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Review Article

Ewing Sarcoma, an enigmatic malignancy of likely progenitor cell origin, driven by transcription factor oncogenic fusions

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Abstract: Since its first description by James Ewing in 1921, Ewing Sarcoma has been a cryptic malignancy. A poorly differentiated tumor of uncertain histogenesis and aggressive biologic behavior, it is the second most common malignancy of bone and soft tissue affecting adolescents and young adults. Some two decades ago, the understanding of Ewing Sarcoma biology took a leap forward with the identification of recurrent EWS/Ets fusions, which drive oncogenesis in this disease. A further leap forward occurred over the last half decade with the application of gene silencing, global expression profiling and primary cell culture technologies to the study of this disease. Resulting work has revealed EWS/Ets fusions to be surprisingly versatile regulators of gene expression, and has narrowed the search for the elusive cell of origin. Improved understanding of EWS/Ets biology and relevant oncogenic pathways has in turn led to the development of targeted therapies, including, recently, small molecules targeting key complexes involving the oncogenic fusion itself. In many respects still remaining an enigma, Ewing Sarcoma is an important model for cancers originating in progenitor-type cells or manifesting progenitor-type cell features, and cancers containing recurrent oncogenic fusions, the latter a surprisingly expanding number.

Keywords: Ewing Sarcoma, fusion oncogene, transcription factor, progenitor cell

Introduction

Since its original description by James Ewing in 1921, Ewing Sarcoma remains an enigmatic malignancy. Ewing Sarcoma is a biologically aggressive, poorly differentiated tumor of bone and soft tissue, and less commonly viscera [1-3]. The current definition encompasses the historical entities of Ewing Sarcoma, Peripheral Primitive Neuroectodermal Tumor, Peripheral Neuroepithelioma and Askin Tumor, all of which share the same EWS/Ets oncogenic fusions and similar biologic behavior. Ewing Sarcoma represents the second most common bone and soft tissue malignancy in adolescents and young adults, peaking in incidence at about 5 per million in this age group. It is slightly more common in males, more common in Caucasians and rarely arises in individuals of African ancestry. As with other bone and soft tissue malignancies, Ewing Sarcoma presents with pain and/or a mass, and a destructive/infiltrative lesion on imaging. The most common bony sites are the long bones of the extremities, pelvis, chest wall and spine. Lesions of long bones typically involve the diaphysis.

Biologically, Ewing Sarcoma is a classic example of a malignancy driven by a fusion oncogene [3-8]. Oncogenic fusions in Ewing Sarcoma arise from specific chromosomal translocations that yield in-frame fusion of the amino terminus of the EWS gene on chromosome 22 and the carboxyl terminus, including the DNA-binding domain, of an Ets gene. EWS is a member of the TET family of proteins, which are ubiquitously expressed in all cells and appear to be involved in transcription and/or RNA processing. Ets genes, which tend to manifest more restricted expression, are tissue-specific transcription factors. The Ets gene is Fli1 in 85% of cases, Erg in 10% of cases, and Etv1, Etv4 or FEV in the remaining 5% of cases. In-frame fusion of EWS to an Ets factor in Ewing Sarcoma yields a highly expressed, non-physiologic, potent transcription factor, which activates an oncogenic program

