**; Supplementary Figure S2F)). (**e**) Venn diagram overlaps of EWS/FLI downregulated or EWS/FLI direct downregulated-genes data sets with the Kauer *et al.* EWS/FLI downregulated-genes data set. In the Kauer *et al.* data set, a molecular function map of Ewing sarcoma was constructed based on an integrative analysis of gene expression profiling experiments following EWS/FLI knockdown in a panel of five Ewing sarcoma cell lines, and 59 primary Ewing sarcoma tumors using mesenchymal progenitor cells (MPC, a suggested cell-of-origin of Ewing sarcoma) as the reference tissue. The χ^2-determined *P*-values are indicated.

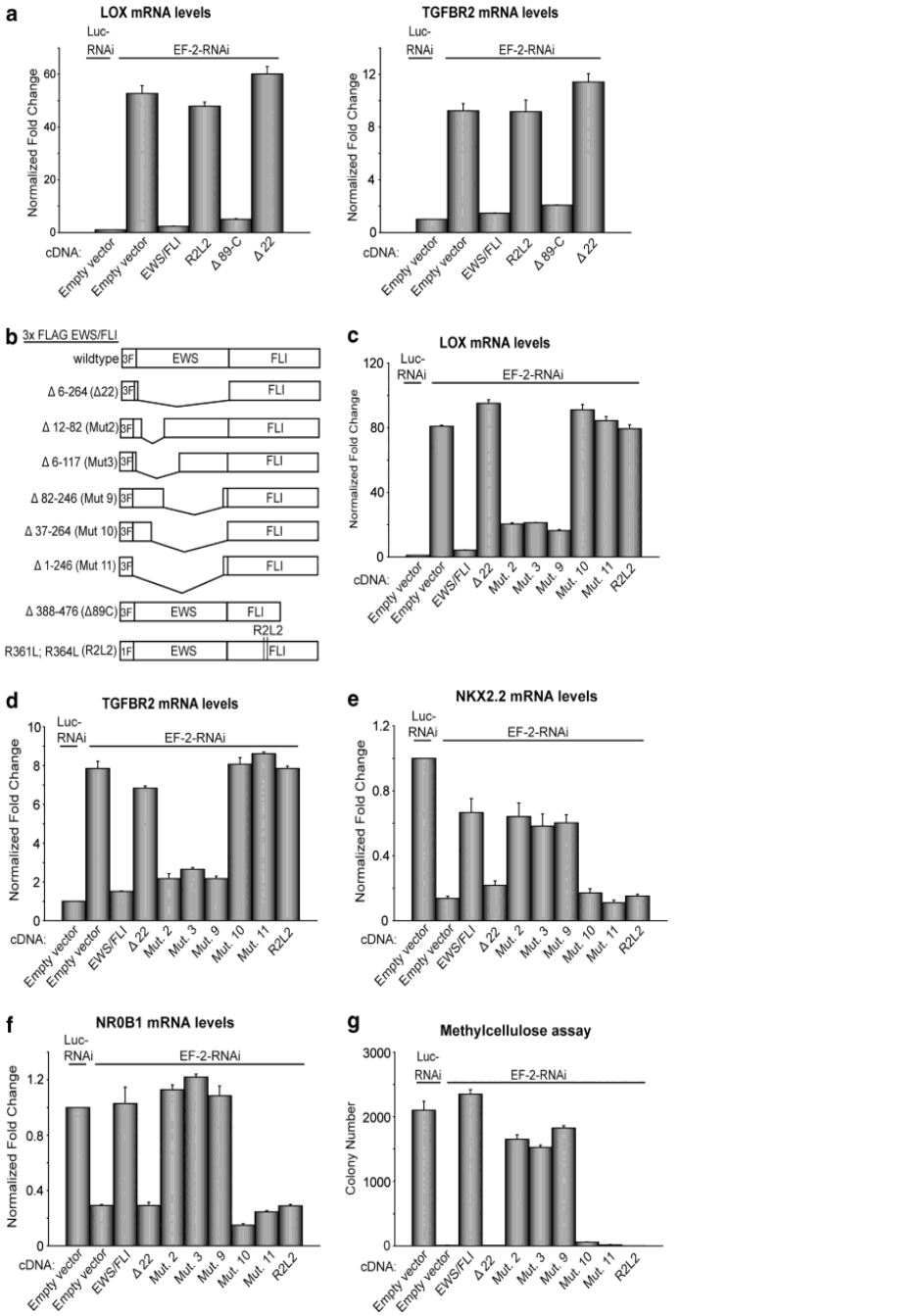

Figure 3. Structure-function analysis of EWS/FLI-mediated repression. (**a**) qRT–PCR analysis of *LOX* and *TGFBR2* expression in A673 cells following knockdown of EWS/FLI (with the EF-2-RNAi construct) and rescue with 3 × -FLAG wild-type EWS/FLI, 3 × -FLAG Δ22, 1 × -FLAG R2L2, 3 × -FLAG Δ89C or an empty vector control. Luc-RNAi is a negative control vector. Normalized fold enrichment was calculated by determining the fold-change of each condition relative to the control Luc-RNAi condition, with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. (**b**) Schematic representation of 3 × -FLAG (3F) EWS/FLI wild-type and mutant constructs and 1 × -FLAG (1F) R2L2 mutant construct. Amino acids deleted/mutated in the EWS and FLI1 domains are indicated. (**c**, **d**) Repression of *LOX* and *TGFBR2*, by wild-type EWS/FLI, or mutants, as analyzed by qRT–PCR. EWS/FLI was knocked-down in A673 cells and rescued with the indicated constructs. Error bars indicate standard deviations. (**e**, **f**) Activation of *NKX2.2* and *NR0B1*, by wild-type EWS/FLI, or mutants, as analyzed by qRT–PCR. EWS/FLI was knocked-down in A673 cells and rescued with the indicated constructs. Error bars indicate standard deviations. (**g**) Quantification of colonies formed in methylcellulose by A673 cells infected with the indicated RNAi and cDNA constructs. Error bars indicate standard deviations of duplicate assays.

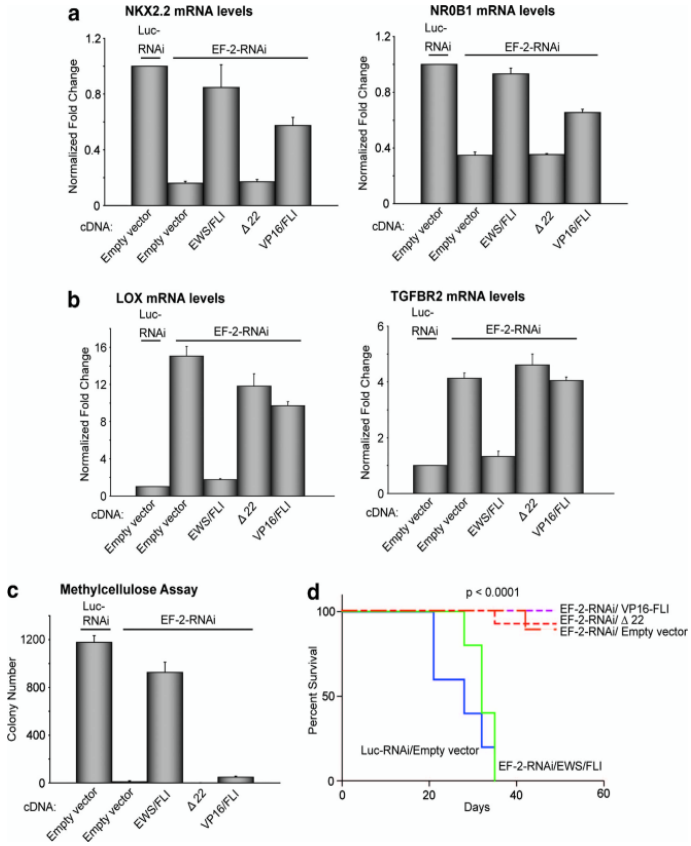

Figure 4. EWS/FLI-mediated transcriptional activation and transcriptional repression are required for oncogenic transformation in Ewing sarcoma cells. (**a**, **b**) qRT–PCR analysis of *NKX2.2* and *NR0B1*, or *LOX* and *TGFBR2*, following knockdown of EWS/FLI (with the EF-2-RNAi construct) and rescue with the indicated cDNAs or an empty vector control. Luc-RNAi is a negative control. Error bars indicate standard deviations. Normalized fold enrichment was calculated by determining the fold-change of each condition relative to the control Luc-RNAi condition, with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. (**c**) Quantification of colonies formed in methylcellulose by A673 cells infected with the indicated RNAi and cDNA constructs. Error bars indicate standard deviations of duplicate assays. (**d**) Survival curves for immunodeficient mice injected with control knockdown A673 cells re-expressing empty vector or EWS/FLI knockdown A673 cells re-expressing empty vector, EWS/FLI cDNA, Δ22 or 2 × VP16/FLI cDNA constructs. Five mice underwent bilateral subcutaneous injection for each condition, and each animal was sacrificed once one of their tumors reached a 2-cm end point. Percent survival was plotted as a Kaplan–Meier survival curve using GraphPad Prism. The log-rank (Mantel–Cox test) *P*-value is indicated.

in Ewing sarcoma cells (Figure 5e). These data were consistent with siRNA experiments that showed that knockdown of HDAC2 or HDAC3, but not HDAC1, derepressed both *LOX* and *TGFBR2* (Supplementary Figures S5C and D).

If HDAC2 and/or HDAC3 were truly involved in EWS/FLI-mediated transcriptional repression, we would predict that these proteins would interact with EWS/FLI mutants that mediate transcriptional repression, but not with inactive mutants. To test this, we knocked-down endogenous EWS/FLI in Ewing sarcoma cells and rescued with RNAi-resistant cDNAs encoding wild-type EWS/FLI, the Δ22 mutant (that does not mediate transcriptional repression), or mutant 9 (that retains transcriptional repression activity). We found that wild-type EWS/FLI and mutant 9 both bind HDAC2 and HDAC3, while the Δ22 mutant does not (Figure 5f). This correlation between transcriptional repression and HDAC binding suggests that repression may be mediated via EWS/FLI-bound HDACs at specific genomic loci. Of interest, we previously found that vorinostat-mediated HDAC blockade also disrupts the growth of Ewing sarcoma cells in tissue culture, and the ability of those cells to form colonies in soft-agar assays.[9]

NuRD interacts with EWS/FLI to mediate transcriptional repression

HDAC2 and HDAC3 are class I HDACs. Class I HDACs serve as catalytic subunits for various multiprotein transcriptional repressor complexes, including Sin3A, NuRD, NCoR/SMRT and CoREST (see Figure 7 for complex composition).[22] To test if any of these complexes are involved in EWS/FLI-mediated transcriptional repression, we asked whether depletion of any of these complexes (using RNAi) resulted in derepression of *LOX* and/or *TGFBR2*. Although we achieved good reduction of both RNA and protein levels for Sin3A, REST and NCoR/SMRT in A673 cells, there were no changes in *LOX* or *TGFBR2* expression with any of these manipulations (Supplementary Figures S6A–C). In contrast, ~50% knockdown of the NuRD complex component chromodomain helicase DNA-binding protein 4 (CHD4) resulted in significant upregulation of both *LOX* and *TGFBR2* (Figure 6a). These data

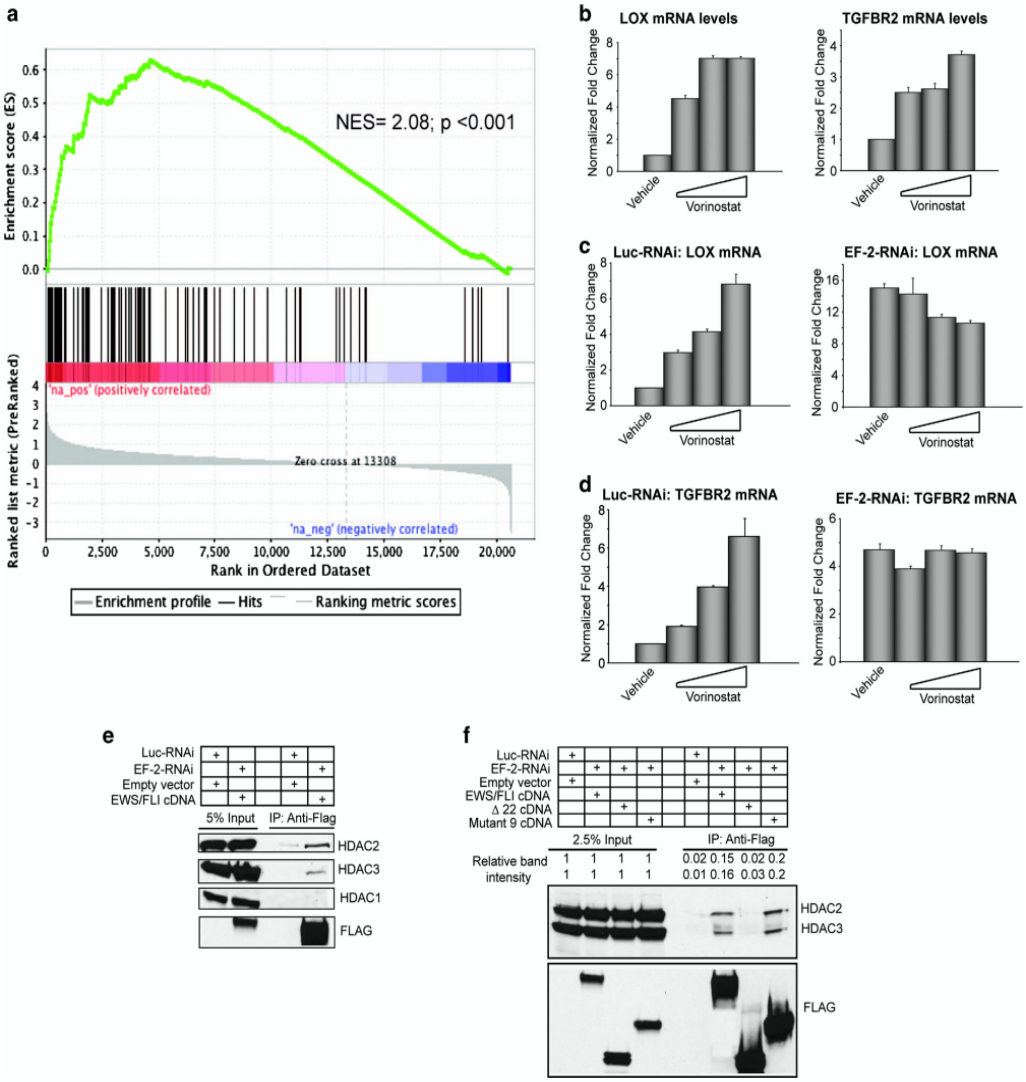

Figure 5. Transcriptional repression by EWS/FLI is mediated by HDACs. (**a**) Gene set enrichment analysis (GSEA) using vorinostat-regulated genes in A673 Ewing sarcoma cells as the rank-ordered data set and the 100 EWS/FLI direct downregulated targets as the geneset. The positions of the 100 genes are indicated as black vertical lines in the center portion of the panel. The normalized enrichment score (NES) and *P*-value are shown. (**b**) qRT–PCR analysis of *LOX* and *TGFBR2* in A673 cells treated with increasing concentrations of the HDAC-inhibitor vorinostat. Normalized fold enrichment was calculated by determining the fold-change of each condition relative to the control vehicle-treated condition, with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. Error bars indicate standard deviations. (**c**, **d**) qRT–PCR analysis of *LOX* and *TGFBR2* in A673 cells expressing a control RNAi or EWS/FLI RNAi construct (EF-2-RNAi) treated with increasing concentrations of the HDAC inhibitor vorinostat. Error bars indicate standard deviations. (**e**, **f**) Co-immunoprecipitation of EWS/FLI and HDACs. Endogenous EWS/FLI was knocked-down in A673 cells (with the EF-2-RNAi) and replaced with 3 × -FLAG-tagged versions of the indicated cDNAs that were resistant to the RNAi construct. Luc-RNAi is a negative control. Relative band intensities were quantified using ImageQuant (GE Healthcare Biosciences, Pittsburgh, PA, USA).

suggest that the NuRD complex is involved in EWS/FLI-mediated transcriptional repression.

The simplest model to explain these findings is that EWS/FLI binds the NuRD complex directly. We found that full-length EWS/FLI and the repressive mutant 9 allele bind both CHD4 and MTA2 NuRD complex components in co-immunoprecipitation experiments, while the non-repressive Δ22 protein does not (Figure 6b). These data support the hypothesis that EWS/FLI interacts (directly or indirectly) with the NuRD complex to mediate transcriptional repression at loci such as *LOX* and *TGFBR2*.

