Chapter 15
Translocations in Ewing Sarcoma

Jason M. Tanner and Stephen L. Lessnick

Contents

Abstract Ewing sarcoma is a bone-associated malignancy arising primarily in childhood and adolescence. It is an aggressive cancer harbouring a characteristic translocation, t(11;22)(q24.3;q12.2). This rearrangement fuses the genes *EWSR1* and *FLI1*, producing a fusion protein (EWS/FLI) that initiates an oncogenic transcription programme. Other rearrangements between similar genes have also been found to be drivers of Ewing sarcoma in a minority of cases. Understanding the

J.M. Tanner
Department of Oncological Sciences, Huntsman Cancer Institute, School of Medicine, University of Utah, Salt Lake City, UT 84112, USA

S.L. Lessnick, M.D., Ph.D. (✉)
Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH 43205, USA

Center for Childhood Cancer and Blood Disorders, The Research Institute at Nationwide Children's Hospital, 700 Children's Drive WA5011, Columbus, OH 43205, USA
e-mail: Stephen.Lessnick@nationwidechildrens.org

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molecular processes governed by these rearrangements promises to generate immediately actionable therapeutic strategies. This chapter discusses the defining role that translocations and their after-effects play in Ewing sarcoma.

Keywords Translocation • EWS/FLI • Ewing sarcoma • Transcription factor • Oncogenesis

15.1 Introduction

Ewing sarcoma was first described by James Ewing (Fig. 15.1) in 1921 as a tumour composed of distinctive sheets of cells with "small hyperchromatic nuclei" [1]. He noted that these tumours were distinguishable from osteogenic sarcoma by their histopathological morphology. Indeed, Ewing sarcoma continues to be characterized by its appearance as a small, round blue cell tumour, and modern molecular biology techniques have enabled scientists to elucidate many mechanistic details important for development of this tumour [2]. One particularly important discovery – made roughly 60 years after Ewing's first description of the disease – was that Ewing sarcoma harbours a recurrent set of chromosomal translocations that drive oncogenesis [3–5]. Further study of this key translocation event and its consequences have led to greater understanding of the disease and promises to provide improved therapies for those who fall victim to this malignancy. In this chapter, we discuss the biology of Ewing sarcoma with a focus on its associated translocations, including the two most common rearrangements t(11;22)(q24.3;q12.2) and t(21;22)(q22.2;q12.2) (which generate the fusion proteins EWS/FLI and EWS/ERG, respectively) as well as other, less common translocations.

Fig. 15.1 James Ewing (ca. 1890; Source: Images from the History of Medicine, National Library of Medicine; record UI: 101414702)

15.2 Clinical Overview

Ewing sarcoma is a relatively broad term for a group of tumours collectively known as the Ewing sarcoma family of tumours. (Previously referred to as "Ewing's" sarcoma, the WHO has opted to avoid possessive nomenclature; hence, "Ewing" sarcoma is the current WHO-accepted term that will be utilized in this chapter.) This family is predominantly composed of classic Ewing sarcoma, which is a bone-associated tumour that harbours one of a set of oncogenic translocations (discussed hereafter), but also includes tumours such as Askin's tumour, primitive neuroectodermal tumours (PNETs), and Ewing tumours arising in soft-tissue, known as extraosseous Ewing sarcoma [6–9]. Despite the nuances distinguishing these different members of the Ewing sarcoma family of tumours, chromosomal rearrangements are a common feature of Ewing family tumours, and are the focus of this chapter [8–11].

Ewing sarcoma is a disease of young people, occurring most commonly in children and adolescents. The mean age at diagnosis is 15 years, and ~80 % of all cases occur in patients under the age of 25 [12, 13]. For reasons that are not understood, the disease occurs at a modestly higher rate in males than females (male-to-female ratio of 1.2) (Fig. 15.2) [13, 14]. Although the disease is relatively rare, with an incidence of ~3 per million per year in the United States, Ewing sarcoma is the second most common childhood bone tumour, after osteosarcoma [15, 16]. It is most commonly encountered in patients of European ancestry, and is exceedingly uncommon in populations of African or East Asian ancestry [17–21].

Ewing sarcoma is an aggressive cancer with a high propensity for metastasis. In fact, up to 25 % of patients already have metastatic disease at the time of diagnosis [22]. This may well be an underestimation, as it is thought that many patients have undetectable micrometastatic disease at diagnosis as well. Indeed, the relapse rate is

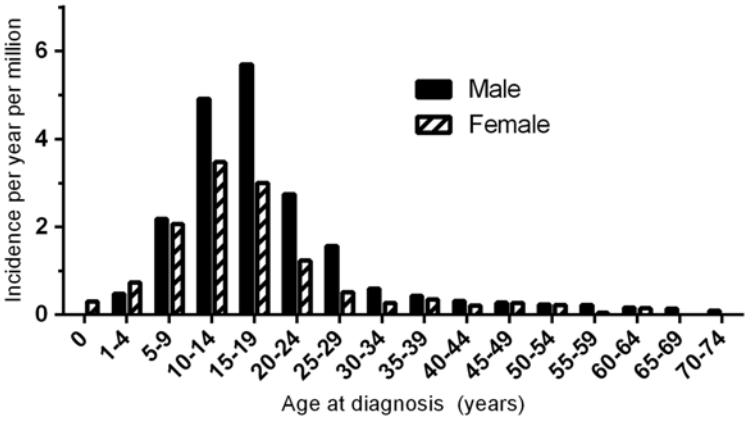

Fig. 15.2 Incidence of Ewing Sarcoma per year per million grouped by age at diagnosis (SEER data, 1973–2010) [13]

