In 1920, during a meeting of the New York Pathological Society, James Ewing described an unusual tumor in a 14-year-old girl as a "diffuse endothelioma of bone." The tumor had initially been diagnosed as an osteosarcoma, but its architecture, the morphologic features of its cells, and its marked sensitivity to radiation therapy led Ewing to consider it as a distinct entity, going so far as to hypothesize an endothelial-cell origin. He later reported similar tumors in other adolescents, which pathologists variously referred to as Ewing's sarcoma, Askin's tumor, and peripheral primitive neuroectodermal tumor, on the basis of their shared morphologic and immunohistochemical features. The first landmark discovery toward unequivocally diagnosing Ewing's sarcoma was made more than 70 years later, when the most frequent of the chromosomal translocations that define the tumor was identified. A century after Ewing's seminal observation, the cancer that bears his name has become a paradigm for solid-tumor development after a single genetic rearrangement.

In this review, we discuss the clinical features and pathogenesis of Ewing's sarcoma, along with current and experimental therapeutic approaches. From the mechanistic point of view, we review the way in which a unique chromosomal translocation harnesses the epigenetic machinery of permissive cells to rewire their transcriptome and initiate a heterogeneous cancer that can elude even the most intensive conventional therapy available.

INCIDENCE AND CLINICAL FEATURES

An aggressive bone and soft-tissue cancer, Ewing's sarcoma arises predominantly in children and young adults, with an incidence of 1 case per 1.5 million population, a frequency among persons of European ancestry that is almost 10 times as high as the frequency among those of African ancestry, a slight predilection for males (ratio of cases among males to cases among females, 1.6:1.0), and a peak incidence at 15 years of age. Ewing's sarcoma accounts for about 2% of cancers in children, is the second most common bone cancer in children, and can occur in any part of the body but most commonly involves the pelvis and proximal long bones. In approximately 20% of patients, tumors are extraosseous and can arise in numerous organs (Fig. 1); extraosseous Ewing's sarcoma occurs much more frequently in adults than in children. The discovery of an undifferentiated round-cell tumor in the soft tissues of an adult warrants inclusion of Ewing's sarcoma in the differential diagnosis.

The clinical features of Ewing's sarcoma are largely nonspecific. Patients may report localized pain, which may be accompanied by swelling that can be mistaken for a minor injury. The pain is often mild, sometimes increasing at night...
Ewing’s Sarcoma

or after exercise, although some patients do not have pain at all. In the absence of pain, the only sign may be the fortuitous palpation of a firm mass. Nevertheless, a pathological fracture is reported in 10 to 15% of cases, and in cases of advanced disease, nonspecific constitutive symptoms may appear, including fever, night sweats, fatigue, and weight loss. Blood tests may show elevated levels of nonspecific markers of inflammation and bone remodeling, including alkaline phosphatase, which are generally not informative; however, an elevated serum lactate dehydrogenase level is reported to correlate with the tumor burden and to have diagnostic and prognostic value.

Radiologic analysis is usually more strongly suggestive, with the typical multiple, confluent, lytic bone lesions giving rise to images described as “moth eaten” on standard films. Subperiosteal growth may translate into two other classic images — Codman's triangle and the “onion peel” — which, respectively, represent the displaced periosteum and the resulting proliferative reaction (Fig. 1). Metastases and response to treatment are typically monitored by means of computed tomography or magnetic resonance imaging.
Ewing's sarcoma carries a low mutational burden and, at 0.15 mutations per megabase, has one of the lower mutational rates of all cancers. The defining genetic alteration is one of several possible reciprocal chromosomal translocations that generate the fusion between the gene encoding Ewing's sarcoma breakpoint region 1 (EWSR1) and a gene encoding a member of the E-twenty-six (ETS) family of transcription factors. About 85 to 90% of cases bear the chromosomal translocation t(11;22)(q24;q12), which leads to the fusion of EWSR1 to the gene encoding Friend leukemia virus integration 1 (FLI1). In roughly a quarter of the cases, the only detectable genetic event is the chromosomal translocation, supporting the notion that the resulting fusion protein is predominantly, if not solely, responsible for transformation. Mutations of other genes, notably STAG2 and TP53, occur in a minority of tumors at diagnosis. Although these virtually rules out the diagnosis of Ewing's sarcoma. A definitive diagnosis relies on the identification of signature chromosomal translocations (discussed below) by means of either in situ hybridization or the more rapid quantitative polymerase chain reaction.