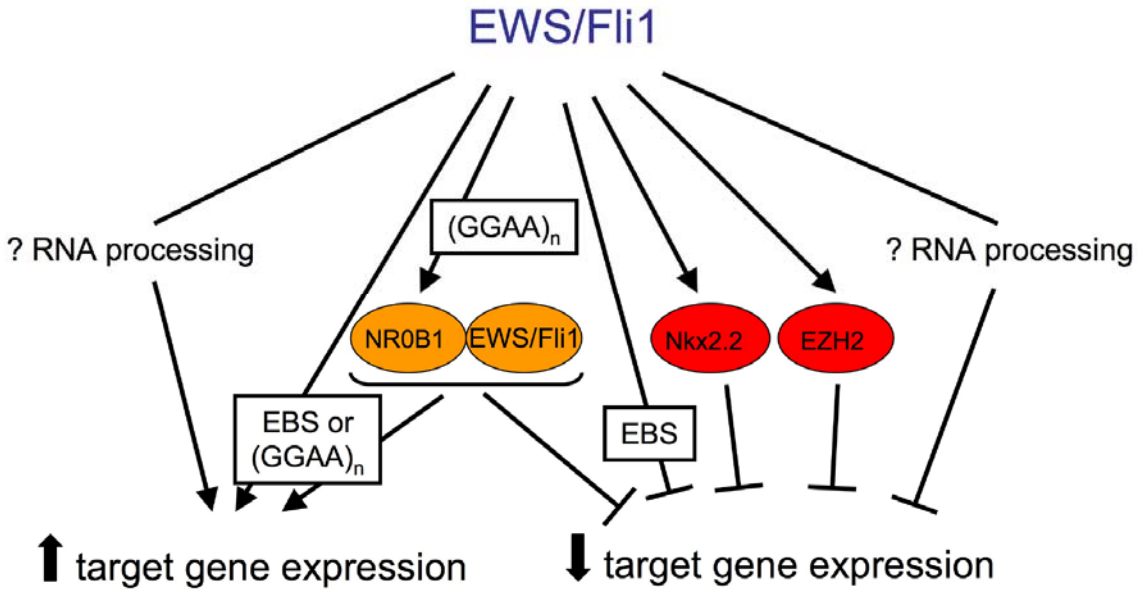

Figure 1. EWS/Ets gene regulatory pathways. EWS/Fli1 and other EWS/Ets fusions both activate and repress gene expression. EWS/Fli1 activates some target genes directly, through Ets DNA-binding sites (EBS) or through GGAA microsatellites [$(GGAA)_n$]. Some target genes are coregulated (activated or repressed) with the transcriptional coregulator NR0B1, itself an EWS/Fli1-induced gene. EWS/Fli1 is also able to repress some target genes directly, through Ets DNA-binding sites (EBS). Additional mechanisms of target gene repression are induction of the transcriptional repressor Nkx2.2, and induction of the histone methyltransferase EZH2. EWS/Ets fusions may also regulate target gene expression through non-transcriptional mechanisms, including RNA processing.

in the cells of origin of the tumor. Clinically, Ewing Sarcoma is an aggressive malignancy with a relatively poor long-term outcome [3]. Multiagent chemotherapy has greatly improved the survival of Ewing Sarcoma patients, and remains the mainstay of therapy. Unfortunately, overall long-term survival with the best conventional therapy remains around 50%. Moreover, one of four patients present with overt metastatic disease, and 75% of these patients do not survive 5 years. Five-year survival of patients with recurrent disease is even worse, at less than 10%.

With advances in technology, namely siRNA-mediated gene silencing, global gene expression profiling, and primary cell isolation, culture and manipulation, understanding of the molecular pathways of pathogenesis in Ewing Sarcoma has greatly advanced in the last decade. This increased understanding has, in turn, led to advances, not yet fully realized but highly promising, in therapy of this aggressive neoplasm. Ewing Sarcoma has been the subject of other excellent recent reviews [3, 5, 6, 8]. The goal of this review is to provide an update of the pathobiology of this disease, with a focus on exciting recent findings related to the mechanisms of regulation of oncogenic pathways by EWS/Ets fusions, histogenesis of this cryptic malignancy, and possibilities for biologically targeted therapy.

Oncogenesis by EWS/Ets fusions I: mechanisms of (dys) regulation of gene expression

Studies to date reveal EWS/Ets fusions to be multifunctional gain-of-function transcriptional regulators, capable of exerting activating and repressive effects on gene expression, both important to oncogenesis in Ewing Sarcoma (**Figure 1**). The ability of EWS/Ets fusions to activate gene expression has been evident from the earliest studies of the function of these oncoproteins, which showed that the EWS component confers a more potent transcription activating domain than present in the parent Ets gene [9]. Moreover, other studies demonstrated a correlation between the potency of transcriptional activation and clinical prognosis for the