~90 % for patients who undergo surgical resection of their primary tumours without adjuvant chemotherapy [23–25]. As Ewing first observed, these tumours are often highly sensitive to radiation therapy, which was thus was a mainstay of treatment for much of the twentieth century [26, 27]. The refinement of chemotherapeutic strategies and improved surgical techniques have led to great improvements in patient survival, and current conventional treatment modalities have achieved 5-year disease-free survival rates of 60–70 % for non-metastatic disease. However, prognosis for metastatic disease remains dismal with a 5-year disease-free survival of only 10–30 % [28–30]. Moreover, survivors frequently must endure morbidities resulting from conventional anti-cancer therapy, such as severe deformities and amputations due to radical surgical resections of their tumour, and increased risk of future malignancy resulting from radiation and chemotherapy [31, 32]. Better treatments are clearly needed to provide greater survival and higher quality of life. To this end, studies continue to seek a better understanding of the molecular processes underlying Ewing sarcoma oncogenesis, including the molecular consequences of its associated chromosomal translocations.

15.3 Translocations in Ewing Sarcoma

In 1983, scientists at the Curie Institute in France identified a balanced reciprocal translocation between chromosomes 11 and 22 in patient samples and cell lines of Ewing sarcoma [3, 4, 33]. This rearrangement, t(11;22)(q24.3;q12.2), was successfully cloned several years later, and the translocation breakpoint was characterized [34]. It was revealed that this translocation resulted in an in-frame fusion of two genes: Ewing Sarcoma Breakpoint Region 1 (*EWSR1*) on chromosome 22 and Friend Leukaemia Virus Integration Site 1 (*FLI1*) on chromosome 11 [34]. The fusion protein encoded by the joining of these two genes is known as EWS/FLI. Approximately 85 % of Ewing sarcoma tumours carry this hallmark cytogenetic abnormality [9, 11, 28, 33]. The remaining 15 % of tumours carry other chromosomal rearrangements resulting in similar fusions of other genes in the same families as *EWSR1* and *FLI1* [35–39]. A list of these chromosomal rearrangements found in Ewing sarcoma is provided in Table 15.1. Details regarding each of these translocations will be discussed in the following sections.

Table 15.1 Chromosomal rearrangements found in Ewing sarcoma

Fusion	Translocation	References
EWSR1/FLI1	t (11;22)(q24;q12)	[3, 4, 33]
EWSR1/ERG	t(21;22)(q22;q12)	[38, 72]
EWSR1/ETV1	t(7;22)(p22;q12)	[35]
EWSR1/ETV4	t(7;22)(q21;q12)	[36]
EWSR1/FEV	t(2;22)(q35;q12)	[37]
FUS/ERG	t(16;21)(p11;q22)	[111]
FUS/FEV	t(2;16)(q35;p11)	[110]

15.4 EWS/FLI

15.4.1 Wild-Type EWS and the FET Family of Proteins

Prior to its cloning as part of *EWSR1/FLI1* in Ewing sarcoma, the *EWSR1* gene had not been identified and hence bears the name of the disease. *EWSR1* encodes a 656-amino acid protein called EWS. EWS is part of the FET (FUS, EWS, TAF15) family of proteins, which are involved in diverse cellular functions including gene expression and RNA processing (Fig. 15.3) [34, 40, 41].

It is ubiquitously expressed and is principally found in the nucleus, although it can be cytoplasmic or localized to the cell membrane [42–44]. The amino terminus of EWS contains a transcriptional activation region comprised of multiple pseudo-repeats rich in serine, tyrosine, glycine, and glutamine (SYGQ) (Fig. 15.3) [45–48]. This SYGQ transactivation domain is critical for interaction between EWS and RNA polymerase II; indeed, wild-type EWS has also been shown to interact with other members of the transcriptional machinery including TFIID and CREBBP/CBP/p300 [45, 46, 49]. The C-terminus of EWS contains arginine-glycine-glycine (RGG) motifs and an RNA recognition motif (RRM), possibly implicating full-length EWS in RNA binding, processing and transcription [41, 50]. The two other members of the FET family of proteins, FUS (also known as TLS) and TAF15, can also be involved in the development of other non-Ewing sarcoma cancers (Table 15.2) [51–58]. These proteins bear striking similarities to EWS, particularly with respect to the domain organization found in the N-termini of EWS and FUS [59–61].