By far the most important prognostic factor is the presence of metastasis at the time of diagnosis. Patients with local disease that responds to multimodal therapy currently have a 5-year survival rate of more than 70%. In contrast, less than 30% of patients presenting with metastases survive for 5 years. The most common metastatic sites are the lungs, bone, and bone marrow, although Ewing's sarcoma can metastasize to a broad panel of organs, including lymph nodes, liver, and brain. Patients with metastasis limited to the lung have a better prognosis than those with metastasis to the bone or bone marrow. In the absence of metastasis, the tumor site constitutes the single most important prognostic factor, with a worse outcome for patients with proximal primary tumors (i.e., in the pelvis and sacrum) than for patients with distal tumors. Other clinical indicators of unfavorable evolution include a large primary neoplasm, older age at diagnosis (>18 years), and elevated serum lactate dehydrogenase levels.

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mutations may accelerate disease progression at late stages, they are not required for tumor initiation or maintenance.15,18

Together with FUS/TLS and TAF15, EWSR1 forms the FET (or TET) gene family and encodes the ubiquitously expressed EWS protein. Although the vast majority of Ewing’s sarcomas harbor a fusion protein containing EWS, about 1% of the tumors bear chromosomal translocations that implicate FUS.17,19 Substitution of FUS for EWSR1 does not appear to alter the phenotype or behavior of Ewing’s sarcoma.

EWS, FUS, and TAF15 are RNA-binding proteins that share a structure composed of an intrinsically disordered, low-complexity, prionlike, SYGQ-rich N-terminal transactivation domain, followed by three arginine-and-glycine–rich (RGG) repeats of different lengths. RGG1 and RGG2 are separated by an RNA recognition motif consisting of 87 amino acids, and RGG2 and RGG3 by a zinc-finger domain20 (Fig. 2). The transactivation domain of EWS, encoded by the first seven exons of EWSR1, is largely silent in the wild-type protein but becomes highly active on loss of the C-terminal region after the chromosomal translocation.21 It is inhibited by the RGG repeats, which is consistent with its activation after their substitution by the fusion partner.21,22 Prionlike domains display phase-transition properties, defined as the ability of a biologic system to undergo a change of phase or state, which may include transition from protein solutions to liquidlike, phase-separated compartments that constitute membraneless organelles.20 Why fusion proteins containing FET family members display oncogenic activity requires further explanation, but one possibility is that the phase-transition properties of FET proteins endow the associated transcription factors with tumor-promoting functions.

In addition, and like most RNA-binding proteins, EWS participates in the regulation of diverse aspects of RNA metabolism,23 and in vivo EWSR1 knockout studies have shown that EWS is implicated in meiosis, B-lymphocyte maturation, hematopoietic stem-cell self-renewal, DNA repair, and cell senescence.24 EWS also plays an important role in neuronal structure, dopaminergic signaling pathways, and motor function in the central nervous system25 and regulates transcription by binding to various transcriptional activators and repressors, as well as RNA polymerase II through its prionlike domain.26 EWSR1 can partner not only with genes encoding ETS family members but also with a broad range of non-ETS genes to generate fusion proteins implicated in the pathogenesis of diverse soft-tissue tumors.27,28 EWSR1-NEATC2, EWSR1-POUF1, EWSR1-PATZ1, EWSR1-SMARCA, and EWSR1-S3 give rise to rare, undifferentiated round-cell tumors resembling Ewing’s sarcoma. Their rarity has hampered detailed characterization, leading to an unresolved debate over whether they should be considered Ewing’s sarcomas. Other EWSR1–non-ETS fusions give rise to well-defined entities, including DSRCT (EWSR1-WT1), myxoid liposarcoma (EWSR1-DDIT3), clear-cell sarcoma (EWSR1-ATF1), and extraskeletal myxoid chondrosarcoma (EWSR1-NR4A3).28 Unrelated chromosomal translocations, which generate the non-FET–non-ETS gene fusions BCOR-CCNB329 and CIC-DUX4,30 give rise to tumors with morphologic features resembling those of Ewing’s sarcoma. These tumors were initially classified as Ewing’s sarcoma, but their pathogenesis and biologic properties are now known to be clearly distinct from those of Ewing’s sarcoma.31

