Signaling and Regulation

Molecular Cancer Research

EWS/FLI1 Regulates EYA3 in Ewing Sarcoma via Modulation of miRNA-708, Resulting in Increased Cell Survival and Chemoresistance

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Abstract

Ewing sarcoma is an aggressive pediatric cancer of the bone and soft tissue, in which patients whose tumors have a poor histologic response to initial chemotherapy have a poor overall prognosis. Therefore, it is important to identify molecules involved in resistance to chemotherapy. Herein, we show that the DNA repair protein and transcriptional cofactor, EYA3, is highly expressed in Ewing sarcoma tumor samples and cell lines compared with mesenchymal stem cells, the presumed cell-of-origin of Ewing sarcoma, and that it is regulated by the EWS/FLI1 fusion protein transcription factor. We further show that EWS/FLI1 mediates upregulation of EYA3 via repression of miR-708, a miRNA that targets the *EYA3* 3′-untranslated region, rather than by binding the *EYA3* promoter directly. Importantly, we show that high levels of *EYA3* significantly correlate with low levels of miR-708 in Ewing sarcoma samples, suggesting that this miR-mediated mechanism of EYA3 regulation holds true in human cancers. Because EYA proteins are important for cell survival during development, we examine, and show, that loss of EYA3 decreases survival of Ewing sarcoma cells. Most importantly, knockdown of EYA3 in Ewing sarcoma cells leads to sensitization to DNA-damaging chemotherapeutics used in the treatment of Ewing sarcoma, and as expected, after chemotherapeutic treatment, EYA3 knockdown cells repair DNA damage less effectively than their control counterparts. These studies identify EYA3 as a novel mediator of chemoresistance in Ewing sarcoma and define the molecular mechanisms of both EYA3 overexpression and of EYA3-mediated chemoresistance. *Mol Cancer Res; 10(8); 1098–108. ©2012 AACR.*

Introduction

Ewing sarcoma is a devastating pediatric cancer of the bone and soft tissue that generally occurs in patients in the second decade of life (1). Ewing sarcomas are characterized by the presence of the nonphysiologic fusion protein transcription factor, EWS/FLI1. This protein results from a chromosomal translocation that brings together the *EWS* gene on chromosome 22, with the *FLI1* gene on chromosome 11 (2), resulting in the fusion of a potent EWS transcriptional activation domain with the FLI1 DNA-binding domain. The EWS/FLI1 fusion protein promotes numerous oncogenic properties, including cell proliferation (3), transformation (4), and *in vivo* tumor growth (5) and is essential to Ewing sarcoma pathogenesis.

Over the past 30 years, outcomes for patients that present with localized disease have improved dramatically. However, the prognosis for patients who present with metastasis, who relapse, or have a poor histologic response to initial therapy, remains poor (6, 7). Indeed, histologic response after preoperative chemotherapy remains a significant indicator of prognosis (7–9). Thus, it is important to understand potential mechanisms of chemoresistance in Ewing sarcoma, in an effort to develop more effective ways to treat this disease. Furthermore, Ewing sarcoma chemotherapeutic treatment regimens are harsh and aggressive, and survivors of Ewing sarcoma are at an especially high risk of death later in life from secondary, treatment-associated malignancies and cardiac dysfunction compared with age-matched, gender-matched controls (10). In addition, it is estimated that 30 years after diagnosis of their primary cancer, 42.4% of childhood cancer survivors exhibit severe, disabling, or life-threatening conditions as a result of their therapy or may even experience death due to long-term complications (11). Therefore, novel therapies targeting mechanisms of chemoresistance in Ewing sarcoma not only have the potential to improve primary disease outcomes but also carry the promise to mitigate late effects associated with treatment toxicities for survivors.

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Although EWS/FLI1 is an attractive target because of its absence in normal cells, there are many challenges to targeting EWS/FLI1 directly. First, the structure of EWS/FLI1 is predicted to be highly disordered (12). Second, the protein has poor solubility because of its overall size. These features make it challenging to determine the structure of EWS/FLI1 and thus rational drug design is difficult. In addition, kinase inhibition has been successful in targeting another nonphysiologic oncogenic fusion protein, BCR/ABL, but the actions of EWS/FLI1 are not dependent on a kinase domain. It is therefore important to understand the role of EWS/FLI1 cofactors as well as target genes in Ewing sarcoma, in an effort to identify potential therapeutic targets.

In this study, we describe a novel target of the EWS/FLI1 fusion protein, EYA3, which belongs to the EYA family of proteins. The EYA proteins are critical developmental regulators that contain 2 domains important for their function: the EYA domain and the transactivation domain (TAD). The EYA domain is a conserved carboxy-terminal region with 2 critical activities: protein-binding activity and tyrosine phosphatase activity. EYA proteins bind to the SIX family of homeoproteins through their Eya domain (13), resulting in a partnering of the EYA TAD with the DNA-binding activity of the SIX family proteins. Thus, the SIX/EYA complex functions as a bipartite transcription factor that is crucial for the normal development of many tissues (14–17), and when reexpressed in adult tissues can drive oncogenesis by reinitiating developmental programs out-of-context (18–24). In addition, EYA proteins have recently been shown to have activities that may be outside of their roles as transcriptional coactivators. One of these functions includes a recently identified role for EYA proteins in DNA repair (25). Because histologic response to chemotherapy, including DNA-damaging agents, remains a key prognostic indicator in Ewing sarcoma, we asked whether EYA proteins, downstream of EWS/FLI1, act as mediators of resistance to DNA-damaging chemotherapeutics in Ewing sarcoma cells.

Materials and Methods

Cell lines and cell culture

Human mesenchymal stem cell (hMSC) lines were obtained from Lonza and ScienCell. hMSCs from Lonza were isolated from adult human bone marrow and purity was determined by flow cytometry and tridifferentiation capabilities. hMSCs from ScienCell were isolated from adult human bone marrow and purity was determined by flow cytometry and adipogenic differentiation. A673 cells were obtained from American Type Culture Collection. EWS502 and TC71 cell lines were obtained from Dr. Steve Lessnick. Cell lines requiring reauthentication were profiled with assistance from the University of Colorado Cancer Center DNA sequencing center at the molecular pathology shared resource. Lentiviral short hairpin RNA (shRNA) constructs targeting human *FLI1* (shEWS/FLI1#1 and shEWS/FLI1#2), *EYA3* (shEYA3#1 and shEYA3#5), and control shRNA construct targeting *EGFP* were obtained from Open Biosystems. Off-target scramble shRNA was obtained from Addgene (plasmid 1864; ref. 26). Preparation of replication-incompetent infectious virus to create stable shRNA-expressing cell lines was carried out as previously described (27). Following infection, cells were selected with 2 μg/mL puromycin.