As will be discussed in the following sections, translocations between FET genes and various partners can result in fusion proteins that alter transcriptional programmes and drive oncogenic transformation. Thus, the aforementioned interactions between FET proteins and members of the transcriptional machinery have important implications for molecular mechanisms underlying Ewing sarcoma tumourigenesis,

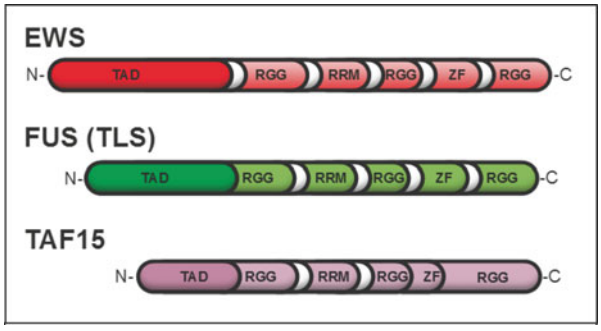

Fig. 15.3 Diagrammatic representation of FET-family proteins and their functional domains. *TAD* Transcriptional activation domain, *RGG* arginine-glycine-glycine motif, *RRM* RNA recognition motif, *ZF* zinc finger

Table 15.2 Representative non-Ewing sarcoma cancers involving translocations of FET-family proteins

	Fusion	Translocation	References
Clear cell sarcoma	EWSR1/ATF1	t(12;22)(q13;q12)	[53]
Desmoplastic small round cell tumour	EWSR1/WT1	t(11;22)(p13;q12)	[54]
Extraskeletal myxoid chondrosarcoma	EWSR1/NR4A3	t(9;22)(q22;q12)	[56]
Extraskeletal myxoid chondrosarcoma	TRF15/NR4A3	t(9;17)(q22;q11)	[52]
Myxoid liposarcoma	FUS/DDIT3	t(12;16)(q13;p11)	[51]
Myxoid liposarcoma	EWSR1/DDIT3	t(12;22)(q13;q12)	[58]
Small round cell sarcoma	EWSR1/ZNF278	t(1;22)(p36.1;q12)	[134]
Acute myelogenous leukaemia	FUS/ERG	t(16;21)(p11;q22)	[55]

as well as other cancers driven by translocations of FET genes. Recent experiments have elegantly demonstrated that FUS and EWS are able to form both homotypic and heterotypic "amyloid-like" polymers via interactions between disordered regions of polypeptides with little diversity in amino acid sequence, termed low complexity domains [49, 62]. Such aggregates could form a platform for intermolecular binding similar to molecular "velcro", leading to alteration of various cellular processes. Indeed, these polymers have been shown to bind to the C-terminal domain (CTD) of RNA polymerase II and induce transcription [49]. Accordingly, improper localization of FET proteins and their corresponding low complexity domains could disrupt gene expression at multiple loci, potentially contributing to an oncogenic phenotype. Such a model remains unproven, but is currently being actively tested.

15.4.2 Wild-Type FLI and the ETS Family of Transcription Factors

The *FLI1* gene encodes the 452 amino acid FLI protein, which is a member of the ETS (E26 transformation-specific) family of transcription factors. ETS transcription factors share a highly conserved DNA binding domain. This binding domain is known as the ETS domain, and is a winged helix-turn-helix that binds to DNA, most avidly at DNA motifs containing a core sequence of GGAA or GGAT [63, 64]. Full-length murine *Fli1* is capable of oncogenic function; indeed, the *Fli1* gene was first characterized as an integration site for the Friend murine leukaemia virus, a function from which the gene derives its name (Friend Leukaemia Virus Integration Site 1) [65]. Integration of the virus at the murine *Fli1* locus results in overexpression of *Fli1* and produces erythroleukaemia in mice [66]. Wild-type FLI appears to play important roles in haematopoiesis, particularly in megakaryocyte development [67]. Deletion of *Fli1* in mice results in dysfunctional megakaryocyte differentiation, and overexpression of *Fli1* in erythroleukaemia cells pushes them toward a megakaryocytic programme of differentiation [68, 69].

15.4.3 The EWS/FLI Fusion

To form EWS/FLI, the 5′ portion of the *EWSR1* gene and the 3′ region of the *FLI1* gene are joined together, allowing transcription of in-frame fusion transcripts and ultimately synthesis of the EWS/FLI fusion protein. The reciprocal fusion of the 5′ end of *FLI1* and the 3′ end of *EWSR1* is not expressed, and the reciprocal derivative chromosome is sometimes lost [9, 70]. Interestingly, the oncogenic *EWSR1*/*FLI1* fusion can result from several distinct translocation breakpoints occuring within introns of *EWSR1* and *FLI1* [71–73]. Classic splicing processes then generate fusion transcripts joining 5′ exons of *EWSR1* with 3′ exons of *FLI1*. EWS/FLI can thus be categorized into subtypes based upon the location of the translocation breakpoint and which exons are fused together [34]. For instance, the most commonly observed translocation in Ewing sarcoma joins exons 1–7 of *EWSR1* to exons 6–10 of *FLI1*. This rearrangement is sometimes termed a "Type I" fusion, but it is more commonly referred to simply as a "7/6" EWS/FLI fusion. Likewise, other fusions of EWS/FLI can be referred to by the exons that are fused, and a partial list of observed EWS/FLI fusions is illustrated in Fig. 15.4.

The functional significance of these subtly different EWS/FLI fusion products remains largely unknown. However, some data exist that suggest that the "7/6" EWS/FLI fusion ("Type I") is more weakly transactivating compared to other EWS/FLI fusion subtypes [74]. This distinction was thought to be potentially useful as a prognostic variable, and retrospective analyses of patient cohorts suggested that patients with "7/6" EWS/FLI fusions had better survival rates compared to patients whose tumours harboured EWS/FLI from other translocation breakpoints [75, 76]. However, recent studies have revealed that prognostic differences no longer exist within current treatment protocols [77, 78]. Hence, the functional significance of different breakpoints, if any exists at all, remains unknown.