At least 5 of the 27 members of the ETS family can fuse to EWS to generate Ewing’s sarcoma: FLI1,2 ERG,32 FEV,33 ETV1,34 and E1AF35; FLI1 is found in 85 to 90% of cases. ETS factors are implicated in differentiation and cell-cycle control,36 and their activity is associated with the development of diverse cancers, including pre-B-cell acute lymphoblastic leukemia and prostate cancer.37 All members of the family share a DNA-binding domain that recognizes the consensual core 5′-GGAA/T-3′ DNA motif, often referred to as the ETS binding motif. FLI1 has two ETS-binding domains separated by an FLI1-specific (FLS) sequence.38,39 The 5′ ETS domain and the FLS sequence form the N-terminal transactivating domain, which is substantially less potent than the EWS N-terminal transactivating domain by which it is replaced in the fusion protein. After chromosomal translocation, the portion of FLI1 containing the 3′ ETS-binding domain that becomes fused to EWS undergoes a conformational change, which allows it to activate a broader repertoire of genes than wild-type FLI1 does.38,39

The range and diversity of tumors arising in response to EWS-bearing fusion protein expres-
sion suggest that EWS plays a major role in cell transformation, whereas its fusion partner bears responsibility for cell-type specificity and tumor phenotype. The same FET-ETS\textsuperscript{40} and FET–non-ETS\textsuperscript{41} fusion proteins can give rise to divergent tumors, supporting the notion that the properties of the cell undergoing transformation modulate fusion protein activity and its consequences.

**Figure 3 (facing page). Role of EWS-FLI1 in Epigenetic Regulation of Gene Expression.**

EWS-FLI1 affects epigenetic control of gene expression by altering histone modifications, DNA methylation, and noncoding RNA expression. Nucleosomal histones, around which DNA is wound, can undergo a variety of posttranslational modifications that contribute to structural chromatin changes associated with gene silencing (e.g., heterochromatin or chromatin compaction) or activation (e.g., euchromatin or chromatin relaxation). The two best-characterized modifications that affect gene activity are acetylation and methylation of lysine residues on histone H3. Histone H3 acetylation of lysine 27 (H3K27ac) is associated with gene activation, whereas H3 methylation can be associated with both activation and silencing. Thus, trimethylation of lysine 9 and lysine 27 (H3K9me3 and H3K27me3, respectively) are repressive marks, whereas monomethylation, bimethylation, and trimethylation of lysine 4 (H3K4me1, H3K4me2, and H3K4me3, respectively) are activation marks.\textsuperscript{46} Chromatin regulators (CR) include histone acetyltransferases (e.g., CBP/p300), histone deacetylases, methyltransferases (e.g., mixed-lineage leukemia [MLL] methyltransferases), and demethylases (e.g., lysine-specific histone demethylase 1 [LSD1]), as well as large complexes such as BAF, which relaxes chromatin by antagonizing the deposition of H3K27me3 by polycomb group proteins.\textsuperscript{46} On binding to GGAA microsatellites, EWS-FLI1 multimers recruit the BAF complex; CBP/p300, which generates H3K27ac; and MLL methyltransferase, which forms H3K4me1, primarily at distal (enhancer) regulatory elements that synergize with promoters (shown here bearing the H3K4me3 and H3K27ac marks) to drive gene expression.\textsuperscript{46} The combined effect of these CR complexes is to relax the compacted heterochromatin (bottom of figure), resulting in euchromatin, in which the nucleosomes appear as beads on a string and where the DNA is accessible to transcription factors. The binding of EWS-FLI1 monomers to single GGAA elements results in the removal of wild-type (WT) ETS factors, along with their associated activating CR complexes, leading to chromatin compaction and silencing of associated genes. EWS-FLI1 can reduce DNA methylation, which acts as a barrier to transcription factor binding. DNA methylation occurs on cytosines residues within CpG islands (characterized by a cytosine preceding a guanine in the 5′ to 3′ strand). In Ewing’s sarcoma, reduction in DNA methylation occurs predominantly within sequences that correspond to enhancers. EWS-FLI1 represses numerous microRNAs (miRNAs) that drive cell differentiation. MicroRNAs guide RNA-induced silencing complexes (RISCs) to target messenger RNAs (mRNAs), resulting in their degradation or in the inhibition of their translation. By repressing miRNA-145, which targets numerous mRNAs encoding proteins that maintain cell plasticity, such as SOX2, EWS-FLI1 impairs differentiation and promotes pluripotency.