Ewing sarcoma human tumor samples

RNA from Ewing sarcoma tumor samples was obtained from the Children's Hospital Colorado Molecular Diagnostics Laboratory according to our COMIRB protocol. cDNA was generated using miScript (Qiagen). Quantitative reverse transcriptase PCR (qRT-PCR) for *EYA3*, hsa-miR-145, and hsa-miR-708 is described below.

Immunoblotting

Whole-cell lysates were obtained using radioimmunoprecipitation assay buffer (28) for Western blot analysis. We used primary antibodies against EYA3 (Santa Cruz Biotechnology; #SC-15101), FLI1 (BD Biosciences; #554266), cleaved PARP (BD Biosciences; #8111KC), β-actin (Sigma-Aldrich; #A5316), and tubulin (Sigma-Aldrich; #T4026). Densitometry, where included, was carried out using Quantity One software (Bio-Rad laboratories).

Transfection of miRNA mimics into Ewing sarcoma cells

A673 cells were plated to be about 50% confluent in 6-cm dishes. The following day cells were transfected with 100 nmol/L miR-145 and 100 or 10 nmol/L miR-708 miRNA mimics or equal amounts of negative control mimic (Dharmacon/Thermo Fisher Scientific) using X-treme GENE siRNA reagent (Roche). For EYA3 protein expression whole-cell lysates were collected as described above 48 hours after transfection. For miR-708 chemosensitivity experiments, cells were plated at 5,000 cells per well in 96-well plates 24 hours after transfection, and experiments were carried out as described below.

Real-time PCR

To detect *EYA3* transcript, RNA was isolated using TRIzol and reverse transcribed using iScript (Bio-Rad laboratories). Real-time PCR was carried out with forward primer: 5′-TGATGCCACTTCTTCCCAAGA-3′, reverse primer: 5′-AAGTGAGTGGAAGATG-ATGATGGTT-3′, and probe: 5′-FAM-AGAACGGG-TATTTCTGTGGGACTTGGATG-TAM-3′. Primers to detect *IGFBP3* transcript were previously described (29). Samples were normalized to *GAPDH* (Applied Biosystems; #Hs99999905_m1) or *cyclophilin* (Applied Biosystems; #Hs01018503_m1). For miRNA detection, RNA was isolated with QIAzol and miRNeasy and reverse transcribed with miScript (Qiagen). Primers for miR-145, miR-708, and miR-28-5p were purchased from Qiagen and qRT-PCR for miRNAs were normalized to U6 RNA (Qiagen).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) for EWS/FLI1 binding to DNA was carried out as previously described (27).

Luciferase reporter assays

For the basic 3′-untranslated region (3′-UTR) luciferase reporter assay, 293T cells were plated at 50,000 cells per well in 24 well plates. The following day, cells were transfected with *EYA3* 3′-UTR-luciferase reporter plasmid or empty vector control (Origene), 20 nmol/L miRNA mimics or negative controls (Dharmacon/Thermo Fisher Scientific), and a *Renilla* construct, using Lipofectamine 2000 (Invitrogen). After 24 hours, lysates were prepared and analyzed with the Dual Luciferase Kit (Promega) on a Modulus Microplate (Turner Biosystems). Transfection efficiency was normalized to *Renilla* activity, and the effect of the mimic on the *EYA3* 3′-UTR-luciferase reporter plasmid was normalized to the effect of the mimic on a control 3′-UTR-luciferase reporter plasmid. The data shown are an average of at least 3 independent experiments and the errors bars represent SEM. Statistical significance was determined using one-way ANOVA with Dunnett post test. 3′-UTR luciferase reporter assays using mutated seed sequences were carried out as described above, with the exception that 5 nmol/L miRNA mimics or controls were used. Furthermore, the miR-145 and miR-708 seed sequence sites were mutated in the *EYA3* 3′-UTR using site-directed mutagenesis II XL kit (Stratagene). miR-708 seed site mutation primers (5′-agccttccccttgagcacgttttcactcctgaagg-3′ and 5′-ccttcagga-gtgaaaacgtgctcaaggggaaggct-3′) were used, and both miR-145 seed sequence sites were mutated consecutively in the same plasmid using primers (5′-gcttgattttctctaagaacttgcatgaggagccttccccttg-3′ and 5′-caaggggaaggctcctcatgcaagttcttagagaaaatcaagc-3′; 5′-actcctgaagggagctggagactagtaccaactgagaa-3′ and 5′-ttctcagttg-gtactagtctccagctcccttcaggagt-3′). For these assays representative data are shown.

Annexin V assays

A total of 10^6 cells were plated in 10-cm dishes. After 48 hours, adherent cells and media were collected and stained with Annexin V–fluorescein isothiocyanate and propidium iodide (BD biosciences) and analyzed using the CyAn flow cytometer (Beckman Coulter). The data shown are the average of 3 independent experiments and the errors bars represent SEM. Statistical significance was determined using one-way ANOVA with Dunnett post test. Representative flow cytometry plots are shown.

Chemosensitivity assays

Cell viability in response to chemotherapeutics was determined using MTS and clonogenic assays. For MTS assays, cells were plated at 5,000 cells per well in triplicate in 96-well plates. The following day, cells were treated with varying concentrations of etoposide (Sigma-Aldrich) or doxorubicin (Sigma-Aldrich). After 72 hours, cell viability was assessed using the MTS assay (Promega). For clonogenic assays, 500 cells per well were plated in triplicate in 6-well plates. The following day, cells were treated with varying doses of etoposide (Sigma-Aldrich) or doxorubicin (Sigma-Aldrich). After 72 hours, cells were washed and fresh media was added. After 7 to 10 days, colonies were stained with crystal violet and counted. To normalize for any survival effects of EYA3 knockdown, independent of chemotherapy treatment, a no-drug-treated control was set to equal 100% viability for each cell line, and each data point was normalized to this control. Statistical significance was determined using one-way ANOVA with Dunnett post test. Representative dose response curves are shown.

