Chapter

Drug Targeting of Chromosomal Translocations in Fusion-Positive Sarcoma

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Abstract

Sarcomas are heterogeneous cancers of bone or soft tissue. They occur in children, adolescents, and young adults (AYAs). Herein, the subgroup of fusion-positive (FP) sarcomas is characterized by chromosomal rearrangements generating pathognomonic fusion transcripts and oncoproteins. In Ewing sarcoma (EwS), FP-rhabdomyosarcomas (FP-RMS) and synovial sarcomas (SyS), the most common and aggressive forms of sarcomas in childhood and adolescence, the oncogenic rearrangements involve transcription cofactors such as by FET-ETS, PAX3/7-FOXO1 or SS18-SSX fusion oncogenes in EwS, FP-RMS, or SyS, respectively causing widespread epigenetic rewiring and aberrant gene expression. Regardless of these translocations, few recurrent mutations are observed in these sarcomas that may contribute to disease; thus, it is of particular interest to consider the consequences of these translocations for tumor development. Results of current research examining the disease, analyzing, and classifying the role of associated rearrangements of chromatin, and investigating possibilities for tumor-specific intervention such as blocking the transcriptional activity of the fusion protein, or the processes caused by this activity are summarized here and some resulting therapeutic opportunities are presented.

Keywords: fusion-positive sarcoma, epigenetic rewiring, aberrant gene expression, targeted therapy

1. Introduction

Sarcomas are heterogeneous cancers of bone or soft tissue. They occur in children as well as adolescents and young adults (AYAs). They are rare among adult malignancies but account for 12–15% of all pediatric tumors [1]. Despite the introduction and continued optimization of multimodal therapies, approximately one-third of sarcoma patients still die from the disease. Current therapies combine surgery, polychemotherapy, radiation, immunotherapy, and/or targeted therapeutics. Scientific advances have enabled more precise molecular characterization of sarcoma subtypes [2–4] and discovered new therapeutic targets and prognostic biomarkers [5]. Patients with primary metastatic disease or recurrence have a very poor prognosis in both age groups [6].

The pathogenesis of many sarcomas is poorly understood, but research over the past 20 years has identified recurrent, characteristic chromosomal translocations in approximately one-third of sarcomas (including most pediatric, adolescent, and

young adult tumors). Chromosomal rearrangements resulting in oncogenic fusion genes are more common in childhood cancers than in adult tumors [7, 8].

The first sarcoma-specific chromosomal translocation was detected in 1982 in patients with alveolar rhabdomyosarcoma [9]. In subsequent years, chromosomal aberrations were identified in additional sarcomas [10]. These translocations are specific to the individual sarcomas and are considered tumor-initiating in those in which they occur [11, 12].

Fusion-positive sarcomas are characterized molecularly by a relatively quiescent genome with recurrent, balanced translocations leading to the formation of novel fusion oncogenes that are key to pathogenesis [13]. In these sarcomas, fusion protein-forming translocations are often the primary driver of disease pathogenesis and are accompanied by very few other mutations [14], although a limited number of recurrent, cooperating mutations have been identified (e.g., STAG2 in Ewing sarcomas and KRAS in synovial sarcomas) [15–19].

With the advent of advanced techniques in molecular genetics and pathology, new translocations in sarcomas are regularly reported, leading to reclassification and adjusted risk stratification. Many sarcomas are now diagnosed and classified or reclassified based on these underlying molecular alterations [2, 4, 20].

The marked tumor specificity, of the individual fusion genes, suggests that their oncogenic roles are specific to a particular cell type and/or developmental stage. Consistent with the consideration that factors related to developmental timing are associated with oncogenesis triggered by the fusion genes, many of these sarcomas occur primarily in children [8].

Unlike other cancers, these diseases contain chimeric and neomorphic proteins that are clonally present, and due to their tumor specificity and demonstrated role in tumorigenesis, these fusion proteins often represent unique and promising targets for therapeutic intervention and robust opportunities to cure these diseases [11, 12, 15, 21].

2. Ewing sarcoma

Ewing sarcoma is a rare, aggressive bone or soft tissue tumor that primarily affects children, adolescents, and young adults (AYAs) with ~1.5 cases per million children and AYAs worldwide. The average age at diagnosis is 15 years. Approximately 20–25% of patients have metastatic disease at diagnosis, which is often unresponsive to intensive therapy [22]. Standard therapy for Ewing sarcoma consists of a multimodality treatment regimen that includes surgical resection and/or local radiation therapy, as well as intensive five-drug chemotherapy and the administration of compressed interval cycles [23].

Most Ewing sarcomas have a chromosomal rearrangement at 22q12 [10]. This led to the identification of the EWSR1 gene, which can be fused to one of several partner genes: FLI1 t(11;22), ERG t(21;22), ETV1 t(7,22), ETV4 t(17,22), or FEV t(2,22). The most common fusion is EWSR1-FLI1, which occurs in ~85% of tumors [24]. In a recent comprehensive study, it was found that in 42% of Ewing sarcomas, the fusion gene results from a loop-like rearrangement, a process known as chromoplexia. These loops always contained the disease-defining fusion at the center, but they interrupted several additional genes and appear to be associated with an aggressive form of Ewing sarcoma [25].

Ewing sarcomas have few other infrequently recurring mutations besides an EWSR1/ETS translocation, including TP53 (5–10%), CDKN2A (10%), and STAG2 (15–20%) [16, 26]. The loss of P53 and STAG2 suggests a rare group of tumors that, together with the translocation, form an aggressive subset of Ewing sarcoma [15, 18]. Furthermore, very little is known about the genetic heterogeneity within the tumor in Ewing sarcoma, its subclonal genetic architecture, and the relationship between these

factors and clinical outcome. The majority of pediatric solid tumors, including Ewing sarcoma, express an active DNA transposase, PGBD5, that can promote site-specific genomic rearrangements in human cells and may promote resistance to therapy [27, 28]. However, whether the genomic landscape of Ewing sarcomas differs in relapse from primary disease is unknown [24]. Recent analyses of DNA methylation status in Ewing sarcoma showed that primary tumors from patients with metastatic disease were more heterogeneous than those with localized disease [29]. However, most Ewing sarcomas have very few additional genetic alterations, suggesting that the fusion is likely the primary cause of disease development. Previous findings suggest that either mesenchymal stem cells or neural crest-derived stem cells are the cell of origin of Ewing sarcoma, although this is still a matter of debate [30, 31].

EWSR1 encodes a protein with a function in RNA binding and transcriptional regulation. The amino terminus of the EWSR1 protein functions as a strong transcriptional activator [32]. All Ewing sarcoma fusion partner genes encode related transcription factors, with conserved DNA-binding ETS domain. These ETS domain transcription factors play an important role in biological development [33]. During each fusion, the amino-terminal transactivation domain of EWSR1 is fused to the ETS domain-containing carboxyl terminus of the corresponding fusion partner. The resulting fusion gene functions primarily as an aberrant transcription factor. The dominant EWSR1/ETS translocation EWSR1-FLI1 results in heterogeneous expression profiles that have different biological implications. Therefore, variable expression of EWSR1-FLI1 has recently been proposed as a source of heterogeneity in these tumors. Cells with high EWSR1-FLI1 expression (EWSR1-FLI1^{high}) are highly proliferative, whereas EWSR1-FLI1^{low} cells have a strong propensity to migrate, invade, and metastasize [34].

EWSR1-FLI1 can act as both a transcriptional activator and a transcriptional repressor, depending on the sequence of DNA binding sites and the presence of additional co-factors [35, 36]. EWSR1-FLI1 acts directly or indirectly on many important cellular processes such as cell cycle, apoptosis, angiogenesis, metabolism, and cell migration by binding to these sites [24]. EWSR1-FLI1 binds to DNA either at ETS-like consensus sites with a GGAA core motif or at GGAA microsatellites (GGAA-mSats). EWSR1-FLI1 multimers directly induce open chromatin at GGAA-mSats by recruiting the nucleosome remodeling BRG1/BRM-associated factor complex (BAF) and establishing de novo enhancers that interact with promoters to drive gene expression [35, 37]. Fusion multimers physically interact with BAF complexes, which appear to be critical for EWS-FLI1 function, as BAF complexes are required for activation of EWS-FLI1 target genes. The variable length of GGAA-mSats in the germline may lead to differential activity of these enhancers and is an important determinant of tumor progression [38].

Conversely, EWSR1-FLI binds to canonical ETS recognition sites without repeats and represses wild-type ETS factors, which can lead to suppression of enhancers and downregulation of nearby genes [35]. The chimeric transcription factor can directly repress certain genes such as LOX and TGFBR2 through direct interaction and recruitment of the nucleosome remodeling and deacetylase repressor complex (NuRD), which includes histone deacetylases and the histone demethylase LSD1 [39].

Interestingly, EWSR1-ETS fusion proteins also bind to DEAD/DEADH box RNA helicases and modulate their activity, thus also affecting the transcription and splicing machinery of tumor cells and causing changes in overall transcriptome processing [40, 41].