most commonly observed EWS/Ets fusions [10]. A number of genes have been shown to be directly activated by EWS/Ets fusions in the context of Ewing Sarcoma cells or mesenchymal progenitor cells, the candidate cell of origin, including IGF-1 and the transcriptional coregulator NR0B1 [11, 12]. The importance of activation of gene expression to Ewing Sarcoma oncogenesis by EWS/Ets fusions has been shown experimentally: the introduction of KRAB-Fli1, which contains a potent repressor domain in place of the EWS component, into Ewing Sarcoma cells, inhibits both oncogenic transformation in vitro and tumorigenicity in vivo [13]. Interestingly, recent ChIP-chip studies indicate that, in addition to utilizing similar DNA-binding sites to their parent Ets factors, EWS/Ets fusions make additional use of GGAA repeat-containing microsatellites to activate target gene promoters [12, 14]. This mechanism of activation requires, at minimum, four to five such repeats and appears to be specific to EWS/Fli1 upregulated genes [12].

The extent and importance of transcriptional repression by EWS/Ets oncogenic fusions have only been appreciated more recently. Indication that EWS/Ets fusions could repress gene expression had existed from studies of some target genes, notably the Transforming Growth Factor beta Receptor II (TGFβRII) [15]. However, the extent of repression of gene expression only became evident from global gene expression profiling studies of Ewing Sarcoma cells with stably silenced EWS/Ets [16, 17]. As evident from studies of TGFβRII, EWS/Ets fusions are capable of transcriptional repression in some contexts. However, gene expression profiling studies have also revealed entirely novel and unexpected additional mechanisms of transcriptional repression operant in Ewing Sarcoma. The homeoprotein Nkx2.2 is one of the most strongly induced genes by EWS/Fli1 in Ewing Sarcoma cells [17]. Functional analyses of Nkx2.2 reveal that it is a transcriptional repressor responsible for down-regulation of a subset of genes by EWS/Ets [18]. Since Nkx2.2 is required for EWS/Ets-mediated transformation [17], this EWS/Ets-Nkx2.2 pathway establishes the biological importance of transcriptional repression in Ewing Sarcoma oncogenesis. The orphan nuclear receptor NR0B1, another highly induced gene by EWS/Fli1, is also required for optimal transformation by EWS/Ets [19], and is a known transcriptional corepressor [20]. Interestingly, ChIP-chip, ChIP and biochemical experiments indicate that EWS/Fli1 and NR0B1 interact on target gene promoters [21]. Thus, NR0B1 is another candidate for target gene repression by EWS/Ets. Analyses of NR0B1 function indicate that it is able to both repress and activate gene expression; moreover, on one studied promoter, EWS/Fli1 appears to inhibit activation by NR0B1 [21]. The EWS/Ets-NR0B1 pathway thus highlights the likely importance of combinatorial protein-protein interactions and overall promoter context in the end-effect of EWS/Ets fusions on target gene expression. An additional, potentially far-reaching, mechanism of repression of gene expression by EWS/Ets fusions involves a histone methyl transferase [22]. The histone methyltransferase enhancer of Zeste, *Drosophila*, Homolog 2 (EZH2) silences gene expression by lysine methylation, as part of the Polycomb Repressor Complex 2 (PRC2). EZH2 is an upregulated target, apparently direct, of EWS/Fli1, and is required for optimal oncogenic transformation, tumor growth and metastasis. Interestingly, siRNA silencing and expression profiling experiments indicate that EZH2 inhibits endothelial, epithelial and neural differentiation in Ewing Sarcoma cells. Like the Nkx2.2 studies discussed above, therefore, these studies again underscore the functional importance of repression of gene expression in Ewing Sarcoma pathogenesis.

Early experiments defining the oncogenic potential of EWS/Ets oncogenic fusions in NIH3T3 cells suggested the existence of a DNA-binding-independent transforming activity [23, 24], in addition to the DNA-binding-dependent activity discussed above. This was quite intriguing, given the known functions of the EWS gene in RNA processing [25]. Interestingly, several studies have shown that EWS/Ets fusions can affect RNA splicing [26-29]. Moreover, EWS/Fli1 and EWS interact in the cell [30, 31]. This leaves open the interesting possibility that oncogenic transformation by EWS/Fli1 in part involves mechanisms related to RNA processing. Such a mechanism is clearly not sufficient for oncogenic transformation, as indicated by the inability of a DNA-binding impaired EWS/Fli1 allele to rescue transformation in Ewing Sarcoma cells with stably silenced endogenous EWS/Fli1 or to induce tumors in primary bone derived cells, in contrast to “wild type” EWS/Fli1 [17, 32]. However, such a mechanism may be needed for optimal transformation and/or for other,

currently less well studied, parameters of Ewing Sarcoma tumor biology.