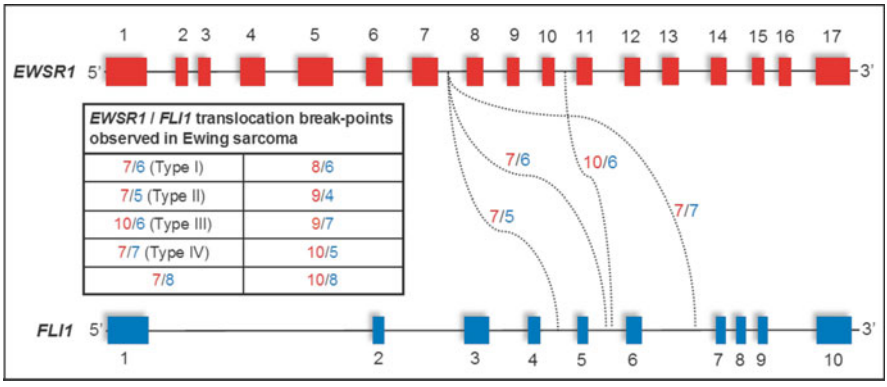

Fig. 15.4 Diagrammatic representation of *EWSR1* and *FLI1* exons. Known translocation breakpoints are indicated

15.4.4 Oncogenic Function of EWS/FLI

EWSR1 and *FLI1* genes are fused in-frame, encoding the EWS/FLI oncoprotein (Fig. 15.5). This translocation-derived oncoprotein contains the N-terminal transactivation domain of EWS fused with the DNA-binding domain of FLI, forming an oncogenic transcription factor that is indispensible for tumourigenesis [34, 42, 79–81]. The first studies implicating EWS/FLI as a driver in Ewing sarcoma observed that overexpression of EWS/FLI in NIH3T3 murine fibroblasts induced oncogenic transformation, measured by anchorage-independent growth in soft agar. This was later confirmed by experiments demonstrating the ability of EWS/FLI-expressing NIH3T3 cells to form tumours in mouse xenografts [80, 82, 83]. Furthermore, studies utilizing patient-derived Ewing sarcoma cell lines have shown that disruption of EWS/FLI expression by RNA interference (RNAi) and other means results in loss of transformation [70, 84–92]. Together, these findings clearly indicate that EWS/FLI is the driver mutation underlying Ewing sarcoma oncogenesis.

This loss of transformation is accompanied by changes in gene expression, including activation and repression of numerous EWS/FLI target genes [70, 89, 92–94]. Importantly, when EWS/FLI is reintroduced after being silenced by RNAi, the oncogenic expression profile and transformed phenotype of Ewing sarcoma are restored, indicating that EWS/FLI is at the head of an oncogenic programme of gene expression [70, 92, 94]. Studies show that thousands of genes are either

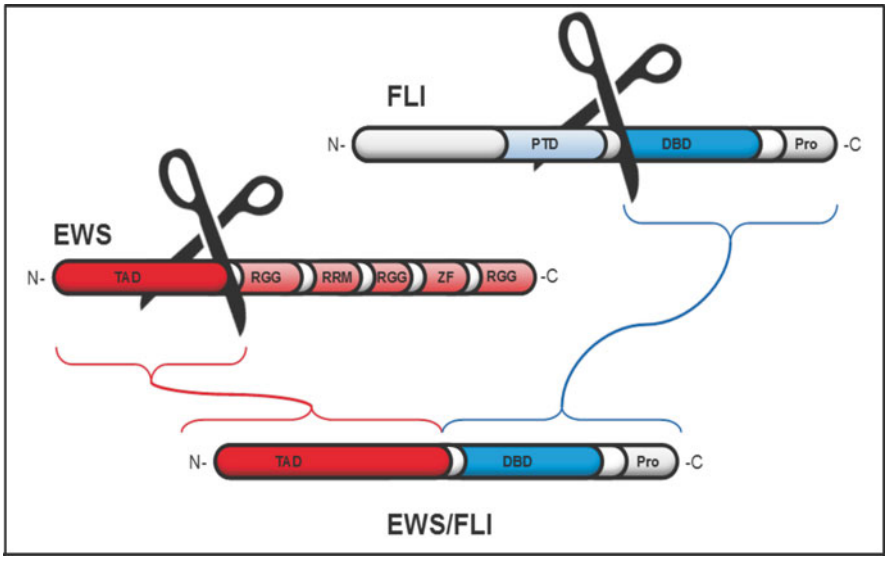

Fig. 15.5 Illustration of the EWS/FLI fusion protein, joining the N-terminal portion of EWS with the C-terminal portion of FLI. *PTD* pointed domain, *DBD* DNA binding domain, *Pro* proline-rich activation domain, *TAD* Transcriptional activation domain, *RGG* arginine-glycine-glycine motif, *RRM* RNA recognition motif, *ZF* zinc finger

upregulated or downregulated by EWS/FLI, leading to "transcriptional mayhem" [70, 81, 95]. This dysregulation of EWS/FLI target gene expression has been the focus of investigations into the mechanisms by which EWS/FLI drives tumourigenesis, and studies have revealed several EWS/FLI-regulated genes that are also required for tumourigenesis, including NR0B1, NKX2.2 and GLI1 [70, 92, 94, 96, 97].