The NuRD complex also contains the LSD1 histone demethylase.[23] To determine if LSD1 activity is required for transcriptional repression mediated by EWS/FLI, we used two recently-described small-molecule reversible inhibitors of LSD1 (HCI-2509 and HCI-2528) (Venkataswamy *et al.*, submitted for publication). Both of these inhibitors decreased the viability of A673 cells (IC_{50} of 0.93

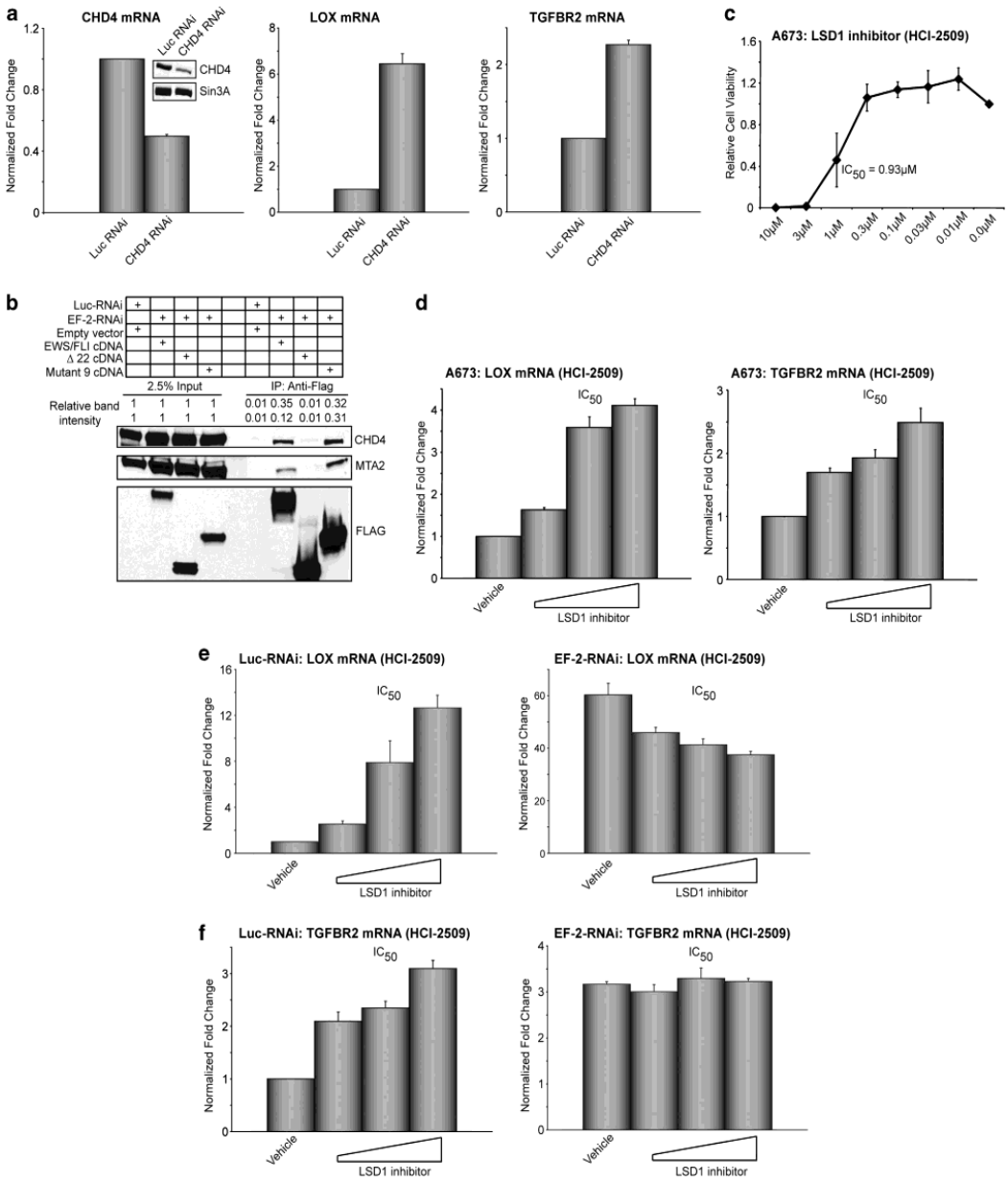

Figure 6. EWS/FLI interacts with the members of the NuRD co-repressor complex. (**a**) qRT–PCR analysis of *CHD4*, *LOX* and *TGFBR2* in A673 cells following knockdown of the NuRD component CHD4. Western blot analysis to demonstrate efficiency of CHD4 knockdown, Sin3A was used as the loading control. Error bars indicate standard deviations. Normalized fold enrichment was calculated by determining the fold-change of each condition relative to the control Luc-RNAi condition, with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. (**b**) Co-immunoprecipitation of 3 × -FLAG EWS/FLI, or the indicated mutants, and NuRD complex components CHD4 and MTA2. Relative band intensities were quantified using ImageQuant. (**c**) Relative cell viability of A673 cells treated with increasing concentrations of the LSD1 inhibitor (HCI-2509). The IC_{50} is indicated. Error bars indicate standard deviations. (**d**) qRT–PCR analysis of *LOX* and *TGFBR2* following 72 h of treatment with increasing concentrations of the LSD1 inhibitor (HCI-2509). The dose corresponding to the IC_{50} is indicated. Error bars indicate standard deviations. (**e**, **f**) qRT–PCR analysis of *LOX* and *TGFBR2* in A673 cells expressing Luc-RNAi (negative control) or EWS/FLI RNAi (EF-2-RNAi) following 72 h of treatment with increasing concentrations of the LSD1 inhibitor HCI-2509. The dose corresponding to the IC_{50} is indicated. Error bars indicate standard deviations.

and 0.47 μM, respectively) and upregulated both *LOX* and *TGFBR2* (Figures 6c and d; Supplementary Figures S6D and E). Similar results were observed in three other Ewing sarcoma cell lines (Supplementary Figures S6F–H). Derepression of *LOX* and *TGFBR2* was absolutely dependent on the expression of EWS/FLI, as the effect was lost following knockdown of the fusion protein (Figures

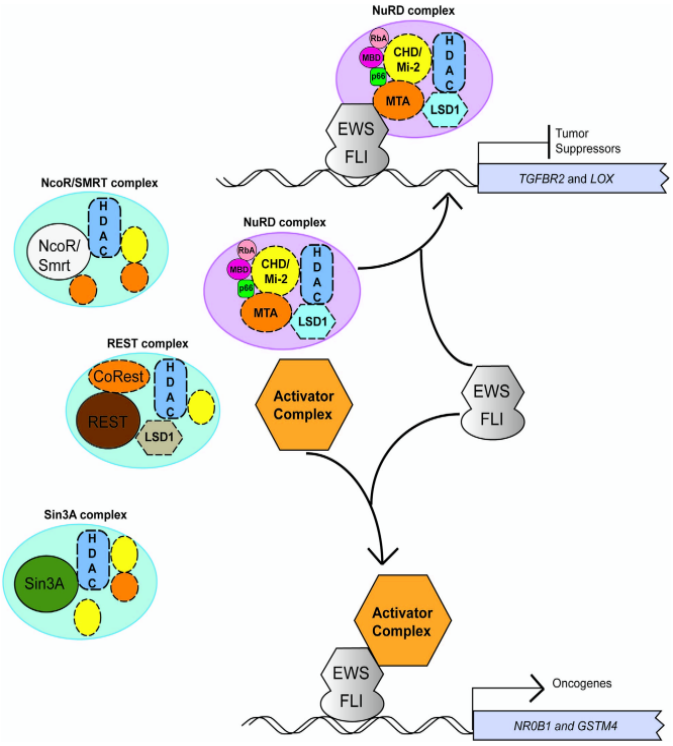

Figure 7. Binary-switch model for EWS/FLI-mediated transcriptional regulation. At directly-repressed genes, such as *LOX* and *TGFBR2*, EWS/FLI may preferentially recruit transcriptional repressor complexes, such as the NuRD complex with its associated HDACs and LSD1, to transcriptionally inhibit gene expression. At other repressed loci, other repressor complexes could be recruited. In contrast, at directly activated genes, such as *NR0B1* and *GSTM4*, EWS/FLI may preferentially recruit (yet to be determined) activator complexes to transcriptionally upregulate gene expression. The mechanism by which preferential recruitment occurs is not yet known.

6e and f), and was not observed in the EWS/FLI-deficient cell line HEK 293 (Supplementary Figure S6I). Dose-dependent increases in expression of both *LOX* and *TGFBR2* with siRNA knockdown of LSD1 in A673 cells confirmed the specificity of the effect observed with the small-molecule inhibitors (Supplementary Figure S6J). Taken together, these data highlight a central role for the NuRD complex in the development of Ewing sarcoma and indicate a possible therapeutic strategy for targeting a critical transcriptional function of EWS/FLI via NuRD/LSD1/HDAC blockade.

DISCUSSION

Early studies using heterologous systems suggested that EWS/FLI functions as a transcriptional activator to mediate oncogenic transformation. Later data from Ewing sarcoma patient-derived cell lines suggested that EWS/FLI might also function as a transcriptional repressor. However, the mechanism of transcriptional repression by EWS/FLI and the role of repression in oncogenesis have been uncertain. We have now demonstrated that EWS/FLI functions as both a transcriptional activator, and as a transcriptional repressor, in Ewing sarcoma. Importantly, we demonstrated that the repressive function of EWS/FLI is absolutely required for the oncogenic function of the fusion. Finally, we showed that transcriptional repression by EWS/FLI is mediated through direct binding of the NuRD complex, and that the NuRD-associated histone deacetylase and LSD1 functions are key components of this activity.

These data have important implications for our understanding of Ewing sarcoma development. First, these data once again indicate that the NIH3T3 model does not recapitulate critical features of Ewing sarcoma oncogenesis, because in that model, transcriptional activation by EWS/FLI was sufficient for oncogenic transformation.[2,6] A recent study indicated that some ETS-family members mimic the RAS/MAPK pathway by activating genes that are regulated by this oncogenic pathway.[24] It is possible that EWS/FLI upregulates a similar set of genes in NIH3T3 cells, but a distinct set in Ewing sarcoma. Indeed, transcriptional profiling studies support the notion that EWS/FLI regulates different genes in each cell type.[18,20]

The second important implication is that in addition to key upregulated genes, such as *NR0B1*, *NKX2.2*, *CAV1*, *GSTM4*, etc., the downregulated transcriptional signature is also of critical importance to the development of Ewing sarcoma. In support of this argument, we validated previous data suggesting an important role for inhibition of *TGFBR2* in Ewing sarcoma development, and extended these data by demonstrating a critical role for the inhibition of *LOX* expression as well. Interestingly, LOX has been suggested to function as a tumor suppressor, or as an oncogene, depending on the cellular context.[25] In Ewing sarcoma, our data indicate its tumor suppresive function dominates. The ability of EWS/FLI to inhibit the expression of tumor suppressors in Ewing sarcoma suggests that mutation of these genes may be unnecessary for the development of this tumor. Indeed, relatively few genes have been identified that are mutationally inactivated in Ewing sarcoma.[26] We speculate that direct

transcriptional repression of tumor suppressors by EWS/FLI diminishes the need for mutational inactivation.

Third, binding of NuRD to EWS/FLI demonstrates that the transcriptional repressive function of EWS/FLI is both direct and active. We previously demonstrated that some EWS/FLI upregulated targets, such as NKX2.2 and NR0B1, function as transcriptional repressors in Ewing sarcoma.[9,10] Transcriptional repression of some genetic loci is therefore 'indirect' (that is, EWS/FLI upregulates transcriptional repressors, and these repressors mediate inhibition at other loci). However, part of the transcriptional repressive signature of EWS/FLI is directly mediated by the fusion protein itself. Further work will be required to determine the relative contributions of direct versus indirect transcriptional repression.

Additionally, a direct contribution by NuRD and its associated HDACs and LSD1 indicate an 'active' transcriptional repressive mechanism mediated by EWS/FLI. Such a mechanism stands in contrast to 'passive' mechanisms of transcriptional repression, such as non-productive competition for ETS-family DNA-binding sites by EWS/FLI, or 'squelching' mechanisms (where EWS/FLI could compete for limiting transcriptional co-activators, thereby preventing these proteins and complexes from being recruited to some target loci). Furthermore, the finding that derepression of *LOX* and *TGFBR2* with an HDAC inhibitor or the LSD1 inhibitor in Ewing sarcoma cells was completely dependent on EWS/FLI expression further supports the 'direct' repression model at these promoters. Identification of an 'active' transcriptional repressive mechanism for EWS/FLI does not negate the possibility for passive repression at some loci. Further work would be required to determine whether such passive mechanisms are operative, and if so, what their relative contributions are to EWS/FLI-mediated oncogenesis in Ewing sarcoma. It is also worth noting that even though the NuRD complex typically contains both HDAC1 and HDAC2, we instead found HDAC2 and HDAC3 associated with EWS/FLI and required for repression of *LOX* and *TGFBR2*. This suggests that there may be EWS/FLI-associated subcomplexes of the NuRD co-repressor in Ewing sarcoma cells that contains HDAC3 instead of HDAC1.

One question that arises from this work is how the choice between EWS/FLI-mediated transcriptional activation versus transcriptional repression is made at specific genetic loci. Our finding that the activation and repressive functions of the fusion protein are currently inseparable suggests that a binary-switch model might be operative (Figure 7). For example, at some loci EWS/FLI would bind transcriptional activators (such as p300/CBP), while at other loci the fusion will bind NuRD (or even other repressors at other loci). This decision could be dictated by the specific EWS/FLI response element present, nearby bound transcriptional regulators, and/or the local chromatin environment. Interestingly, we previously demonstrated that EWS/FLI binds GGAA-microsatellite elements near genes that are transcriptionally activated by the oncoprotein, and that GGAA-microsatellites are enriched near EWS/FLI-upregulated genes, but are depleted near downregulated genes.[12] Thus, the GGAA-microsatellite appears to serve as an EWS/FLI-activating response element. Similarly, using an unbiased motif identification tool (MEME), we were unable to identify any significant enrichment of the consensus high-affinity ETS site at the EWS/FLI direct-repressed gene promoters (data not shown). Most members of the ETS family bind to DNA at sequences containing either a GGAA or GGAT core sequence.[3] At the EWS/FLI direct-repressed gene promoters analyzed there were several such potential ETS core motifs with flanking sequences that resembled low-affinity binding sites for other transcription factors (composite sites). It is tempting to speculate that such composite sites function as *in vivo* EWS/FLI binding elements at direct-repressed genes. In support of this concept are genome-wide localization studies that have identified two classes of *in vivo* ETS-binding sites,[1] high-affinity binding sites and[2] lower-affinity binding sites found in close proximity to low affinity binding sites for other transcription factors, allowing for co-operative binding and regulation.[27] Our current data do not address whether a specific EWS/FLI-repressive response element exists in the promoter or enhancer regions of downregulated target genes. Additional studies will be required to characterize the EWS/FLI binding sites at repressed genes.

Finally, the importance of EWS/FLI-mediated transcriptional repression to the oncogenic phenotype of Ewing sarcoma suggests that inhibition of the repressive function might be a therapeutic strategy for this disease. Indeed, the contribution of HDACs and LSD1 to the repressive function suggests an immediate avenue to exploit this strategy via the administration of HDAC and/or LSD1 inhibitors to patients with Ewing sarcoma. HDAC inhibition has already been shown to block Ewing sarcoma cell growth, transformation and survival in *ex vivo* settings.[9] *In vivo*, HDAC inhibitors have shown efficacy in some preclinical xenograft models[28] but not in others.[29] Our data show that LSD1-specific inhibitors also block growth and survival of multiple Ewing sarcoma patient-derived cell lines. Of note, a recent report documented LSD1 expression in Ewing sarcoma tumors, and demonstrated that inhibition of LSD1 activity (with a monoamine oxidase inhibitor) blocked Ewing sarcoma cell growth in tissue culture.[30] LSD1 inhibition in xenograft models has yet to be reported. It is possible that combination approaches would be useful, such as a combination of HDAC and LSD1 inhibitors, or combinations of these inhibitors with other established therapeutic approaches (such as chemotherapy). Additional preclinical studies are warranted to test this hypothesis.

In summary, we have shown that EWS/FLI functions as a transcriptional repressor, and that this function is also critical for oncogenic transformation mediated by this fusion oncoprotein. This provides a mechanistic explanation for recent transcriptional profiling data and the occasional report of putative EWS/FLI direct-down-regulated genes. These data also provide a biochemical rationale for the evaluation of NuRD inhibitors (such as HDAC and LSD1 inhibitors) as a new therapeutic approach for Ewing sarcoma.