These data demonstrate that EWSR1-FLI1 utilizes distinct chromatin regulatory mechanisms whose interplay at the right time and in the right cellular context leads to the transformed phenotype of Ewing sarcoma.

3. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children and adolescents, comprises a diverse group of cancers [42]. There are several subtypes: embryonal, alveolar, and pleomorphic rhabdomyosarcoma. Embryonal RMS occurs in infants and children, and as patients age, the proportion of embryonal RMS decreases. Conversely, the proportion of alveolar and pleomorphic types increases in adolescents and older patients. Currently available multimodal therapy results in an overall survival rate of approximately 65% in children and adolescents diagnosed with RMS [43]. However, cure rates have stagnated since the 1990s. Rhabdomyosarcoma is very sensitive to cytotoxic combination chemotherapy [44]. For low- or intermediate-risk RMS patients (who are mostly pediatric patients with embryonal-type tumors), a high cure rate can be expected with current standard treatment. In adolescents and elderly patients, most of whom have had alveolar or pleomorphic type RMS, the prognosis is poor [6].

Chromosomal translocations are observed in alveolar rhabdomyosarcomas in two translocation patterns: The DNA binding site of PAX, a member of the paired-box family of transcription factors, is fused to a transactivation domain on FOXO1 (FKHR), a member of the forkhead transcription factor family [45, 46]. The t(2;13) translocation results in the fusion of the PAX3 gene with FOXO1, whereas the t(1;13) translocation fuses PAX7 with FOXO1, both of which now serve as important prognostic biomarkers for this disease (Barr et al. 1995). The O subgroup of the FOX family includes four members (FOXO1, FOXO3, FOXO4, and FOXO6). FOXO factors are considered tumor suppressors that are inactivated by the phosphatidylinositol 3-kinase (PI3K)-AKT pathway, which is regulated by several microRNAs [47]. The prevalence of the translocation with PAX3-FOXO1 is higher than that with PAX7-FOXO1 [48].

PAX3-FOXO1 is an aberrant transcription factor that disrupts gene regulatory networks that control myogenic differentiation, proliferation, cell death, and invasiveness [49, 50]. The translocation product overlaps with wild-type PAX3 function while modifying it through changes in abundance, transcriptional activity, target gene recognition, and chromatin regulation [51–56]. Patients with fusion-positive alveolar rhabdomyosarcoma (FP-ARMS) have a strikingly low somatic mutation burden and are also associated with a significantly higher rate of metastasis and lower survival compared to FP-negative RMS [56]. Metastatic FP-RMS remains essentially incurable [57].

PAX3/7:FOXO1-positive RMS (FP-RMS) is associated with alveolar histology [58]. Silencing of PAX3/7:FOXO1 (P3F) *in vitro* has been associated with decreased growth of human FP-RMS cells [59]. The effects of the fusion on tumor induction have been studied by ectopic expression and conditional activation in various cell types [60–62]. The fusion was necessary but not sufficient to induce FP+ myogenic tumors, as the fusion oncoprotein alone did not reliably induce tumor formation [60–62]. When combined with additional oncogenic hits, only those cells that expressed the fusion prior to the introduction of additional events formed tumors [62]. These observations are consistent with genomic subclonality analyses identifying PAX3/7:FOXO1 as a founding event and driver in FP-RMS [63]. Cooperating genetic events in FP-ARMS include amplification of MYCN or CDK4 or loss of CDKN2A, TP53, or ARF [17, 61].

PAX-FOXO1 fusions are thought to contribute to the phenotype and malignancy of ARMS by dysregulating PAX-specific target genes such as the epigenetic regulator JARID2, the receptor tyrosine kinases MET and FGFR4, and IGF2, Hippo and their downstream signaling pathways [64–67]. In addition, rearrangement of the PAX gene

is thought to lead to dysregulation and amplification of a shared receptor tyrosine kinase/RAS/PIK3CA signaling axis [17]. PAX-FOXO1 fusion is also thought to affect normal FOXO function and its regulation of TGF-β signaling [68]. Recently, PAX3-FOXO1 was shown to directly establish super-enhancers in cooperation with the master transcription factors MYOG, MYOD, and MYCN to drive a myogenic transcriptional program in ARMS [55]. Thus, as in Ewing sarcoma, both aberrant transcriptional and epigenetic regulation drive the development and maintenance of FP-ARMS.

4. Synovial sarcoma

Synovial sarcoma (SyS) is a rare malignancy of soft tissue near the joints that occurs in patients of all ages but is particularly common in children and young adults. Synovial sarcoma accounts for 10% of soft tissue malignancies diagnosed annually [69]. The incidence of this disease has increased over the past three decades, while survival rates (~56%) have remained stagnant [69, 70]. Treatment of this disease consists of radical surgical resection, radiotherapy, and adjuvant chemotherapy, which offers a chance of cure in localized disease. However, the disease is prone to relapse, and metastases are common and almost always fatal [70].

Synovial sarcoma is associated with the occurrence of a chromosomal rearrangement, t(X;18) [71]. This aberration results in the formation of a fusion gene involving SS18 (also known as SYT) and one of three related genes: SSX1, SSX2, or SSX4. The presence of an SS18-SSX fusion gene is the characteristic genomic abnormality associated with the development of Synovial sarcoma [71–73]. Similar to Ewing sarcoma, Synovial sarcoma is characterized by low somatic mutation rates and no chromosomal aberrations other than the pathognomonic fusion [74, 75]. Some genes are mutated in more than 5% of Synovial sarcoma cases, including TP53, PTEN, CTNNB1, and APC [74]. Histologically, Synovial sarcoma shows a unique pattern with variable mesenchymal and epithelial components [74]. Expression of an SS18-SSX fusion leads to transformation of cultured fibroblasts and development of high-penetrance synovial sarcoma-like disease in mice when expressed in muscle progenitor cells [76, 77]. On the other hand, knockdown of the fusion protein in Synovial sarcoma cells results in the death of these cells [78].

SS18-SSX fusions do not act as transcription factors because neither SS18 nor the SSX proteins contain DNA-binding domains. Instead, they function as transcriptional regulators, aberrant chromatin regulators that drive oncogenesis by deregulating epigenetic processes and gene expression [79, 80]. SS18 is a member of the BAF complex (also known as the SWI/SNF complex) that directly interacts with the catalytic subunit of this nucleosome remodeling complex, BRM [81, 82]. BAF complexes promote gene activation through nucleosome remodeling that opens DNA for access by transcription factors and the transcription machinery. SSX proteins, on the other hand, have been shown to colocalize with Polycomb group proteins, which tend to function as gene repressors [83]. Current models suggest two potentially competing mechanisms of transforming activity in synovial sarcomas: SS18-SSX displaces wild-type SS18 and BAF47 (also known as SMARCB1, SNF5, or INI1) from the BAF complex, which may then drive Sox2-mediated proliferation/differentiation [79]. Alternatively, there is evidence that SS18-SSX can directly recruit Polycomb repressor complex 2 (PRC2) and Histone-Deacetylases (HDAC) to ATF2 targets, silencing transcription at these sites [84]; other studies have implicated SS18-SSX fusion genes in epigenetic regulation and modification of target genes [85, 86]. Treatment with a selective inhibitor of the histone methyltransferase EZH2, the enzymatic component

of the PRC2, reverses gene expression of synovial sarcomas and leads to growth inhibition and cell death in SS18-SSX-positive cells [87].

Most recently, two studies have further elucidated mechanisms underlying the retargeting of SS18-SSX-containing BAF complexes. Using CRISPR/Cas9-mediated epitope tagging, Banito et al. were able to investigate SS18-SSX1 occupancy and its effects on gene expression genome-wide. They observed that SS18-SSX1 is recruited to unmethylated CpG-rich sequences on DNA through interaction with lysine demethylase 2B (KDM2B), a core component of the non-canonical PRC1.1, also known as the BCOR complex. Recruitment of SS18-SSX to these PRC1.1 targets results in abnormal induction of genes that constitute a gene signature of Synovial sarcoma, including transcription factors associated with neurogenesis and development [88]. Second, McBride et al. have shown that SS18-SSX targets BAF complexes in bivalent chromatin regions to genes marked by H3K4me3 and H3K27me3, repressing PRC2 and abnormally activating a gene program essential for Synovial sarcoma survival. Loss of SS18-SSX results in decreased binding of the BAF complex to genes that depend on the fusion for their continued expression decreased chromatin accessibility at these sites, and mesenchymal differentiation [89].

5. Targeting fusion oncoproteins

Fusion proteins of the sarcomas shown here appear to block the differentiation potential of these cells. This is achieved by hijacking transcriptional regulatory mechanisms to maintain the expression of stem cell transcriptional programs or by repressing differentiation programs. In Ewing sarcomas, the EWSR1-FLI1 protein upregulates EZH2 by binding to its promoter, thereby blocking its endothelial and neuronal differentiation capabilities [90]. Recent data show that in this process EZH2-containing PRC2 complexes interact with HDAC1, 2 and this HDAC activity mediates the immature, tumorigenic phenotype of Ewing sarcoma [91]. However, in the alveolar RMS HDAC1,2,3 also appears to serve an essential function of P3F-driven super-enhancers, as appropriate inhibitors disrupt the activity of these tumor-specific super-enhancers [92].

