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Review Article

Druggable upregulated proteins in EWS-FLI-driven Ewing sarcoma as emerging new therapeutic targets

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Abstract: Ewing sarcoma (ES) is a highly aggressive soft tissue tumor that primarily affects the long bones of children and young adults. It is distinguished by a characteristic chromosomal translocation between the Ewing sarcoma breakpoint region 1 (EWS) gene and the erythroblast transformation-specific (ETS) family of genes, most commonly resulting in the EWS-friend leukemia integration 1 (EWS-FLI1) fusion gene. This translocation is observed in approximately 80%-85% of ES cases. This fusion gene encodes a non-physiological chimeric fusion protein that plays a central role in tumorigenesis by interacting with numerous partner proteins. Several studies have demonstrated the tumorigenic potential of the EWS-FLI1 protein when transfected into non-cancer cell lines. However, targeting EWS-FLI1 directly remains a significant challenge, as no drug to date has been reported to bind to and inhibit its activity effectively. An alternative therapeutic strategy involves targeting key overexpressed protein complexes implicated in ES tumorigenesis, many of which may be downstream interacting partners of EWS-FLI1. This review explores emerging protein targets as potential therapeutic avenues in ES treatment.

Keywords: Ewing sarcoma, gene translocation, pediatric cancer, EWS-FLI1, chimeric fusion protein, emerging alternative targets

Introduction

Ewing sarcoma (ES) is a rare, aggressive malignancy that predominantly arises in the bones or soft tissues of children and adolescents. Despite accounting for less than 1% of all pediatric cancers [1], ES is associated with poor prognoses, particularly in cases presenting metastasis or relapse [2, 3]. According to the World Health Organization (WHO), ES is categorized under soft tissue and bone tumors [4]. The hallmark genetic event in ES is a chromosomal translocation between *EWSR1* on chromosome 22 and *FLI1* on chromosome 11. This translocation generates the EWS-FLI1 fusion protein, a driver of oncogenic transformation and disease progression [5]. EWS-FLI1 promotes tumorigenesis by dysregulating cell cycle progression [5] and epigenetic mechanisms [6] irrespective of the disease stage [7], thereby activating several oncogenic pathways and repressing tumor suppressor genes [8, 9]. Clinically, ES is classified into three categories: primary non-metastatic, metastatic, and recurrent disease. Conventional treatments, including surgery, chemotherapy, and radiation, are employed to treat all ES grades [4] and have demonstrated improved survival rates in 20%-30% of metastatic cases [10]. At diagnosis, the disease can be further classified into localized and metastatic stages [11]. Patients with localized ES exhibit favorable outcomes, with survival rates of approximately 70% following treatment with multimodal therapies [11]. By contrast, metastatic ES often presents a significant therapeutic challenge, with worse outcomes and frequent relapses even under aggressive treatment protocols [11]. Several factors influence disease progression, including tumor location (e.g., axial skeleton, pelvis, or extremities) [12], specific gene fusions, and clinical risk factors such as elevated lactic dehydrogenase (LDH) levels, fever, and age (over 12 years) [13]. Additionally, relapse occurs in approximately 30% of patients, for which treatment options are limited and typically unsuccessful [11]. Although these factors do not represent formal clinical grading, they significantly shape the ov-

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erall prognosis and clinical profile of ES patients [13]. According to some studies, grading the histological response to chemotherapy, a critical predictor of prognosis in ES, is significant. Tumor necrosis following preoperative chemotherapy is graded into four categories: grade I (necrosis: ≤50%), grade II (50%-90%), grade III (90%-99%), and grade IV (100%) [14, 15]. Higher grades of necrosis strongly correlate with improved event-free and overall survival rates [14-16]. For instance, Wunder et al. reported a 5-year event-free survival rate of 84% for patients with grade III/IV responses compared to 0% for those with grade I responses. Tumor size and surgical margins are also significant predictors of event-free survival in ES patients [15]. The treatment approach for ES varies depending on its stage and location. For localized ES, a multidisciplinary approach integrating chemotherapy, surgery, and radiotherapy is typically recommended [17]. When complete surgical resection with clear margins is feasible, surgery alone may suffice. Radiotherapy serves as an alternative or adjunct in cases where surgery is contraindicated [18]. Chemotherapy regimens incorporating alkylating agents and topoisomerase-II inhibitors such as vincristine, doxorubicin, cyclophosphamide, ifosfamide, and etoposide, are standard for both localized and metastatic ES [19, 20]. However, these drugs induce nonspecific damage DNA, leading to severe side effects. This highlights the need for novel therapeutic strategies targeting specific molecular drivers of ES. Recent research has attempted to explore the molecular landscape of ES to identify new therapeutic targets.

EWS-FLI1: exclusive yet untargetable

ES is characterized by the hallmark t(11; 22) (q24; q12) translocation, which fuses *EWSR1* with *FLI1*, driving pathogenesis in 85%-90% of cases [20]. The resultant EWS-FLI1 fusion protein acts as an aberrant transcription factor, reprogramming gene expression to promote uncontrolled cell proliferation, resistance to apoptosis, and inhibition of differentiation [21-23]. The EWS-FLI fusion protein is expressed in all stages of ES [24] and binds non-canonical GGAA-microsatellite sequences in DNA, altering the expression of genes involved in cell cycle regulation, DNA damage repair, and metabolic pathways [25, 26]. Preclinical studies have demonstrated that silencing or reducing EWS-FLI1 expression using RNA interference or CRISPR-Cas9 gene editing results in significant tumor regression both in *vitro* and in *vivo* models. EWS-FLI1 not only drives oncogenesis but also plays a pivotal role in maintaining the malignant phenotype of ES [27, 28]. Although efforts have been made to inhibit EWS-FLI1 transcriptional activity, disrupt its interactions with partner proteins, or degrade its mRNA using antisense oligonucleotides and siRNA, these methods have shown limited success clinically. EWS-FLI-1 is involved in the direct transcription of several crucial oncogenic and tumor-suppressive genes. The EWS-FLI1 protein upregulates genes such as cyclin D1 [29] (involved [30] in the G1/S cell cycle transition) [30]; c-Myc [30] cell receptors such as VEGF [31], IGF-1R [32], and CAV-1 [33] and the growth factor PDGF-C [34]. These factors collectively drive tumor proliferation, angiogenesis, and intracellular signaling. High cyclin D expression in tumors is associated with poor patient outcome [31, 34]. CAV-1 regulates numerous intracellular signaling pathways [33]. IL1RAP, a direct target highly expressed in ES, helps in maintaining the cystine and glutathione pool within cells [35]. EWS-FLI1 downregulates the expression of key tumor suppressor genes such as FOXO1 [36], $p21^{WAF1/CIP1}$ [37], $P57^{KIP2}$ [30] PHLDA1 [38], SPRY [39], and TGF-RII [40]. In many tumors, FOXO1 downregulation is linked to loss of tumor suppressor activity and increased neoplastic potential. FOXO1 also participates indirectly in cell cycle regulation by downregulating the expression of cyclin D1, which is highly expressed in ES [36]. $P57^{KIP2}$ and $P21^{WAF1/CIP1}$ are cell cycle-dependent kinase inhibitors, which are downregulated in ES [30, 37]. According to in *vitro* and in *vivo* studies, PHLDAI exhibits anti-apoptotic activity [38]. In ES, SRPY1 is indirectly downregulated through promotor methylation [39] (**Figure 1**).