MATERIALS AND METHODS

Constructs and retroviruses

The Luc-RNAi, EF-2-RNAi, EWS/FLI, Δ22, R2L2, mutants 2, 3 and 9 have been described previously.[6,7,15] The mutants were 3 × -FLAG tagged, R2L2 was 1 × -FLAG tagged and subcloned into the Murine Stem Cell Virus (MSCV) retroviral system (Clontech, Mountain View, CA, USA). 3 × -FLAG mutants 10 and 11 were generated by PCR and subcloned into the MSCV vector. The 2 × -VP16/FLI construct in the SRα retroviral vector was previously described.[6] 3 × -FLAG cDNAs of LOX and TGFBR2 were generated and cloned into the MSCV retroviral vector. REST shRNA was designed and cloned into the pMKO.1 retroviral vector. SMARTpool siRNAs targeting NcoR/SMRT, HDAC1, HDAC2, HDAC3 and LSD1 were purchased from Dharmacon (Lafayette, CO, USA).

Cell culture

HEK 293EBNA cells and Ewing sarcoma cell lines (A673, TC71, TC32 and SK-N-MC) were infected with retrovirus, and polyclonal populations were grown in the appropriate selection media, as previously described.[31,32] Growth assays (3T5) were performed as previously described.[31]

Soft agar and methylcellulose assays

Soft agar assays were performed as described previously.[31] Methylcellulose assays were performed by seeding 1×10^5 cells per 6-cm plate in the absence or presence of appropriate antibiotic selection media in 1% final concentration of methylcellulose.

Xenograft assay

TC32 cells infected and selected with an empty vector, LOX or TGFBR2 cDNA were injected into the flanks of nude mice at 1×10^6 cells per flank.

A673 cells infected with a control or EWS/FLI RNAi and re-expressing an empty vector, RNAi-resistant EWS/FLI, Δ22 or 2× VP16/FLI cDNA constructs were selected and injected into the flanks of nude mice at 5×10^5 cells per flank. Five mice were used per condition. Tumors were measured using digital calipers and three-dimensional tumor volumes were calculated using the equation (length × width × depth)/2. Survival curve was plotted using GraphPad Prism (La Jolla, CA, USA). Animal experiments were performed following approval from the University of Utah Institutional Animal Care and Use Committee.

Quantitative reverse-transcriptase polymerase chain reaction

Total RNA from cells was amplified and detected using SYBR green fluorescence for quantitative analysis.[31] Normalized fold enrichment was calculated by determining the fold-change of each condition relative to the control condition (either Luc-RNAi or empty vector), with the data in each condition normalized to an internal housekeeping control gene *GAPDH*. Primer sequences used for qRT–PCR analysis for all target genes are included in the supplementary information (Supplementary Table S2).

Antibodies

The following antibodies were used for immunodetection: M2-anti-FLAG (HRP; Sigma, St Louis, MO, USA; A8592), anti-FLI-1 (Santa-Cruz, Santa Cruz, CA, USA; sc-356 ×), anti-α-Tubulin (Calbiochem, Billerica, MA, USA; CP06), anti-HDAC1 (Santa-Cruz sc-7872), anti-HDAC2 (Santa-Cruz sc-7899), anti-HDAC3 (Santa-Cruz sc-11417), anti-CHD4/Mi2 β (BETHYL Laboratories Inc., Montgomery, TX, USA; A301-081A), anti-MTA-2 (Abcam, Cambridge, MA, USA; A8106), anti-LOX (Santa-Cruz sc-66947), anti-EWS (Santa-Cruz sc-48404), anti-REST (Santa-Cruz sc-374611), anti-NcoR (Abcam ab80856), anti-LSD1 (Cell Signaling, Danvers, MA, USA; C69G12), anti-NKX2.2 (Santa-Cruz sc-15015) and anti-NR0B1 (Abcam ab24552).

Directed ChIP

Directed ChIPs were performed as previously described[27] using anti-FLI-1 and anti-ELK-1 antibodies (sc-356X and sc-355 respectively; Santa Cruz Biotechnology, Inc). Quantitative RT–PCR was performed with *LOX*, *TGFBR2* primers and with *ALB* and *BCL2L1* as normalization controls.[12] See Supplementary Table S2 for primer sequences.

Electrophoretic mobility shit assay

Nuclear extracts were prepared from 293EBNA cells transfected with 3 × -FLAG wild-type EWS/FLI, 3 × -FLAG deletion mutants of EWS/FLI, or empty vector control expression plasmids. Twenty milligrams of nuclear extract protein, 5 nM [^{32}P]-labeled probe called DNA duplex (I), containing a high-affinity EWS/FLI-binding site, called 'ETS2 probe',[2] 100-fold excess (500 nM) of specific unlabeled competitor DNA duplex (I) and 1 × Gel Shift Binding Buffer (Promega Corporation, Madison, WI, USA) were used in each reaction to determine specific binding. For details refer to Gangwal *et al.*[12]

Co-immunoprecipitation assays

A673 cells infected with the Luc-RNAi or EF-2-RNAi construct were transduced with an empty vector or 3 × -FLAG EWS/FLI cDNA. Nuclear extracts were prepared. Co-immunoprecipitation experiments were conducted as previously described[33] using anti-FLAG-M2-Magnetic Beads (Sigma M8823).

Cell viability assay

ATPlite from Perkin-Elmer (Waltham, MA, USA) was used to determine cell viability. Ewing sarcoma cell lines were seeded in 384-well plates (1000 cells per well) and treated with different concentrations of the inhibitors (0.1% final dimethyl sulfoxide (DMSO) concentration). After 96-h of incubation, an equal volume of ATPlite was added directly to the culture well. Luminescence was read 5 min later on an Envision plate reader. Viability was calculated relative to untreated cells, and IC_{50} values were calculated using GraphPad Prism.

Microarray and ChIP-Chip analysis

Overlaps between the different gene sets were performed using the Venn Master program (Universität Ulm, Helmholtzstr, Ulm, Germany; http://www.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/vennm/doc.html). Statistical significance of the overlaps was determined using Chi square analysis. Gene set enrichment analysis (GSEA) was performed using GSEA2.07 program (Broad Institute, Cambridge, MA, USA; http://www.broad.mit.edu/gsea/).[34] Analysis of the Schaefer sarcoma data set was done using ONCOMINE (Compendia Bioscience, Ann Arbor, MI, USA; https://www.oncomine.com/resource/login.html).

CONFLICT OF INTEREST

Sorna Venkataswamy, Hariprasad Vankayalapati, Steven L Warner, Sunil Sharma and David J Bearss filed United States patent application 61/523 801 on 08/15/2011: Substituted (E)-N′-(1-Phenylethylidene)Benzohydrazide Analogs as Histone Demethylase Inhibitors.

ACKNOWLEDGEMENTS

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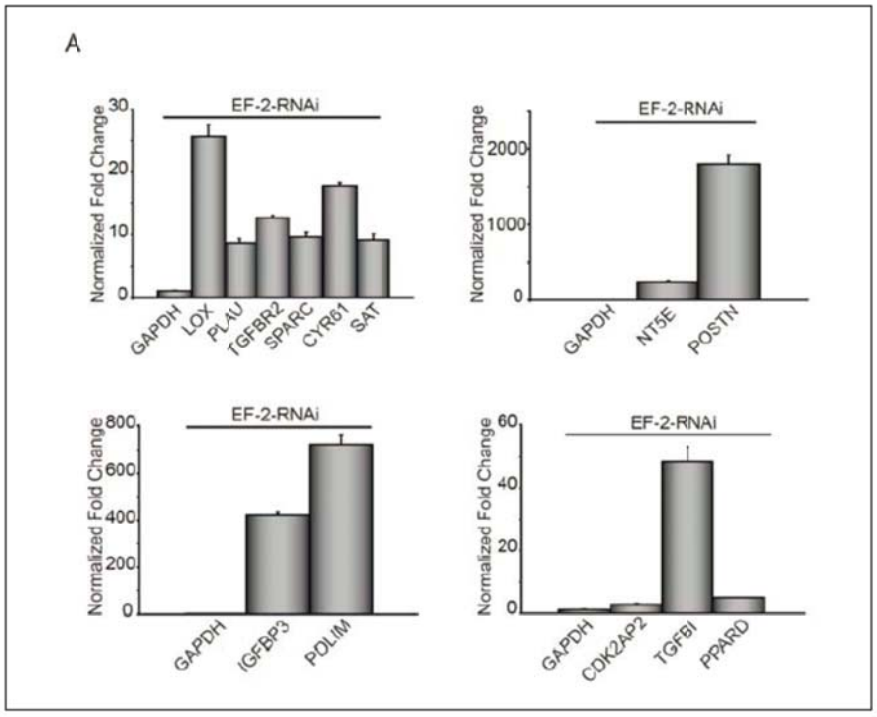
Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Supplemental Information

Supplementary Table S7.1 Primer sequences for qRT-PCR analysis of RNA and ChIP DNA.

Gene/Locus	Forward primer	Reverse Primer
GAPDH primer set	CCGAGCCACATCGCTCAGACA	GCCTTCTCCATGGTGGTGAAG
LOX primer set	GCGACGACCCTTACAACC	GGACGCCTGGATGTAGTAGG
TGFBR2 primer set	CATCTGTGAGAAGCCACAGG	TGCACTCATCAGAGCTACAGG
TGFBI primer set	ATGGCGCTCTTCGTGCGGCTG	TCATATCCAGGACAGCACTCGTAGC
SPARC primer set	AACCACCACTGCAAACACG	AGAAGTGGCAGGAAGAGTCG
PLAU primer set	GTCGCTCAAGGCTTAACTCC	GGTCTGTATAGTCCGGGATGG
POSTN primer set	CCATGTTTATGGCACTCTGG	TGCTCTCCAAACCTCTACGG
NT5E primer set	GTCCAGGCCTATGCTTTTGG	ATGATTGAGAGGAGCCATCC
CYR61 primer set	GTAAGGTCTGCGCCAAGC	CCCGTTTTGGTAGATTCTGG
SAT primer set	TACTGCGGCTGATCAAGGAG	GCAAAACCAACAATGCTGTG
CDK2AP2 primer set	ATGTCCTACAAACCCATCGCCCC	CCCATCTCCTCTATGACTGACAGC
IGFBP3 primer set	CATCTACACCGAGCGCTGTGGC	GGAACTTGGGATCAGACACCCG
PDLIM primer set	CGAGCAGCCTCTCGCCATTTCC	CCCTTCCTCCGTCACCAGAGG
PPARD primer set	AGCAGCCACAGGAGGAAGCCC	GTGGAAGCCCGATGCCTTGTCC
LOX ChIP	CTGTGTGTAGGTAATTGAGAAATGGG	CCGGCATTTTGAAAAAGAGACAGG
LOX 5Kb Upstream ChIP	GAGTCTGGCTCTGTCACCCAG	GGAGAATGGGTCTTTGAAGTACAGG
LOX 5Kb Downstream ChIP	GAGACTGTTCTCAAAGAAAACTGTAG	GGATCATCAAGCCATCATTTGCC
TGFBR2 ChIP	GAAGCAAATGAACACTTAGAATGACAGG	CTTCTAGCAATTCATTTAATGAATTCCTTTACC

Supplementary Figure S7.1 Validation of EWS/FLI direct downregulated genes. (A) qRT-PCR validation of the EWS/FLI downregulated gene expression signature in A673 cells following knockdown of endogenous EWS/FLI with the EF-2-RNAi retroviral construct. Error bars indicate standard deviation (SD). *GAPDH* is shown as a control gene unaffected by the knock-down. (B) qRT-PCR validation of EWS/FLI mediated repression of *TGFBR2* in A673, TC71 and TC32 Ewing sarcoma cells following knockdown of endogenous EWS/FLI (with EF-2-RNAi) and rescue with an EWS/FLI cDNA construct that is resistant to the RNAi effect. Luc-RNAi is a negative control. Error bars indicate SD. (C) ChIP of EWS/FLI at the *TGFBR2* promoter in A673 cells using antibodies against FLI (which recognizes EWS/FLI) or ELK1 (negative control). Data are plotted as fold enrichment compared to the average enrichment of two negative control genes. The error bars indicate standard error of means of five independent experiments. We were unable to demonstrate loss of binding following knock-down of EWS/FLI, and restoration of binding following re-expression likely due to the efficacy of the RNAi effect. Even with ~80% EWS/FLI knockdown in RNA and protein expression the remaining 20% of protein likely still binds to relevant target sites, albeit at reduced levels overall. While this should be sufficient to observe reduced binding, the level of "background" binding to irrelevant sites in the genome is likely reduced by a similar level. Thus, normalizing gene-specific binding to background binding causes the ratio to remain consistent.


A
EF-2-RNAi
Normalized Fold Change
GAPDH
LOX
PLAU
TGFBR2
SPARC
CYR61
SAT
GAPDH
NT5E
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GAPDH
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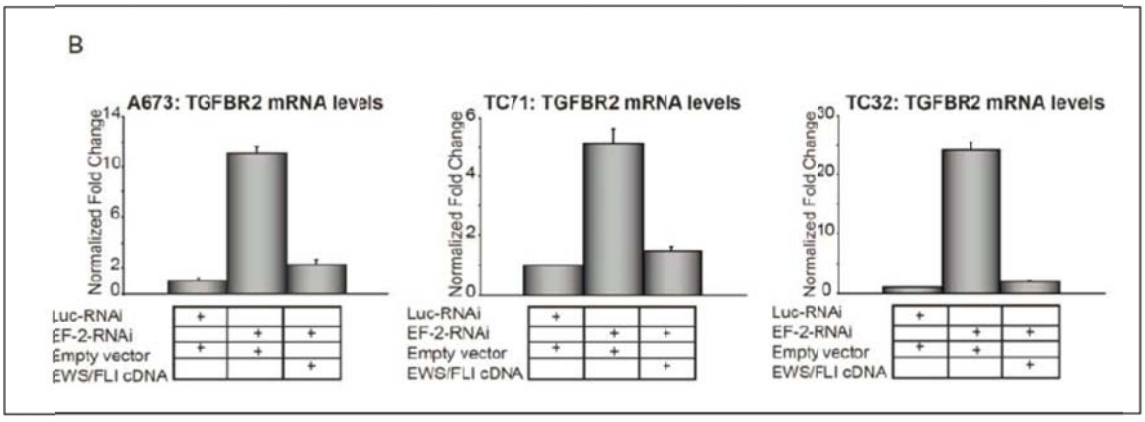
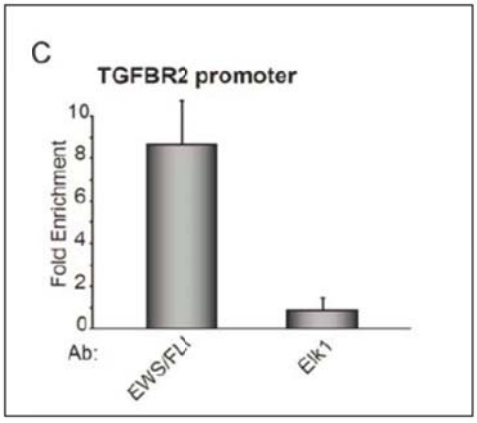
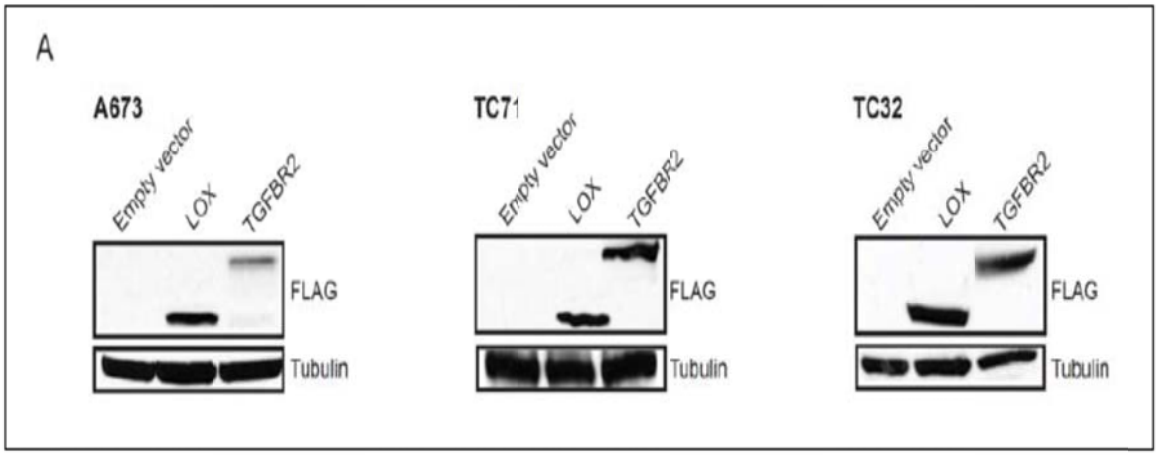
PDLIM
GAPDH
CDK2AP2
TGFBI
PPARD