COMET assays

A673-shscramble, A673-shEYA3#1, and A673-shEYA3#5 cells were grown to about 30% to 40% confluency in 6-cm dishes and then treated with 10 μmol/L etoposide for 48 hours. Cells were then treated as per the Oxiselect COMET assay kit protocol (Cell Biolabs). Images were analyzed for olive tail moment using CASPlab software (http://casplab.com). Results shown are the average of 4 independent experiments and are normalized to the control cell line set to equal 1. Statistical significance was determined by carrying out a one-way ANOVA with Dunnett post test on the natural log-transformed normalized olive tail moments.

γ-H2AX flow cytometry

A673-shscramble, A673-shEYA3#1, and A673-shEYA3#5 cells were grown to be about 40% to 60% confluent in 6-cm dishes and then treated with 100 μmol/L etoposide for 2 hours. Cells were then washed twice with PBS and fresh media was added. Cells were collected at 0, 1, and 3 hours and analyzed for γ-H2AX using the FlowCellect DNA damage histone H2AX dual detection kit (Millipore) and CyAn flow cytometer (Beckman Coulter). The data shown represent the average of 3 independent experiments, and statistical significance was determined using one-way ANOVA with Dunnett post test. Representative flow cytometry plots are shown.

Caspase-3/7 assays

To evaluate caspase-3 and caspase-7 activation in A673 cells expressing miR-708, we plated 5,000 cells per well in 96-well white-walled tissue culture plates. The following day, cells were transfected with 100 nmol/L miR-708 mimic or negative control (Dharmacon/Thermo Fisher Scientific) using X-treme GENE siRNA reagent (Roche). Twenty-four hours later, the media was changed and the next day, cells were treated with Caspase-Glo-3/7 substrate according to the protocol for the Caspase-Glo-3/7 assay (Promega). Plates were analyzed on a Modulus Microplate (Turner Biosystems) and caspase-3/7 activity was proportional to luminescent signal. The data shown represent the average of 3 independent experiments, and statistical significance was determined using one-way ANOVA with Dunnett post test.

Results

EYA3 is expressed in Ewing sarcoma cell lines and Ewing sarcoma tumor samples at higher levels than in hMSCs

EYA proteins are implicated in adult tumors (21, 24) and have recently also been linked to pediatric malignancies (20, 23). Therefore, we asked whether EYA family members are overexpressed in the pediatric tumor Ewing sarcoma, in which EYA proteins had not previously been implicated. To this end, we used qRT-PCR to compare the expression level of all 4 *EYA* genes in Ewing sarcoma cell lines to that in hMSCs, the presumed cell-of-origin of Ewing sarcoma (30–32). In this analysis, only *EYA3* was consistently upregulated across all 3 tested Ewing sarcoma cancer cell lines, when compared with hMSCs (Supplementary Fig. S1A). We further showed that the EYA3 protein is indeed overexpressed in Ewing sarcoma cell lines when compared with hMSCs (Fig. 1A). As a control in these experiments, we showed that a well-validated, repressed gene in Ewing sarcoma, *IGFBP3* (29), is highly repressed in Ewing sarcoma cell lines when compared with hMSCs (Supplementary Fig. S1B). We further extended these findings to 23 human Ewing sarcoma tumor samples, in which *EYA3* mRNA levels are again increased when compared with hMSCs (Fig. 1B). Indeed, *EYA3* mRNA levels within Ewing sarcoma tumor samples are almost always higher than the levels observed in 2 samples of unique human bone marrow–derived MSCs obtained from independent sources [Lonza (L) and ScienCell (SC)] and are comparable with those seen in the human Ewing sarcoma tumor-derived A673 cell line. Together, these data suggest that EYA3 is overexpressed in Ewing sarcoma.

EYA3 is a target of EWS/FLI1

Because Ewing sarcoma is driven by the nonphysiologic fusion protein transcription factor, EWS/FLI1, we asked whether EYA3 is regulated by this fusion protein. We thus knocked down the EWS/FLI1 fusion protein in A673 cells, using 2 different lentiviral shRNA constructs (Fig. 2A). Importantly, *EYA3* mRNA and protein levels were decreased in a manner proportional to the extent of EWS/FLI1 knockdown (Fig. 2B and C). Because published ChIP–chip data suggested that *EYA3* might be a direct transcriptional target of EWS/FLI1 (33), we examined whether EWS/FLI1 could indeed be found at putative EWS/FLI1-binding sites on the *EYA3* promoter *in vivo*. However, we were unable to show binding of EWS/FLI1 to the *EYA3* promoter using ChIP in A673 cells (Supplementary Fig. S2).

EWS/FLI1 regulates EYA3 expression through regulation of miR-708

Because *EYA3* does not seem to be a direct transcriptional target of EWS/FLI1, we explored additional mechanisms by which EWS/FLI1 may regulate EYA3. Analysis of the *EYA3* 3′-UTR using miRNA prediction software (microRNA.org) revealed that 3 EWS/FLI1 downregulated miRNAs (34, 35) are predicted to bind the *EYA3* 3′-UTR: miR-145, miR-28-5p, and miR-708 (Fig. 3A). Indeed, knockdown of EWS/FLI1 in A673 cells led to a substantial increase in miR-145 and miR-708 levels and also moderately increased miR-28-5p levels (Fig. 3B), confirming the regulation of these miRs by EWS/FLI1. As expected, this response correlated with the efficiency of EWS/FLI1 knockdown. Furthermore, miR-145 and miR-708 mimics were able to repress the *EYA3* 3′-UTR. However, from our results, we are unable to conclude that miR-28-5p represses the *EYA3* 3′-UTR in this system, despite successful expression of miR-28-5p mimic (Supplementary Fig. S3). To confirm the action of miR-145 and miR-708 on the *EYA3* 3′-UTR, we mutated the seed sequences for these miRNAs in the *EYA3* 3′-UTR reporter construct and found that miR-145 and miR-708 are unable