Transcription factors such as EWSR1-FLI1 can bind to DNA target sites on chromatin and initiate chromatin remodeling by recruiting other transcription factors and coactivator complexes. One way to achieve this chromatin remodeling is through association with BAF complexes. These multimembered complexes use ATP to move, displace, or exchange nucleosomes on chromatin. In Ewing sarcomas, BAF complexes can directly interact with the N-terminal EWSR1 protein of the fusion protein to promote and direct its tumor-specific activity at GGAA microsatellites. This binding activity is attributed to a specific prion-like domain in the N-terminal EWSR1 protein that is sufficient to drive chromatin remodeling and oncogenic gene transcription when fused to FLI1 [37]. In alveolar RMS, no direct interaction of P3F with the BAF complex has yet been shown. However, prion-like domains are suspected in a growing class of genes involved in oncogenic fusions, including FOXO1 and SS18 [93]. In synovial sarcomas, the SS18-SSX fusion also relocalizes and disrupts the BAF complex. The SS18-SSX fusion protein not only displaces wild-type SS18 binding and the tumor suppressor BAF47 from the complex [79]. Moreover, the SS18-SSX-containing BAF complexes interact with various repressive polycomb complexes in a context-dependent manner, thereby promoting the transcription of oncogenic genes [89], or alternatively, SS18-SSX and the BAF complex can localize and activate target genes via interaction with KDM2B and the PRC1.1 complex [88], as described above.

Despite these preclinical and clinical data, to date, there are few examples of targeted therapies that directly target these fusion transcription factors in solid tumors. However, all of these examples do not directly target structures of these chimeric transcription factors that are considered undruggable but attempt to identify processes or proteins that are essential for the activity or stability of these fusion proteins. An example is the observed interaction of EWSR1-FLI1 with RNA helicase A: YK-4-279 interferes with the interaction of EWSR1-FLI1 with RNA helicase A and thereby efficiently impairs both the activity of the fusion protein and cell proliferation of Ewing sarcoma cells [40]. Based on these data, the derivative TK-216 is now being tested in a clinical trial in patients with relapsed or refractory Ewing sarcoma. Another example is BAF complexes in which the SS18-SSX fusion protein is present in synovial sarcomas. Recent studies have shown that targeting the BRD9 protein, which is a component of SS18-SSX-containing complexes, provides potent antitumor effects in this context [94, 95]. BRD9 and SS18-SSX bind together to regions of the synovial sarcoma genome, and small molecule-triggered targeted degradation of BRD9 prevents oncogenic transcriptional programs in cell lines and blocks tumor progression in vivo [94]. These results will form the basis for future clinical trials in patients with synovial sarcomas. Furthermore, efforts are underway to identify downstream target genes that have critical roles in mediating the oncogenic effects of fusion transcription factors. Examples of these are described below.

6. Combining targeted drugs with protein degradation

To identify potential targeted therapeutic compounds that can promote fusion protein degradation, high-throughput chemical (HTS) screening can be used in a model system that reports on the stability of the target protein ([96]. Thus, cell-based systems expressing a fluorescent dye-labeled protein of interest and a different color fluorescent control can be used for image-based screening that can identify compounds that measure the stability of the fluorescently labeled protein. The identified compounds can be further investigated and the mechanism affecting protein stability can be identified [96].

An example of the successful use of such a system was recently published for Ewing sarcomas: Using a high-throughput drug screen (HTS) enriched with FDA-approved drugs coupled with global protein stability (GPS) approach revealed that the dual HDAC and phosphatidylinositol 3-kinase (PI3K) inhibitor Fimepinostat (CUDC-907) is an excellent candidate to modulate EWSR1-FLI1 stability. Fimepinostat greatly reduced the amount of fusion protein, decreased the viability of several Ewing sarcoma cell lines and PDX primary cells, and delayed tumor growth in a xenograft mouse model, while not significantly affecting healthy cells. They demonstrated that EWSR1-FLI1 protein levels were mainly regulated by the HDAC activity of Fimepinostat [97].

A second approach to degradation of fusion oncoproteins is their targeted protein degradation mediated by degradation molecules or proteolysis targeting chimeras (PROTACs). While there are several strategies for targeted protein degradation [98–100], PROTACs are small molecule-based and thus a drug-like method to degrade a target protein of interest. The methodology combines small molecules that can bind directly to E3 ligases such as CRBN and VHL [101–103] with molecules that bind to the desired target protein-coupled through a chemical linker such as polyethylene glycol. Thus, these compounds bring the target protein and an E3 ligase complex into close proximity, resulting in polyubiquitination of the target protein, followed by proteasome-mediated degradation [100].

This strategy requires a small molecule that can bind to the desired fusion protein but does not necessarily need to enter the enzyme pocket or specifically inhibit the activity of the target protein, which has historically been an obstacle to the development of drugs targeting transcription factors. Small molecule inhibitors of proteins with bromodomains and extra terminal domains (BET) such as JQ1 and OTX015 have been successfully converted into degraders [104, 105].

On the other hand, there are ways to directly tag fusion proteins for proteasomal degradation. For example, it has been shown that EWSR1-FLI1 degradation involves polyubiquitination at lysine-380, which marks the fusion protein for proteasomal degradation [106]. Lysine-380 is located within the DNA-binding domain and is also present in wild-type FLI1 and conserved in several other members of the ETS family like ETS1. However, this may limit specificity [107]. Although, given the short half-life of EWSR-FLI1, a PROTAC targeting a lysine-380-containing motif could create a therapeutic window [106]. On the other hand, EWSR1-FLI1-specific PROTACs have not yet been developed. However, PROTACs targeting fusion protein interacting with BET or CK proteins have been successfully tested in Ewing sarcoma cells [108, 109].

In the search for small molecules that can bind to a protein of interest, the HTS method is now being used very successfully. A wide variety of target-specific HTS methods and assay formats can be used (see review in Coussens et al. [110]). With improvements in stability and delivery of PROTACS targeting fusion proteins, they may represent a viable approach to identify new drugs for targeted therapy of FP sarcomas.

7. Ways to block oncogenic transcription

The basic mechanism by which fusion-positive sarcomas promote and maintain tumorigenicity is through the activation of pathogenic transcriptional programs. They mediate this (as described above) through direct regulation of genes at promoters, the establishment of de novo enhancers, and aberrant recruitment of transcription cofactors [111]. Pathogenic transcriptional activity is also achieved through dysregulation of epigenetic programs, including the generation of super-enhancers characterized by extended stretches of acetylation at histone H3 lysine 27 (H3K27ac) [112]. These histone marks are recognized by members of the BET family (BRD2, BRD3, BRD4) [113]. They have an essential role in regulating transcription by interacting with various proteins such as RNA polymerase II [114]. This allows multiple approaches to intervene pharmacologically in this pathogenic transcriptional program. For example, the first published inhibitor of BET proteins, JQ1, has also shown much noted antitumor activity against various tumor cells [115]. We demonstrated that the BET inhibitor JQ1 reverses the EWSR1-FLI1 transcriptional signature of Ewing sarcoma cells and inhibits tumor growth of Ewing sarcoma xenografts [116]. These results have been confirmed or further investigated in other studies [109, 117–119] Thus, Jacques et al. confirmed the effect of JQ1 on Ewing sarcoma xenografts and additionally observed their decreased vascularization [117]. The effect on angiogenesis was confirmed by another study that examined rhabdomyosarcoma in addition to Ewing sarcomas and showed a reduction in the expression of tumor-associated angiogenic factors [118]. Finally, EWSR1-FLI1 or EWSR1-ERG were studied in a functional complex with BRD4, MED1, and RNAPII [109], and impairment of this complex was observed either by RNA interference of BRD4 expression or by BET inhibitors [109]. In alveolar rhabdomyosarcoma, PAX3-FOXO1 was shown to recruit BRD4 to establish de novo

super-enhancers at myogenic transcription factors. These FP-RMS cells were highly sensitive to JQ1, as it selectively silenced PAX3-FOXO1-driven transcription [55].

Another way to interfere with the pathogenic transcriptional program of FP sarcomas is to pharmacologically inhibit transcription-dependent cyclin kinases (CDKs) CDK7, 8, 9, 12, and 13. These CDKs have an essential role in transcription by phosphorylating the C-terminal domain of RNA polymerase II, thereby regulating transcription initiation and elongation [120]. Indeed, profiling of cancer cell lines with the covalent CDK7/12/13 inhibitor, THZ1, showed exceptional sensitivity in cancer cell lines dependent on dysregulated transcriptional programs [121]. Using chemical genomics screening, Iniguez et al. 2018 found that Ewing sarcomas are particularly sensitive to THZ1. Further, they observed that the selective CDK12/13 inhibitor THZ531 elicited DNA damage repair in an EWSR1-FLI1-dependent manner. Combining these molecules with the PARP inhibitor Olaparib resulted in tumor volume reduction and prolonged survival in both cell lines and patient-derived xenografts without hematopoietic toxicity [122].