Targeting EWS-FLI1 directly has proven challenging due to several intrinsic factors. The protein's large size complicates in *vitro* study [41]. Furthermore, EWS-FLI1 is predicted to be an intrinsically disordered protein, lacks a stable three-dimensional structure, making it insoluble and difficult to analyze it structurally. The structure of EWS-FLI1 is unavailable in the Protein Data Bank, further hindering structure-guided design. Moreover, it does not have specific Ramachandran angles in its backbone and exhibits polymorphism in the bound state [42].

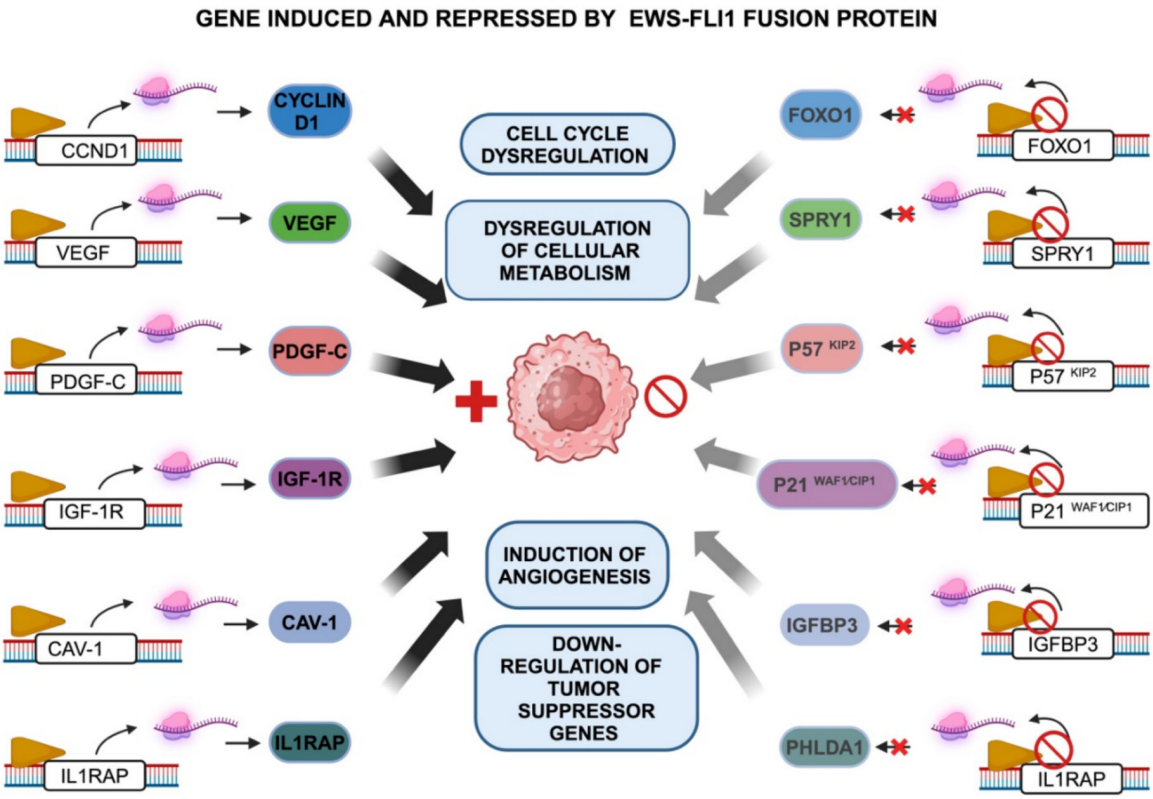

Figure 1. Graphical illustration of genes induced and repressed by the EWS-FLI1 fusion protein. "Created in BioRender. J, M. (2025) https://BioRender.com/b08o291".

These characteristics hinder the development of drugs specifically targeting EWS-FLI1. To accelerate drug discovery, computer-aided design tools have been used to predict the protein structure, related genes, and pathways [43, 44]. However, the benefits of these computational advancements and virtual drug design were limited, as many of the developed structure-guided drugs led to severe toxic side effects. YK-4-279, a small molecule inhibitor, disrupts the interaction between RNA helicase A and EWS-FLI, effectively limiting tumor progression. However, the therapeutic potential of YK-4-279 in patients remains under investigation [45]. The development of ES mouse models has also faced hurdles, as EWS-FLI1 expression often results in embryonic lethality or developmental defects, complicating in vivo studies [46, 47]. Meanwhile, a more promising approach is targeting of downstream pathways of EWS-FLI1. Several potential downstream targets of EWS-FLI1, such as insulin-like growth factor 1 receptor (IGF1R), poly (ADP-ribose) polymerase (PARP), and the Wnt/β-catenin signaling pathway, have been identified [48, 49]. These pathways promote tumor growth and survival, but they represent promising avenues for alternative therapies aimed at mitigating disease progression. This review elaborates on the emerging alternative therapeutic targets, exploring their potential to offer new perspectives and improve treatment efficacy in comparison to established therapy.

Limitations of current standard therapies

Current treatment protocols for ES involve multi-agent chemotherapy, including doxorubicin, vincristine, cyclophosphamide, ifosfamide, and etoposide. In a clinical trial involving children with cancer who were treated with doxorubicin, vincristine, cyclophosphamide, ifosfamide, and etoposide, no significant difference was observed between the dose-intense treatment group and normal dose treatment group [50]. Adding ifosfamide and etoposide to the doxorubicin vincristine cyclophosphamide regime improved outcome in non-metastatic ES, but not in metastatic ES [19]. Dose-intensified chemotherapy with vincristine, doxorubicin,

and cyclophosphamide along with ifosfamide and etoposide exhibited increased efficacy and reduced toxicity compared with the vincristine, ifosfamide, doxorubicin, and etoposide regime [51]. YK-4-279 sensitizes ES cells to vincristine [52]. Most anticancer drugs are associated with adverse effects. Doxorubicin, an anthracycline family of anticancer drug is a cornerstone of ES therapy, exhibits its anticancer activity by inhibiting topoisomerase II but causes severe cardiotoxicity [53], particularly in patients with pre-existing heart conditions such as hypertension and myocardial infraction (MI). Liposomal formulations of doxorubicin significantly reduce adverse effects than free doxorubicin but remain suboptimal [54]. Most combination treatment regimes for ES involve doxorubicin as a key agent. Hence, a suitable alternative drug with limited adverse effects and higher efficacy must be identified for ES treatment. Many clinical trials involving combination therapies have achieved only limited success as they have often resulted in several adverse effects such as cytotoxicity, and systemic, hematological, and renal toxicities (**Table 1**). Hence, we intend to suggest some of the emerging therapeutic targets as an alternative.