B
A673: TGFBR2 mRNA levels
TC71: TGFBR2 mRNA levels
TC32: TGFBR2 mRNA levels
Normalized Fold Change
Luc-RNAi
EF-2-RNAi
Empty vector
EWS/FLI cDNA


C
TGFBR2 promoter
Fold Enrichment
Ab:
EWS/FLI
Elk1

Supplementary Figure S7.2 TGFβR2 has tumor suppressive roles in Ewing sarcoma. (A) Western blot analysis of A673, TC71, and TC32 cells expressing 3X-FLAG LOX, 3X-FLAG TGFBR2 cDNA or an empty vector control. Expressed proteins were detected with an anti-FLAG antibody, and anti-Tubulin was used as a loading control. (B) Quantification of colonies formed in soft agar by A673, TC71 and TC32 cells expressing 3X-FLAG TGFBR2 cDNA as compared to cells expressing an empty vector control. Error bars indicate SD of duplicate assays. (C) Growth assays (3T5) for A673, TC71 and TC32 cells expressing 3X-FLAG TGFBR2 cDNA as compared to cells expressing an empty vector control. (D) Tumor volumes were measured using digital calipers and plotted for immunocompromised mice subcutaneously injected with TC32 Ewing sarcoma cells expressing an empty vector control or 3X-FLAG LOX cDNA. Tumor volumes are plotted at two time points, Day 12 and Day 16 postinjection. Five mice were used per group, both flanks of each mouse were injected, therefore, 10 total tumors were measured. The three TC32 clones with modulation of EWS/FLI used in Fig. 7.1 of the primary manuscript could have been a useful tool to address what fraction of EWS/FLI tumorigenicity is LOX-mediated. However, given that the survival curves comparing empty vector versus LOX cDNA expressing TC32 cells are only separated by a week (Fig. 7.2C), this would be a very challenging experiment to interpret. Furthermore, we have previously shown that xenograft experiments using EWS/FLI knockdown exhibit an "escapee" effect, whereby the RNAi knockdown effect is lost over time (7). Thus, comparisons between EWS/FLI knockdown and LOX expression would be confounded by this effect as well, making direct comparisons essentially uninterpretable. (E) Survival curves for immunodeficient mice injected with TC32 cells expressing 3X-FLAG TGFBR2 cDNA or an empty vector construct. The log-rank (Mantel-Cox Test) determined p-value using GraphPad Prism is indicated. (F) Western blot analysis of tumors excised from immunodeficient mice injected with TC32 cells expressing the indicated 3X-FLAG cDNAs. (G) Graphical representation of *TGFBR2* expression levels in 27 primary Ewing sarcoma patient-derived tumors and ten Ewing sarcoma cell lines in the Schaefer *et al*. dataset. The EWS/FLI or EWS/ERG translocation fusion status for each sample is indicated. The Schaefer *et al.* dataset compared Ewing sarcoma specimens to analyze differential gene expression between metastatic and localized tumors. There was no normal tissue used as a baseline reference. Thus, the *LOX* and *TGFBR2* levels were direct expression levels obtained on the Affymetrix microarray platform. However, we note that our own microarray datasets of EWS/FLI-mediated transcriptional profiles (7, 9, 32) using Affymetrix microarray platforms, each demonstrated low *LOX* and *TGFBR2* expression in the presence of EWS/FLI expression, and increased *LOX* and *TGFBR2* expression following EWS/FLI knockdown. Thus, the low absolute expression levels observed by Schaefer *et al*. is well-correlated to our own published microarray data, supporting our assertion that primary Ewing sarcoma tumors also exhibit low-level expression of these two genes.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LOX
TGFBR2
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LOX
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FLAG
Tubulin
TC32
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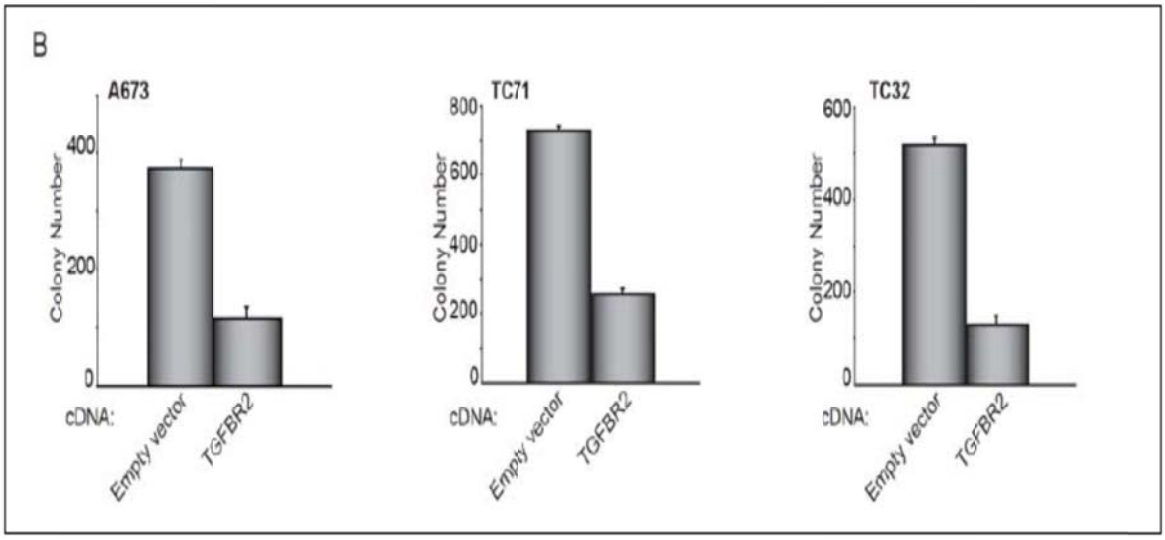
LOX
TGFBR2
FLAG
Tubulin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TGFBR2
TC71
Colony Number
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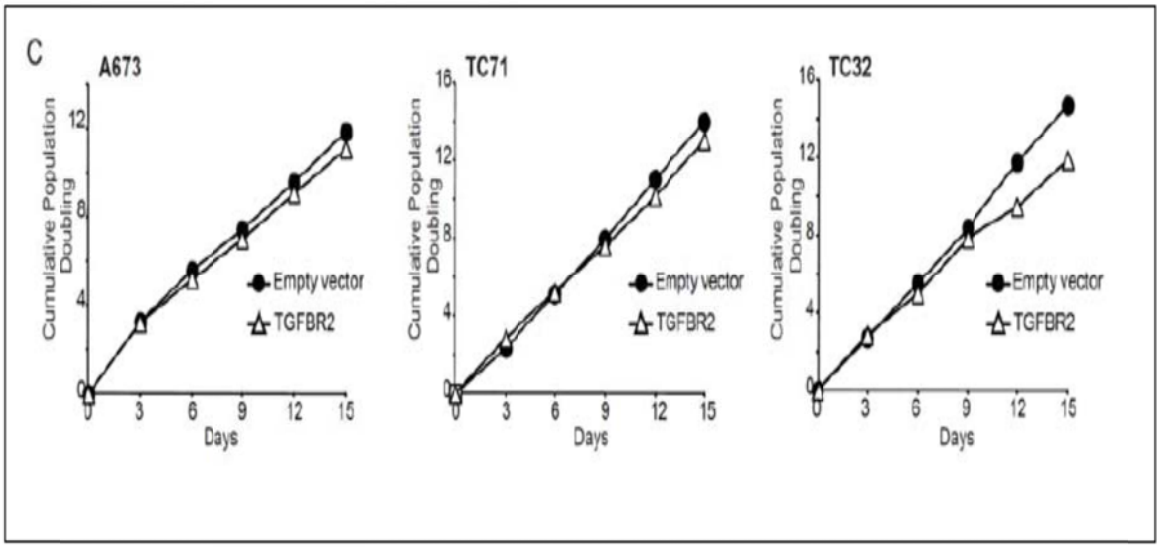
TGFBR2


C
A673
Cumulative Population Doubling
12
8
4
0
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TGFBR2
0
3
6
9
12
15
Days
TC71
Cumulative Population Doubling
16
12
8
4
0
Empty vector
TGFBR2
0
3
6
9
12
15
Days
TC32
Cumulative Population Doubling
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TGFBR2
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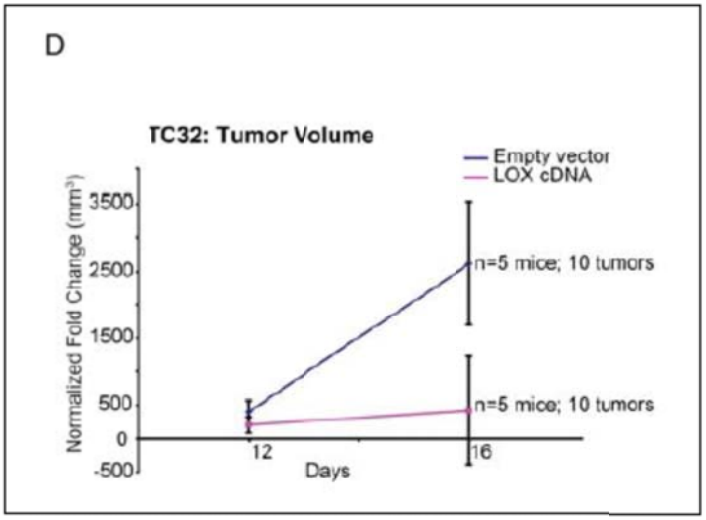
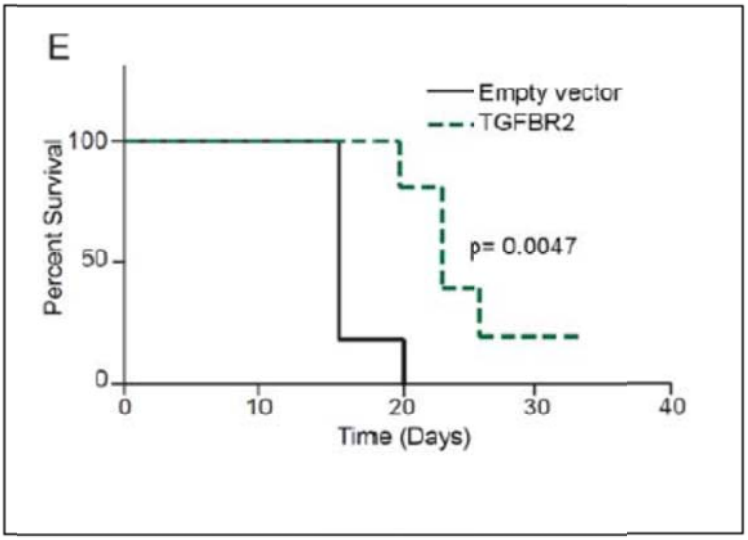
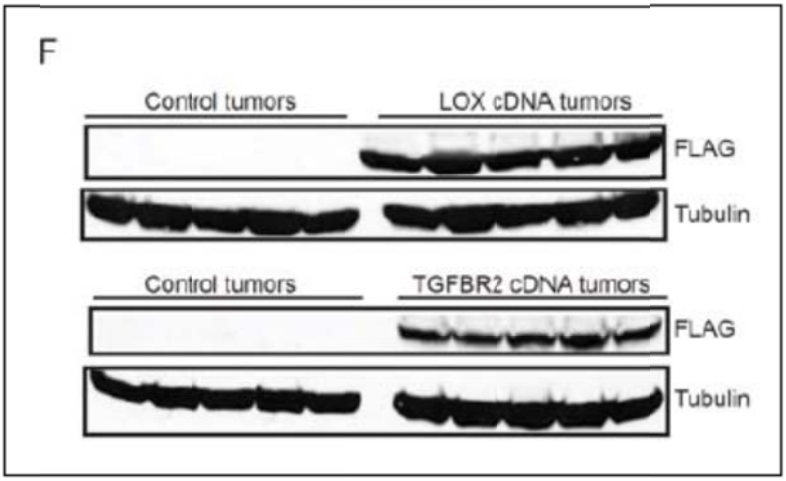
3
6
9
12
15
Days

(Supplementary Figure S7.2 continued)







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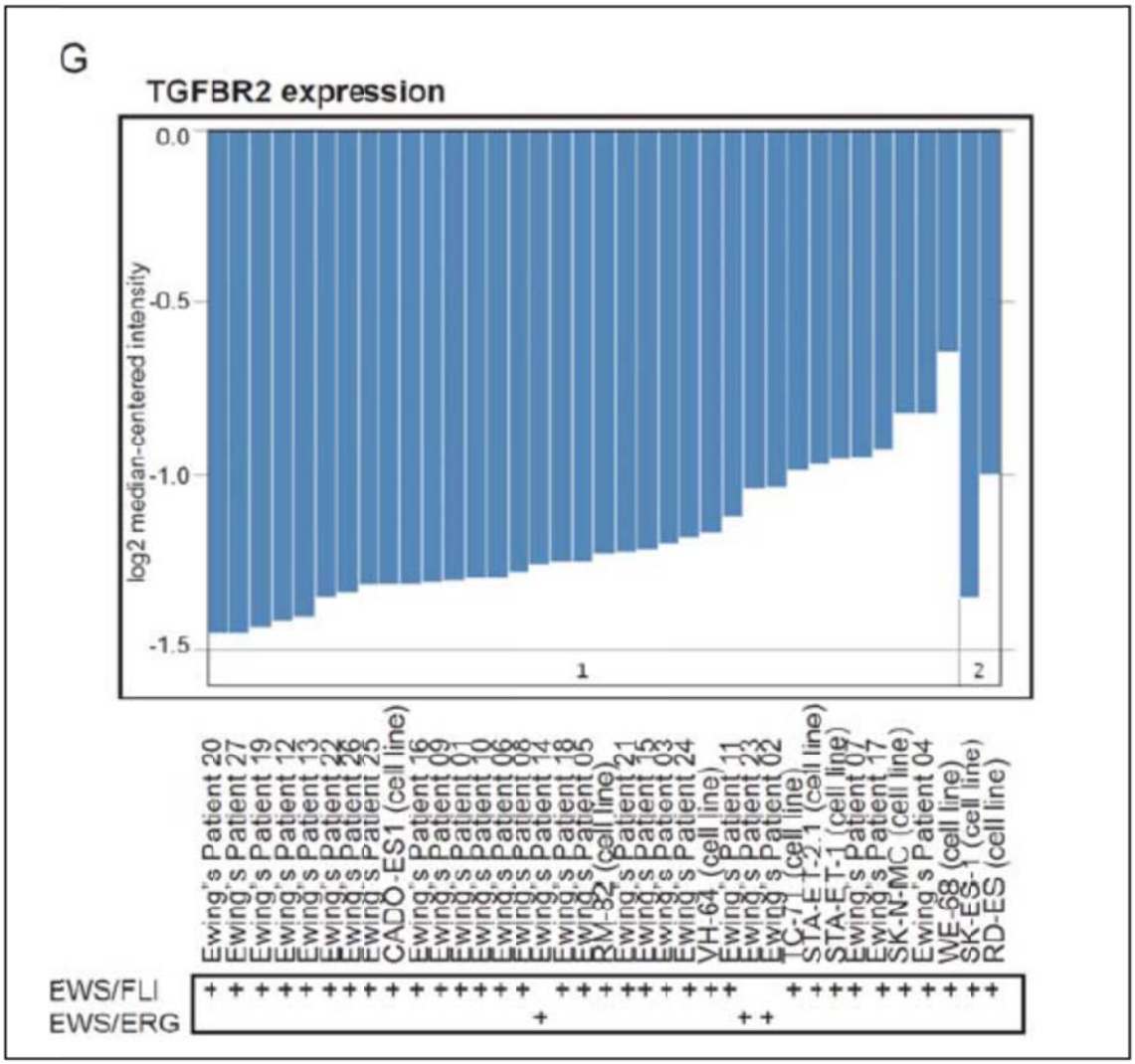
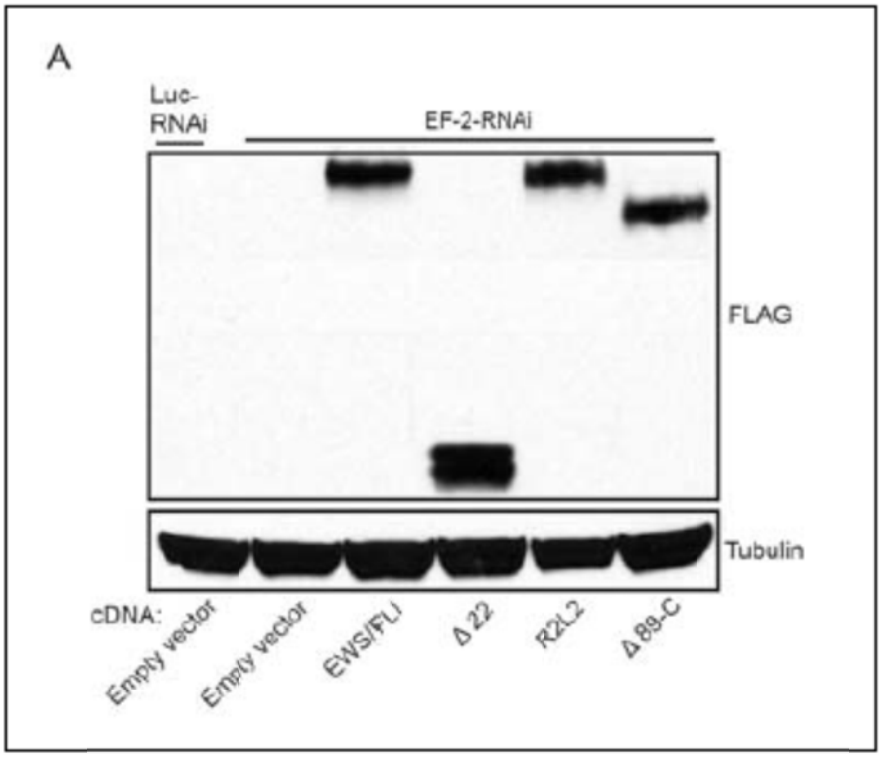