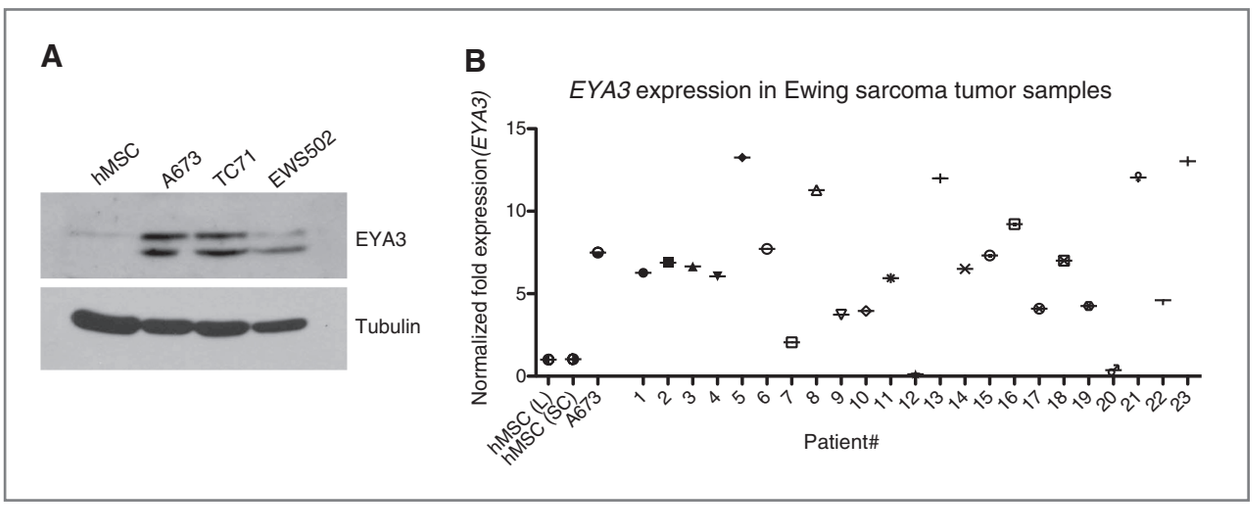

Figure 1. EYA3 is overexpressed in Ewing sarcoma cell lines and Ewing sarcoma tumor samples when compared with hMSCs. A, Western blot analysis showing the level of EYA3 protein in a panel of Ewing sarcoma cell lines as well as in hMSC. B, graphic representation of the expression of *EYA3* mRNA in Ewing sarcoma tumor samples compared with both the A673 cell line and with 2 unique human bone marrow–derived MSC samples obtained from 2 independent sources: Lonza (L) and ScienCell (SC).

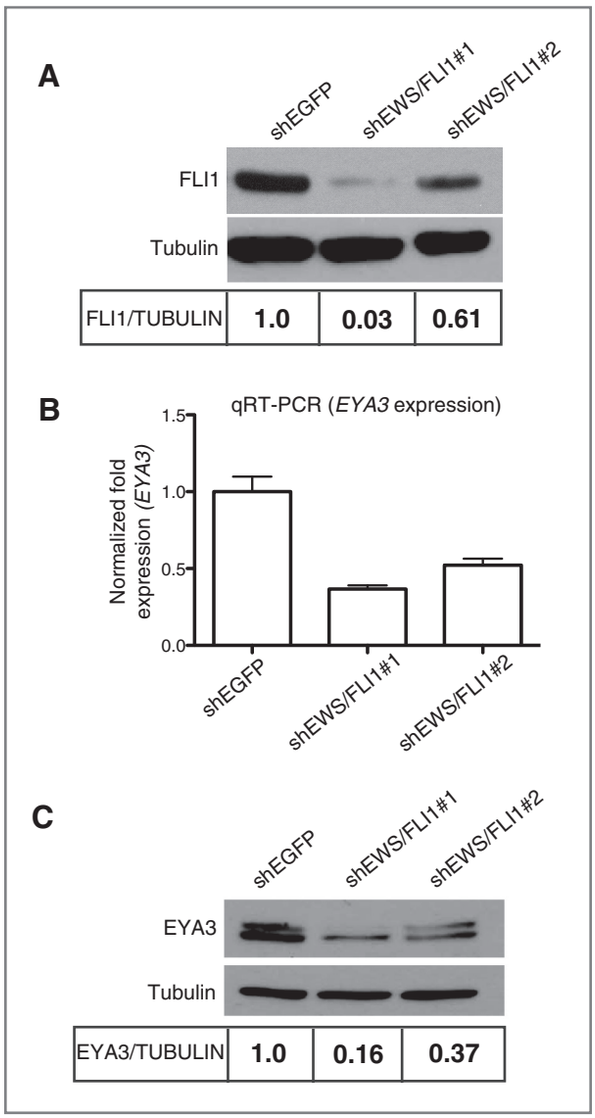

Figure 2. EYA3 is a downstream target of EWS/FLI1. A, Western blot analysis using an anti-FLI1 antibody shows stable EWS/FLI1 knockdown in A673 cells. B, qRT-PCR analysis shows levels of *EYA3* mRNA in stable EWS/FLI1 knockdown cells. Duplicate samples were assessed for each cell line. C, EYA3 protein expression levels in EWS/FLI1 knockdown cells as shown by Western blot analysis using an anti-EYA3 antibody.

to repress the *EYA3* 3′-UTR when their respective seed sequences are mutated (Fig. 3C). In addition, expression of miR-708, and to a lesser extent miR-145, in A673 cells led to a decrease in EYA3 protein levels (Fig. 3D). This decrease in EYA3 protein levels could also be observed when much lower levels of the miR-708 mimic were transfected into A673 cells (Supplementary Fig. S4). We thus examined whether this mechanism of regulation of EYA3 would be relevant to human Ewing sarcomas. Indeed, we found that miR-708 expression inversely correlates with *EYA3* expression in human Ewing sarcoma tumor samples (Pearson correlation; $P < 0.05$, $R = -0.40$; Fig. 3E). In contrast, expression of miR-145 did not correlate with *EYA3* expression in these samples (Supplementary Fig. S5). Together, these data suggested that the major mechanism by which EYA3 is overexpressed in Ewing sarcoma is via EWS/FLI1 repression of miR-708, which targets the *EYA3* 3′-UTR.

EYA3 plays a role in cell survival and chemoresistance in Ewing sarcoma cells

Because EYA proteins are important for progenitor cell survival during organogenesis (36–38), we explored the role of EYA3 in Ewing sarcoma cell survival. Introduction of 2 individual lentiviral shRNA constructs targeting *EYA3* into A673 Ewing sarcoma cells led to greater than 50% reduction in EYA3 levels resulting in increased apoptosis, as measured by PARP cleavage (Fig. 4A) and Annexin-positive/propidium iodide (PI) positivity (Fig. 4B and C). Thus, as expected, the survival phenotypes of EYA proteins are conserved in the context of cancer.