The exact mechanisms by which EWS/FLI causes up-regulation of target genes is an area of active study. It is known that EWS/FLI alters expression of some genes in a direct manner, while it dysregulates other genes indirectly [98, 99]. Nevertheless, it has been definitively shown that the ability of EWS/FLI to bind DNA is essential for Ewing sarcoma oncogenesis [79]. Chromatin immunoprecipitation experiments followed by microarray analysis (ChIP-chip) and deep sequencing (ChIP-seq) have clearly demonstrated that EWS/FLI binds to high-affinity ETS sequences (ACCGGAAGTG) [63, 64, 100, 101]. Interestingly, it was also revealed that EWS/FLI binds to microsatellite repeats of the sequence GGAA [102, 103]. In fact, binding of EWS/FLI to microsatellites is required for upregulation of *NR0B1*, *CAV1*, and *GSTM4*; genes that are critical downstream effectors of EWS/FLI-driven tumourigenesis [102, 103].

Furthermore, as previously mentioned, it has been shown that wild-type EWS is capable of forming a molecular "velcro"-like polymer that facilitates protein-protein interactions between EWS and other proteins, including RNA polymerase II [49, 62]. The low complexity domain in the N-terminal region of wild-type EWS is retained in the EWS/FLI fusion protein, fused to the DNA-binding ETS domain of FLI. It is tempting to speculate, therefore, that the DNA-binding domain of FLI acts to re-direct the molecular "velcro" of EWS to different loci throughout the genome, leading to disruption of regulatory protein complexes and transcriptional activation of EWS/FLI target genes. For instance, GGAA microsatellite repeats could facilitate EWS/FLI polymerization as multiple DNA sequence repeats could permit EWS/FLI to bind in series, forming a scaffold of EWS low complexity domains to which coactivator complexes and transcriptional machinery (e.g., RNA polymerase II) could bind, thus upregulating that locus. Similarly, such a phenomenon could allow EWS/FLI to recruit repressive regulatory complexes to various loci, resulting in down-regulation of target genes. This model, while intriguing, remains unproven, and further testing will shed light on the true mechanisms underlying EWS/FLI-mediated transcriptional dysregulation.

EWS/FLI also down-regulates thousands of genes in Ewing sarcoma. This is particularly interesting considering the presence of the N-terminal transactivation domain of EWS in the EWS/FLI oncoprotein. The mechanisms by which such a transactivator-containing transcription factor causes direct repression of genes remains another active area of study, and several mechanistic insights have been revealed. For instance, it has been demonstrated that a corepressor complex called the Nucleosome Remodelling and Deacetylase (NuRD) complex plays an important role in repression of EWS/FLI targets. Interestingly, disruption of NuRD complex function by vorinostat treatment (a histone deacetylase inhibitor) or RNAi-mediated silencing of *CHD4* (a core NuRD component) resulted in de-repression of EWS/FLI-repressed target genes [104]. Additionally, inhibition of lysine-specific demethylase 1

(LSD1) resulted in de-repression of EWS/FLI-regulated target genes. This effect was lost upon silencing of EWS/FLI, implicating EWS/FLI-mediated disruption of associated epigenetic factors in Ewing sarcoma oncogenesis [104, 105]. Continued investigation of these phenomena is likely to generate a clearer mechanistic understanding of EWS/FLI-driven up- and down-regulation of target genes, potentially providing targets for new and better therapeutics.

15.5 EWS/ERG

In 1993 it was found that a distinct translocation event between the *EWSR1* gene and another ETS family member, *ERG* (ETS-Related Gene), also generated a fusion protein, termed EWS/ERG [38]. The t(21;22)(q22.2;q12.2) rearrangement producing this alternate fusion oncoprotein is present in approximately 10 % of Ewing tumours, making it the most common alternate translocation in Ewing sarcoma [38, 72]. Tumours carrying the EWS/ERG mutation do not carry the EWS/FLI fusion, indicating that EWS/ERG likely drives Ewing sarcoma oncogenesis in ways very similar to EWS/FLI. Indeed, the DNA-binding ETS domain of ERG is shares 98 % amino acid identity with the ETS domain of FLI, and the full-length proteins are 68 % similar [38, 106]. Furthermore, EWS/ERG-harbouring Ewing sarcoma tumours were no different compared to cases of EWS/FLI-containing tumours with respect to age at diagnosis, primary site, metastasis, as well as overall and event-free survival [107].

Like EWS/FLI, EWS/ERG induces oncogenic transformation when it is expressed in NIH3T3 cells [83]. Functionally, EWS/ERG is presumed to bind similar, if not identical, sets of loci as EWS/FLI, likely dysregulating expression of target genes in similar ways. This presumption is supported by evidence indicating that EWS/FLI and EWS/ERG dysregulate the same core subset of genes when introduced into NIH 3T3 cells, although these results must be interpreted cautiously considering the inaccuracies of this model [70, 108].