Oncogenesis by EWS/Ets fusions II: cell of origin and mechanisms of oncogenic transformation

The cell of origin of Ewing Sarcoma has long been a subject of debate, especially among pathologists! A number of studies have recently converged on mesenchymal progenitor cells as a likely cell of origin. On the one hand, Riggi et al showed that stable expression of an EWS/Ets oncogenic fusion in mouse mesenchymal progenitor cells was sufficient to give rise to poorly differentiated tumors resembling Ewing Sarcoma [33]. The authors introduced an EWS/Fli1 fusion into a primary culture of mouse mesenchymal progenitor cells (mMPCs) using a retroviral expression vector and obtained Ewing Sarcoma-like tumors upon injection of the manipulated cells into SCID mice. Importantly, the tumorigenic mMPCs apparently maintained intact p53 and $p16^{INK4A}/p19^{ARF}$ function (as seen in most Ewing Sarcomas [34, 35]), thus suggesting that, in the appropriate cell type, EWS/Ets expression may be s*ufficient* to initiate and support tumorigenesis. In a similar study, Castillero-Trejo et al introduced the EWS/Fli1 fusion into primary bone-derived cells and also obtained Ewing Sarcoma-like tumors [32]. The efficiency of tumor formation increased with passage number and, in this study, some tumors acquired p53 mutations. Thus, mouse progenitor-type cells from bone and/or bone marrow are permissive to tumor formation upon expression of EWS/Fli1 (**Table 1**).

Tirode et al, on the other hand, demonstrated that stable silencing of EWS/Fli1 in Ewing Sarcoma cells results in a gene expression signature that overlaps with mesenchymal progenitor cells [36]. Furthermore, the authors demonstrated that the resulting cells are able to differentiate along adipocytic, chondrocytic and osteocytic lineages under appropriate growth conditions, a defining property of mesenchymal progenitor cells. Interestingly, control cells (without EWS/Fli1 silencing) showed a similar ability to differentiate along the same lineages, albeit to a lesser degree. In a similar analysis, Potikyan et al compared the gene expression profile of Ewing Sarcoma cells with silenced EWS/Fli1 to the expression profiles of a panel of diverse cells lines, and obtained the closest match with human fetal lung fibroblasts, thus further supporting a mesenchymal origin [37]. These studies thus suggest that Ewing Sarcoma cells possess mesenchymal progenitor cell-type properties, which are enhanced upon loss of expression of the EWS/Ets oncogenic fusion. This idea is further supported by the observation that expression of EWS/Ets fusions in pluripotent marrow stromal cells inhibits their ability to differentiate along osteogenic and adipogenic lineages [38]. Interestingly, the mesenchymal progenitor properties of Ewing Sarcoma cells appear to be particularly prominent in $CD133^+$ cancer stem cells isolated from primary tumors, compared to their $CD133^-$ counterparts [39].