Because EYA3 is important for Ewing sarcoma cell survival and because EYA3 contributes to DNA repair in response to DNA damage in human embryonic kidney cells (25), we asked whether inhibition of EYA3 could sensitize Ewing sarcoma cells to DNA-damaging chemotherapeutics. A673 EYA3 knockdown cell lines were treated with 2 drugs typically used in the Ewing sarcoma chemotherapeutic treatment regimen: etoposide and doxorubicin. Indeed, knockdown of EYA3 sensitized cells to etoposide and doxorubicin, as shown by a decrease in viability using an MTS assay (Fig. 5A and B). These findings were validated using clonogenic chemosensitivity assays (Fig. 5C and D). In addition, we showed that this phenotype is conserved when EYA3 is stably inhibited in the SKES-1 Ewing sarcoma cell line, as shown by clonogenic chemosensitivity assays to etoposide (Supplementary Fig. S6). Furthermore, we examined whether miR-708, as the key regulator of EYA3 in Ewing sarcoma, was important for cell survival and if miR-708, alone, is able to sensitize Ewing sarcoma cells to chemotherapeutics. We found that addition of miR-708 to the A673 Ewing sarcoma cell line does in fact increase markers of apoptosis: PARP cleavage and activated caspases-3 and caspases-7 (Supplementary Fig. S7A and B). Furthermore, miR-708 expression sensitizes A673 cells to etoposide (Supplementary Fig. S7C). Because disease relapse is an important clinical outcome associated with chemoresistance, among other factors, we examined our Ewing sarcoma tumor samples and although our sample numbers were limited, we observed a clear trend that patients with low levels of miR-708 and high levels of *EYA3* have a worse 3-year relapse-free survival (Supplementary Fig. S8).

EYA3 is known to enhance DNA repair in response to DNA damage. Thus, we asked whether the mechanism by which EYA3 mediates chemoresistance in Ewing sarcoma cells is via its ability to increase DNA repair. To this end, EYA3 knockdown cells were treated continuously with 10 μmol/L etoposide for 48 hours, and the cells were examined for the presence of DNA damage using COMET assays. Importantly, EYA3 knockdown cells had statistically larger tail moments than their control counterpart, indicative of