Emerging alternative therapeutic targets

In the pursuit of novel therapeutic approaches for ES, an in *silico* differential gene expression analysis was conducted using ES cell-line and tumor datasets from the Gene Expression Omnibus (GEO) [GSE132966, GSE17674]. The analysis, performed with GEO2R using the criteria of $P < 0.01$ and |log2 fold-change|> 1,with DESeq2 software package which uses negative binomial distribution that identified upregulated genes, including Structure-Specific Recognition Protein 1 (SSRP1), Forkhead Box Protein M1 (FOXM1), Nuclear Receptor Subfamily 0 Group B Member 1 (NR0B1), androgen receptor (AR), transcription factor JUN (JUN), Murine Double Minute 2 (MDM2), Ephrin Type-A Receptor 2 (EphA2), Paired Box 5 (PAX5), and Polo-Like Kinase 1 (PLK1), in either or all of the differential transcriptome data from TC-71, A-673, and ES tumors. This review explores these major upregulated genes, their roles in ES, and their potential as alternative therapeutic targets based on research from the past decade (**Figure 2**).

Facilitates Chromatin Transcription (FACT) complex

SSRP1, a key subunit of the FACT complex, is overexpressed in many cancers [55]. Earlier studies reported that SSRP1 is expressed only in fetal rat kidneys and renal cell carcinoma but not in adult kidneys [56]. However, recent reports have demonstrated that FACT is highly expressed in the cancer tissues of most patients compared with normal tissues [55, 57]. SSRP1 is significantly overexpressed in human ovarian cancers relative to normal ovarian tissues [58]. FACT expression is higher in human and mouse tumor cell lines than in normal human and mouse cell lines [59, 60]. Prognosis is poor in pediatric patients with neuroblastoma who exhibit elevated FACT complex expression, which indicates the significance of the FACT complex in tumor prognosis [61]. In hematological malignancies, inhibiting this complex led to apoptosis and decreased cell cycle progression by downregulating WNT and Hedgehog pathways [62]. Interestingly, FACT inhibition was also used as a treatment strategy in ES. Specifically, curaxins, SSRP1 inhibitors, have shown efficacy in ES, inducing DNA damage, cell death, and suppression of EWS-FLI1-related gene expression [22, 63, 64]. While these findings are encouraging, further research is required to solidify the therapeutic potential of FACT complex inhibitors in ES.

FOXM1

FOXM1 is overexpressed in numerous cancers, including carcinomas and sarcomas [65-78]. FOXM1 activity is regulated by phosphorylation [79], ubiquitination, SUMOylation [80], acetylation [81] and methylation. FOXM1 regulates cell proliferation, migration, metastasis, and angiogenesis [82]. Its dysregulation is implicated in tumorigenesis and cancer progression, with high FOXM1 expression correlating with poor prognosis across multiple malignancies [83]. *FOXM1* knockdown significantly increases cancer cell sensitivity to chemotherapeutic and targeted agents, including thiostrepton, honokiol, bortezomib, siomycin A [84] curcumin, SR-T100, FDI-6 [85], RCM-1, and DFS lignan [81, 85-88]. In ES, FOXM1 overexpression supports cell cycle regulation, and its inhibition significantly reduces tumor cell proliferation [88].

Table 1. Summary of the outcomes of clinical trials conducted between 2014 and 2024

S.No.	PHASE	STUDY DESIGN	FINDINGS	TARGET/MODE OF ACTION	Drug	CLINICAL ACTIVITY (OBSERVATION)	REFERENCE
1	-	Evaluating the age-related toxicity of Busulfan-Melphalan (BuMel) compared to conventional chemotherapy Vincristine Actinomycin-D Ifosfamide (VAI)	Severe acute toxicity was observed in all groups of patients and was more common in younger patients.	Alkylating agent Microtubule assembly disruption Transcription inhibitor Alkylating agent	Busulfan-Melphalan Vs Vincristine Actinomycin-D Ifosfamide	No (Severe toxicity)	[163]
2	Phase II	Non-comparative, randomized, double-blind, placebo-controlled, multicenter trial to determine the efficacy and safety of regorafenib with relapsed disease	Trial results suggested that Regorafenib might modestly delay tumor progression.	Multikinase inhibitor	Regorafenib	Yes	[164]
3	Phase Ib	Open-label, dose expansion study to assess the safety and maximum tolerance dose (MTD) recommends the dose for Phase II clinical trial and to evaluate the pharmacokinetics (PK) of Regorafenib and Irinotecan	Regorafenib combined with Vincristine and Irinotecan showed clinical activity in patients with EWS.	Multikinase inhibitor Microtubule assembly disruption Topoisomerase I inhibitor	Regorafenib Vincristine Irinotecan	Yes	[165]
4	Phase II	Open-label, non-randomized, study of Palbociclib and Ganitumab in patients with relapsed EWS	The combination lacked adequate therapeutic activity.	CDK4/6 inhibitor IGF1 Antagonist	Palbociclib Ganitumab	No	[166]
5	Phase I	Assessing the MTD of Simvastatin with Topotecan and Cyclophosphamide	Hematologic toxicity was observed.	HMG CoA reductase inhibitor Topoisomerase 1 inhibitor Alkylating agent	Simvastatin Topotecan Cyclophosphamide	No	[167]
6	Phase III	Assessing the effect of Ganitumab added to interval-compressed chemotherapy	No significant change in outcome compared to a previous study with the IGF1 inhibitor. Increased toxicity was observed with an increased dose of Ganitumab.	IGF1 Antagonist Alkylating agent Topoisomerase II inhibitor Microtubule assembly disruption	Ganitumab Vincristine Doxorubicin Cyclophosphamide Ifosfamide Etoposide	No	[168]
7	Phase II	Randomized controlled study to assess the response rate and safety of long vs short schedule VIT	No significant survival benefit was identified, but long VIT schedule produced a superior response rate compared to the shorter VIT schedule.	Microtubule assembly disruption Topoisomerase 1 inhibitor Alkylating agent	Vincristine Irinotecan Temozolomide	Inconclusive	[169]
8	Phase III	Open-label, randomized trial assessing the efficacy of European versus US regimen	Dose-intensive chemotherapy with the US regimen was more effective, less toxic, and shorter in duration.	Microtubule assembly disruption Alkylating agent Topoisomerase II inhibitor Transcription inhibitor	Vincristine Ifosfamide Doxorubicin Etoposide Actinomycin-D Cyclophosphamide Busulfan-Melphalan	Yes (Comparative study between two regimens)	[51]
9	-	Assessing the late toxicity of alkylating agent-based regime	Persistent kidney toxicity and gonadal toxicity were observed in majority of patients.	Microtubule assembly disruption Transcription inhibitor Alkylating agent	VAC-Vincristine sulfate Actinomycin-Dcyclophosphamide VAI-VincristineActinomycin-D Ifosfamide	Inconclusive	[170]
10	-	Assessing the safety and response at RP2D of Ipilimumab and testing the combination with Nivolumab.	Long exposure to these drugs increases toxicity and leads to adverse effects.	Anti-PD1 receptor antibody Anti-CTLA-4 antibody	Nivolumab Ipilimumab	Yes (Partial response)	[171]