15.6 EWS/ETV1, EWS/ETV4, EWS/FEV

In addition to EWS/FLI and EWS/ERG, other EWS/ETS translocations have also been described in Ewing sarcoma. These alternate rearrangements result in the fusion of the *EWSR1* gene with *ETV1* (ETS variant gene 1), *ETV4* (ETS variant gene 4) and *FEV* (fifth Ewing sarcoma variant) (Table 15.1) [35–37, 39]. Each of these additional fusion proteins occurs in <1 % of all Ewing sarcoma cases, making them exceptionally rare. Being members of the same family of transcription factors, ERG, ETV1, ETV4 and FEV are all highly similar, particularly in their ETS DNA-binding domains. In fact, ETS domains of FLI, ERG and FEV are 98 % similar. ETV1 and ETV4 are also similar to other ETS proteins, but are more similar to each other because they have identical DNA-binding domains.

These rare alternate fusions have been less well studied than EWS/FLI. However, their structural similarities suggest that they share much of the same oncogenic functions required for Ewing sarcoma tumourigenesis. Indeed, the mutually exclusive nature of these different types of EWS/ETS fusions suggests that they may be largely interchangeable. Notwithstanding the relative paucity of data regarding these uncommon rearrangements, some functional differences have been observed in experiments utilizing NIH3T3 cells. Using this model, it was shown that EWS/FLI, EWS/ERG and EWS/FEV were capable of inducing anchorage-independent growth in soft agar assays, whereas EWS/ETV1 and EWS/ETV4 were incapable of inducing such transformation [108]. Interestingly, each fusion protein enabled tumour formation by NIH3T3 cells in murine xenografts. The mechanism and relevance of these differences remain unknown. It has also been suggested that EWS/FEV, EWS/ETV1 and EWS/ETV4 exist predominantly in extraosseous Ewing sarcoma [109]. However, insufficient data exists at the present time to draw any definitive conclusions about this potential correlation. It is also unknown whether these different fusion proteins have any significance with regard to outcome.

15.7 FUS/ERG and FUS/FEV

EWSR1 is the founding member of the FET (*FUS*, *EWSR1*, *TAF15*) family of RNA-binding proteins involved in Ewing sarcoma translocations. However, in rare instances, other members of the family are involved. Chromosomal rearrangements between *FUS* (also known as *TLS*) and *ERG* or *FEV*, both ETS family member genes, have been identified in rare cases of Ewing sarcoma [110, 111].

The FUS protein has a similar domain structure to that of EWS, containing an N-terminal transactivation domain with SYGQ repeats, and C-terminal RGG and RRM motifs (Fig. 15.5). Considering these shared structural features, it is likely that FUS/ETS fusions drive oncogenesis via mechanisms similar to those utilized by EWS/FLI. However, this hypothesis has not been thoroughly tested, in large part due to the relative scarcity of these alternate chromosomal rearrangements. Nevertheless, some functional similarities have been observed. For instance, both EWS/FLI and FUS/ERG have been shown to disrupt RNA splicing by similar mechanisms [112]. Expression of insulin-like growth factor 1 (*IGF1*) is also induced by several FET/ETS fusion proteins, including FUS/ERG [113]. However, these data must be interpreted with some caution as they are based largely on murine cells, which may lack some features important for EWS/FLI function [114].

Currently, only FUS/ERG and FUS/FEV fusions have been described, but it is possible that other FET/ETS fusions could exist in Ewing sarcoma. However, such instances would be exceedingly rare. The uncommon nature of such alternate fusions makes it difficult to elucidate whether specific rearrangements have important implications for prognosis, probability of relapse, or other factors. As mentioned before, these alternate translocations do pose a potential complication for molecular diagnosis of the disease, as a tumour that appears negative for all known

translocations may harbour an oncogenic FET/ETS rearrangement that has not yet been characterized and thus evades detection. These fusions are so scarce, however, that only a small minority of patients would be impacted by such a scenario.

15.8 "Ewing-Like Sarcomas" and Their Translocations

The existence of multiple alternate chromosomal rearrangements in Ewing sarcoma raises the question of how best to molecularly define the disease. In general, histopathological features and patient presentation give good pre-test probability for diagnosis, and definitive diagnosis commonly given by detection of CD99, a cell surface marker found on most Ewing sarcoma cells [115]. Biopsies are often subjected to molecular tests detecting the presence of the t(11;22)(q24.3;q12.2) translocation. Presence of EWS/FLI transcript are detected with RT-PCR, and translocations involving *EWSR1* are detected via breakapart FISH assays. These methods will detect almost all known FET/ETS chromosomal rearrangements in Ewing sarcoma. However, a family of tumours exists in which non-FET/ETS fusions are present (Table 15.3). These cancers are termed "Ewing-like sarcomas".

One such "Ewing-like" tumour was first reported in 2009 as a new t(20;22)(q13;q12) rearrangement between *EWSR1* and *NFATC2* (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2) [116]. The wild-type NFATC2 protein is a member of the NFAT family of transcription factors and is a key player in T-cell and neuronal development. NFATC2 binds DNA cooperatively with Fos and Jun, members of the activator protein 1 (AP1) family of regulatory transcription factors [117–120]. Interestingly, ETS proteins and the EWS/FLI fusion protein are also capable of cooperative DNA binding with AP1 proteins [121–123]. Also, NFAT proteins, like ETS proteins, recognize DNA sequences with a core motif of GGAA/T [116]. Together these findings suggest possible shared mechanisms of oncogenesis between EWS/ETS and EWS/NFATC2 fusions.

