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Promiscuous Partnerships in Ewing's Sarcoma

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Abstract

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

Introduction

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

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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 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) (Fig. 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.

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*, *TGFBR2*, *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 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 (*NROB1*, *CAVI*, 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) (Fig. 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 is data to suggest that the EWS/FLI type 1 fusion has reduced transactivation potential in Ewing’s sarcoma cell lines when compared 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, a recent study from large cooperative group trials concluded that fusion subtypes no longer have prognostic significance. 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 (72).

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 (73, 74). 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) (75–79) (Fig. 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 (80–83). Indeed, the few studies that have looked specifically at these alternate fusions support this notion.

The most common of the alternate translocations is t(21;22)(q22;q12), found in approximately 10% of Ewing’s sarcoma tumors. This chromosomal translocation encodes the EWS/ERG fusion protein (67, 75). ERG shares 68% overall amino acid identity with FLI and 98% identity within their ETS DNA-binding domains (75, 84). 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 (85). Like the EWS/FLI subtypes in Ewing's sarcoma, different EWS/ERG fusions have been described in the literature, varying in their breakpoints across different introns in the *EWSR1* and *ERG* genes (Fig. 2).

The EWS/ETV1, EWS/ETV4 and EWS/FEV fusions each occur in <1% of Ewing's sarcoma tumors (76–79). 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) (86). 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 (87–89).

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 (83). 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 (90). 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* (Fig. 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 (91, 92). 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” (93). In all cases identified, an in-frame chimeric fusion between exon 8 of *EWSR1* and exon 3 of *NFATc2* gene was identified (Fig. 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 (94) 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 (95) and is normally regulated by calcium signaling (96). 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) (97, 98). Interestingly, cooperative DNA binding with AP1 proteins is a shared feature of NFAT and ETS proteins, including ETS1, ERG, and EWS/FLI (87–89, 97). 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 NFATc2, suggesting that the EWS/ERG fusion either directly or indirectly may be able to regulate NFATc2 expression in Ewing’s sarcoma cells (93). 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 (99). This translocation created an EWS/POU5F1 chimeric protein through fusion of exon 6 of *EWSR1* with part of exon 1 of *POU5F1* (Fig. 3). POU5F1 is a member of the POU homeodomain transcription factor family, and is often referred to by its alternate name OCT4 (100). POU5F1 expression is restricted predominantly to embryonic stem cells and germ cells, and it has clear roles in regulating pluripotency/stemness (101, 102). 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 (99) (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 (103). 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 (Fig. 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 (104–108). 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 (109).

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 (103). 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 (83). 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 (110, 111). 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 (112). In AT/RT, loss of the *SMARCB1* gene causes aberrant cell cycle progression, partly via the downregulation of p16INK4a, a tumor suppressor (113). Furthermore, *SMARCB1* binds the promoter of Cyclin D1 and regulates its overexpression (112).

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 (*i.e.*, a Ewing’s sarcoma) (114). 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 Cys₂-His₂ 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 (115), 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* (Fig. 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 (116). 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.

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* (Fig. 4) (90). 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 (117–121). On the other hand, several observations are consistent with a neural crest cell-of-origin (122–124). 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 (125, 126).

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, 127). 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 (83).

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 (128). 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 (129, 130). 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) (131–133). 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 (134, 135). 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 (136). This model also did not give rise to sarcomas spontaneously and did so only when the p53 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 (120). 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 (137)) 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*, *CAVI*, 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 (138). 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 (139). 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 (140). 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 (141).

A newer hypothesis is that DNA strand breaks occur specifically at sites of active transcription. It has been shown that topoisomerase II β (TOP2B) is recruited to sites of active transcription by nuclear hormone receptors (142, 143). 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 (143). 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 *NROB1*, *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*, *TGFBR2*, *VEGF*, and *CAVI* (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 (*i.e.*, 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 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 (144). 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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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 *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.

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.

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; SNF₂: 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.

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: Cys₂-His₂ 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.

Table 1

Clinicopathologic features of non-TET/ETS “Ewing’s-like tumors”. M: Male; F: Female; +: Positive staining; -: Negative staining; NSE: Neuron specific enolase; SYN: Synaptophysin.

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