Supplementary Figure S7.3 Expression and DNA-binding of EWS/FLI deletion mutants. (A) Western blot analysis of A673 cells infected with an empty vector control retrovirus, or retroviruses expressing 3X-FLAG wild-type EWS/FLI, or the Δ22, R2L2 or Δ89-C mutants. Tubulin was used as the loading control. (B) Western blot analysis of samples from Figure S3.A demonstrating the efficiency of the EWS/FLI RNAi. The 3X-FLAG tagged mutant constructs that are re-expressed are indicated with a red asterisk, while the band position of the endogenous EWS/FLI is indicated. Tubulin was used as the loading control. Because some of the EWS/FLI deletion mutants are approximately the same size as endogenous EWS/FLI, it can be difficult to evaluate the endogenous EWS/FLI band by Western blotting. Therefore, we have also included the qRT-PCR (Figure S7.3C) data for all the EWS/FLI knock-down/rescues to demonstrate efficiency and maintenance of the EWS/FLI RNAi effect. (C) Quantitative RT-PCR analysis to demonstrate efficiency and maintenance of EWS/FLI knock-down in A673 cells with the EF-2-RNAi construct, re-expressing an empty vector, 3X-FLAG tagged EWS/FLI or mutant constructs. Luc-RNAi is a negative control. (D) Western blot analysis of A673 cells infected with empty vector or 3X-FLAG EWS/FLI or the indicated mutants. Tubulin was used as the loading control. (E) Western blot analysis of samples from Figure S3.D to demonstrate the efficiency of the EWS/FLI RNAi. The 3X-FLAG tagged mutant constructs that are re-expressed are indicated with a red asterisk while the band position of endogenous EWS/FLI is indicated. Tubulin was used as the loading control. (F) EMSA with a DNA duplex (I) containing a high-affinity EWS/FLI-binding site, called "ETS2 probe." A specific EWS/FLI band indicated by a red asterisk is present when 3X-FLAG EWS/FLI or 3X-FLAG deletion mutants of EWS/FLI from nuclear extracts are included. The specific band in each case is supershifted with the anti-FLAG antibody and competed with an excess of unlabeled DNA duplex (I) A control nuclear extract that does not contain wild-type EWS/FLI or deletion mutants of EWS/FLI as well as the DNA binding mutant (R2L2) nuclear extract generate only nonspecific binding (indicated by "ns").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FLAG
Tubulin
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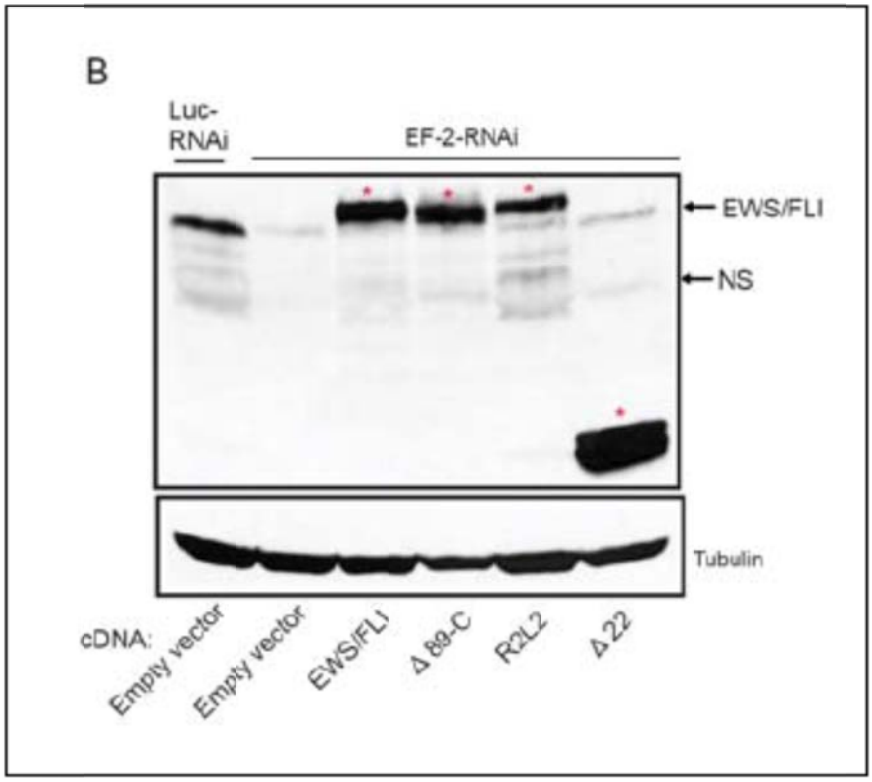
Empty vector
EWS/FLI
Δ 22
R2L2
Δ 89-C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EF-2-RNAi
EWS/FLI
NS
Tubulin
cDNA:
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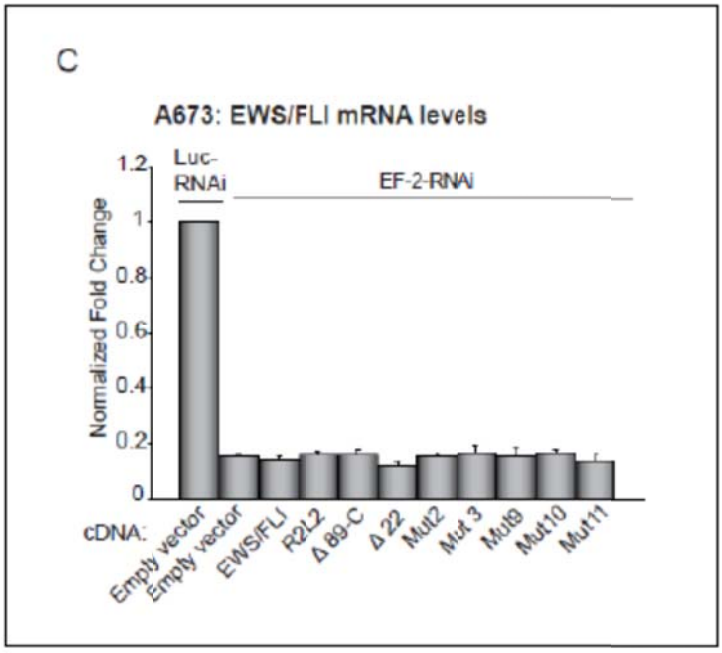
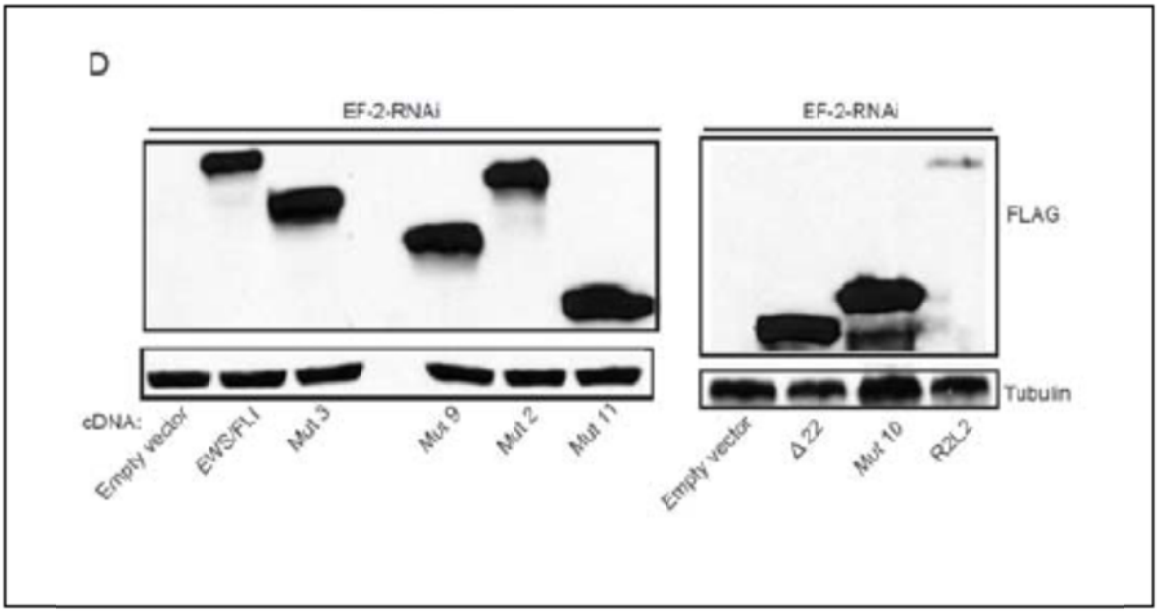
Empty vector
EWS/FLI
Δ 89-C
R2L2
Δ 22

(Supplementary Figure S7.3 continued)





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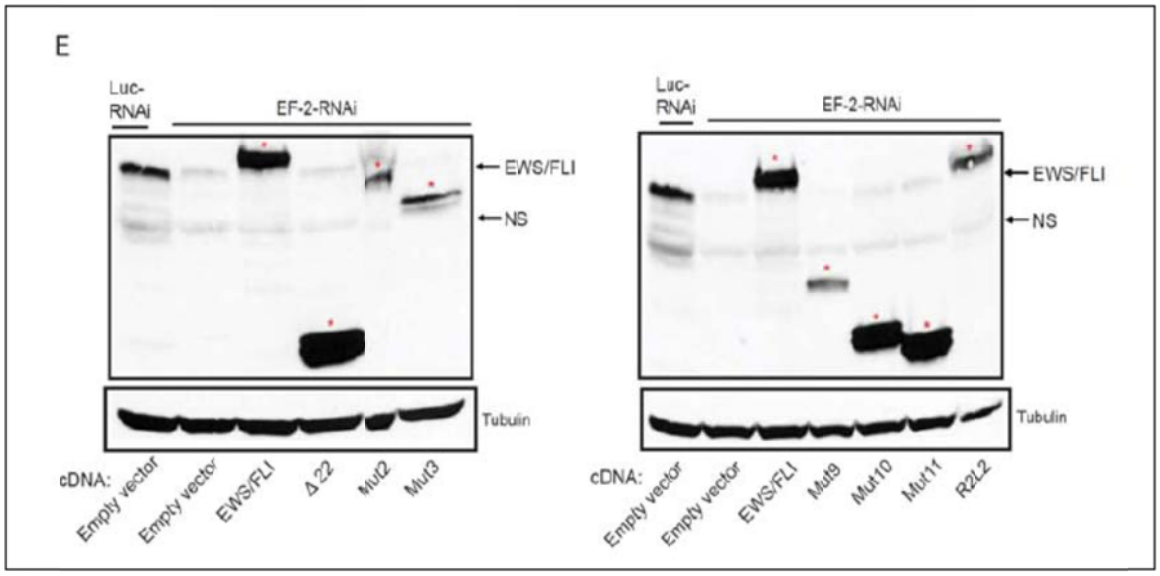
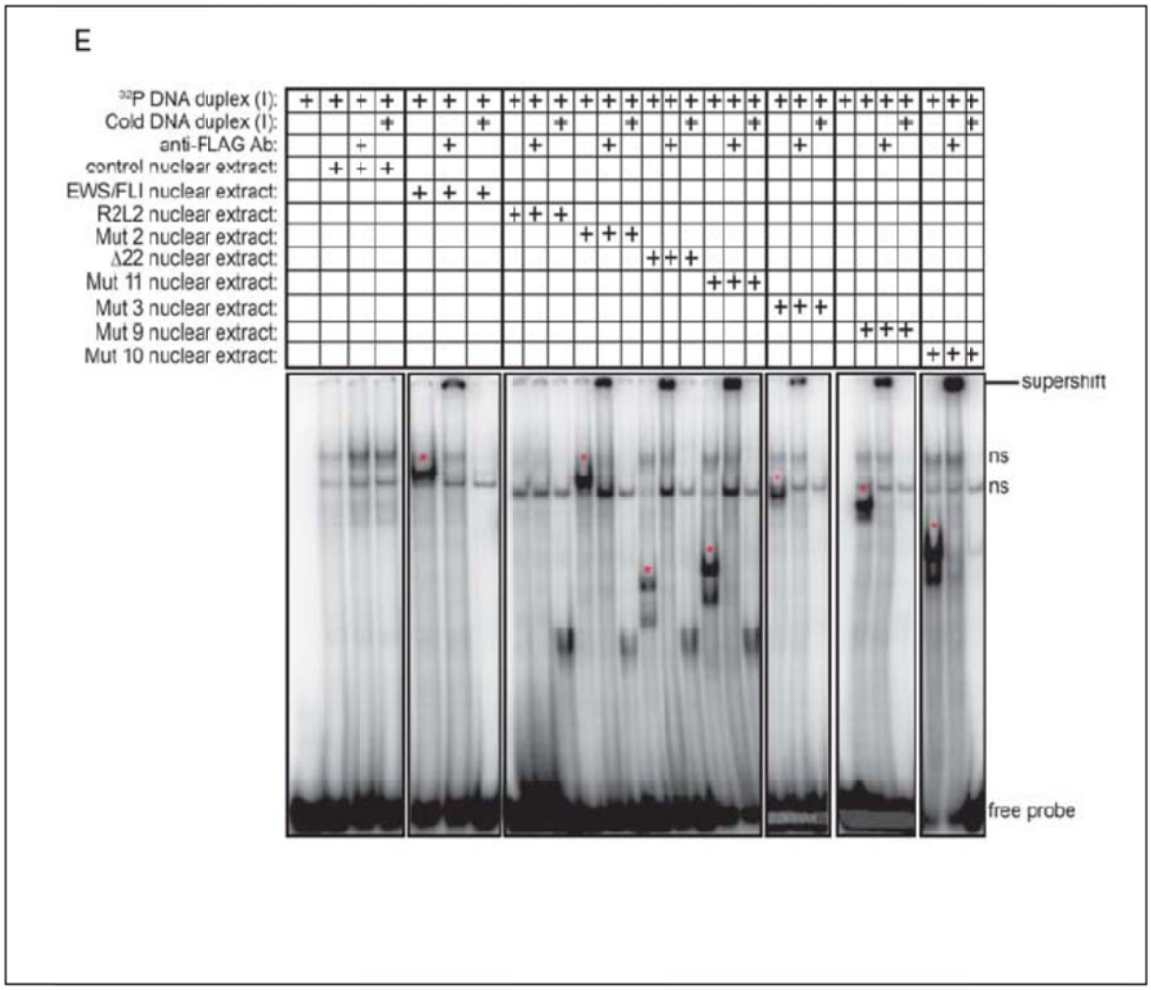
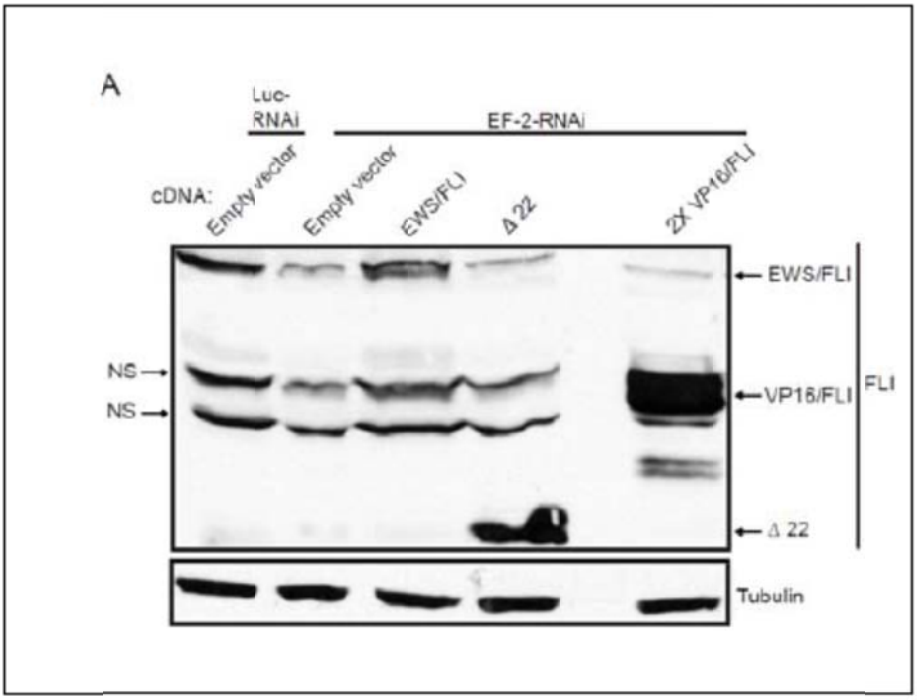