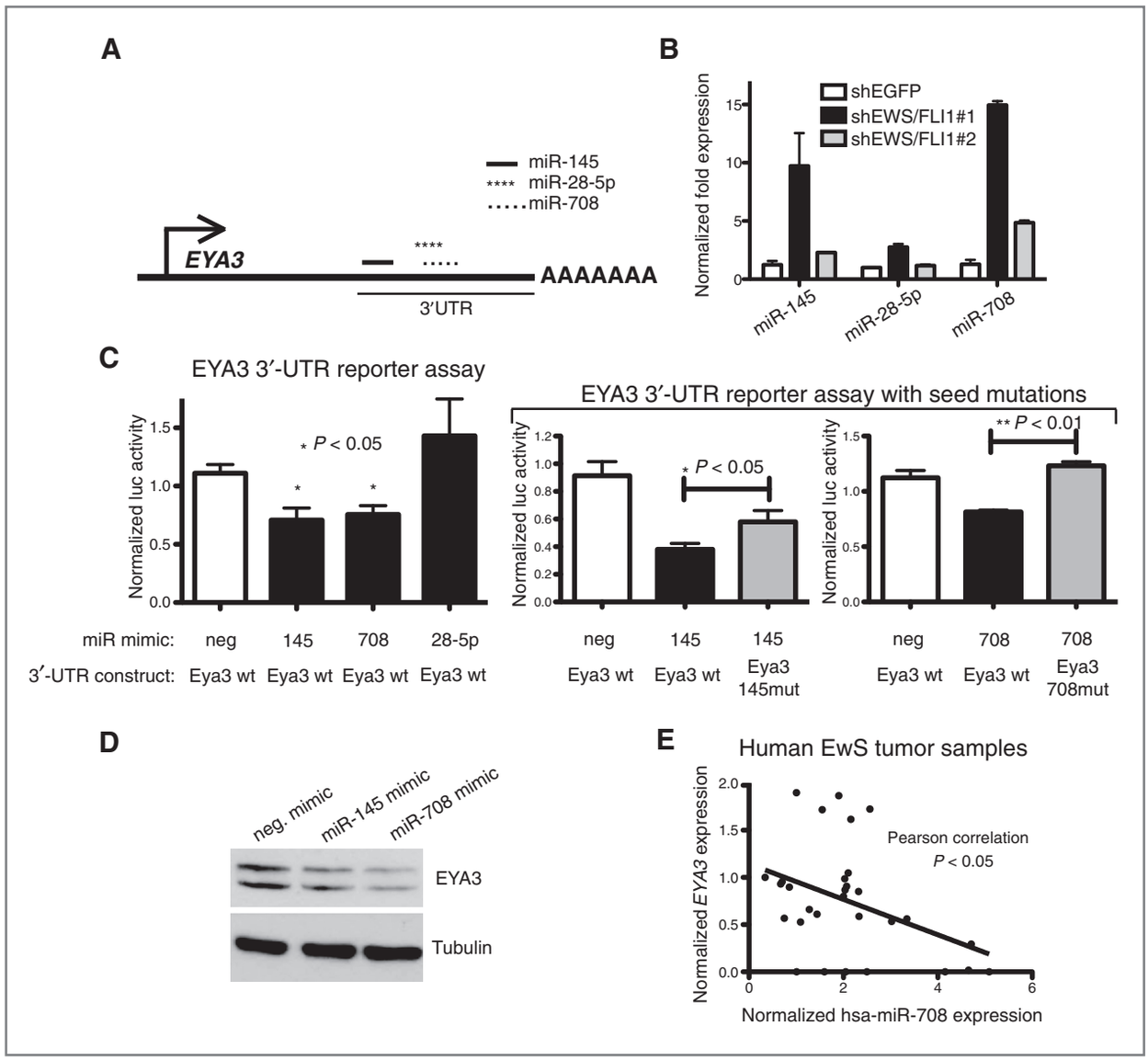

Figure 3. EWS/FLI1 increases EYA3 expression through repression of miR-708. A, three EWS/FLI1 downregulated miRNAs, miR-145, miR-28-5p, and miR-708 (34, 35) are predicted to bind the EYA3 3′-UTR (microRNA.org). B, qRT-PCR for miR-145, miR-28-5p, and miR-708 in EWS/FLI1 knockdown cell lines. Duplicate samples were assessed for each cell line. C, data shown are from *EYA3* 3′-UTR luciferase reporter assays with miR-145, miR-708, and miR-28-5p miRNA mimics and *EYA3* 3′-UTR luciferase reporter assays with miRNA mimics and seed sequence mutations. For miR-145, two seed sequences were found within close proximity to one another and both sequences were mutated. For assays with the wild-type 3′-UTR, experiments shown are an average of 3 replicates and statistical significance was determined using one-way ANOVA with Dunnett post test. For the *EYA3* 3′-UTR luciferase reporter assays in which the seed sequences were mutated for miR-145 and miR-708, the experiments were repeated at least twice and representative graphs are shown. D, Western blot analysis to examine EYA3 protein levels after introduction of exogenous miRNA mimics, miR-145 and miR-708, in Ewing sarcoma cells. These experiments were carried out at least twice and representative Western blots are shown. E, correlation analysis of hsa-miR-708 expression with *EYA3* mRNA expression in human Ewing sarcoma tumor samples, as shown by qRT-PCR. Statistical significance was determined using Pearson correlation.

increased DNA damage in response to etoposide treatment (Fig. 6A). We then examined the presence of γ−H2AX, as a measure of ongoing DNA repair, to more directly assess the effects of EYA knockdown on the DNA repair process. To this end, EYA3 knockdown cells were treated with 100 μmol/L etoposide for 2 hours after which the etoposide was washed off and the cells were allowed time to undergo DNA repair for 1 and 3 hours. Figure 6B and C shows that EYA3 knockdown cells have significantly more γ−H2AX at 1 and 3 hours after treatment, indicating the continued presence of DNA damage and thus suggesting that the repair process is less efficient in these cells. These data are consistent with the described role of EYA3 in DNA repair (25) and suggest that the role of EYA3 in DNA repair may be one means by which it mediates chemoresistance. Furthermore, because miR-708 targets EYA3, and as, similar to EYA3 knockdown,

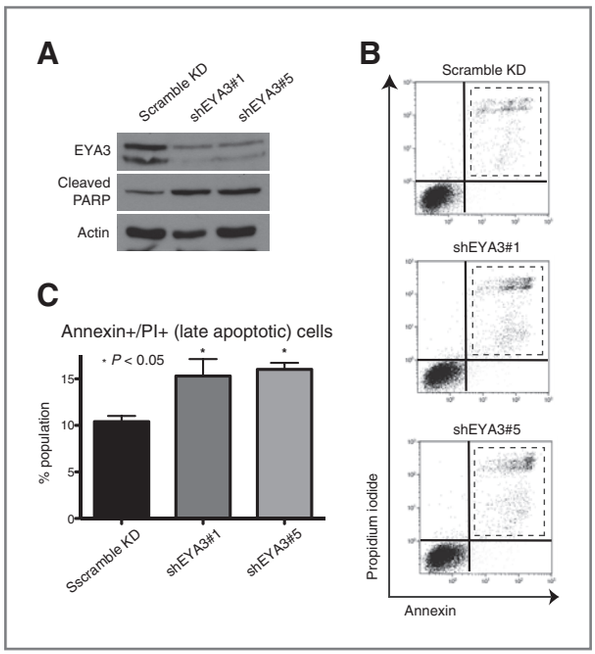

Figure 4. EYA3 is important for Ewing sarcoma cell survival. A, Western blot analysis showing EYA3 protein levels in A673 EYA3 stable knockdown cell lines and corresponding increases in baseline PARP cleavage. Representative Western blots are shown. B, representative flow cytometry plots of Annexin/PI staining in A673 EYA3 knockdown cell lines. C, graphic representation of Annexin V staining in A673 EYA3 knockdown cells. These graphs represent the average of 3 independent experiments, and statistical significance was determined using one-way ANOVA with Dunnett post test.

expression of miR-708 sensitizes Ewing sarcoma cells to etoposide, we showed that miR-708–expressing cells also repair DNA less effectively than their control counterparts following etoposide treatment (Supplementary Fig. S7D).

Discussion

Although the prognosis for Ewing sarcoma patients has improved over the last 3 decades, it remains poor, and unfortunately, patients that present with advanced disease or whose cancer is refractory to chemotherapy have especially poor outcomes (6–9). In addition, there is significant concern around the use of high-dose conventional chemotherapies on pediatric patients, as patients that survive a childhood cancer may live several decades after their disease is cured. Thus, in addition to more acute and shorter term toxicities relevant to the treatment of adult oncology patients, pediatric patients may experience later and more long-term toxicities associated with these drugs. In this study, we examined the role of a novel downstream target of EWS/FLI1, EYA3, in mediating chemoresistance, as a means to identify possible new drug targets that if inhibited, may mitigate the effects of standard chemotherapy.

EYA family members have been implicated in numerous cancers. For example, EYA1-3 plays an important role in breast cancer via the ability of the EYA tyrosine phosphatase activity to mediate migration, invasion, and transformation, as well as metastasis (21). EYA2 is a required SIX1 cofactor to enable the induction of cancer stem cell characteristics and TGF-ß signaling, and patients who have high levels of EYA2 in addition to high levels of SIX1 have an especially poor prognosis (39). EYA2 is amplified and overexpressed in