11	Phase I	Assessing the MTD, toxicity, pharmacokinetics and determining the RP2D for Metformin in combination with Vincristine, Irinotecan, and Temozolomide in children with relapsed or refractory solid and central nervous system tumors	The MTD was not determined due to study closure with less than six patients enrolled at Dose Level 4. Hematological and gastrointestinal toxicities were observed.	mTORC1 inhibitor Microtubule assembly disruption Topoisomerase 1 inhibitor Alkylating agent	Metformin Vincristine Irinotecan Temozolomide	Inconclusive	[172]
12	Phase II	Assessing the progression-free survival rate for Regorafenib	Regorafenib exhibited modest activity in the Ewing family sarcomas.	Multikinase inhibitor	Regorafenib	Yes (Modest activity)	[173]
13	Phase I	Open-label, multicenter, dose-escalation study of TB-403 to determine the efficacy, MTD, and PK of TB-403.	Good tolerance was observed for TB-403	Anti-PlGF antibody	TB-403	Inconclusive	[174]
14	Basket Phase II	Open-label, single-arm study to assess the MTD of Lurbinectedin.	Lurbinectedin is safe and showed clinical activity.	Transcription inhibitor	Lurbinectedin	Yes	[175]
15	Phase III	Assessing the efficacy and toxicity of two therapy arms of the standard four-drug therapy [VAIA] versus six-drug regimen [CEVAIE].	An intensified six drug regimen showed no significant improvement than standard four-drug therapy.	Alkylating agent Microtubule assembly disruption Topoisomerase II inhibitor.	VAIA-Vincristine Ifosfamide Adriamycin Dactinomycin CEVAIE-Carboplatin Epirubicin VincristineDactinomycin Ifosfamide Etoposide	No	[176]
16	Phase III	Open-label, prospective, multicenter, randomized controlled clinical trial. Eligible patients had disseminated EWS with metastases to the bone and/or other sites, excluding patients with only pulmonary metastases	In patients with very high-risk EWS, additional TreoMel-HDT was of no benefit for the entire cohort. TreoMel-HDT may have benefitted children aged < 14 years.	Alkylating agents	Treosulfan Melphalan	No	[177]
17	Phase III	Randomized trial to test whether the addition of Vincristine, Topotecan, and Cyclophosphamide (VTC) to interval-compressed chemotherapy improved survival outcomes for patients with previously untreated nonmetastatic ES	While VTC added to five-drug interval compressed chemotherapy did not improve survival, these outcomes represent the best survival estimates to date for patients with previously untreated nonmetastatic ES.	Microtubule assembly disruption Topoisomerase 1 inhibitor Alkylating agent	Vincristine Topotecan Cyclophosphamide	No	[178]
18	Phase Ib/II	In total, 41 patients finally received the treatment regimen, including 29 in cohort A and 12 in cohort B. For cohort A, the first five patients were treated at the initial level of 20 mg/m^2/d d × 5 × 2, and two of them subsequently had a dose-limiting toxicity (DLT). An additional six patients were then treated at 15 mg/m^2 without any DLT, and RP2D was determined.	The combination of Vincristine, Irinotecan, and Anlotinib demonstrated an acceptable toxicity profile and promising clinical efficacy in patients with advanced EWS.	Multi-kinase inhibitor Microtubule assembly disruption Topoisomerase I inhibitor	Anlotinib Vincristine Irinotecan	Yes	[179]
19	Phase I	Assessing the MTD, PK, and PD and recommend the phase II dosage	All patients showed progression in first two cycles, except one patient with ependymoma with stable disease.	HDAC inhibitor	Entinostat	Yes	[180]
20	Phase I	Non-randomized, open-label study evaluated the DLT, safety, PK, and antitumor activity of ASP3026.	ASP3026 at a 200-mg dose may provide therapeutic benefit for patients with solid tumors.	ALK inhibitor	ASP3026	Yes (Partial response)	[181]