Several other studies, however, suggest that tumor initiation in Ewing Sarcoma may be more complex than single step transformation of mesenchymal progenitor cells by EWS/Ets fusions. First, expression of EWS/Fli1 in primary *human* mesenchymal progenitor cells yields a Ewing Sarcoma-like expression signature, but no tumors in a mouse xenograft model [40]. Since this analysis was performed by the same group that did the analogous mouse experiments, the difference in results is not likely to be due to different technique. Rather, it may be due to differences in the precise cells used, species differences, or exact fusion expression levels. Second, in experiments also performed by this same group, expression of similar levels of EWS/Erg, the second most common Ewing Sarcoma oncogenic fusion, is not well tolerated by mouse mesenchymal progenitor cells, in contrast to EWS/Fli1 [11]. Further, in a mouse genetic model, induced expression of EWS/Fli1 in the bone marrow compartment, including mesenchymal progenitor cells, leads to the rapid development of leukemia, but not Ewing Sarcoma-like tumors [41]. In a different mouse genetic model, targeted expression of EWS/Fli1 in developing embryonic limb bud mesenchyme, does give rise to tumors with some resemblance to Ewing Sarcoma, but only in combination with inactivation of p53 [42]. Possible explanations for the discrepancy between the xenograft and genetic models include differences in the precise nature and/or abundance of the isolated/targeted cells, differences in EWS/Fli1 expression levels, and possible longer latency of Ewing Sarcoma compared to leukemia. Lastly, even the EWS/Fli1-driven mouse mesenchymal progenitor cell-derived tumors showed deviations from Ewing Sarcoma [33]. Namely, at the molecular level, the mMPC-EWS/Fli1 tumors

Table 1. Mouse xenograft models of Ewing Sarcoma using primary, unmodified (non-transformed and non-immortalized) cells

Author	Cells/ source	Growth medium	Cell characterization	EWS fusion	Time of introduction	*In vitro* assays	Mice	Route	time post infection	tumors	p53/Ink/Arf
Riggi et al, 2005	mMPC (C57BL/6 BM)	custom	Flow cytometry; differentiation assay	Fli1, "t2"	?	None	SCID	sc	5 days	100% at 6 wks	Intact
Riggi et al, 2008	hMPC (femoral head BM)	IMDM/ 10% FCS/ PDGF	Flow cytometry; differentiation assay	Fli1, "t2"	PD #8	Cell growth: no change	"immuno-compromised"	renal capsule	?	None	?
Castillero-Trejo et al, 2005	mPBDC (BALB/c BM)	DMEM/ 10% FCS	(Flow cytometry of *tumor* cells)	Fli1, t1	P0	Soft agar (EWS/Fli1-dependent growth)	syngeneic	sc/ip/iv	?	<P15: rare; P17-18: 40%; >P28: 100%	p53 mutation in some tumors

Abbreviations used: m=mouse; h=human; MPC=mesenchymal progenitor cells; PBDC=primary bone-derived cells; BM=bone marrow; t1=type 1 EWS/Fli1 fusion; t2=type 2 EWS/Fli1 fusion (written in quotation marks because the SK-N-MC cell line, from which this fusion was reported to be derived from, harbors the type 1 fusion); PD=population doubling; P=cell passage number following isolation; sc=subcutaneous; ip=intraperitoneal; iv=intravenous.