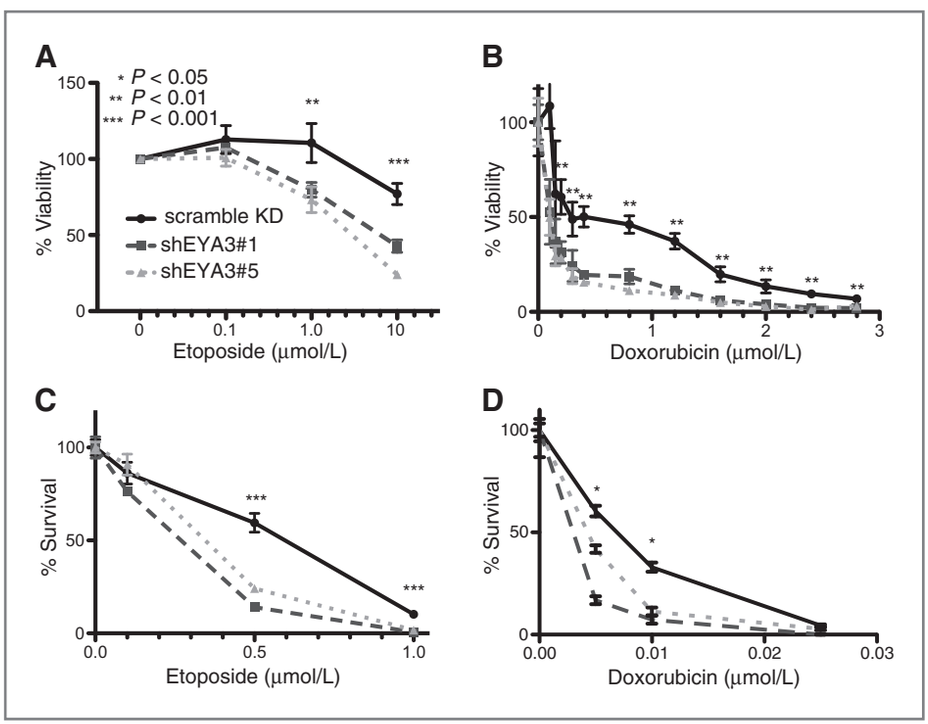

Figure 5. Inhibition of EYA3 sensitizes Ewing sarcoma cells to drugs used in the Ewing sarcoma chemotherapeutic treatment regimen: etoposide and doxorubicin. A and B, etoposide and doxorubicin sensitivity, as shown by MTS assay, in A673 EYA3 stable knockdown cell lines. C and D, etoposide and doxorubicin sensitivity, as shown by clonogenic assays, in A673 EYA3 stable knockdown cell lines. Experiments were carried out at least 3 times and representative dose response curves are shown. Statistical significance was determined using one-way ANOVA with Dunnett post test on the representative experiment shown.

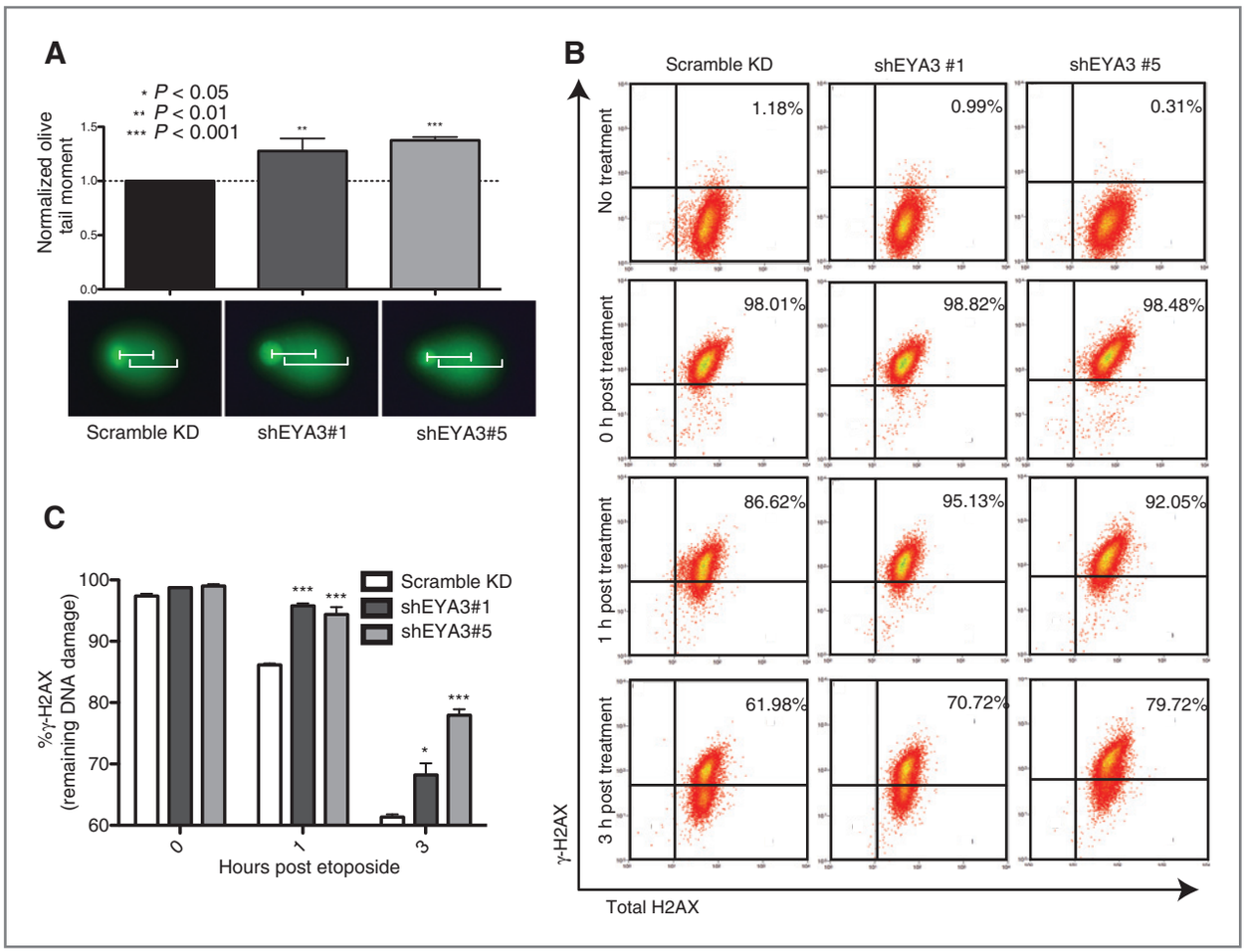

Figure 6. EYA3 knockdown cells inefficiently repair DNA damage after etoposide treatment. A, COMET assays of A673-shscramble, A673-shEYA3#1, and A673-shEYA3#5 cells after 48 hours of continuous treatment with 10 μmol/L etoposide. The graph shown represents the average of 4 independent experiments, and statistical significance was determined by carrying out a one-way ANOVA with Dunnett post test on the natural log-transformed normalized olive tail moments. B, representative flow cytometry plots of γ-H2AX/total H2AX staining in A673 EYA3 knockdown cell lines. C, graphic representation of γ-H2AX DNA repair assay following 2-hour treatment with 100 μmol/L etoposide. The data shown represent the average of 3 independent experiments, and statistical significance was determined using one-way ANOVA with Dunnett post test.

ovarian cancer and correlates with decreased survival (24), in which SIX1 also plays a role (18).