21	-	Evaluating the safety and dosing of the PARP1/2 inhibitor Niraparib (NIR) with Temozolomide (TMZ; arm 1) or Irinotecan (IRN; arm 2)	The combination of NIR and TMZ or IRN was tolerable, at lower doses in comparison with conventional cytotoxic combinations.	PARP Inhibitor Alkylating agent Topoisomerase I inhibitor	Niraparib Temozolomide Irinotecan	Yes	[182]
22	Phase I	Cohort study assessing Talazoparib and Irinotecan with/without Temozolomide in pediatric patients with recurrent/ refractory solid malignancies	The combination of Talazoparib and Irinotecan with/without Temozolomide is feasible and ES.	PARPi Topoisomerase I inhibitor Alkylating agent	Talazoparib Irinotecan Temozolomide	Yes	[183]
23	Phase II	Pediatric patients with recurrent or refractory ES, neuroblastoma, or rhabdomyosarcoma received 240 mg/m^2 of nab-paclitaxel on days 1, 8, and 15 of each 28-day cycle.	Limited activity was observed; however, the safety of nabpaclitaxel in pediatric patients was confirmed.	Interfering with microtubule dynamics Anticancer activity	nab-paclitaxel	Yes (Limited activity)	[184]
24	Phase II	Using a Simon's two-stage design to identify a response rate of ≥ 35%, patients received nab-paclitaxel 125 mg/m^2, followed by Gemcitabine 1000 mg/m^2 i.v. on days 1, 8, and 15 of 4-week cycles.	Only one patient had a partial response that was confirmed on subsequent imaging,	Deoxycytidine analog	Gemcitabine nab-paclitaxel	Yes (Partial response)	[185]
25	-	Multicenter, open-label, single-arm, dose-confirmation and dose-expansion, phase 1-2 trial in 23 hospitals. The primary outcomes were the tolerability, systemic exposure, MTD, and the antitumour activity of Nivolumab at the adult recommended dose in children and young adults.	Nivolumab was safe and well tolerated in children and young adults and showed clinical activity in lymphoma. Nivolumab showed no significant single-agent activity.	Anti-PD1 antibody	Nivolumab	No	[186]
26	Phase II	Multicenter, single-arm, two-stage, phase 2 trial in patients with advanced ES or osteosarcoma recruited from 10 centers in the French Sarcoma Group. Key eligibility criteria were age of ≥12 years.	Cabozantinib has antitumor activity in patients with advanced ES and osteosarcoma and was generally well-tolerated.	Receptor tyrosine kinases inhibitor	Cabozantinib	Yes	[187]
27	Phase II	Determining the efficacy and safety of multimodal treatment including standard chemotherapy with Vincristine, Doxorubicin, Cyclophosphamide, Ifosfamide, and Etoposide.	Multimodal treatment with standard VDC-IE chemotherapy improved the prognosis but statistical confirmation of efficacy compared to historical control was not achieved.	Microtubule assembly disruption Topoisomerase II Inhibitor Alkylating agent Topoisomerase II Inhibitor	Vincristine Doxorubicin Cyclophosphamide Ifosfamide Etoposide	No	[188]
28	Phase I	Determining the MTD, toxicities, and response of Sirolimus combined with oral metronomic therapy in pediatric patients with recurrent and refractory solid and brain tumors.	The results showed good tolerance in children. The recommended phase II dose of Sirolimus was 2 mg/m^2.	mTOR complex inhibitor	Sirolimus	Yes	[189]
29	Phase I	3+3 escalation design, five-dose cohorts of the combination of Adavosertib and Irinotecan were studied. PK and analysis of peripheral blood γH2AX were performed.	Adavosertib (85 mg/m^2) in combination with Irinotecan (90 mg/m^2) administered orally for 5 days was the MTD.	WEE1 inhibitor Topoisomerase I inhibitor	Adavosertib Irinotecan	Yes	[190]
30	Phase I/II	Assessing the DLT, RP2D, and PK of the poly (ADP-ribose) polymerase 1/2 inhibitor Talazoparib in combination with low-dose Temozolomide	No antitumor activity was observed in ES.	Alkylating agent	Talazoparib Temozolomide	No	[191]

31	-	Cohort study evaluating the effect of an intensified pilot protocol, SCMCIE 94.	5-year EFS and OS were significantly improved in localized disease. No survival benefit was found for metastatic disease.	Microtubule assembly disruption Transcription inhibitor Alkylating agent Topoisomerase II inhibitor	Vincristine Cyclophosphamide Actinomycin-D Doxorubicin Ifosfamide Etoposide	Yes	[192]
32	Phase I	Assessing the PK and MTD and analyzing exploratory biomarkers in children with refractory solid tumors.	MTD of Axitinib was 2.4 mg/m^2/dose.	tyrosine kinase inhibitor	Axitinib	Yes (Stable disease in ES)	[193]
33	Phase I	Determining the MTD and RP2D of nab-paclitaxel in patients with recurrent/ refractory extracranial solid tumors.	nab-paclitaxel 240 mg/m^2 qw3/4 was determined as the RP2D	Interfering with microtubule dynamics	nab-paclitaxel	Yes	[194]
34	Phase I	Determining DLT, RP2D, and PK of Eribulin in children with refractory or recurrent solid tumors.	The RP2D of Eribulin was 1.4 mg/ m^2/dose on days 1 and 8 of a 21-day cycle.	Microtubule inhibitor	Eribulin mesylate	Yes (Partial response)	[195]
35	Phase I	Determining the MTD, PK, PD, and preliminary activity of Cabozantinib in children with refractory or relapsed solid tumors.	The recommended dose of cabozantinib in pediatric patients with refractory solid tumors was 40 mg/m^2/day.	Tyrosine kinase inhibitors	Cabozantinib	Inconclusive	[196]
36	Phase I	Assessing the MDT and determining the recommended dose for Phase II trial.	The PK of Ontuxizumab in children was not significantly different from that in adults.	Anti-alpha 4 integrin antibody	Ontuxizumab	Inconclusive	[197]
37	Phase II	Two-cohort, single-arm, open-label study to assess the overall response of Pembrolizumab.	Pembrolizumab had no activity in ES, which could be related to the highly suppressive immune microenvironment in tumors.	PD-1 receptor inhibitor	Pembrolizumab	No	[198]
38	Phase II	Determining the efficacy of Gemcitabine and Docetaxel (G/D) in newly diagnosed high-risk patients.	G/D regimen provides clinical benefit to newly diagnosed HR-ES patients.	Deoxycytidine analog	Gemcitabine Docetaxel	Yes	[199]
39	Phase I/II	Determining the DLT, MTD PK, and activity of Mithramycin in children with refractory solid tumors.	Hepatotoxicity precluded the administration of a Mithramycin at a dose required to inhibit EWS-FLI1.	Transcription inhibitor	Mithramycin	No (Hepatotoxicity)	[200]
40	Phase I	Evaluating the safety and tolerability of Perifosine monotherapy in pediatric patients with recurrent or refractory CNS and solid tumors .	The recommended Phase II dose was 50 mg/m^2/day	c-Akt inhibitor	Perifosine	Inconclusive	[201]
41	Phase II	Assessing the safety and clinical efficacy of Robatumumab in resectable osteosarcoma, unresectable osteosarcoma, and ES metastases.	Low response rate with metastatic disease.	Anti-insulin like growth factor receptor-1 antibody	Robatumumab	Yes (Low response rate)	[202]

Table illustrates the details of clinical trials conducted in the last 10 years with study design, and outcome and therapeutic drug response observed in ES patients treated with various drugs and drug combinations. Majority of the Phase I and II trials (21 out of 36) have demonstrated a clear clinical activity. However, majority of Phase III trials (4 out of 5) have failed to exhibit a proven therapeutic efficacy.

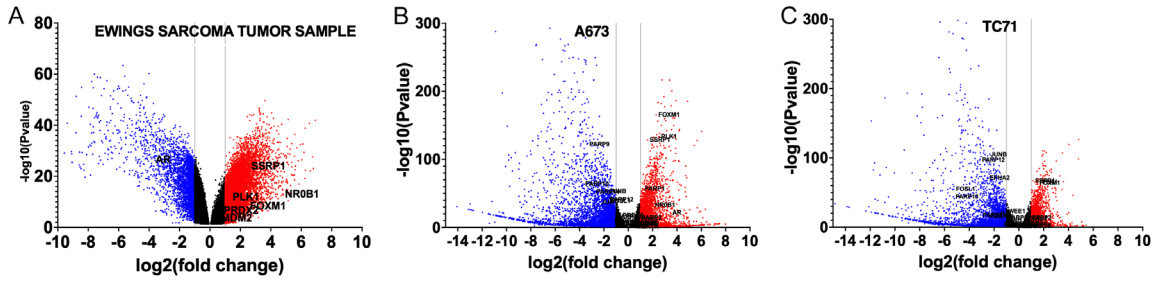

Figure 2. Volcano plot depicting the upregulated (red), downregulated (blue), and nonsignificant (black) genes in (A) ES tumors, (B) A-673, and (C) TC-71, with $P < 0.01$ and $|\log2 \text{ fold-change}| > 1$.