Synergistic effects were also observed with a sequential targeting approach using the histone demethylase inhibitor GSK-J4 and the CDK inhibitor THZ1 [123]. We observed that CDK9 binds to EWSR1-FLI1 via the BET protein BRD4. The combination of the CDK9 inhibitor CDKI-73 with the BET inhibitor JQ1 was more effective in reducing Ewing sarcoma cell proliferation and tumor volume in xenografts than either agent alone [124]. Another study also demonstrated synergy between the EWSR1-FLI inhibitor mithramycin and the CDK9 inhibitor PHA-767491. Importantly, the synergy was observed at clinically relevant concentrations of mithramycin [125]. Finally, in synovial sarcomas, Li et al. 2019 observed that inhibition of CDK9, with either siRNA or the CDK9 inhibitor LDC067, impaired synovial sarcoma cell growth and proliferation in a dose-dependent manner. This was also associated with a decrease in RNA polymerase II phosphorylation and an increase in the expression of anti-apoptotic proteins. In addition, inhibition of CDK9 decreased sarcoma cell spheroid formation and cell motility [126].

8. Inhibition of key players of the fusion-positive interactome

Fusion oncoproteins remodel the transcriptional machinery of cells, silencing genes and activating others by creating new enhancers, remodeling chromatin, and critically altering the epigenetic profile of sarcoma cells. By cooperating with histone deacetylases (HDACs) in transcriptional regulatory complexes, fusion oncoproteins affect histone acetylation and chromatin remodeling. For these chromatin remodeling complexes, they recruit BAF complexes as in the case of Ewing sarcoma [37] or alter their function as in the case of synovial sarcoma [79] to enforce pathogenic transcriptional programs. Binding of EWSR1-FLI1 to GGAA mSATs leads to the binding of histone acetyltransferase p300 at many of these sites and an increase in H3K27ac [35, 36]. On the other hand, the PAX3-FOXO1 fusion oncogene of alveolar rhabdomyosarcoma recruits master transcription factors MYOG, MYOD, and MYCN to activated gene loci and alters their histone acetylation which enables binding and manipulation of reader proteins such as BRD4 [55]. In synovial sarcomas, SS18-SSX fusion oncogenes, cause epigenetic restructuring involving HDACs [127]. Conversely, EWSR1-FLI1 translocation recruits histone deacetylases and histone demethylase LSD1 to specific gene loci through direct interaction with the NuRD complex, thereby suppressing their expression in Ewing sarcoma [39].

However, downstream processes also appear to be important for the epigenetic expression profile in FP sarcomas. For example, EWSR1-FLI1 binds to the promoter of the histone methyltransferase EZH2, upregulating its expression and thereby blocking its endothelial and neuronal differentiation abilities [90, 128]. But, chemical inhibitors of EZH2 activity cannot reproduce the results after RNA interference (unpublished). Yet, recent data show that EZH2-containing PRC2 complexes interact with HDAC1, 2 and this HDAC activity mediates the immature, tumorigenic phenotype of Ewing sarcoma [91].

The involvement of HDACs in key mechanisms of sarcoma cell transformation has paved the way for the investigation of HDACi for therapeutic intervention. Preclinical studies have not found significant therapeutic benefits in solid tumors, including sarcomas. Nevertheless, in combination therapies based on HDACi, sarcomas were represented in most cases as an unclassified group [129]. More recent studies are now specifically examining individual sarcomas and attempting to identify meaningful combination therapies based on known/identified mechanisms: In Ewing sarcomas, we observed that CRISPR/Cas9 knockout of individual HDACs such as HDAC1 and HDAC2 inhibited the invasiveness of Ewing sarcomas and blocked local tumor growth of xenografts. RNA analyses showed that treatment with single HDAC inhibitors (HDACi) blocked an EWSR1-FLI1-specific expression profile, and EwS cells in the presence of HDAC inhibitors (HDACi) such as entinostat and romidepsin had increased susceptibility to treatment with chemotherapeutic agents including doxorubicin. HDACi acted synergistically with the EED inhibitor A-395 and together inhibited tumor growth of Ewing sarcoma xenografts [91]. Similarly, the dual HDAC and phosphatidylinositol 3-kinase (PI3K) inhibitor Fimepinostat can thus also provide simultaneous and sustained inhibition of multiple oncogenic pathways in Ewing sarcoma and reduce EWSR1-FLI1 levels and transcriptional activity [97]. Inhibition of HDAC activity largely affects Ewing sarcoma cell proliferation and survival, alone or in combination with DNA-damaging agents, through a variety of pathways that include induction of apoptosis, cell cycle arrest, and prevention of tumor invasion and metastasis [130–133]. Fimepinostat is currently being tested in children and young adults with relapsed or refractory solid tumors (NCT03893487).

In alveolar RMS, class I HDACs such as HDAC1, 2, and 3 appear to play an essential function in PAX3-FOXO1 driven super-enhancers, as corresponding inhibitors disrupt the activity of these tumor-specific super-enhancers and block transcription and cell proliferation [92]. Recent data show that entinostat affects in vivo growth of FP-RMS and inhibits PAX3-FOXO1 via a multistep and indirect process through an HDAC3-SMARCA4-miR-27a axis [134]. Interestingly, the HDAC inhibitor Entinostat is now being clinically tested in pediatric rhabdomyosarcomas (NCT02780804).

Previous studies have shown that HDAC inhibitors disrupt the oncoprotein complex of synovial sarcoma, leading them to apoptosis. Transcriptome analysis showed that HDAC inhibition blocks the cell cycle, neuronal differentiation promotes polycomb repressor complexes and proapoptotic factors were reactivated. HDAC inhibition resulted in a lower tumor burden in the mouse model [135]. In another study, the response of synovial sarcoma to HDACi was consistently characterized by activation of ERKs, EGR1, and the β-endoglycosidase heparanase. Disruption of HDAC-induced ERK-EGR1-heparanase pathway by concomitant treatment of cells with an MEK inhibitor (trametinib) or a heparanase inhibitor (SST0001/Roneparstat) enhanced the antiproliferative and proapoptotic effects. HDAC and heparanase inhibitors had opposite effects on histone acetylation and heparanase core levels. The combination

of SAHA with SST0001 prevented the upregulation of ERK-EGR1 heparanase, induced by HDACi, and promoted caspase-dependent cell death. In the mouse model, combined treatment with SAHA and SST0001 enhanced the antitumor effect compared with single-agent administration. [127]. Thus, it seems very reasonable to advance mediators of epigenetic processes as treatment targets for FP sarcomas. Pracinostat (SB939) a potent pan-HDAC inhibitor is now being tested in pediatric patients with refractory solid tumors and leukemias (NCT01184274).

9. Conclusions

The FP sarcomas presented here are characterized by chromosomal rearrangements that generate pathognomonic fusion transcripts and oncoproteins. It is certainly desirable to primarily block or destroy the translocation products of the sarcomas themselves with targeted therapeutic approaches. However, this has not yet been possible for the fusion transcription factors EWSR1-ETS, PAX3/7-FOXO1, and the SS18-SSX fusion oncogene. But it seems promising to prevent important binding partners of these fusion oncogenes, which are essential for mediating the oncogenic processes, from successfully binding to these fusion oncogenes. An example of this is the observed blockade of the interaction of EWSR1-FLI1 with RNA helicase A by YK-4-279, and the results of initial therapeutic interventions are of great interest here.

Currently, the greatest progress seems to be promised by approaches that address mediators of the fusion-positive interactome. Essential here seems the pathological takeover of the transcriptional machinery by these fusion oncogenes and the manifestation of their epigenetic state by histone deacetylases. Approaches that block epigenetic reader proteins such as BRD4 or transcription-specific cyclin kinases such as CDK 9 and 12 indicate promising results. The remarkable efficacy of HDAC inhibitors is highly interesting. Also, the use of these inhibitors seems to significantly reduce the stability of fusion oncogenes. On the other hand, particularly high therapeutic effects were achieved experimentally where these inhibitors were used in combination. It can therefore be assumed that targeted therapeutic approaches will be particularly successful in the future where they specifically address pathological processes of the fusion oncogenes and block several identified processes simultaneously. In doing so, the existing plasticity of the tumor must be kept in mind or synergistic processes must be identified by combining the drugs, which will probably make it possible to reduce their concentration and thus toxicity of individual doses.

Acknowledgements

The author would like to thank Angelika Eggert, Nicole Hübener, Katharina Pardon, and Beat W. Schäfer for their support. This work was supported by grants from the Berliner Krebsgesellschaft (RIFF201913), and KINDerLEBEN e.V. Berlin. It is part of the EpiCycle Consortium(01KC2012E), Funding Program of the Federal Ministry of Education and Research (BMBF), Germany.

Conflict of interest

The author declares no conflict of interest.