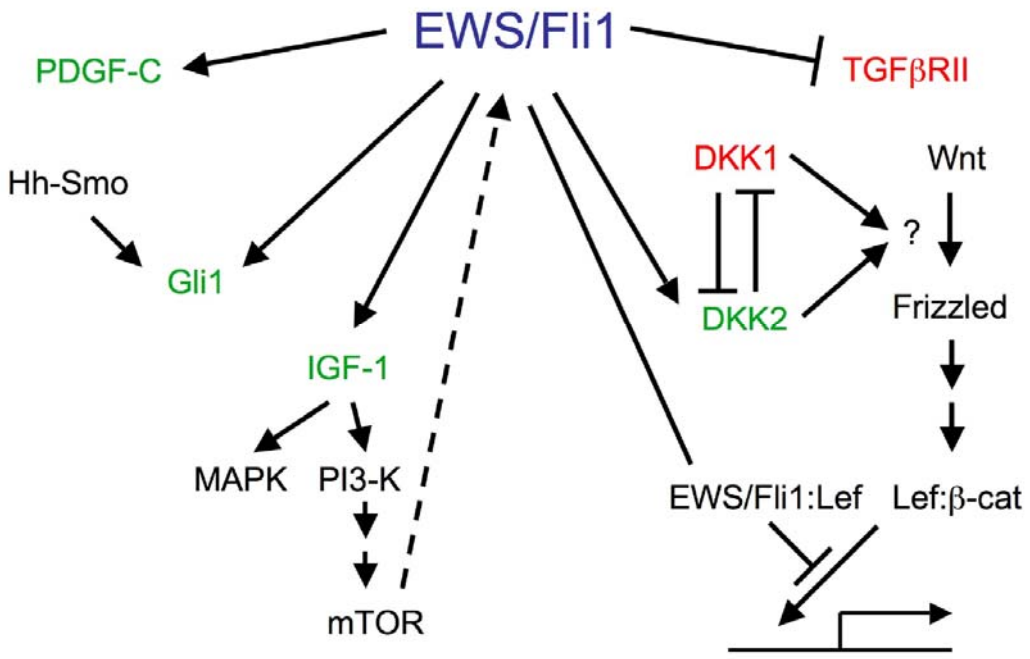

Figure 2. Growth factor pathways regulated by EWS/Ets oncogenic fusions with demonstrated functions in Ewing Sarcoma oncogenesis. Pro-oncogenic (green) and tumor suppressive (red) targets of EWS/Ets fusions in growth factor pathways. Pro-oncogenic targets include PDGF-C, the mediator of Hedgehog-Smoothened signaling Gli1, IGF-1 and DKK2, a regulator of Wnt signaling. mTOR, downstream of IGF-1 signaling, may positively regulate EWS/Ets fusion levels. EWS/Fli1 antagonizes nuclear β-catenin transcriptional activity, possibly by sequestering Lef. EWS/Ets-regulated tumor suppressive targets with demonstrated function in Ewing Sarcoma include TGFβRII and DKK1. The expression of DKK1 and DKK2 appears to be mutually antagonistic.

strongly activated the expression of a number of genes of potential pathogenic importance including IGF-1, Myc, Id2 and MMP3. However, of these genes, only IGF-1 is identified as an EWS/Fli1-regulated target in fusion silencing systems in Ewing Sarcoma cell lines [16, 17, 43]. Thus, while all of the above studies represent progress toward an understanding of Ewing Sarcoma histogenesis, the issue of the precise *human* cell of origin and the question of a requirement for second "hits" remain unresolved.

Oncogenesis by EWS/Ets fusions III: EWS/Ets-regulated oncogenic pathways and targeted therapy

Given the pivotal importance of EWS/Ets fusions in Ewing Sarcoma pathogenesis, these oncogenes have been referred to as the "perfect target without a therapeutic target" [44]. One possible way to target EWS/Ets fusions is inhibition of expression. Such as approach, using small interfering RNA (siRNA) technology, has been successful in inhibiting the growth of Ewing Sarcoma cells in a mouse xenograft metastatic tumor model [45]. Another possible approach is targeting of the fusion proteins themselves. However, this approach has been hampered by difficulties with biochemical purification and analysis of the fusion proteins, due to the presence of unstructured regions and poor solubility [44]. A related approach, and one that has borne the most fruit thus far, is targeting of critical protein-protein interactions of EWS/Ets fusions.

A number of different studies have identified protein-protein interaction partners for EWS/Ets oncogenic fusions. EWS/Fli1 interacts with the transcription factor CBP, and a dominant-negative form of CBP sensitizes Ewing Sarcoma cells to apoptosis [46]. Promoter interactions with the AP-1 (fos/jun) transcription complex may be important for transcriptional regulation

by EWS/Ets fusions, as a C-terminal deletion mutant of EWS/Fli1, defective in cooperative interactions with AP-1, is impaired in cellular transformation [47]. As discussed above, EWS/Fli1 forms a complex with the transcriptional co-regulator NR0B1 [21], which is itself a transcriptional target of EWS/Fli1 and is essential for EWS/Fli1-mediated oncogenic transformation [19]. Similarly, EWS/Fli1 forms complexes with RNA Helicase A, and this interaction stimulates EWS/Fli1 promoter activation and cellular transformation [48]. Additional protein-protein interactions of EWS/Fli1, currently with unknown functional significance in Ewing Sarcoma, include the POU homeodomain protein Brn-3a [49], the BRCA-interacting protein BARD1 [50], hyperphosphorylated RNA Polymerase II (Pol II) [29], RNA Pol II subunit hsRPB7 [51], and serum response factor (SRF) [52]. The interaction between EWS/Fli1 and RNA Helicase A (RHA) is the first to have been successfully "drugged" thus far. Using surface plasmon resonance screening and recombinant EWS/Fli1, Erkizan et al identified a small molecule able to interfere with the EWS/Fli1-RHA interaction, induce apoptosis of Ewing Sarcoma cells and inhibit tumor growth in a mouse xenograft model [53].