NR0B1 (DAX1)

In cervical cancer tissues, Dax1 protein expression is approximately four times higher than in normal cervical tissues. Dax1 plays a significant role in tumor prognosis by activating the Wnt/β-catenin pathway and exhibits a positive correlation with its target genes. Inhibiting DAX1 suppresses the Wnt/β-catenin pathway, thereby reducing cancer progression in cervical cancer [89]. DAX1 is also a direct target of EWS-FLI1 and is critical in regulating cell cycle progression in ES [90]. Studies have indicated that ectopic expression of EWSR1-FLI1 increases NR0B1 levels in various cell types, while its inactivation decreases NR0B1 expression [91]. Functional studies have further revealed that DAX1 is upregulated in ES, and silencing it with siRNA leads to reduced DAX1 mRNA and protein levels [92]. NR0B1 expression is crucial for maintaining the growth and tumorigenic potential of ES cells, highlighting its importance in ES development and progression [93]. A study demonstrates that NR0B1 is tightly linked to Nuclear factor erythroid-2-Related Factor 2 (NRF2) activation, with NR0B1 downregulation significantly inhibiting cancer cell growth. NR0B1 interacts with other proteins in an NRF2-dependent manner. Targeting a specific cysteine residue (C274) within NR0B1 using covalent ligands like BPK-26 and BPK-29 disrupts these interactions, effectively suppressing cancer cell proliferation. This novel approach, demonstrated in non-small-cell lung cancers, highlights the potential of selectively targeting NR0B1 for cancer therapy [94]. NR0B1 activation is primarily driven by histone modifications, particularly histone H4 acetylation and the removal of histone H3K9 and H3K27 methylation [95]. Consequently, epigenetic therapies such as the histone deacetylase (HDAC) inhibitor such as Vorinostat [96] and histone methyltransferase inhibitors may hold therapeutic promise. However, resistance to epigenetic therapies can develop in cancer cells. Combining these therapies with other targeted treatments or immunotherapies might help overcome such resistance.

Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2)

Angiogenesis is crucial for tumor cell proliferation within the tumor microenvironment. Studies have demonstrated that inhibiting ES family tumors with anti-VEGFR-2 agents shows promise as a standalone treatment for ES or in combination with other drugs [97]. VEGFR-2 inhibition significantly decreases tumor growth and microvessel density [98, 99]. A combination treatment study evaluated the effects of Adnectins CT-322, a VEGFR-2 inhibitor, and AT-580Peg40, an IGF1 inhibitor. The results showed a significant reduction in tumor growth, vessel density, and angiogenesis, with a pronounced antitumor effect when both pathways were simultaneously inhibited compared with either inhibitor alone. Further analysis unveiled that this combinatorial inhibition of IGF-1R and VEGFR2 led to the downregulation of IGF-binding protein 2 and compensatory upregulation of VEGF levels. Immunohistological analysis also indicated a normalized tumor vasculature, characterized by increased intervascular and inter-branching distances [100].

AP-1

Inhibiting RRM1, a subunit of ribonucleotide reductase, has been shown to upregulate Activator Protein-1 (AP-1) transcription factors. SLFN11, a direct transcriptional target of EWS-FLI1, is highly expressed in ES and upregulates AP1 expression in ES [101, 102]. AP-1 plays a critical role in regulating cellular processes

such as proliferation, migration, differentiation, and survival. AP-1 activity dysregulation is implicated in cancer and inflammatory diseases. Its activity is tightly regulated by upstream signaling pathways, primarily mitogen-activated protein kinases (MAPKs) and NF-κB pathways, which modulate AP-1's transcriptional functions. AP-1 is a key inducible transcription factor composed of proteins from the Fos, Maf, Jun, and ATF families. AP-1 has emerged as a promising therapeutic target for suppressing tumor progression and improving cancer therapy outcomes [103]. AP-1 affects tumorigenesis in colorectal cancer cells in *vitro* and in *vivo* through the vimentin promoter, broadening its potential in targeted therapy [104]. A multi-omics approach has disclosed diverse downstream effects of AP-1, suggesting that targeting individual AP-1 subunits based on their specific functions could enhance the precision and effectiveness of oncogenic inhibition. However, the lack of specific inhibitors remains a limitation [105]. A small molecule inhibitor T-5224, which disrupts c-Fos/AP-1 binding to DNA, has shown promise in preclinical cancer models, including preventing metastasis in head and neck cancers [106]. However, AP-1's dual role as an oncogene and tumor suppressor necessitates carefully designed therapeutic strategies. Combining AP-1 inhibition with other targeted therapies, such as PARP [107] or cyclin-dependent kinase (CDK) 4/6 [108] inhibitors, might augment efficacy and overcome resistance mechanisms. In ES, exploring AP-1's role in immune checkpoint regulation and its interactions within the tumor microenvironment [109, 110] highlights its potential as a target in combination therapies, particularly for enhancing responses to immunotherapy or other molecular-targeted treatments.

WEE1

WEE1 Overexpression is observed in many cancers [111, 112]. WEE1 is a pivotal player in cell cycle regulation that controls the G2-M checkpoint. Its inhibition forces cells into premature mitosis, leading to cell cycle arrest at the G2 phase [113]. WEE1 kinase catalyzes the inhibitory phosphorylation of CDK1 (Cdc2), thereby preventing inappropriate mitotic entry in response to DNA damage [114]. WEE1 is an attractive drug target in ES because tumor cells often rely heavily on G2 checkpoint repair mechanisms due to frequent p53 mutations that impair G1 checkpoint control [115]. The WEE1 inhibitor AZD1775 (previously MK-1775) has shown promise in preclinical studies, exhibiting synergy with DNA-damaging agents such as carboplatin, cytarabine, and PARP inhibitors. This synergy is achieved through mitotic catastrophe and enhanced sensitivity of tumor cells to chemotherapy [116]. Preclinical data also suggest that WEE1 inhibitors disrupt DNA damage repair mechanisms in various cancers, including ES cells, which are highly dependent on S-phase kinase activity [117]. In ES, the dual inhibition of Dbf4-dependent kinase and WEE1 induces premature mitotic entry, resulting in mitotic catastrophe and subsequent apoptosis [118]. This combination strategy significantly reduces the viability of ES cells. Moreover, WEE1 inhibition sensitizes tumor cells to antimetabolite chemotherapeutics, independent of their p53 status, laying a strong foundation for its clinical use in combination therapies [118]. However, further studies are warranted to optimize inhibitor selectivity and dosing to enhance therapeutic efficiency and minimize adverse effects.