EWSR1 can fuse to a number of other non-ETS proteins to drive formation of "Ewing-like" tumours. Another such fusion is EWS/POU5F1 [124]. POU5F1 (POU class 5 homeobox 1) is also known as OCT4 (octamer-binding transcription factor 4), and is a transcription factor important for regulating pluripotency of stem cells [125–127]. It is thought that this fusion protein functions as an aberrant transcription

Table 15.3 Non-FET/ETS chromosomal rearrangements found "Ewing-like sarcomas"

Fusion	Translocation	References
EWSR1/NFATC2	t(20;22)(q13;q12)	[116]
EWSR1/POU5F1	t(6;22)(p21;q12)	[124]
EWSR1/SMARCA5	t(4;22)(q31;q12)	[133]
EWSR1/PATZ1	t(22;22)(q12;q12)	[134]
EWSR1/SP3	t(2;22)(q31;q12)	[109]
CIC/DUX4	t(4;19)(q35;q13)	[136]
BCOR/CCNB3	inv(X)(p11.4p11.22)	[137]

factor in these tumours, transcriptionally reprogramming cells and generating an oncogenic phenotype.

Fusions between *EWSR1* and *PATZ1* (POZ (BTB) and AT Hook Containing Zinc Finger 1, also known as *ZSG*) or *SP3* are also found in some "Ewing-like" tumours [109, 128]. Both ZSG and SP3 are zinc finger-containing transcription factor proteins and, therefore, potentially function by binding DNA and allowing the EWS portion of the fusion to dysregulate gene expression profiles, similar to EWS/FLI and other Ewing sarcoma rearrangements [109, 129]. Wild-type SP3 also contains an inhibitory domain that is lost in the translocation event generating EWS/SP3, potentially contributing to its oncogenic function.

EWSR1 can also fuse with *SMARCA5* (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 5), an ATPase found in various chromatin remodelling complexes [130–135]. While the EWS/SMARCA5 fusion protein does not directly bind DNA in a sequence-specific manner, it alters expression of key target genes perhaps by altering a chromatin remodelling function. Interestingly, SMARCA5 can function as part of the NuRD complex, which plays an important role in EWS/FLI-mediated repression of target genes (discussed previously) [104]. Whether any relationship exists between EWS/SMARCA5 and NuRD has not been tested.

CIC/*DUX4* and *BCOR*/*CCNB3* fusions have also been described [136, 137]. However, it has not been fully determined whether these tumours represent Ewing sarcoma, "Ewing-like" sarcoma, or a distinct type of bone sarcoma. More in-depth study of the molecular mechanisms underlying these oncogenic chromosomal rearrangements must be undertaken to answer this question. Indeed, a clear molecular-based definition of Ewing sarcoma and its variations may hinge upon achieving a clearer picture of how these fusions generate an oncogenic phenotype.

15.9 Molecular Definitions of Ewing Sarcoma and Diagnostic Challenges

15.9.1 Defining the Disease

The classic diagnostic definition of Ewing sarcoma relies largely upon histopathological features of these tumours, assessed by light microscopy and/or immunohistochemistry [138, 139]. This cancer appears as a small, round cell cancer with hyperchromatic nuclei when viewed by light microscopy after H&E staining [140]. Immunohistochemical staining often reveals high levels of CD99 at the cell membrane, and is used as another diagnostic marker of Ewing sarcoma cells [115, 138].

Additionally, the presence of a balanced translocation involving *EWSR1* and one of the *ETS* family of transcription factors are considered pathognomonic for the disease [138]. However, as discussed in this chapter, a number of different translocations involving FET family members other than *EWSR1* (e.g., *FUS*) also exist.

Additionally, several "Ewing-like" cancers have been found with fusions of EWSR1 to non-ETS proteins. These alternate molecular lesions, rare as they may be, add complexity to the question of how to properly define this disease and its variations.

Generally, Ewing sarcoma can be broadly subdivided into three groups, based on the type of translocation present in the tumour cells: (1) *EWSR1*/*FLI1* and functionally similar translocations (i.e., FET/ETS fusions), (2) non-FET/ETS fusions (e.g., EWS/SMARCA5), and (3) totally distinct translocations (e.g., *CIC*/*DUX4*). Furthermore, tumours of EWS/FLI and other FET/ETS fusions (e.g., EWS/ERG) can be considered classic Ewing sarcoma, while rarer, non-FET/ETS fusions and distinct translocations can be generally termed "Ewing-like" sarcomas. These definitions provide a useful categorical structure for the various molecular lesions driving oncogenesis in these tumours, but definitions will be continuously refined as our understanding of the molecular mechanisms of this disease expands.

Accurate and useful definitions are important insomuch as they may assist in grouping patients in clinically useful ways, such as into groups receiving different treatments or with different prognoses. These goals are especially challenging considering the rarity of non-EWS/FLI fusion variants, and little variation currently exists in the clinical management of different types of fusions.