Supplementary Figure S7.4 Expression of EWS/FLI mutant constructs. (A) Western blot analysis of A673 cells infected with the indicated RNAi constructs and rescued with an empty vector or the indicated constructs. Protein expression was detected with an anti-FLI antibody, and the positions of endogenous EWS/FLI, Δ22, and 2xVP16/FLI are indicated. Note that re-expressed EWS/FLI is 3xFLAG tagged, and thus runs slightly slower than endogenous EWS/FLI. NS indicates nonspecific bands. Tubulin was used as the loading control. (B) Western blot analysis of A673 cells infected with the indicated constructs used for the *in vivo* xenograft tumor formation assay. Protein expression was detected with an anti-FLI antibody, and the positions of endogenous EWS/FLI, Δ22, and 2xVP16/FLI are indicated (with 3xFLAG EWS/FLI running slightly slower than endogenous EWS/FLI). NS indicates nonspecific bands. Tubulin was used as the loading control.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EF-2-RNAi
cDNA:
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Empty vector
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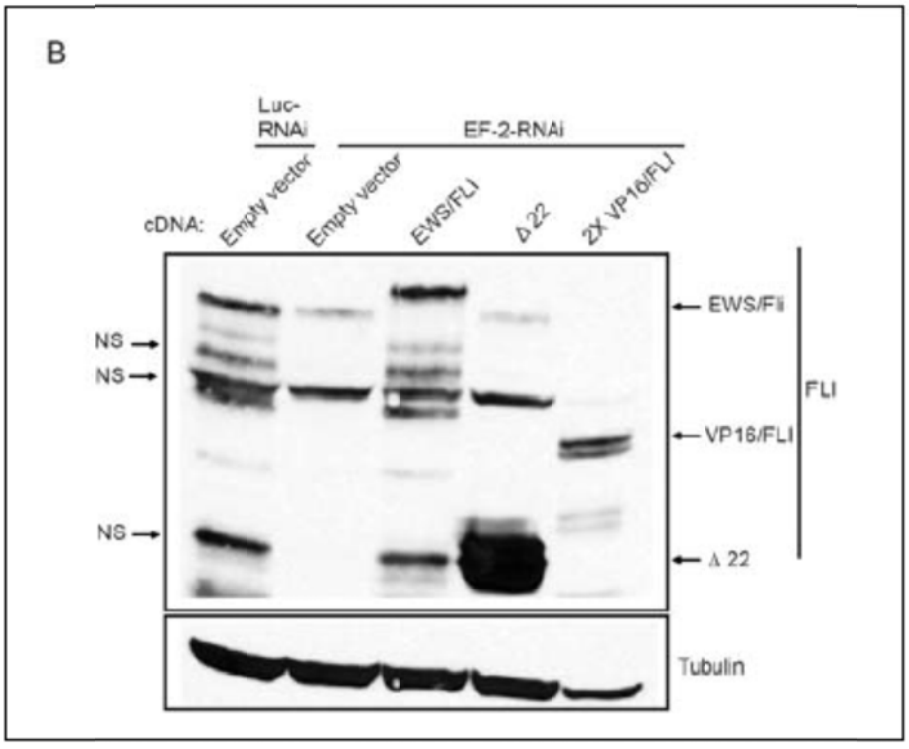
Δ 22
2X VP16/FLI
EWS/FLI
NS
NS
VP16/FLI
FLI
Δ 22
Tubulin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EF-2-RNAi
cDNA:
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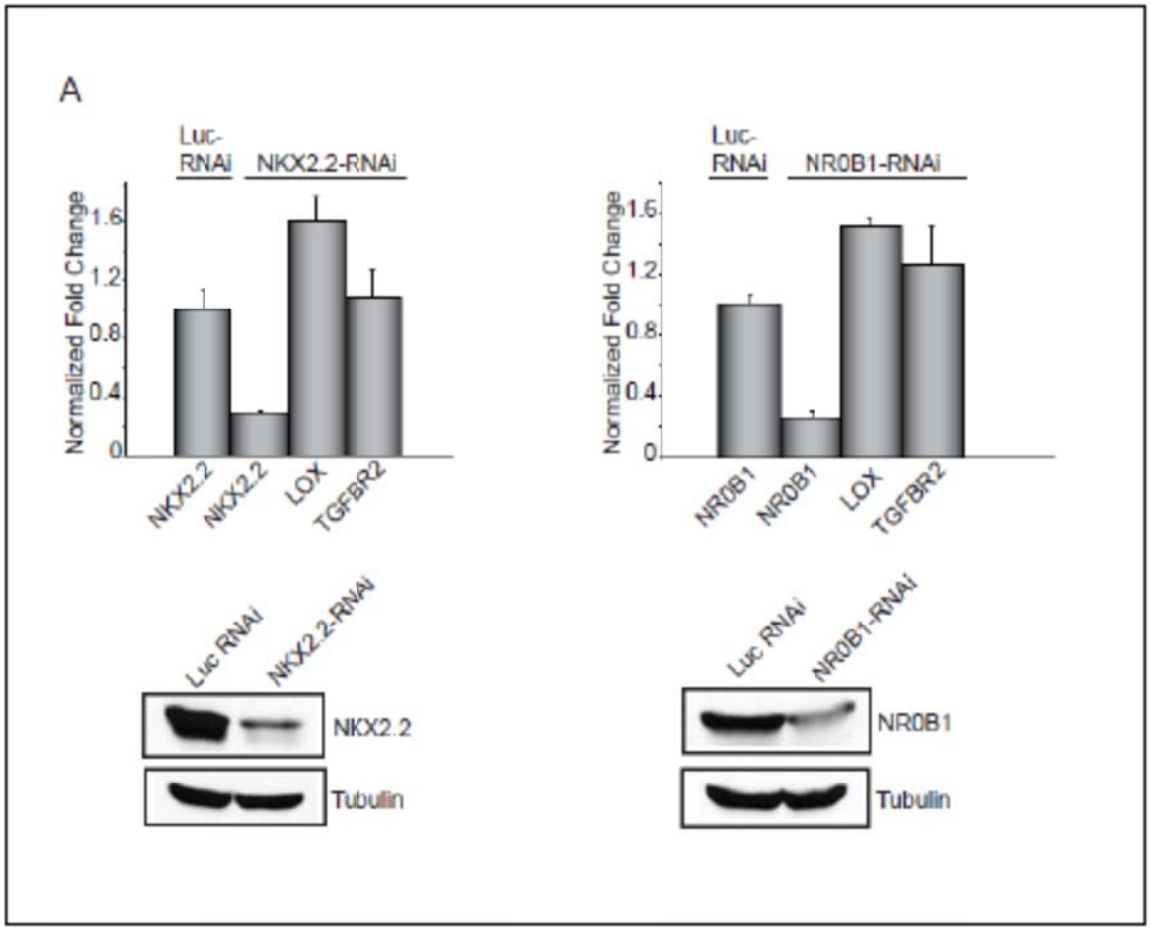
Empty vector
EWS/FLI
Δ 22
2X VP16/FLI
EWS/Fli
NS
NS
NS
VP16/FLI
FLI
Δ 22
Tubulin

Supplementary Figure S7.5 Validation of the role of HDACs in EWS/FLI-mediated target gene repression. (A) qRT-PCR analysis of *LOX* and *TGFBR2* expression following retroviral knockdown of *NKX2.2* or *NR0B1* transcripts in A673 cells. Error bars indicate SD. Western blots indicate levels of NKX2.2 and NR0B1 knock-down in A673 cells. Tubulin was used as the loading control. (B) qRT-PCR analysis of *LOX* and *TGFBR2* expression following treatment of HEK293 cells with increasing doses of the HDAC inhibitor vorinostat. (C) qRT-PCR analysis of *LOX* and *TGFBR2* expression following siRNA-mediated knock-down of HDAC1, HDAC2 or HDAC3 in A673 cells compared to a control siRNA knock-down. Knock-down of HDAC1 and HDAC2 results in a significant increase in expression of *LOX* and *TGFBR2* indicated by the p-values, n.s. indicates nonsignificant p-value. (D) Western blot analysis of HDAC1, HDAC2 and HDAC3 levels in A673 cells following siRNA-mediated knock-down compared to a control siRNA sample. Tubulin was used as the loading control.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Luc-RNAi
NKX2.2-RNAi
Normalized Fold Change
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1.2
0.8
0.4
0
NKX2.2
NKX2.2
LOX
TGFBR2
Luc-RNAi
NR0B1-RNAi
Normalized Fold Change
1.6
1.2
0.8
0.4
0
NR0B1
NR0B1
LOX
TGFBR2
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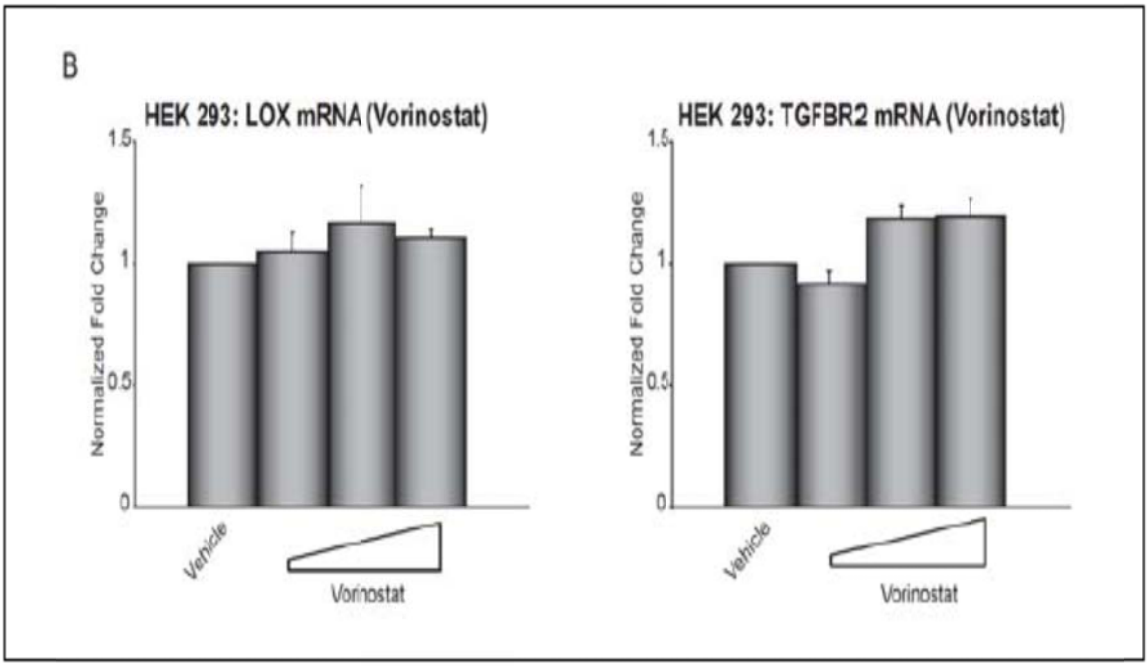
Tubulin
Luc RNAi
NR0B1-RNAi
NR0B1
Tubulin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HEK 293: LOX mRNA (Vorinostat)
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0.5
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Vehicle
Vorinostat
HEK 293: TGFBR2 mRNA (Vorinostat)
Normalized Fold Change
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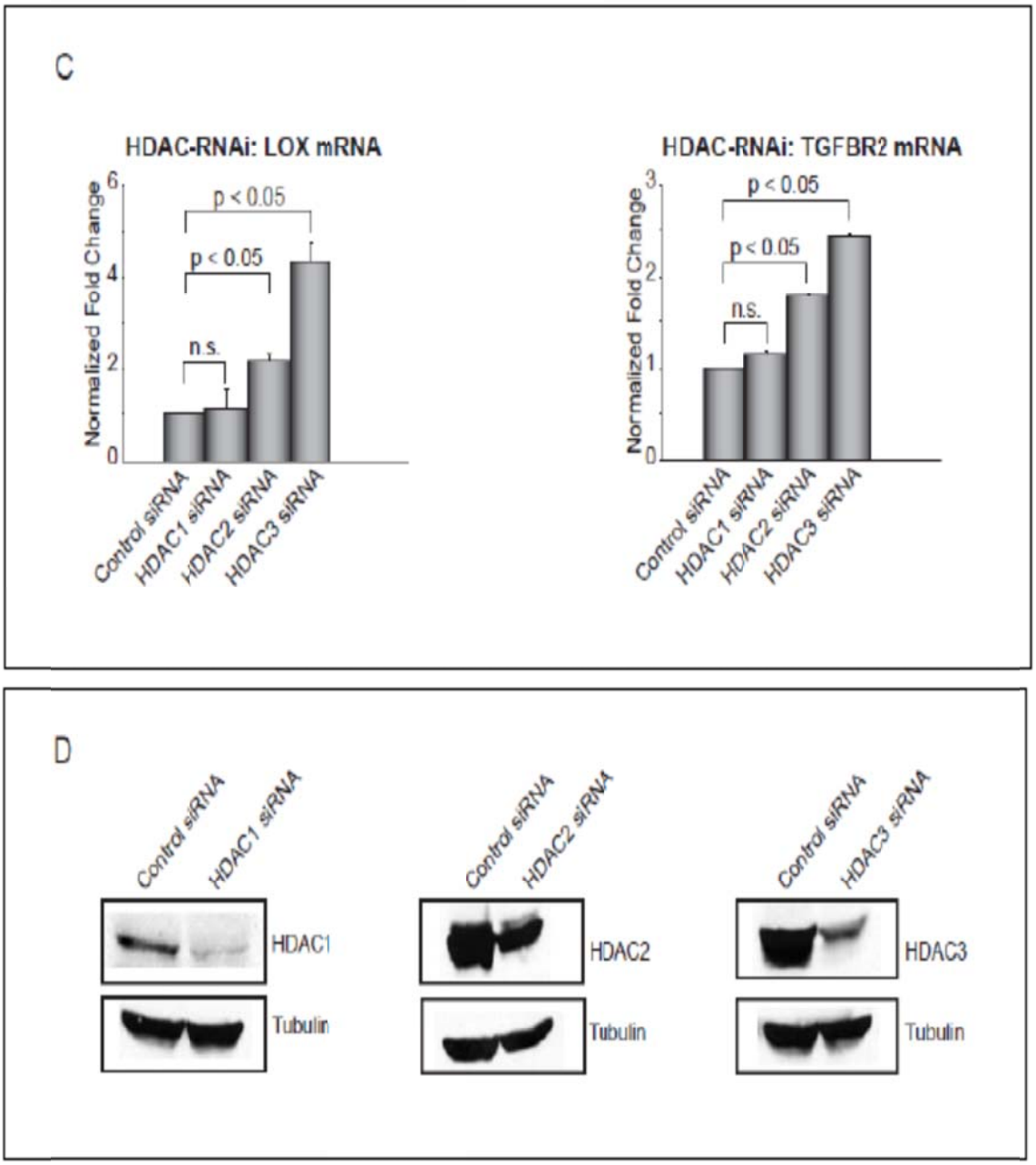
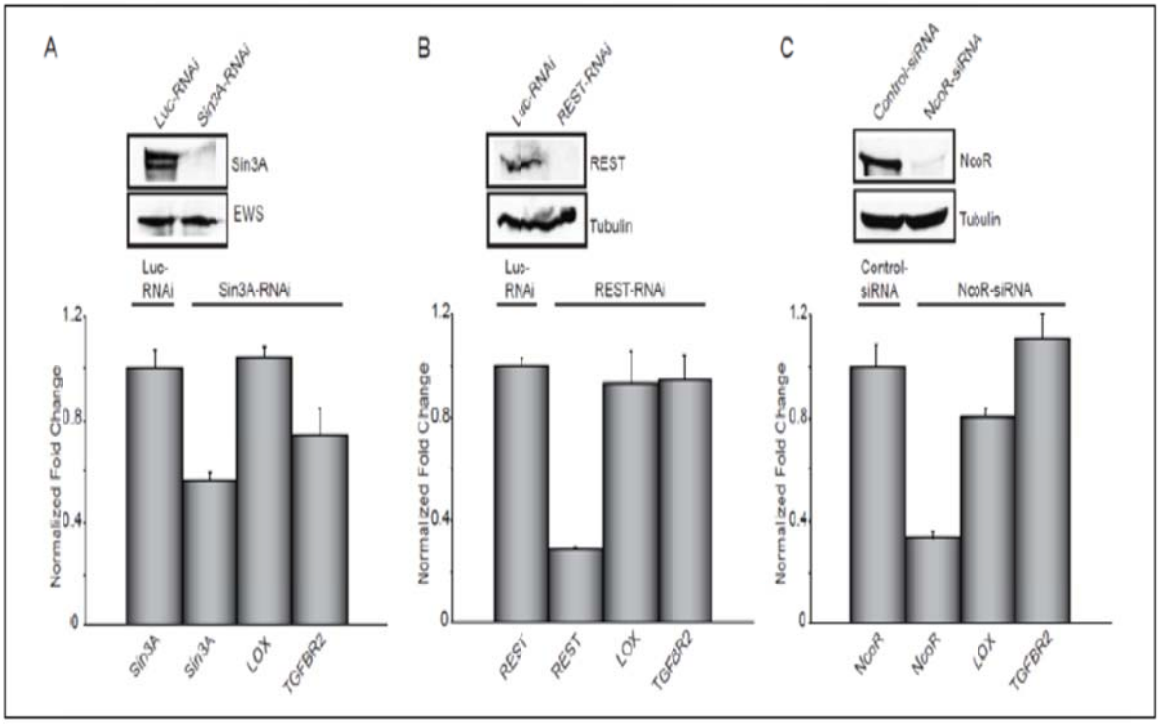
0
Vehicle
Vorinostat