**EWS-FLI1 Fusion Protein and Regulation of the Epigenome**

Shortly after its discovery, EWS-FLI1 was observed to act as an aberrant transcription factor, inducing the expression of genes with oncogenic properties but also repressing numerous genes through mechanisms that remained obscure.\textsuperscript{9} Regardless of its mechanism of action, it became clear that EWS-FLI1 plays a central role in the pathogenesis of Ewing’s sarcoma. Its depletion from cell lines resulted in their inability to grow\textsuperscript{42} and to form tumors in mice,\textsuperscript{43} whereas immortalized fibroblasts engineered to express the fusion protein formed colonies in soft agar and tumors resembling Ewing’s sarcoma in vivo.\textsuperscript{44}

The observations that EWS-FLI1 causes both induction and repression of selected gene repertoires suggested a mode of action that may depend on epigenetic mechanisms. Epigenetic control of gene expression does not implicate nucleotide sequence changes but instead relies on modification of chromatin structure and DNA accessibility to transcription factors and transcriptional regulators.\textsuperscript{45-47} Such control is orchestrated by chromatin remodeling (resulting primarily from histone modification), DNA methylation, and noncoding RNA expression.\textsuperscript{45} Although EWS-FLI1 affects all these events, the manner in which it harnesses chromatin-remodeling mechanisms has set a new paradigm for oncogene-mediated epigenetic instruction of cell transformation (Fig. 3).

EWS-FLI1 binds consensus core GGAA ETS-binding sites\textsuperscript{49,50}, which are found either in isolation or as microsatellites (multiple repeats) throughout the genome. However, the effects of the binding of EWS-FLI1 to single or repeat GGAA elements are in stark opposition. On recognizing GGAA microsatellites, the phase-transition properties of the EWS prionlike domain allow EWS-
FLI1 multimers to bind and open these otherwise inaccessible genomic regions by recruiting the major ATP-dependent chromatin-remodeling complex SWI/SNF (switch/sucrose nonfermentable), also known as BAF (BRG1/BRM–associated factor), which regulates genomic architecture and DNA accessibility. In addition, EWS-FLI1 multimers recruit enzymes that catalyze methylation or acetylation of specific lysine residues on histone 3, inducing chromatin relaxation, including the mixed-lineage leukemia (MLL) methyltransferase complex and the histone acetyltransferase p300. By orchestrating the activities of BAF, MLL, and p300, EWS-FLI1 behaves like a pioneer factor, converting silent chromatin regions into fully active enhancers (Fig. 3). These normally quiescent GGAA microsatellite–containing regions, which show no baseline activity in any physiological setting, now become the major drivers of the fusion protein's target gene repertoire. GGAA-repeat-length polymorphisms appear to affect EWS-FLI1 function. Long repeats, predominantly found in populations of African ancestry, are associated with decreased transcriptional activation properties of EWS-FLI1, possibly explaining the lower frequency of Ewing's sarcoma in populations of African ancestry than in populations of European ancestry.
Conversely, on reaching single GGAA elements, EWS-FLI1 monomers can displace physiologically bound ETS factors along with their associated chromatin regulators, which establish active chromatin marks, resulting in the silencing of genes driven by wild-type ETS factors. The precise mechanism of gene silencing by EWS-FLI1 remains to be elucidated, but a possible explanation lies in the ability of EWS-FLI1 to interact with the corepressor complex NuRD (nucleosome remodeling and deacetylase); the histone deacetylase and lysine-specific histone demethylase 1 (LSD1) activities associated with the NuRD complex remove acetyl and methyl groups, respectively, from histones, reducing DNA accessibility. The overall effect of EWS-FLI1–mediated establishment of new enhancers in parallel with the silencing of a repertoire of ETS-driven genes is to rewire a portion of the genome, leading to the expression of a gene repertoire that causes transformation of permissive cells.