MDM2

ABCA6 overexpression has been implicated in various cancers such as lung cancer [119], acute myeloid leukemia (AML) [120] and aggressive ES. In ES, ABCA6 influences malignancy by increasing MDM2 expression through cholesterol-mediated inhibition of the IGF1R/AKT/MDM2 axis, which elevates intracellular cholesterol levels and reduces p53 expression [121]. The p53 tumor suppressor-regulated cellular stress response pathway plays a critical role in maintaining genomic integrity and preventing oncogenic transformation [122]. p53 activity is tightly autoregulated by MDM2, an E3 ubiquitin ligase that promotes the proteasomal degradation of p53 via ubiquitination [123]. MDM2 overexpression is frequently observed in various tumors and is associated with reduced p53 factivity. While this may drive oncogenesis, the prognostic significance of MDM2 expression varies across tumor types [124]. While MDM2 possesses oncogenic potential in certain malignancies, evidence suggests that MDM2 overexpression is associated with poor prognosis or unexpectedly, correlates with better outcomes in other types of malignancies. These variabilities therefore underline

the complexity of MDM2 use as a biomarker [124]. Targeting the p53-MDM2 interaction, especially in wild-type p53 cancers, is a promising therapeutic strategy [125].

Several MDM2 inhibitors, such as RG7112, idasanutlin, and AMG-232, are under clinical investigation and have shown potential in reactivating p53-mediated tumor suppression [126]. However, the regulation of MDM2, including its splice variants, remains complex and necessitates cancer-specific therapeutic approaches [127]. In ES, MDM2 gene amplification has been reported in approximately 10% of cases, both in primary tumors and metastases [128]. This genetic alteration often occurs together with the co-amplification of the CDK4 gene, disrupting cell cycle control mechanisms [128]. Small molecules such as RITA (NSC-652287) can reactivate p53, induce apoptosis, and effectively target ES cell lines. RITA also downregulates IGF-1R, a critical factor in tumor cell survival and growth [129]. The continued development and clinical exploration of MDM2 inhibitors like RG7112 and idasanutlin highlight their potential as targeted therapies for ES, especially in cases involving MDM2 amplification [126].

Peroxiredoxins (PRDXs)

PRDX are a family of thiol-specific antioxidant enzymes that protect cells by scavenging of H_2O_2, alkyl hydroperoxides, and peroxynitrites [130]. PRDXs are key components of redox signaling pathways and contribute to the pathophysiology of various diseases, including cancer [131]. Their roles are context-dependent, with isoforms acting either as tumor-suppressors or oncogenes. While their protective functions in cardiovascular and neurological diseases are well-established, PRDX involvement in cancer is highly complex [132]. Many studies have emphasized that the association between high PRDX levels and increased resistance of cancer cells to reactive oxygen species (ROS), enhancing their survival and potentially contributing to therapeutic resistance [133]. PRDX4 overexpression in various cancers has been linked to tumorigenesis and may serve as a potential diagnostic marker and therapeutic target [134]. In ES, PRDX2 knockdown by siRNA has been shown to suppress cell motility and promote apoptosis, suggesting that PRDX2 contributes to disease progression through the AKT/mTOR signaling pathway [130]. This finding opens new opportunities for targeting PRDX2 in combination treatments. Similar studies in leukemia and gastric cancer have demonstrated that targeting PRDX2 can induce cancer cell apoptosis or differentiation [135, 136].

PARP inhibitors

PARPs are critical enzymes in the DNA damage response, particularly in base excision repair and two DNA double-strand break repair pathways: homologous recombination and nonhomologous end-joining [137]. Inhibiting PARP1, the most studied PARP family member, has emerged as an effective strategy, especially in cancers with BRCA mutations, such as breast and ovarian cancers [138, 139]. Preclinical studies in ES have shown promising results, including complete tumor regression in xenograft models when PARP inhibitors (PARPi) like olaparib are combined with DNA-damaging agents such as temozolomide, radiation, and trabectedin, which disrupts EWS-FLI1 transcription [140]. PARPi enhance the efficacy of radiation and chemotherapy by causing irreparable DNA damage, leading to apoptosis and cell death, particularly in cells with defective DNA repair mechanisms [141]. However, challenges remain in clinical development, such as optimizing dosing, overcoming resistance mechanisms, and managing off-target effects. Refining PARP-targeted therapies, particularly in combination with other agents [142], holds substantial potential for augmenting outcomes in ES patients, especially for tumors that are difficult to remove surgically.

Mer tyrosine kinase (MERTK)

MERTK is an aberrantly expressed TAM family receptor tyrosine kinase implicated in many malignancies, including ES. Studies have demonstrated that MERTK is critical for oncogenic progression, promoting growth factor independence, cell cycle progression, proliferation, resistance to apoptosis, and metastasis [143]. MERTK is expressed in nearly all ES lines and patient samples, and CRISPR screens have confirmed that ES cells rely on MERTK for survival and proliferation. MERTK signaling also facilitates immune evasion by inducing an anti-inflammatory cytokine profile and altering immune cell function [144]. Inhibition of MERTK

reverses these oncogenic properties, as evidenced in preclinical studies using MRX-2843, a selective MERTK inhibitor. MRX-2843 effectively suppresses MERTK phosphorylation and its downstream pathways, showing potent antitumor activity in several ES cell lines. Combination treatments have shown promising results; for example, MRX-2843 combined with B-cell lymphoma 2 (BCL-2) inhibitors, such as venetoclax or navitoclax, exhibited synergistic effects, producing superior therapeutic outcomes compared to single-agent treatments. MERTK further activates key oncogenic pathways, including MAPK, PI3K, and Janus kinase/signal transducers and activators of transcription. These findings underscore the potential of MERTK as a therapeutic target in ES, with ongoing clinical trials exploring MRX-2843 as a monotherapy and in combination with BCL-2 inhibitors to improve outcomes in ES patients [145].

PLK1

PLK1 is a serine/threonine kinase with a critical role in mitotic progression, regulation of cell cycle checkpoints at the G2/M transition, DNA damage response, and cell death pathways. PLK1 expression peaks during the G2/M-phase, where it ensures the accuracy of mitotic events. In many cancers, including ES, PLK1 is frequently overexpressed, leading to disrupted mitotic control and genomic instability [146]. Beyond its cell cycle-related functions, PLK1 has recently been linked to inflammatory and immune responses. PLK1 inhibition has emerged as a promising therapeutic strategy, as inhibitors induce mitotic arrest and apoptosis in cancer cells. However, resistance to these inhibitors and limited clinical success remain significant challenges [147]. In ES, combining PLK1 inhibitors with agents like eribulin has shown promise, inducing cancer cell death via the intrinsic apoptotic pathway [148]. Additionally, targeting PLK1 in conjunction with other protein regulators such as PRC1 has demonstrated specificity in ES, offering new opportunities for combinatorial strategies to overcome resistance and improve therapeutic outcomes by targeting chemoresistant cells [149].