(Supplementary Figure S7.5 continued)



Supplementary Figure S7.6 Identifying corepressors involved in EWS/FLI-mediated gene repression; validating the role of LSD1 in transcriptional repression by EWS/FLI. (A-C) qRT-PCR of the indicated transcripts following knockdown of the Sin3A repressor complex, the REST repressor complex or the NCoR/SMRT repressor complex, respectively. Error bars indicate SD. Inset panels show Western blot analysis to demonstrate significant knock-down of the Sin3A, REST and NcoR/SMRT repressor complexes in A673 cells. Wild-type EWS protein and Tubulin were used as loading controls for the Western blot analysis. (D) Relative cell viability assay of A673 cells treated with the indicated concentrations of the LSD1 inhibitor HCI-2528. IC_{50} (as determined by GraphPad Prism) was 0.47μM. Error bars indicate SD. (E) qRT-PCR analysis of *LOX* and *TGFBR2* mRNA expression in A673 cells after 72 hours of treatment with the indicated concentrations of the LSD1 inhibitor HCI-2528. The dose corresponding to the IC_{50} is indicated. Error bars indicate SD. (F-H) Cell viability assays performed on TC71, TC32 and SK-N-MC cell lines treated with the indicated concentrations of LSD1 inhibitors HCI-2509 and HCI-2528. The IC_{50} for each inhibitor (as determined by GraphPad Prism) is shown. Error bars indicate SD. (I) qRT-PCR analysis of *LOX* and *TGFBR2* expression following 72 hours of treatment of HEK 293 cells with increasing doses of the LSD1 inhibitor (HCI-2509). Error bars indicate SD. (J) qRT-PCR analysis of *LOX* and *TGFBR2* expression at 72 hours following siRNA-mediated knock-down of LSD1 in A673 cells. Increasing concentrations of the LSD1 siRNA resulted in a dose dependent increase in expression of *LOX* and *TGFBR2*. Western blot analysis demonstrating the efficiency of the LSD1 siRNA, although the dose dependent decrease in the LSD1 protein expression is less obvious in the scanned image (the Western blot film shows a slight dose dependent decrease in the LSD1 protein with increasing concentrations of the LSD1 siRNA). Error bars indicate SD.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Sin3A-RNAi
Sin3A
EWS
Luc-RNAi
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Normalized Fold Change
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0.8
0.4
0
Sin3A
Sin3A
LOX
TGFBR2
B
Luc-RNAi
REST-RNAi
REST
Tubulin
Luc-RNAi
REST-RNAi
Normalized Fold Change
REST
REST
LOX
TGFBR2
C
Control-siRNA
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Normalized Fold Change
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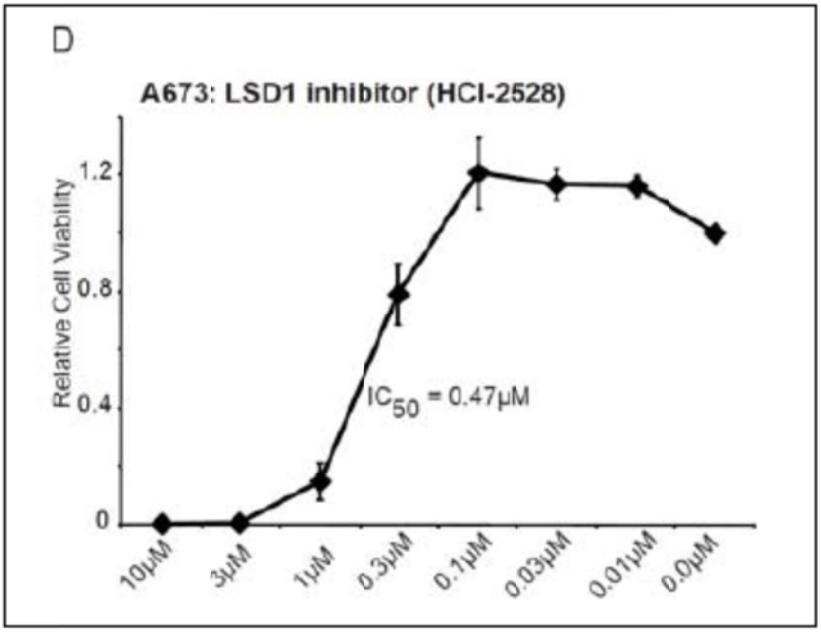
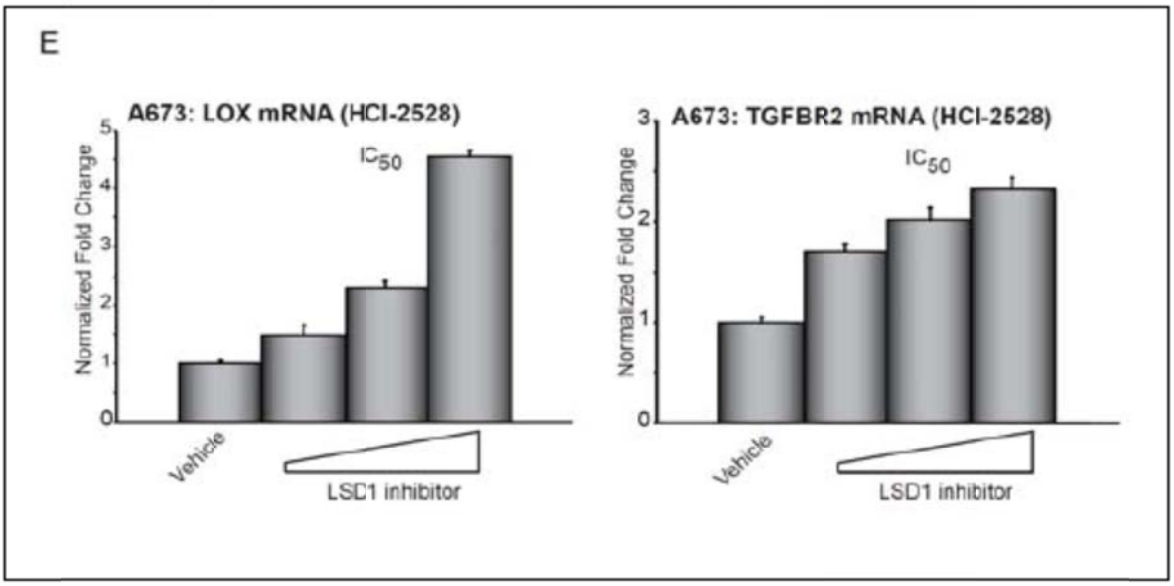
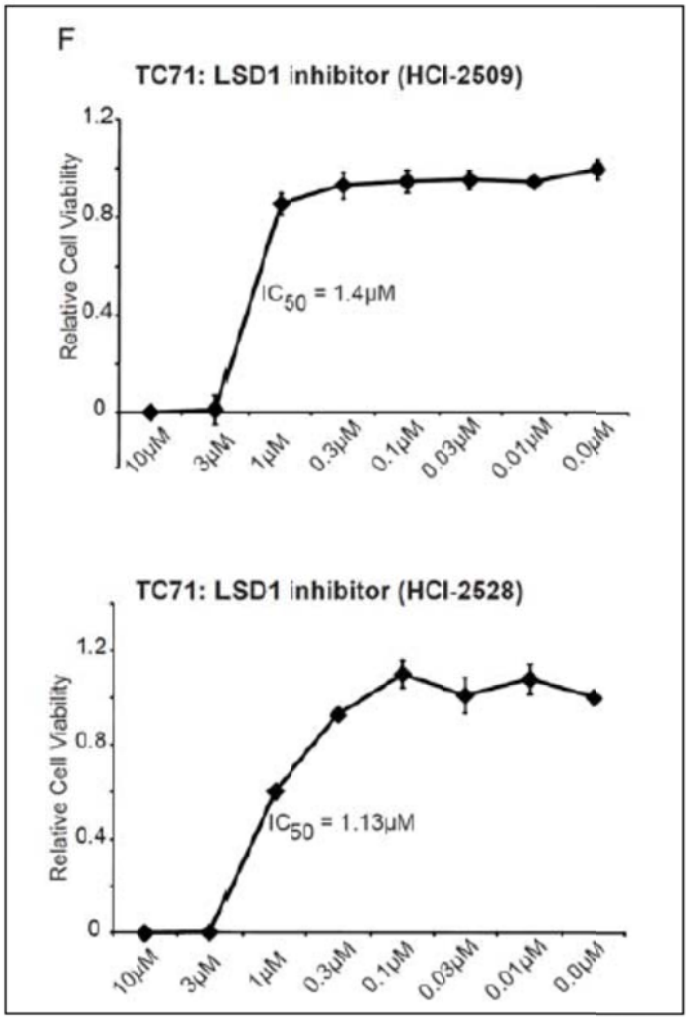
LOX
TGFBR2


D
A673: LSD1 inhibitor (HCI-2528)
Relative Cell Viability
1.2
0.8
0.4
0
IC50 = 0.47μM
10μM
3μM
1μM
0.3μM
0.1μM
0.03μM
0.01μM
0.0μM

(Supplementary Figure S7.6 continued)





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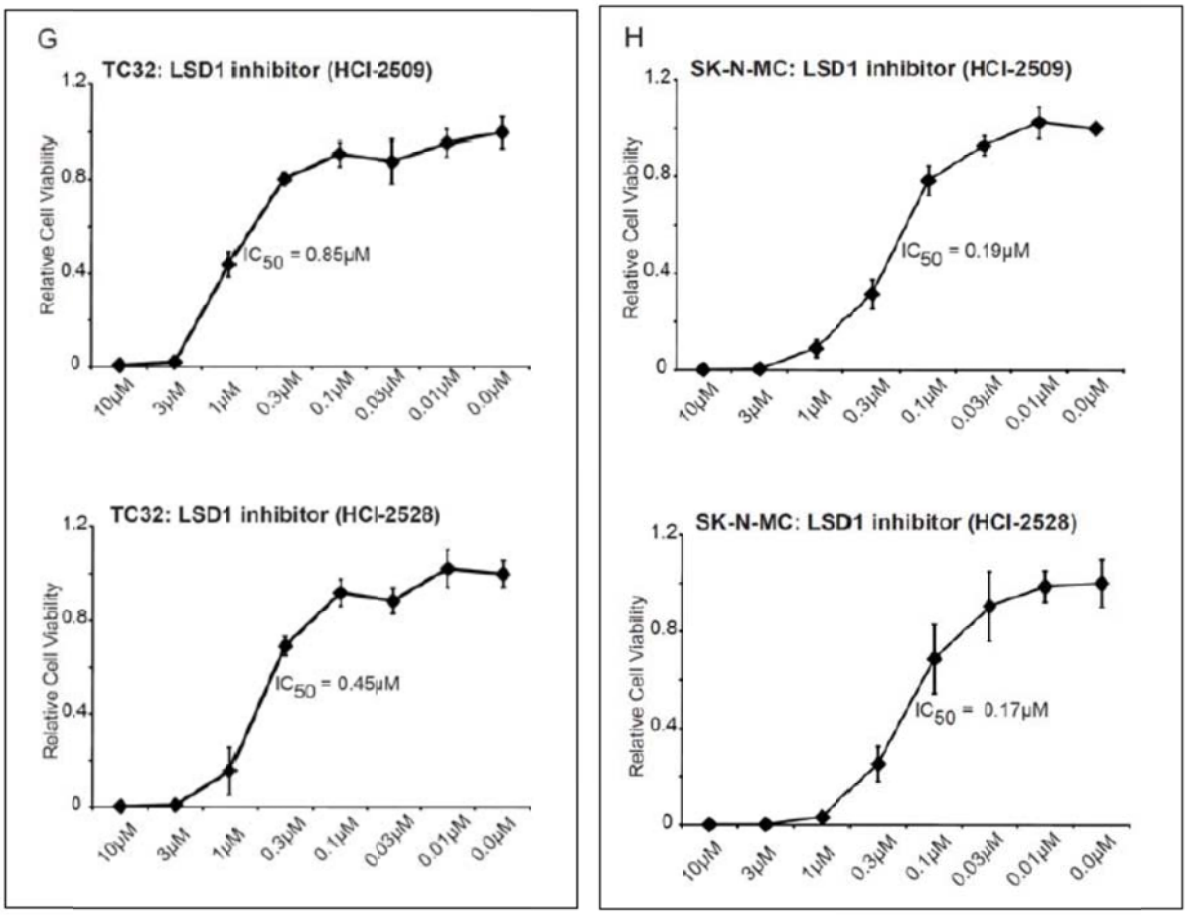

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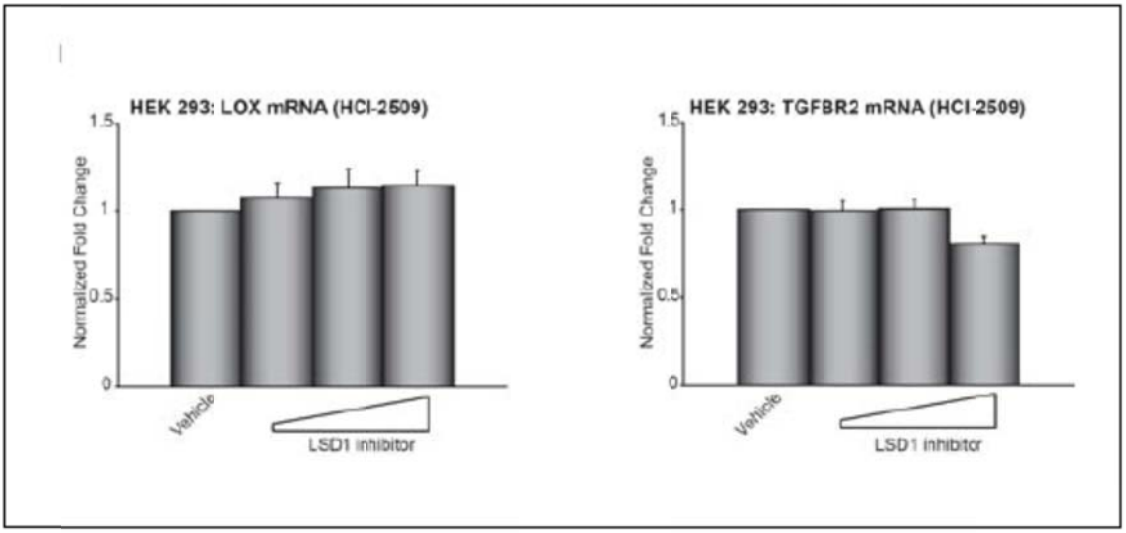