DNA methylation undergoes a marked decrease in regions of the genome that correspond to putative enhancer elements, which is consistent with the generation of active enhancers through chromatin relaxation and suggests synchrony between DNA methylation and chromatin remodeling. The DNA methylation patterns associated with EWS-FLI1 expression are highly specific to Ewing's sarcoma. EWS-FLI1 also affects noncoding RNA expression in diverse ways. One mechanism entails partial inhibition of TAR RNA-binding protein 2 (TARBP2) activity, implicated in microRNA (miRNA) processing, which leads to decreased maturation of a broad panel of miRNAs. Another, more direct effect appears to target the expression or maturation of selected miRNAs, including let-7a, miR-30, and miR-145. The net result of TARBP2 and miR-145 inhibition is to impair cell differentiation and contribute to the establishment and maintenance of tumor-cell subpopulations that display a high degree of plasticity, driving both tumor initiation and heterogeneity in Ewing's sarcoma. EWS-FLI1 can also induce the expression of long, noncoding RNAs (lncRNAs), one of which, EWSAT1, facilitates the development of Ewing's sarcoma by repressing selected target genes. Although epigenetic rewiring is arguably the most prominent mechanism underlying the development of Ewing's sarcoma, its extent depends, at least in part, on the cellular context in which the translocation arises.

Expression of EWS-FLI1 induces senescence or apoptosis in most nontransformed primary cells, suggesting that a specific, permissive environment is required for the maintenance of EWS-FLI1 expression and function. Divergent approaches have shown that mesenchymal stem or stromal cells, which provide the permissive environment required for EWS-FLI1 to induce transformation, constitute a candidate cell of origin in Ewing's sarcoma (Fig. 4). These cells originate in the mesenchyme, the mesodermal part of the embryo that evolves into connective and skeletal tissues, and display a high degree of heterogeneity, as well as the plasticity required to differentiate into a broad range of lineages in vitro. However, Ewing's sarcoma may also arise from other primary cells characterized by pluripotency, with neural crest cells as an additional candidate. Ewing's sarcoma displays marked intratumor heterogeneity generated by a subpopulation of poorly differentiated cells, which can initiate tumor growth and give rise to nontumorigenic progeny. These cells, which depend at least in part on noncoding RNA regulation by EWS-FLI1, have the functional properties of cancer stem cells and express the neural stem-cell markers CD133 and SOX2. Single-cell expression profiling, used to assess the intratumor heterogeneity in Ewing's sarcoma models, has highlighted cell-to-cell fluctuation in EWS-FLI1 expression. EWS-FLI1hi cells are associated with high proliferation but low migration, whereas EWS-FLI1low cells migrate but have a low proliferation rate. Transcriptional assessment of Ewing's sarcoma models at the single-cell level revealed that EWS-FLI1hi cells display proliferation and strong oxidative phosphorylation, whereas EWS-FLI1low subpopulations display a signature associated with hypoxia. Thus, both developmental trajectories and variation in expression levels of the genetic driver may contribute to the phenotypic heterogeneity of Ewing's sarcoma cells, possibly posing an additional challenge for clinical management.
Current management of primary Ewing’s sarcoma, which relies on a combination of cytotoxic drugs and local reduction through surgery, radiotherapy, or both, according to feasibility, has improved the 5-year survival rate among patients with localized disease from 10% in the era before chemotherapy to about 70% currently. Current regimens include intensive induction chemotherapy, comprising doxorubicin, etoposide, cyclophosphamide, vincristine, and ifosfamide, to reduce the size of the primary tumor and target micrometastatic disease, followed by consolidation chemotherapy to eliminate residual cells.69-71 European centers have designed trials of dose intensification through high-dose therapy, with autologous stem-cell rescue,69,71 whereas the Children’s Oncology Group has tested dose intensification through shortened intervals between doses (interval compression).70 Comparison of the two strategies suggests that the approach based on interval compression may be more effective and associated with fewer toxic effects.72 However, successfully treated patients are at risk for the development of long-term disabilities73 and other cancers, particularly chemotherapy-associated myeloid dysplastic syndrome or leukemia74 and radiation-associated sarcoma.75 Moreover, recurrent disease is currently incurable.