Abbreviations

APC	Adenomatous Polyposis Coli regulator of WNT signaling pathway
AKT	AKT serine/threonine kinase
ARF	ADP-ribosylation factor
ATF2	Activating transcription factor 2
ATP	Adenosin-triphosphate
AYA	adolescents, and young adults
BAF	ATP-dependent BRG1/BRM associated factor
BAF47	is SMARCB1: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
BET	Bromodomain and extraterminal domain
BRG	BRM/SWI2-related gene is SMARCA4 SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4
BRD	Bromodomain containing
BRM	Brahma is a core, ATPase subunit of the chromatin-remodeling complex
Cas9	Type II CRISPR RNA-guided endonuclease
CDK	Cyclin dependent kinase
Co-IP	Co-immuno-precipitation
CRBN	Cereblon
CRISPR	Clustered regularly interspaced short palindromic repeats
CTNNB1	Catenin beta 1
DEAD	Contains the amino acid sequence D-E-A-D (asp-glu-ala-asp)
EED	Embryonic ectoderm development
ERG	Erythroblast transformation specific (ETS) related gene
ETV	ETS variant transcription factor
EWSR1	Ewing sarcoma breakpoint region 1/EWS RNA binding protein 1
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FET	Fused in sarcoma (FUS) RNA binding protein, EWSR1 and TATA-box binding protein associated factor 15 (TAF15) family of genes
FEV	Fifth Ewing variant transcription factor, ETS family member
FGFR4	Fibroblast growth factor receptor 4
FLI1	Friend leukemia virus integration 1 proto-oncogene, ETS transcription factor
FOXO1	Forkhead box O1
HDAC	Histone deacetylase
Hippo	Protein kinase hippo (hpo) is part of a signaling pathway that controls organ size through the regulation of cell proliferation and apoptosis
IGF2	Insulin like growth factor 2
INI1	Integrase interactor 1 (INI1) is SMARCAB1
LSD1	Lysine-specific demethylase 1A
KDM2B	Lysine (K)-specific demethylase 2B
KRAS	Kirsten rat sarcoma viral oncogene homolog (KRAS) proto-oncogene, GTPase
LOX	Lysyl oxidase
MET	Mesenchymal-epithelial transition factor (MET) proto-oncogene, receptor tyrosine kinase

miR-27a	microRNA-27a
MYCN	MYCN proto-oncogene, BHLH transcription factor
MYOD	Myogenic differentiation
MYOG	Myogenin
PAX	Paired box
p21	is CDKN1A: cyclin dependent kinase inhibitor 1A
PCR2	Polycomb repressor complex 2
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PTEN	Phosphatase and tensin homolog
RAS	Rat sarcoma proto-oncogene, GTPase
RNAi	RNA interference
RNAPII	RNA Polymerase II
SAHA	Suberoylanilide hydroxamic acid
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
SNF5	Sucrose nonfermenting 5 is SMARCB1
SS18	Synovial sarcoma translocation, chromosome 18, subunit of BAF chromatin remodeling complex
SSX	Synovial sarcoma, X breakpoint
STAG2	Stromal antigen 2
SWI/SNF	SWItch/Sucrose Non-Fermentable is a subfamily of ATP-dependent chromatin remodeling complexes
TGFBR2	Transforming growth factor-beta receptor 2
TP53	Tumor protein 53
TGF-β	Transforming growth factor beta
VHL	von Hippel-Lindau tumor suppressor

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References

[1] Stiller CA, Trama A, Serraino D, Rossi S, Navarro C, Chirlaque MD, et al. Descriptive epidemiology of sarcomas in Europe: Report from the RARECARE project. European Journal of Cancer. 2013;**49**(3):684-695

[2] Baldauf MC, Gerke JS, Orth MF, Dallmayer M, Baumhoer D, de Alava E, et al. Are EWSR1-NFATc2-positive sarcomas really Ewing sarcomas? Modern Pathology. 2018;**31**(6):997-999

[3] Koelsche C, Hartmann W, Schrimpf D, Stichel D, Jabar S, Ranft A, et al. Array-based DNA-methylation profiling in sarcomas with small blue round cell histology provides valuable diagnostic information. Modern Pathology. 2018;**31**(8):1246-1256

[4] Watson S, Perrin V, Guillemot D, Reynaud S, Coindre JM, Karanian M, et al. Transcriptomic definition of molecular subgroups of small round cell sarcomas. The Journal of Pathology. 2018;**245**(1):29-40

[5] Grunewald TG, Alonso M, Avnet S, Banito A, Burdach S, Cidre-Aranaz F, et al. Sarcoma treatment in the era of molecular medicine. EMBO Molecular Medicine. 2020;**12**(11):e11131

[6] Sultan I, Qaddoumi I, Yaser S, Rodriguez-Galindo C, Ferrari A. Comparing adult and pediatric rhabdomyosarcoma in the surveillance, epidemiology and end results program, 1973 to 2005: An analysis of 2,600 patients. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2009;**27**(20):3391-3397

[7] Downing JR, Wilson RK, Zhang J, Mardis ER, Pui CH, Ding L, et al. The pediatric cancer genome project. Nature Genetics. 2012;**44**(6):619-622

[8] Rahal Z, Abdulhai F, Kadara H, Saab R. Genomics of adult and pediatric solid tumors. American Journal of Cancer Research. 2018;**8**(8):1356-1386

[9] Seidal T, Mark J, Hagmar B, Angervall L. Alveolar rhabdomyosarcoma: A cytogenetic and correlated cytological and histological study. Acta Pathologica, Microbiologica, et Immunologica Scandinavica. Section A. 1982;**90**(5):345-354

[10] Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature. 1992;**359**(6391):162-165

[11] Rabbitts TH. Commonality but diversity in cancer gene fusions. Cell. 2009;**137**(3):391-395

[12] Mertens F, Antonescu CR, Mitelman F. Gene fusions in soft tissue tumors: Recurrent and overlapping pathogenetic themes. Genes, Chromosomes & Cancer. 2016;**55**(4):291-310

[13] Mertens F, Johansson B, Fioretos T, Mitelman F. The emerging complexity of gene fusions in cancer. Nature Reviews. Cancer. 2015;**15**(6):371-381

[14] Grobner SN, Worst BC, Weischenfeldt J, Buchhalter I, Kleinheinz K, Rudneva VA, et al. The landscape of genomic alterations across childhood cancers. Nature. 2018;**555**(7696):321-327

[15] Crompton BD, Stewart C, Taylor-Weiner A, Alexe G, Kurek KC, Calicchio ML, et al. The genomic landscape of pediatric

Ewing sarcoma. Cancer Discovery. 2014;**4**(11):1326-1341

[16] Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013;**499**(7457):214-218

[17] Shern JF, Chen L, Chmielecki J, Wei JS, Patidar R, Rosenberg M, et al. Comprehensive genomic analysis of rhabdomyosarcoma reveals a landscape of alterations affecting a common genetic axis in fusion-positive and fusion-negative tumors. Cancer Discovery. 2014;**4**(2):216-231

[18] Tirode F, Surdez D, Ma X, Parker M, Le Deley MC, Bahrami A, et al. Genomic landscape of Ewing sarcoma defines an aggressive subtype with co-association of STAG2 and TP53 mutations. Cancer Discovery. 2014;**4**(11):1342-1353

[19] Vlenterie M, Hillebrandt-Roeffen MH, Flucke UE, Groenen PJ, Tops BB, Kamping EJ, et al. Next generation sequencing in synovial sarcoma reveals novel gene mutations. Oncotarget. 2015;**6**(33):34680-34690

[20] Koelsche C, Schrimpf D, Stichel D, Sill M, Sahm F, Reuss DE, et al. Sarcoma classification by DNA methylation profiling. Nature Communications. 2021;**12**(1):498

[21] Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW. Cancer genome landscapes. Science. 2013;**339**(6127):1546-1558

[22] Gaspar N, Hawkins DS, Dirksen U, Lewis IJ, Ferrari S, Le Deley MC, et al. Ewing Sarcoma: Current management and future approaches through collaboration. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2015;**33**(27):3036-3046

[23] Pappo AS, Dirksen U. Rhabdomyosarcoma, Ewing Sarcoma, and Other Round Cell Sarcomas. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2018;**36**(2):168-179

[24] Grunewald TGP, Cidre-Aranaz F, Surdez D, Tomazou EM, de Alava E, Kovar H, et al. Ewing sarcoma. Nature Reviews. 2018;**4**(1):5

[25] Anderson ND, de Borja R, Young MD, Fuligni F, Rosic A, Roberts ND, et al. Rearrangement bursts generate canonical gene fusions in bone and soft tissue tumors. Science. 2018;**361**:6405

[26] Agelopoulos K, Richter GH, Schmidt E, Dirksen U, von Heyking K, Moser B, et al. Deep sequencing in conjunction with expression and functional analyses reveals activation of FGFR1 in Ewing Sarcoma. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2015;**21**(21):4935-4946