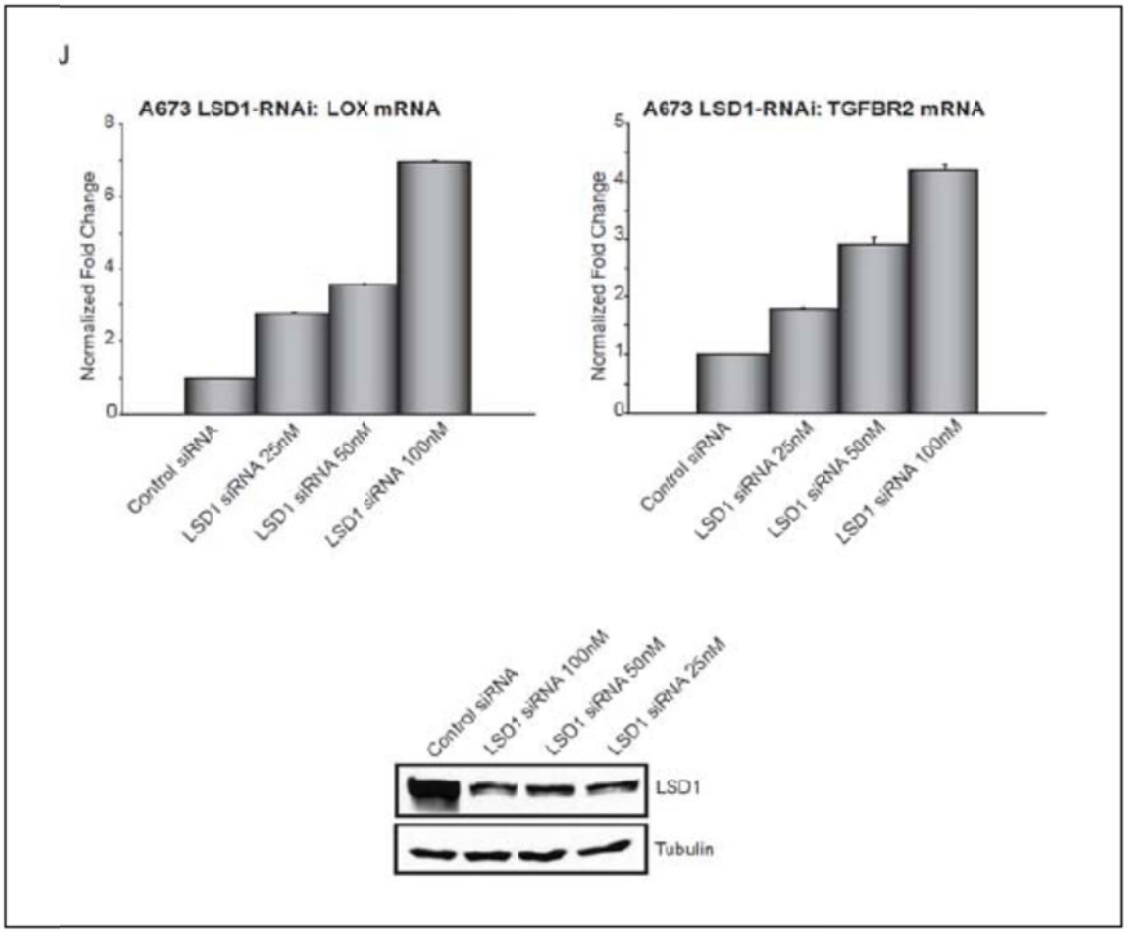

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

James Ewing first described the pediatric bone and soft tissue associated malignancy called Ewing sarcoma in 1921 (1). In the mid-1980s the identification and characterization of the reciprocal translocation, t(11;22)(q24;q12), in the majority of Ewing sarcoma tumors was the first big step in determining the molecular genetics underlying the disease (2). The chromosomal rearrangement was found to encode the fusion protein EWS/FLI, which functions as an aberrant oncogenic transcription factor (3, 4). Since then, several other EWS/ETS and TET/ETS based fusion proteins have been identified in Ewing sarcoma. Hence, Ewing sarcoma is predominantly a TET/ETS driven malignancy (5).

The Ewing sarcoma genome is relatively stable. Indeed, very few if any secondary mutations have been identified in Ewing sarcoma (6, 7). In addition, few copy number gains and losses have been reported in this disease (8), further highlighting that the EWS/FLI fusion (and other TET/ETS fusions) is the main gain-of-function mutation in Ewing sarcoma, and that transcriptional changes mediated by EWS/FLI are critical effectors of the disease process. The lack of secondary genetic changes, such as activating mutations in the PI3K, RAS pathways etc., or inactivating mutations in known tumor suppressors like RB etc., further highlight the central role of EWS/FLI, the

initiating oncogenic event, in Ewing sarcoma. Therefore, the working model is that EWS/FLI (and other TET/ETS fusions) is the major driver of Ewing sarcoma development, and it does so by functioning as a central mediator of a hierarchy of transcriptional networks, by activating and repressing critical target genes in Ewing sarcoma. The EWS/FLI target genes then contribute to various aspects of oncogenic transformation and maintenance, including anchorage-independent growth [e.g., *NKX2.2* (9), *NR0B1* (10), *GLI1* (11), *LOX* (12)], selfsufficiency of growth signals [e.g., *IGF1* (13)], insensitivity to antigrowth signals [e.g., *TGFBR2* (12, 14)], resistance to chemotherapy [e.g., *GSTM4* (15)], tissue invasion and metastasis [e.g., *EZH2* (16)], evasion of apoptosis [e.g., *IGFBP3* (17)], limitless replicative potential [e.g., *hTERT* (18)], sustained angiogenesis [e.g., *VEGF* (19)], thus, bypassing the need for accumulating secondary mutations to drive oncogenesis (20). This model is further supported by the fact that ongoing EWS/FLI protein expression is required for the transformed phenotype of Ewing sarcoma cells (9, 10, 21).

Much effort has been focused on deciphering the molecular targets of EWS/FLI and determining how these critical target genes contribute to the pathogenesis of Ewing sarcoma (22). However, the big unanswered question in the field was, mechanistically, how does the transcription factor EWS/FLI regulate its target genes? Furthermore, what are the EWS/FLI binding elements at target gene promoters? Also, how does the same fusion oncoprotein activate and repress target genes in Ewing sarcoma? The goal of this thesis was to understand the mechanisms by which EWS/FLI directly upregulates and downregulates critical target genes in Ewing sarcoma. It is hoped that an enhanced understanding of the mechanisms utilized by EWS/FLI to dysregulate gene expression,

and insight as to the proteins it interacts with to achieve this function, will lead to the development of new and effective targeted therapies.

DNA binding by EWS/FLI

In the process of identifying direct EWS/FLI target genes in Ewing sarcoma we identified microsatellite repeats as novel EWS/FLI response elements (23). As detailed in Chapters 3 and 4, combining genome wide localization of EWS/FLI with transcription profiling data we determined that the mechanism of regulation of a subset of EWS/FLI target genes, including critical targets like *NR0B1* and *GSTM4* that are absolutely necessary for oncogenic transformation, is through GGAA-microsatellite repeats (15, 23). The identification of GGAA-microsatellites as EWS/FLI response elements was surprising given that these repeats were previously considered “genomic junk” with no biological function (24). These studies have since been independently validated by several research groups using more recent ChIP-sequencing techniques (25, 26). Interestingly, the *NR0B1* GGAA-microsatellite response element has recently been used as a tool to screen for drugs that inhibit EWS/FLI activity (27), further highlighting the wide-ranging applicability and significance of this research.

Among the EWS/FLI direct targets, only about 10% harbor GGAA microsatellites as the EWS/FLI response element (23). Of the remaining 90% of direct targets, a small but significant proportion harbors the high-affinity ETS consensus site as the EWS/FLI response element. Bioinformatics analysis revealed that both the microsatellites and the high-affinity ETS sites were only enriched in the promoters of EWS/FLI direct “activated” target genes. Using ChIP-sequencing approach, a recent study identified

frequent occurrences of combinations of two ETS binding sites in several EWS/FLI direct-activated target genes (25). This data raises the possibility that EWS/FLI may activate transcription from some promoters as a homo- or heterodimer with other ETS transcription factors. In addition to microsatellites and high affinity ETS sites, composite sites for transcription factors including, E2F, NRF1 and NFY with ETS sites are also overrepresented in a subset of EWS-FLI direct activated targets (28), suggesting that cooperative interactions may occur between EWS-FLI and specific cognate transcription factors to regulate transcription from a subset of these promoters.

Interestingly, EWS/FLI direct “repressed” target genes lack both the GGAA microsatellites and the high-affinity ETS consensus sites. Therefore, EWS/FLI binding to GGAA microsatellites or the high-affinity ETS sites is associated solely with gene activation. Early inspection of the promoter regions of direct-repressed EWS/FLI targets both by ChIP-Chip (Sankar, unpublished observation) and later by ChIP-sequencing (26) revealed an enrichment of ETS/AP1 composite binding sites. EWS/FLI (and other EWS/ETS proteins) has previously been shown to interact with AP1 proteins (29). Additionally, this cooperative interaction was shown to be necessary for transformation by the EWS/ETS fusion proteins. These data suggest that ETS/AP1 motifs may act as functional sites for EWS/FLI binding at promoters of repressed genes. However, additional work is required to identify and characterize EWS/FLI binding motifs at direct repressed target genes. Also, future studies focused on more detailed analysis of the EWS/FLI binding sites at target gene promoters and enhancers, identified by increasing the number of sequencing reads for EWS/FLI in a ChIP-sequencing based approach, may reveal other new binding motifs for the fusion protein in the Ewing sarcoma genome.

Transcriptional activity of EWS/FLI

An important question raised by these studies is: what are the key determinants of transcriptional outcome following EWS/FLI binding? How does EWS/FLI decide between transcriptional activation versus transcriptional repression? Several models can explain this functional dichotomy in the EWS/FLI fusion protein and these models are not mutually exclusive. As alluded to previously, one model involves “the binding motif” and the “local protein concentration”; the presence of GGAA-microsatellite repeats or the consensus high-affinity ETS site at promoters of upregulated genes may allow for EWS/FLI binding and subsequent recruitment of coactivator proteins, to achieve higher local concentration at these sites, leading to gene activation. Conversely, the absence of these motifs may allow for EWS/FLI binding at variant low-affinity sites, subsequent recruitment of corepressor proteins, which may be abundant at these sites, leading to gene repression.

A second model which highlights a key determinant of transcriptional outcome is the “chromatin architecture and the histone modifications” present at target gene promoters. EWS/FLI bound GGAA-microsatellite repeat regions are nucleosome depleted, RNA polymerase II bound and harbor the chromatin signature of enhancer elements characterized by the enrichment of histone H3 lysine 4 mono- and di-methylation (H3K4me1/2) (26). Interestingly, silencing of EWS/FLI in Ewing sarcoma cells leads to increased nucleosome occupancy at the GGAA repeat elements and, conversely, ectopic expression of EWS/FLI in non-Ewing sarcoma cells like primary endothelial cells leads to nucleosome depletion at a subset of GGAA-microsatellite regions that are normally associated with repressive chromatin (26). These data suggest

that the EWS/FLI fusion protein acquires chromatin-altering activity, leading to chromatin disruption and ultimately, transcriptional dysregulation. Furthermore, despite their identical DNA-binding domains, the tumor-specific genomic retargeting of the EWS/FLI fusion protein is achieved in comparison to the parental transcription factor, FLI1 in Ewing sarcoma cells (26). These data suggest that EWS/FLI may function as a "pioneer factor" capable of inducing and maintaining either open or closed chromatin architecture by virtue of its interactions with chromatin remodeling proteins.

In the process of identifying direct-repressed targets of EWS/FLI, and understanding the mechanism of EWS/FLI-mediated transcriptional repression, we identified that EWS/FLI interacts with the NuRD (nucleosome remodeling and histone deacetylase) chromatin remodeling complex to directly repress a subset of critical target genes in Ewing sarcoma (refer to Chapter 7) (12). NuRD is a repressor complex, but interestingly, a recent study demonstrated that some of the NuRD complex members may be recycled into an activating complex called the NuRF complex (nucleosome remodeling factor) (30), thereby allowing for a "switch" between transcriptional activation and repression.

These new findings raise the possibility of a third "dynamic switch" model, whereby, in Ewing sarcoma a switch between the NuRD and NuRF complexes may exist. The NuRD complex may function as the "epigenetic reader" of chromatin modifications at target gene promoters, and through a coordinated effect of the local epigenetic architecture, and the DNA motif EWS/FLI is bound to, the NuRD complex may be stabilized to cause transcriptional repression or may be destabilized, allowing EWS/FLI to then interact with the NuRF complex leading to transcriptional activation. The same

domains within EWS/FLI may be able to interact either with the NuRF (activator) or NuRD (repressor) complexes in Ewing sarcoma cells making these two functions inseparable on the EWS/FLI molecule and, suggesting that competition may occur for cofactor interaction with EWS/FLI. In support of this are data presented in Chapter 7 (12), demonstrating our inability to separate activation and repression functions, using deletion mapping analysis, on the EWS/FLI molecule. These data suggest that the same domains within the protein may interact with coactivators or corepressor proteins, depending on the promoter context. Importantly, both activation and repression by EWS/FLI are necessary for full oncogenic transformation in Ewing sarcoma (12).

Collectively, the work outlined in this dissertation is a step toward understanding the mechanisms of EWS/FLI mediated transcriptional regulation of target genes, and opens up several interesting questions as potential future directions. What is the epigenetic landscape of Ewing sarcoma cells and how does EWS/FLI affect the histone modifications and the chromatin signature at target gene promoters and enhancers? Since EWS/FLI has the ability to modulate nucleosome occupancy, which chromatin remodeler proteins does it interact with in Ewing sarcoma? Is the NuRD complex the predominant remodeler and repressor complex recruited by EWS/FLI? Is there a switch between the NuRD and the NuRF complexes that determine the transcriptional fate of the EWS/FLI bound target genes? LSD1 is part of the NuRD complex, and LSD1 has both transcriptional activation and repressive functions. Since LSD1 activity is important in Ewing sarcoma (Chapter 7), the next important question in this direction is: does LSD1 play a more central role in EWS/FLI-mediated transcription; does it affect EWS/FLI-mediated activation and repression? What fraction of EWS/FLI targets is affected by

modulating LSD1 activity in Ewing sarcoma? Apart from the NuRD complex, identification of a functional interaction between the REST corepressor complex and EWS (refer to Chapter 6), also raises the possibility that EWS/FLI, by virtue of the N-terminal EWS domain, may also recruit the REST complex to repress a subset of its target genes in Ewing sarcoma.

The overarching goal in our lab is to identify new and more effective treatments for Ewing sarcoma patients through detailed molecular studies. Previous efforts in the lab were focused on understanding the function of individual target genes of EWS/FLI as a step towards this goal (9, 10). However, given that EWS/FLI dysregulates thousands of genes in Ewing sarcoma, blockade of EWS/FLI activity itself would be a more ideal approach for the treatment of Ewing sarcoma. In contrast to diseases like Chronic Myelogenous Leukemia (CML), where Gleevac was developed to target a single kinase dependent signaling pathway, the absence of a single aberrant signaling pathway driving Ewing sarcoma pathogenesis precludes the development of a “Gleevac-like” drug for this disease.

Therefore, taking a step back and understanding the mechanisms of gene regulation by EWS/FLI is necessary to develop new and effective targeted therapy for Ewing sarcoma. Based on my dissertation work, we have identified that EWS/FLI utilizes HDAC and LSD1 activities for repression of critical tumor suppressor genes. HDAC inhibitors are FDA approved for several cancers and offer promise for the treatment of Ewing sarcoma. Furthermore, LSD1 is a viable therapeutic target. We have demonstrated that two selective and targeted LSD1 inhibitors, HCI-2509 and HCI-2528, not only reverse the expression of candidate tumor suppressor genes but also lead to cell death of

multiple patient-derived Ewing sarcoma cell lines (12). These studies highlight that LSD1 inhibitors also have the potential to be developed as effective therapeutic agents for patients afflicted with Ewing sarcoma. A clinically effective therapeutic strategy may necessitate administration of the HDAC and/or LSD1 inhibitors as a combination therapy with currently used chemotherapeutic agents for effective treatment of Ewing sarcoma.

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