Although the obvious treatment strategy for Ewing’s sarcoma would be direct inhibition of the FET-ETS fusion protein, its lack of enzymatic activity and disordered structure make it difficult to target with currently available technology. Effective therapy will therefore have to rely on alternative mechanism-based approaches such as inhibition of effector molecules of the FET-ETS fusion protein, reversion of the FET-ETS–induced epigenetic modifications, targeting of molecules and signaling pathways that support and cooperate with fusion protein function, or a combination of these approaches. Several candidate effector molecules have been targeted, including the receptor tyrosine kinase insulin-like growth factor I receptor (IGF-IR), which is induced by EWS-FLI176 and is required for the transformation of fibroblasts.77 Despite the sensitivity of Ewing’s sarcoma cells to IGF-IR inhibition,78,79 in vivo studies using anti–IGF-IR antibodies have shown limited effectiveness.80,81 Poly(adenosine diphosphate–ribose) polymerase (PARP), which is implicated in DNA single-strand break base excision repair, is highly expressed in Ewing’s sarcoma, and in preclinical models, the response to PARP inhibitors was promising.82,83 However, the results of a clinical trial were disappointing.84

The small molecule YK-4-279 has shown promising results in Ewing’s sarcoma cell lines in vitro, as well as in xenografts.85 YK-4-279 inhibits the direct interaction between RNA helicase A and EWS-FLI1, disrupting EWS-FLI1 interactions within the spliceosomes and leading
to an alternative splicing pattern that mimics EWS-FLI1 reduction. However, limited bioavailability and acquired resistance to the drug hampered its usefulness. The antibiotic enoxacin, which enhances TARBP2 activity and restores miRNA maturation, leads to the elimination of Ewing's sarcoma–initiating cells in preclinical models, with a preeminent synergistic activity in combination with chemotherapy. Finally, inhibitors of the histone demethylase LSD1, implicated in transcriptional repression by EWS-FLI1, induce apoptosis selectively in Ewing's sarcoma cell lines and are currently being tested in a clinical study.

### CONCLUSIONS

A century after the seminal discovery of Ewing's sarcoma, the prognosis for patients with localized forms of the tumor has improved dramatically, thanks to aggressive multimodal therapy. However, recurrent and metastatic disease remains a major challenge, and the inability to effectively target the fusion protein that drives the malignant process has led to continued exploration of alternative mechanism-based approaches. Although success has been limited thus far, investigation at the single-cell level is holding promise for the definition of subpopulations of cells that are responsible for driving the tumor and identification of their potential vulnerabilities. The lessons learned from Ewing's sarcoma are not only forging new lines of thought in terms of therapeutic approaches but also providing a road map for addressing the pathogenesis of additional solid cancers in children driven by unique chromosomal translocations and aberrant fusion proteins.

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Ewing’s Sarcoma


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