[27] Henssen AG, Koche R, Zhuang J, Jiang E, Reed C, Eisenberg A, et al. PGBD5 promotes site-specific oncogenic mutations in human tumors. Nature Genetics. 2017;**49**(7):1005-1014

[28] Henssen AG, Reed C, Jiang E, Garcia HD, von Stebut J, MacArthur IC, et al. Therapeutic targeting of PGBD5-induced DNA repair dependency in pediatric solid tumors. Science Translational Medicine. 2017;**9**:44

[29] Sheffield NC, Pierron G, Klughammer J, Datlinger P, Schonegger A, Schuster M, et al. DNA methylation heterogeneity defines a disease spectrum in Ewing sarcoma. Nature Medicine. 2017;**23**(3):386-395

[30] Tirode F, Laud-Duval K, Prieur A, Delorme B, Charbord P, Delattre O. Mesenchymal stem cell features of Ewing tumors. Cancer Cell. 2007;**11**(5):421-429

[31] von Levetzow C, Jiang X, Gwye Y, von Levetzow G, Hung L, Cooper A, et al. Modeling initiation of Ewing sarcoma in human neural crest cells. PLoS One. 2011;**6**(4):e19305

[32] Kovar H, Hyde D. The two faces of the FUS/EWS/TAF15 protein family. Sarcoma. 2011;**2011**:837474

[33] Sharrocks AD. The ETS-domain transcription factor family. Nature Reviews. Molecular Cell Biology. 2001;**2**(11):827-837

[34] Franzetti GA, Laud-Duval K, van der Ent W, Brisac A, Irondelle M, Aubert S, et al. Cell-to-cell heterogeneity of EWSR1-FLI1 activity determines proliferation/migration choices in Ewing sarcoma cells. Oncogene. 2017;**36**(25):3505-3514

[35] Riggi N, Knoechel B, Gillespie SM, Rheinbay E, Boulay G, Suva ML, et al. EWS-FLI1 utilizes divergent chromatin remodeling mechanisms to directly activate or repress enhancer elements in Ewing sarcoma. Cancer Cell. 2014;**26**(5):668-681

[36] Tomazou EM, Sheffield NC, Schmidl C, Schuster M, Schonegger A, Datlinger P, et al. Epigenome mapping reveals distinct modes of gene regulation and widespread enhancer reprogramming by the oncogenic fusion protein EWS-FLI1. Cell Reports. 2015;**10**(7):1082-1095

[37] Boulay G, Sandoval GJ, Riggi N, Iyer S, Buisson R, Naigles B, et al. Cancer-Specific Retargeting of BAF Complexes by a Prion-like Domain. Cell. 2017;**171**(1):163-178

[38] Musa J, Grunewald TGP. Interaction between somatic mutations and germline variants contributes to clinical heterogeneity in cancer. Molecular & Cellular Oncology. 2020;**7**(1):1682924

[39] Sankar S, Bell R, Stephens B, Zhuo R, Sharma S, Bearss DJ, et al. Mechanism and relevance of EWS/FLI-mediated transcriptional repression in Ewing sarcoma. Oncogene. 2013;**32**(42):5089-5100

[40] Erkizan HV, Kong Y, Merchant M, Schlottmann S, Barber-Rotenberg JS, Yuan L, et al. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. Nature Medicine. 2009;**15**(7):750-756

[41] Cai W, Xiong Chen Z, Rane G, Satendra Singh S, Choo Z, Wang C, et al. Wanted DEAD/H or alive: Helicases winding up in cancers. Journal of National Cancer Institute. 2017;**109**(6):1-15

[42] Parham DM. Pathologic classification of rhabdomyosarcomas and correlations with molecular studies. Modern Pathology. 2001;**14**(5):506-514

[43] Hettmer S, Li Z, Billin AN, Barr FG, Cornelison DD, Ehrlich AR, et al. Rhabdomyosarcoma: Current challenges and their implications for developing therapies. Cold Spring Harbor Perspectives in Medicine. 2014;**4**(11):a025650

[44] Raney RB, Maurer HM, Anderson JR, Andrassy RJ, Donaldson SS, Qualman SJ, et al. The Intergroup Rhabdomyosarcoma Study Group (IRSG): Major Lessons From the IRS-I Through IRS-IV Studies as Background for the Current IRS-V Treatment Protocols. Sarcoma. 2001;**5**(1):9-15

[45] Galili N, Davis RJ, Fredericks WJ, Mukhopadhyay S, Rauscher FJ 3rd,

Emanuel BS, et al. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nature Genetics. 1993;**5**(3):230-235

[46] Shapiro DN, Sublett JE, Li B, Downing JR, Naeve CW. Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. Cancer Research. 1993;**53**(21):5108-5112

[47] Coomans de Brachene A, Demoulin JB. FOXO transcription factors in cancer development and therapy. Cellular and Molecular Life Sciences. 2016;**73**(6):1159-1172

[48] Fletcher CD. The evolving classification of soft tissue tumours—An update based on the new 2013 WHO classification. Histopathology. 2014;**64**(1):2-11

[49] Loupe JM, Miller PJ, Bonner BP, Maggi EC, Vijayaraghavan J, Crabtree JS, et al. Comparative transcriptomic analysis reveals the oncogenic fusion protein PAX3-FOXO1 globally alters mRNA and miRNA to enhance myoblast invasion. Oncogene. 2016;**5**(7):e246

[50] Pandey PR, Chatterjee B, Olanich ME, Khan J, Miettinen MM, Hewitt SM, et al. PAX3-FOXO1 is essential for tumour initiation and maintenance but not recurrence in a human myoblast model of rhabdomyosarcoma. The Journal of Pathology. 2017;**241**(5):626-637

[51] Bennicelli JL, Advani S, Schafer BW, Barr FG. PAX3 and PAX7 exhibit conserved cis-acting transcription repression domains and utilize a common gain of function mechanism in alveolar rhabdomyosarcoma. Oncogene. 1999;**18**(30):4348-4356

[52] Davis RJ, Barr FG. Fusion genes resulting from alternative chromosomal translocations are overexpressed by gene-specific mechanisms in alveolar rhabdomyosarcoma. Proceedings of the National Academy of Sciences of the United States of America. 1997;**94**(15):8047-8051

[53] Bennicelli JL, Fredericks WJ, Wilson RB, Rauscher FJ 3rd, Barr FG. Wild type PAX3 protein and the PAX3-FKHR fusion protein of alveolar rhabdomyosarcoma contain potent, structurally distinct transcriptional activation domains. Oncogene. 1995;**11**(1):119-130

[54] Fredericks WJ, Galili N, Mukhopadhyay S, Rovera G, Bennicelli J, Barr FG, et al. The PAX3-FKHR fusion protein created by the t(2;13) translocation in alveolar rhabdomyosarcomas is a more potent transcriptional activator than PAX3. Molecular and Cellular Biology. 1995;**15**(3):1522-1535

[55] Gryder BE, Yohe ME, Chou HC, Zhang X, Marques J, Wachtel M, et al. PAX3-FOXO1 establishes myogenic super enhancers and confers BET bromodomain vulnerability. Cancer Discovery. 2017;**7**(8):884-899

[56] Missiaglia E, Williamson D, Chisholm J, Wirapati P, Pierron G, Petel F, et al. PAX3/FOXO1 fusion gene status is the key prognostic molecular marker in rhabdomyosarcoma and significantly improves current risk stratification. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2012;**30**(14):1670-1677

[57] Williamson D, Missiaglia E, de Reynies A, Pierron G, Thuille B, Palenzuela G, et al. Fusion gene-negative alveolar rhabdomyosarcoma is clinically and molecularly indistinguishable from embryonal rhabdomyosarcoma. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2010;**28**(13):2151-2158

[58] Barr FG. Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma. Oncogene. 2001;**20**(40):5736-5746

[59] Kikuchi K, Tsuchiya K, Otabe O, Gotoh T, Tamura S, Katsumi Y, et al. Effects of PAX3-FKHR on malignant phenotypes in alveolar rhabdomyosarcoma. Biochemical and Biophysical Research Communications. 2008;**365**(3):568-574

[60] Graf Finckenstein F, Shahbazian V, Davicioni E, Ren YX, Anderson MJ. PAX-FKHR function as pangenes by simultaneously inducing and inhibiting myogenesis. Oncogene. 2008;**27**(14):2004-2014

[61] Keller C, Hansen MS, Coffin CM, Capecchi MR. Pax3:Fkhr interferes with embryonic Pax3 and Pax7 function: Implications for alveolar rhabdomyosarcoma cell of origin. Genes & Development. 2004;**18**(21):2608-2613

[62] Naini S, Etheridge KT, Adam SJ, Qualman SJ, Bentley RC, Counter CM, et al. Defining the cooperative genetic changes that temporally drive alveolar rhabdomyosarcoma. Cancer Research. 2008;**68**(23):9583-9588

[63] Chen L, Shern JF, Wei JS, Yohe ME, Song YK, Hurd L, et al. Clonality and evolutionary history of rhabdomyosarcoma. PLoS Genetics. 2015;**11**(3):e1005075