EWS/Ets fusions also regulate growth factor pathways (**Figure 2**), which may lend themselves as potential drug targets, either alone or in combination with inhibitors of EWS/Ets activity. The Insulin-like Growth Factor 1 (IGF-1) signaling pathway is critical to Ewing Sarcoma oncogenesis, via autocrine, and likely endocrine, pathways [54-56]. EWS/Fli1 activates IGF-1 expression [11, 43], while downregulating the expression of the IGF-binding protein IGFBP3 [16], thus presumably stimulating the autocrine pathway. Blockade of the IGF-1 receptor (IGF-1R) is inhibitory to Ewing Sarcoma growth *in vitro* and in *in vivo* mouse xenograft models [55-59], and clinical trials involving both small molecule and antibody inhibitors of IGF-1R in Ewing Sarcoma are ongoing (reviewed in [3]). Additional inhibition of pathway components downstream of the growth factor/ receptor interaction, including MAPK/MEK, PI3-K and mTOR [60-63], may have added efficacy to IGF-1R blockade. Interestingly, by an undefined mechanism, the mTOR inhibitor Rapamycin reduces cellular levels of EWS/Fli1 [64], and may thus have "double efficacy" in Ewing Sarcoma therapy [65]. EWS/Ets fusions also regulate Wnt signaling in a complex manner. EWS/Ets fusions regulate members of the Dickkopf family, secreted modulators of Wnt signaling. Specifically, they induce DKK2, apparently directly, while repressing DKK1 [36, 66, 67]. DKK1 is suppressive to Ewing Sarcoma tumor growth, while DKK2 may be somewhat tumor promoting [66]. Interestingly, EWS/Fli1 also generally antagonizes the activation of β-catenin responsive genes, possibly by sequestration of TCF/Lef [67]. Additionally, Platelet-Derived Growth Factor C (PDGF-C) is an EWS/Fli1-upregulated gene in NIH3T3 cells ([68]; but, interestingly, not in an EWS/Fli1 silencing/rescue model [17]), and a dominant negative form of PDGF-C inhibits Ewing Sarcoma cell growth [69]. EWS/Fli1 also upregulates the effector of Hedgehog pathway signaling Gli1, which is required for optimal cellular transformation [70]. Finally, restoration of TGFβRII expression levels, normally downregulated by EWS/Ets, is inhibitory to Ewing Sarcoma tumor growth [71].

Other known oncogenesis-modifying pathways, currently not known to be regulated by EWS/Ets fusions, are also operant in Ewing Sarcoma, and may be amenable to drug targeting. These include Notch signaling [72] and the NF-kB pathway [70, 73]. Lastly, the identification of presumptive cooperating pathways in EWS/Ets-mediated cellular transformation may present additional opportunities for targeted therapy in Ewing Sarcoma.

Conclusions and perspectives

Over the past two decades, much has been learned about the biology of Ewing Sarcoma, including the pivotal role of EWS/Ets oncogenic fusions and the pathways they regulate, the importance of cellular context for their action and the likely cell of origin, and mechanisms of regulation of downstream gene expression. This information has opened the door to targeted therapies for this aggressive malignancy, including blockade of IGF signaling, and more recently, interference with key EWS/Ets protein-protein interactions. Information learned from the study of Ewing Sarcoma biology has been, and will continue to be, of relevance to other cancers, given the prevalence of activated IGF signaling in other malignancies and the surprising occurrence, in growing numbers of tumors, of oncogenic fusions between EWS family members and transcription factors. Similarly, application of growing knowledge of other highly con-

served pathways in cancer is likely to suggest additional ways to attack Ewing Sarcoma therapeutically. Perhaps in some of our lifetimes, Ewing Sarcoma will become a historical curiosity rather than the taker of the lives of children and young adults in their prime.

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