15.9.2 Challenges of Molecular Diagnosis

The existence of alternate chromosomal rearrangements has clear implications for the diagnosis of Ewing sarcoma. Current diagnostic methods utilized to identify the EWS/FLI fusion may not identify the less common translocations. For instance, breakapart FISH (fluorescence in situ hybridization) probes for *EWSR1* are commonly utilized to determine that a translocation involving EWSR1 exists and are, thus, useful for detecting the most common rearrangements in Ewing sarcoma (i.e., EWS/FLI and EWS/ERG) [138, 139]. This method, however, is unable to detect Ewing sarcoma driven by non-*EWSR1* rearrangements, such as the rarer FUS/ERG and FUS/FEV fusions (Fig. 15.6).

Reverse-transcriptase (RT)-PCR assays have also been utilized to detect the fusion transcript [139]. Such an approach suffers from the same weakness as the EWSR1 breakapart FISH assay in that it is unable to detect transcripts of all possible gene fusions. For instance, primers designed to amplify specific *EWSR1*/*FLI1* fusions will not anneal to *EWSR1*/*ERG* or other alternate transcripts. Despite this weakness, one potential benefit to using a PCR-based assay is the ability to detect specific breakpoints, although this may not be clinically useful, as discussed earlier [75, 76, 78].

Hence, the rare cases of Ewing sarcoma driven by alternate translocations may theoretically result in delayed or incorrect diagnosis in uncommon cases. Clearly, the correct diagnosis of Ewing sarcoma must not rely on one single test but rather on a collection of various criteria, including patient presentation, imaging studies (e.g., X-ray, CT, MRI), histopathology, and pathognomonic molecular lesions such

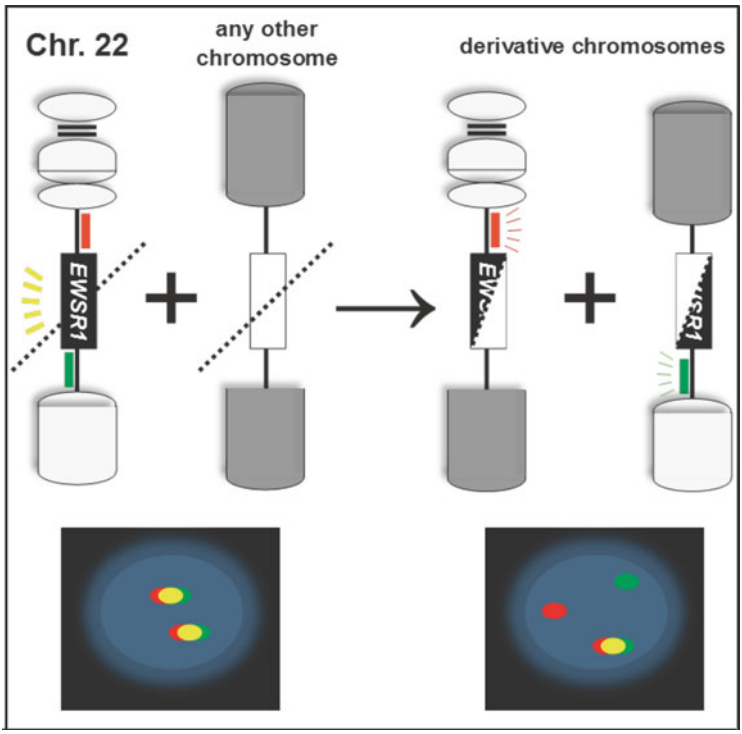

Fig. 15.6 Diagrammatic representation of a breakapart fluorescence in situ hybridization (FISH) assay for *EWSR1*. Fluorescent red and green probes flank the *EWSR1* gene. Intact *EWSR1* with both probes appears *yellow*. A translocation splits the gene, resulting in split *red* and *green* signals. In diploid cells, separate *red* and *green* signals result from the split chromosome, and the normal second allele appears *yellow*

as EWS/FLI. Such a practice of integrating distinct pieces of data to come to a definitive diagnosis is the current practice, allowing for prompt and accurate diagnosis in almost all cases.

15.10 Conclusions

Although it is rare compared to other malignancies, Ewing sarcoma is a devastating disease affecting many young people, resulting in many years of life lost to morbidity and mortality. Over the past 30 years, scientists have made great strides in understanding the molecular mechanisms underlying this cancer. Nevertheless, the increased knowledge gained through studying the cellular and molecular biology of this disease has not yet led to improvements in clinical management. Current standards of care rely on conventional therapies like surgery and chemotherapy, and

improved usage of these treatment modalities have achieved remarkable success in overall survival. No molecularly targeted therapy has been found to be efficacious against Ewing sarcoma, despite increased understanding of the molecular biology of the disease.

The EWS/FLI fusion protein, and the other fusions found in Ewing sarcoma, clearly offer a unique pathogenic feature of this disease that could be targeted. However, transcription factors have proven to be extraordinarily challenging targets for inhibition, often earning them the epithet "undruggable". Thus most efforts have focused on developing deeper understanding of the functions of critical effectors of EWS/FLI-driven oncogenesis. Although progress has been slow, a few promising targets have recently emerged [141]. Future work will continue to pursue a clearer understanding of the oncogenic consequences of the chromosomal rearrangements discussed in this chapter. Understanding why these translocations drive oncogenesis will assist in developing new therapies, likely increasing the odds of survival and bettering post-survival quality of life in these patients.

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