[64] Cao L, Yu Y, Bilke S, Walker RL, Mayeenuddin LH, Azorsa DO, et al. Genome-wide identification of PAX3-FKHR binding sites in rhabdomyosarcoma reveals candidate target genes important for development and cancer. Cancer Research. 2010;**70**(16):6497-6508

[65] Crose LE, Galindo KA, Kephart JG, Chen C, Fitamant J, Bardeesy N, et al. Alveolar rhabdomyosarcoma-associated PAX3-FOXO1 promotes tumorigenesis via Hippo pathway suppression. The Journal of clinical investigation. 2014;**124**(1):285-296

[66] Ginsberg JP, Davis RJ, Bennicelli JL, Nauta LE, Barr FG. Up-regulation of MET but not neural cell adhesion molecule expression by the PAX3-FKHR fusion protein in alveolar rhabdomyosarcoma. Cancer Research. 1998;**58**(16):3542-3546

[67] Walters ZS, Villarejo-Balcells B, Olmos D, Buist TW, Missiaglia E, Allen R, et al. JARID2 is a direct target of the PAX3-FOXO1 fusion protein and inhibits myogenic differentiation of rhabdomyosarcoma cells. Oncogene. 2014;**33**(9):1148-1157

[68] Schmitt-Ney M, Camussi G. The PAX3-FOXO1 fusion protein present in rhabdomyosarcoma interferes with normal FOXO activity and the TGF-beta pathway. PLoS One. 2015;**10**(3):e0121474

[69] Wang S, Song R, Sun T, Hou B, Hong G, Mallampati S, et al. Survival changes in patients with Synovial Sarcoma, 1983-2012. Journal of Cancer. 2017;**8**(10):1759-1768

[70] Sultan I, Rodriguez-Galindo C, Saab R, Yasir S, Casanova M, Ferrari A. Comparing children and adults with synovial sarcoma in the Surveillance, Epidemiology, and End Results program, 1983 to 2005: An analysis of 1268 patients. Cancer. 2009;**115**(15):3537-3547

[71] Clark J, Rocques PJ, Crew AJ, Gill S, Shipley J, Chan AM, et al. Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. Nature Genetics. 1994;**7**(4):502-508

[72] de Leeuw B, Balemans M, Olde Weghuis D. Geurts van Kessel A.

Identification of two alternative fusion genes, SYT-SSX1 and SYT-SSX2, in t(X;18)(p11.2;q11.2)-positive synovial sarcomas. Human Molecular Genetics. 1995;**4**(6):1097-1099

[73] Skytting B, Nilsson G, Brodin B, Xie Y, Lundeberg J, Uhlen M, et al. A novel fusion gene, SYT-SSX4, in synovial sarcoma. Journal of the National Cancer Institute. 1999;**91**(11):974-975

[74] Merchant MS, Mackall CL. Current approach to pediatric soft tissue sarcomas. The Oncologist. 2009;**14**(11):1139-1153

[75] Joseph CG, Hwang H, Jiao Y, Wood LD, Kinde I, Wu J, et al. Exomic analysis of myxoid liposarcomas, synovial sarcomas, and osteosarcomas. Genes, Chromosomes & Cancer. 2014;**53**(1):15-24

[76] Haldar M, Hancock JD, Coffin CM, Lessnick SL, Capecchi MR. A conditional mouse model of synovial sarcoma: Insights into a myogenic origin. Cancer Cell. 2007;**11**(4):375-388

[77] Nagai M, Tanaka S, Tsuda M, Endo S, Kato H, Sonobe H, et al. Analysis of transforming activity of human synovial sarcoma-associated chimeric protein SYT-SSX1 bound to chromatin remodeling factor hBRM/hSNF2 alpha. Proceedings of the National Academy of Sciences of the United States of America. 2001;**98**(7):3843-3848

[78] Carmody Soni EE, Schlottman S, Erkizan HV, Uren A, Toretsky JA. Loss of SS18-SSX1 inhibits viability and induces apoptosis in synovial sarcoma. Clinical Orthopaedics and Related Research. 2014;**472**(3):874-882

[79] Kadoch C, Crabtree GR. Reversible disruption of mSWI/SNF (BAF) complexes by the SS18-SSX oncogenic fusion in synovial sarcoma. Cell. 2013;**153**(1):71-85

[80] Tamaki S, Fukuta M, Sekiguchi K, Jin Y, Nagata S, Hayakawa K, et al. SS18-SSX, the Oncogenic Fusion Protein in Synovial Sarcoma, Is a Cellular Context-Dependent Epigenetic Modifier. PLoS One. 2015;**10**(11):e0142991

[81] Middeljans E, Wan X, Jansen PW, Sharma V, Stunnenberg HG, Logie C. SS18 together with animal-specific factors defines human BAF-type SWI/SNF complexes. PLoS One. 2012;**7**(3):e33834

[82] Thaete C, Brett D, Monaghan P, Whitehouse S, Rennie G, Rayner E, et al. Functional domains of the SYT and SYT-SSX synovial sarcoma translocation proteins and co-localization with the SNF protein BRM in the nucleus. Human Molecular Genetics. 1999;**8**(4):585-591

[83] Soulez M, Saurin AJ, Freemont PS, Knight JC. SSX and the synovial-sarcoma-specific chimaeric protein SYT-SSX co-localize with the human Polycomb group complex. Oncogene. 1999;**18**(17):2739-2746

[84] Su L, Sampaio AV, Jones KB, Pacheco M, Goytain A, Lin S, et al. Deconstruction of the SS18-SSX fusion oncoprotein complex: Insights into disease etiology and therapeutics. Cancer Cell. 2012;**21**(3):333-347

[85] de Bruijn DR, Allander SV, van Dijk AH, Willemse MP, Thijssen J, van Groningen JJ, et al. The synovial-sarcoma-associated SS18-SSX2 fusion protein induces epigenetic gene (de)regulation. Cancer Research. 2006;**66**(19):9474-9482

[86] Garcia CB, Shaffer CM, Eid JE. Genome-wide recruitment to Polycomb-modified chromatin and activity

regulation of the synovial sarcoma oncogene SYT-SSX2. BMC Genomics. 2012;**13**:189

[87] Kawano S, Grassian AR, Tsuda M, Knutson SK, Warholic NM, Kuznetsov G, et al. Preclinical evidence of anti-tumor activity induced by EZH2 inhibition in human models of Synovial Sarcoma. PLoS One. 2016;**11**(7):e0158888

[88] Banito A, Li X, Laporte AN, Roe JS, Sanchez-Vega F, Huang CH, et al. The SS18-SSX Oncoprotein Hijacks KDM2B-PRC1.1 to Drive Synovial Sarcoma. Cancer Cell. 2018;**34**(2):346-348

[89] McBride MJ, Pulice JL, Beird HC, Ingram DR, D'Avino AR, Shern JF, et al. The SS18-SSX Fusion Oncoprotein Hijacks BAF complex targeting and function to drive Synovial Sarcoma. Cancer Cell. 2018;**33**(6):1128-1141

[90] Richter GH, Plehm S, Fasan A, Rossler S, Unland R, Bennani-Baiti IM, et al. EZH2 is a mediator of EWS/FLI1 driven tumor growth and metastasis blocking endothelial and neuro-ectodermal differentiation. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**(13):5324-5329

[91] Schmidt O, Nehls N, Prexler C, von Heyking K, Groll T, Pardon K, et al. Class I histone deacetylases (HDAC) critically contribute to Ewing sarcoma pathogenesis. Journal of Experimental & Clinical Cancer Research. 2021;**40**(1):322

[92] Gryder BE, Wu L, Woldemichael GM, Pomella S, Quinn TR, Park PMC, et al. Chemical genomics reveals histone deacetylases are required for core regulatory transcription. Nature Communications. 2019;**10**(1):3004

[93] March ZM, King OD, Shorter J. Prion-like domains as epigenetic regulators, scaffolds for subcellular organization, and drivers of neurodegenerative disease. Brain Research. 1647;**2016**:9-18

[94] Brien GL, Remillard D, Shi J, Hemming ML, Chabon J, Wynne K, et al. Targeted degradation of BRD9 reverses oncogenic gene expression in synovial sarcoma. eLife. 2018;**7**:1-26

[95] Michel BC, D'Avino AR, Cassel SH, Mashtalir N, McKenzie ZM, McBride MJ, et al. A non-canonical SWI/SNF complex is a synthetic lethal target in cancers driven by BAF complex perturbation. Nature Cell Biology. 2018;**20**(12):1410-1420

[96] Blay V, Tolani B, Ho SP, Arkin MR. High-throughput screening: Today's biochemical and cell-based approaches. Drug Discovery Today. 2020;**25**(10):1807-1821

[97] Pedot G, Marques JG, Ambuhl PP, Wachtel M, Kasper S, Ngo QA, et al. Inhibition of HDACs reduces Ewing sarcoma tumor growth through EWS-FLI1 protein destabilization. Neoplasia. 2022;**27**:100784