EphA2

EphA2 is a receptor tyrosine kinase highly expressed in ES [150]. Studies have shown a strong association between EphA2 protein and angiogenesis-supporting protein CAV1. The EphA2-CAV1 complex promotes angiogenesis in conditions such as meningiomas [151] and prostate cancer [152]. The Eph/ephrin system has been implicated in various pathological conditions, including cancer, although its mechanisms remain incompletely understood [153]. EphA2 is overexpressed in several cancer types, both in cancer cells and in the stromal elements of the tumor microenvironment, where it is associated with aggressive disease features and poor prognosis [154]. For instance, high EphA2 expression in gastric cancer-associated stromal cells (GCSCs) correlates with increased relapse risk and reduced survival, highlighting its value as a prognostic marker [155]. In ES, EphA2 plays a key role in tumor processes such as angiogenesis and metastasis. Preclinical models have demonstrated the antitumor effects of EphA2 inhibitors, suggesting that targeting EphA2 could effectively address primary tumor growth and metastasis [156]. Further research into the EphA2-ephrin axis in ES and other cancers underscores its potential as a promising therapeutic target [157].

Beta-adrenergic receptors (β-ARs)

A high number of β-ARs are found in ES cells [158]. Of them, β3-ARs are particularly notable for regulating cellular response to oxidative stress. Inhibiting β3-ARs induces ROS-induced cell death. A β-AR blockade has shown potential for improving cancer outcomes, as demonstrated in retrospective analyses and case reports involving reproductive cancers, angiosarcoma, and multiple myeloma [159]. Both genomic and non-genomic pathways, including matrix metalloproteinases, mitogen-activated protein kinase pathways, and oxidative stress, are implicated in β-AR-mediated tumorigenesis [160, 161]. Preliminary data suggest that β3-AR expression in ES tumors and their surrounding stromal cells may serve as a marker for disease recurrence and malignancy in the tumor stroma. These findings highlight the role of β-ARs in ES pathology and suggest β-AR blockers as potential therapeutic agents for future clinical applications [162].

Discussion

Despite extensive research efforts worldwide, no single agent or combination of drugs has been able to directly bind and inhibit the non-physiological chimeric fusion protein associated with ES. This underscores the urgent need

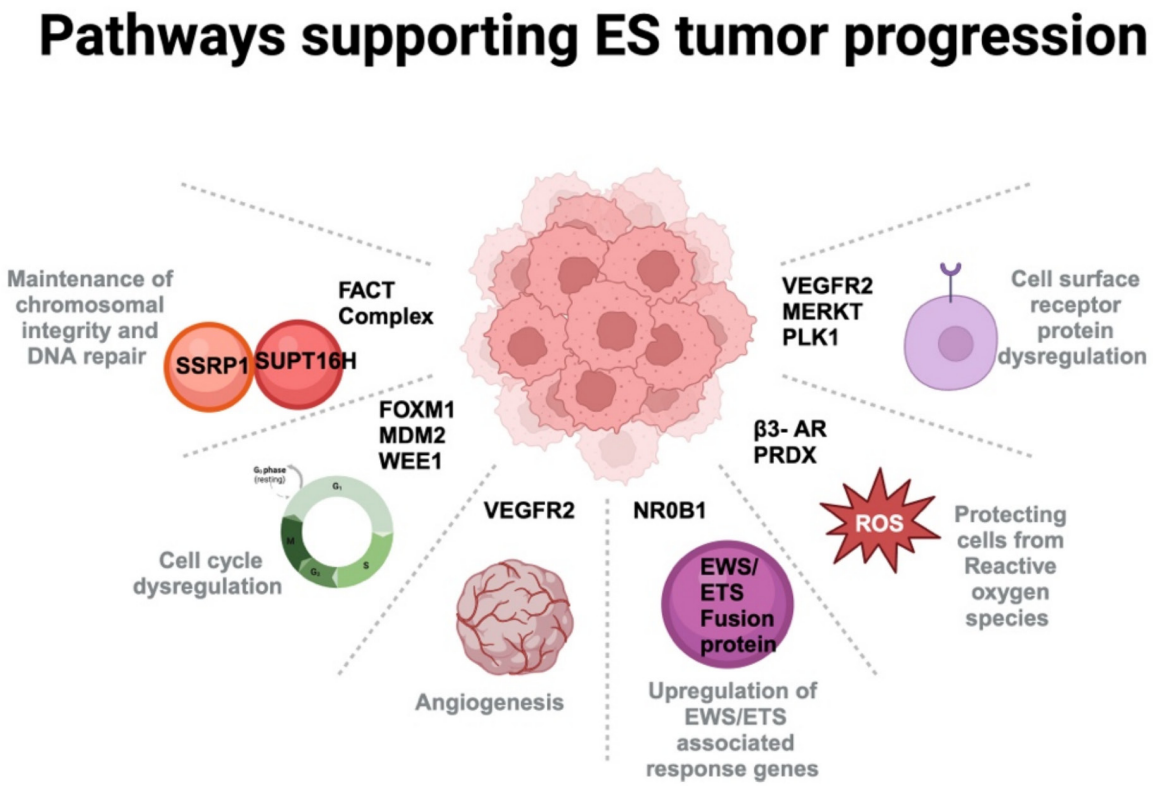

Figure 3. Schematic illustration of pathways involved in ES pathology/progression/tumorigenesis. "Created in BioRender. J, M. (2025) https://BioRender.com/z35u196".

for identifying alternative therapeutic targets that are significantly overexpressed in the presence of EWS-FLI or its altered configurations. These new targets could pave the way for developing functionally specific treatments against ES. This review highlights a list of promising emerging drug targets for ES and critically examines the roles of crucial molecular pathways and proteins, such as SSRP1, FOXM1, NR0B1, and VEGFR2, which contribute significantly to tumor growth and survival (**Figure 3**). Targeting transcription factors such as AP-1 and kinases such as WEE1 and PLK1 has shown potential in disrupting oncogenic processes. Additionally, therapeutic targeting of proteins such as MDM2, PRDX, and MERTK, together with the inhibition of PARP and beta-adrenergic receptors, offers a promising strategy to enhance sensitivity compared to existing treatments. These approaches may also provide a means to overcome drug resistance.

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Disclosure of conflict of interest

None.

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