[98] Bondeson DP, Crews CM. Targeted protein degradation by small molecules. Annual Review of Pharmacology and Toxicology. 2017;**57**:107-123

[99] Toure M, Crews CM. Small-molecule PROTACS: New approaches to protein degradation. Angewandte Chemie (International Ed. in English). 2016;**55**(6):1966-1973

[100] Nalawansha DA, Crews CM. PROTACs: An emerging therapeutic modality in precision medicine. Cell Chemical Biology. 2020;**27**(8):998-1014

[101] Buckley DL, Van Molle I, Gareiss PC, Tae HS, Michel J, Noblin DJ,

et al. Targeting the von Hippel-Lindau E3 ubiquitin ligase using small molecules to disrupt the VHL/HIF-1alpha interaction. Journal of the American Chemical Society. 2012;**134**(10):4465-4468

[102] Kronke J, Udeshi ND, Narla A, Grauman P, Hurst SN, McConkey M, et al. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. Science. 2014;**343**(6168):301-305

[103] Lu G, Middleton RE, Sun H, Naniong M, Ott CJ, Mitsiades CS, et al. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. Science. 2014;**343**(6168):305-309

[104] Lu J, Qian Y, Altieri M, Dong H, Wang J, Raina K, et al. Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. Chemistry & Biology. 2015;**22**(6):755-763

[105] Winter GE, Buckley DL, Paulk J, Roberts JM, Souza A, Dhe-Paganon S, et al. DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation. Science. 2015;**348**(6241):1376-1381

[106] Gierisch ME, Pfistner F, Lopez-Garcia LA, Harder L, Schafer BW, Niggli FK. Proteasomal degradation of the EWS-FLI1 fusion protein is regulated by a single lysine residue. The Journal of Biological Chemistry. 2016;**291**(52):26922-26933

[107] Ducker C, Shaw PE. Ubiquitin-mediated control of ETS transcription factors: Roles in cancer and development. International Journal of Molecular Science. 2021;**22**(10):1-17

[108] Su S, Chen J, Jiang Y, Wang Y, Vital T, Zhang J, et al. SPOP and OTUD7A Control EWS-FLI1 Protein Stability to Govern Ewing Sarcoma Growth. Advanced Science (Weinh). 2021;**8**(14):e2004846

[109] Gollavilli PN, Pawar A, Wilder-Romans K, Natesan R, Engelke CG, Dommeti VL, et al. EWS/ETS-Driven Ewing Sarcoma Requires BET Bromodomain Proteins. Cancer Research. 2018;**78**(16):4760-4773

[110] Coussens NP, Braisted JC, Peryea T, Sittampalam GS, Simeonov A, Hall MD. Small-molecule screens: A gateway to cancer therapeutic agents with case studies of food and drug administration-approved drugs. Pharmacological Reviews. 2017;**69**(4):479-496

[111] Bradner JE, Hnisz D, Young RA. Transcriptional addiction in cancer. Cell. 2017;**168**(4):629-643

[112] Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell. 2013;**153**(2):307-319

[113] Zeng L, Zhou MM. Bromodomain: An acetyl-lysine binding domain. FEBS Letters. 2002;**513**(1):124-128

[114] Bechter O, Schoffski P. Make your best BET: The emerging role of BET inhibitor treatment in malignant tumors. Pharmacology & Therapeutics. 2020;**208**:107479

[115] Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell. 2013;**153**(2):320-334

[116] Hensel T, Giorgi C, Schmidt O, Calzada-Wack J, Neff F, Buch T, et al. Targeting the EWS-ETS transcriptional program by BET bromodomain

inhibition in Ewing sarcoma. Oncotarget. 2016;**7**(2):1451-1463

[117] Jacques C, Lamoureux F, Baud'huin M, Rodriguez Calleja L, Quillard T, Amiaud J, et al. Targeting the epigenetic readers in Ewing sarcoma inhibits the oncogenic transcription factor EWS/Fli1. Oncotarget. 2016;**7**(17):24125-24140

[118] Bid HK, Phelps DA, Xaio L, Guttridge DC, Lin J, London C, et al. The bromodomain BET inhibitor JQ1 suppresses tumor angiogenesis in models of childhood sarcoma. Molecular Cancer Therapeutics. 2016;**15**(5):1018-1028

[119] Loganathan SN, Tang N, Fleming JT, Ma Y, Guo Y, Borinstein SC, et al. BET bromodomain inhibitors suppress EWS-FLI1-dependent transcription and the IGF1 autocrine mechanism in Ewing sarcoma. Oncotarget. 2016;**7**(28):43504-43517

[120] Larochelle S, Amat R, Glover-Cutter K, Sanso M, Zhang C, Allen JJ, et al. Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II. Nature Structural & Molecular Biology. 2012;**19**(11):1108-1115

[121] Kwiatkowski N, Zhang T, Rahl PB, Abraham BJ, Reddy J, Ficarro SB, et al. Targeting transcription regulation in cancer with a covalent CDK7 inhibitor. Nature. 2014;**511**(7511):616-620

[122] Iniguez AB, Stolte B, Wang EJ, Conway AS, Alexe G, Dharia NV, et al. EWS/FLI confers tumor cell synthetic lethality to CDK12 inhibition in Ewing Sarcoma. Cancer Cell. 2018;**33**(2):202-216

[123] Heisey DAR, Jacob S, Lochmann TL, Kurupi R, Ghotra MS, Calbert ML, et al. Pharmaceutical interference of the EWS-FLI1-driven transcriptome by cotargeting H3K27ac and RNA polymerase activity in Ewing Sarcoma. Molecular Cancer Therapeutics. 2021;**20**(10):1868-1879

[124] Richter GHS, Hensel T, Schmidt O, Saratov V, von Heyking K, Becker-Dettling F, et al. Combined inhibition of epigenetic readers and transcription initiation targets the EWS-ETS transcriptional program in Ewing Sarcoma. Cancers. Basel; 2020;**12**(2):1-17

[125] Flores G, Everett JH, Boguslawski EA, Oswald BM, Madaj ZB, Beddows I, et al. CDK9 blockade exploits context-dependent transcriptional changes to improve activity and limit toxicity of Mithramycin for Ewing Sarcoma. Molecular Cancer Therapeutics. 2020;**19**(5):1183-1196

[126] Li X, Seebacher NA, Xiao T, Hornicek FJ, Duan Z. Targeting regulation of cyclin dependent kinase 9 as a novel therapeutic strategy in synovial sarcoma. Journal of Orthopaedic Research. 2019;**37**(2):510-521

[127] Lanzi C, Favini E, Dal Bo L, Tortoreto M, Arrighetti N, Zaffaroni N, et al. Upregulation of ERK-EGR1-heparanase axis by HDAC inhibitors provides targets for rational therapeutic intervention in synovial sarcoma. Journal of Experimental & Clinical Cancer Research. 2021;**40**(1):381

[128] Burdach S, Plehm S, Unland R, Dirksen U, Borkhardt A, Staege MS, et al. Epigenetic maintenance of stemness and malignancy in peripheral neuroectodermal tumors by EZH2. Cell Cycle. 2009;**8**(13):1991-1996

[129] Lanzi C, Cassinelli G. Combinatorial strategies to potentiate the efficacy of HDAC inhibitors in fusion-positive sarcomas. Biochemical Pharmacology. 2022;**198**:114944

[130] Tang F, Choy E, Tu C, Hornicek F, Duan Z. Therapeutic applications of histone deacetylase inhibitors in sarcoma. Cancer Treatment Reviews. 2017;**59**:33-45

[131] Souza BK, da Costa Lopez PL, Menegotto PR, Vieira IA, Kersting N, Abujamra AL, et al. Targeting histone deacetylase activity to arrest cell growth and promote neural differentiation in Ewing Sarcoma. Molecular Neurobiology. 2018;**55**(9):7242-7258

[132] El-Naggar AM, Somasekharan SP, Wang Y, Cheng H, Negri GL, Pan M, et al. Class I HDAC inhibitors enhance YB-1 acetylation and oxidative stress to block sarcoma metastasis. EMBO Reports. 2019;**20**(12):e48375

[133] Sampson VB, Vetter NS, Kamara DF, Collier AB, Gresh RC, Kolb EA. Vorinostat enhances cytotoxicity of SN-38 and temozolomide in Ewing Sarcoma cells and activates STAT3/AKT/MAPK pathways. PLoS One. 2015;**10**(11):e0142704

[134] Bharathy N, Berlow NE, Wang E, Abraham J, Settelmeyer TP, Hooper JE, et al. The HDAC3-SMARCA4-miR-27a axis promotes expression of the PAX3:FOXO1 fusion oncogene in rhabdomyosarcoma. Science Signaling. 2018;**11**(557):1-13

[135] Laporte AN, Poulin NM, Barrott JJ, Wang XQ, Lorzadeh A, Vander Werff R, et al. Death by HDAC inhibition in synovial sarcoma cells. Molecular Cancer Therapeutics. 2017;**16**(12):2656-2667