However, EYA has recently been shown to have functions outside of its role as a transcription cofactor, and, indeed, there is evidence that in some contexts, EYA is tumor suppressive rather than tumor promotional, although the dependence of EYA on SIX1 in this context is unknown. For example, EYA4 is hypermethylated in colon cancer (40) and esophageal adenocarcinoma (41). In this study, we compared the expression of EYA3 in Ewing sarcoma cell lines and human tumors to its expression in hMSCs, the presumed cell-of-origin of Ewing sarcoma, and show that EYA3 is upregulated in this cancer, suggesting that it may, as is observed in many cancers, have a tumor promotional role in the context of Ewing sarcoma.

Because greater than 85% of Ewing sarcomas express the EWS/FLI1 fusion protein, we asked whether EYA3 may be downstream of EWS/FLI1 in this disease. For these studies, we used the A673 human tumor-derived Ewing sarcoma cell line, in which we see high levels of EYA3 expression and show that knockdown of EWS/FLI1 in these cells results in a concomitant decrease in *EYA3* mRNA and protein expression. Our data showed that EYA3 is indeed a target of EWS/FLI1; however, to our surprise, EYA3 is not directly transcriptionally regulated by EWS/FLI1 as we were unable to show that the fusion protein binds at the promoter of *EYA3*, despite previously published ChIP–chip data which suggested that EWS/FLI1 may indeed be bound at the *EYA3* promoter (33). Indeed, the 2 potential sites for EWS/FLI1 in the *EYA3* promoter, identified by ChIP–chip, contain only loose EWS/FLI1 consensus-binding sites. Although the core FLI1 consensus-binding sequence is GGAA, this GGAA sequence is generally preceded by a sequence of ACA nucleotides, in which the cytosine is very highly conserved (42). In addition, EWS/FLI1 often regulates critical target genes via GGAA microsatellite repeats (33, 43). The regions

identified by Gangwal and colleagues in a ChIP–chip experiment contained only the core GGAA sequence, without preceding ACA nucleotides or GGAA microsatellite repeats.

Because we were unable to show that EWS/FLI1 binds at the *EYA3* promoter, we explored alternate mechanisms by which EWS/FLI1 may regulate EYA3. We determined that EWS/FLI1 upregulates EYA3 through repression of a miRNA that targets the *EYA3* 3′-UTR, miR-708. Interestingly, we did not observe consistent effects on the *EYA3* 3′-UTR by miR-28-5p, despite being part of the same seed family as miR-708. These results led us to focus our studies on miR-708; however, it remains possible that miR-28-5p is an important regulator of EYA3, yet this regulation is simply not observed in the systems used for our experiments. Furthermore, although miR-145 also regulates EYA3 in our model cell systems, miR-145 does not correlate with *EYA3* expression in human Ewing sarcoma tumor samples. These data suggested that although miR-145 is able to target *EYA3* and miR-145 is repressed by EWS/FLI1, it does not regulate EYA3 in the context of Ewing sarcoma tumors. However, it is also possible that miR-145 is important for regulation of EYA3 in Ewing sarcoma tumors, but the sample size of our Ewing sarcoma tumor dataset is too small to observe this correlation. Nonetheless, our data lead us to conclude that miR-708 repression by EWS/FLI1 is a major mechanism of EYA3 upregulation in Ewing sarcoma.

Interestingly, our studies surrounding the mechanism of EYA3 upregulation highlight a possible means for targeting the action of EYA3 in Ewing sarcoma, in addition to targeting EYA3 directly. Because EYA3 is upregulated in Ewing sarcoma through the repression of miR-708 by the EWS/FLI1 fusion protein, miRNA replacement may serve as a valuable alternative approach to targeting the actions of EYA3 in Ewing sarcoma. Notably, Saini and colleagues recently showed that miR-708 expression is lost in human renal cell carcinomas (RCC) and described loss of miR-708 as important for cell survival, among other things, in this disease (44). In addition, Saini and colleagues further showed that intratumoral injection of miR-708 represses *in vivo* RCC tumor growth (44). In our studies, we showed that the addition of miR-708 mimic to A673 cells is sufficient to increase markers of apoptosis and sensitize Ewing sarcoma cells to etoposide (Supplementary Fig. S7). These data suggest that miR-708 replacement may serve as a potential Ewing sarcoma therapy.

In addition to understanding the mechanism by which EWS/FLI1 upregulates EYA3, we further asked what the functional consequence is of having increased EYA3 levels in Ewing sarcoma cells. During development, EYA proteins are critical for cell survival, and we thus explored the role of EYA3 in this process. Importantly, EYA3 knockdown leads to an increase in PARP cleavage and an increase in Annexin V positivity, suggesting that EYA3 is required for survival even in the absence of any death-inducing stimuli. Because of the newly described role of EYA3 as a mediator of efficient DNA repair through its EYA domain tyrosine phosphatase activity, we further asked whether EYA3 knockdown would lead to a decrease in survival following treatment with DNA-damaging chemotherapeutics that are used to treat Ewing sarcoma clinically. Indeed, EYA3 knockdown significantly sensitizes cells to etoposide and doxorubicin, and this phenotype results from a decreased ability to repair the DNA damage inflicted by these chemotherapeutics when EYA3 levels are decreased. However, it is possible that knockdown of EYA3 sensitizes cells to chemotherapeutics through alternate mechanisms as well. In breast cancer, for example, EYA2 is a required cofactor for the homeobox transcription factor, SIX1, to mediate expansion of tumor-initiating cell populations (39), which have also been linked to chemoresistance (45, 46). Therefore, it will be of interest to focus future studies on the relative contribution of EYAs DNA repair versus transcriptional activities on its ability to mediate chemoresistance in Ewing sarcoma.

Our studies suggest a model by which EWS/FLI1 represses miR-708, resulting in EYA3 overexpression. EYA3 overexpression then contributes to Ewing sarcoma cell chemoresistance through increased DNA repair (Fig. 7). These data thus suggest that inhibitors of EYA3 and/or reintroduction of miR-708 have the potential to sensitize Ewing sarcomas to DNA-damaging chemotherapeutics and to improve relapse-free survival, and indeed, small molecule inhibitors of EYA are currently under development by our group and others (47). It is important to note that we do not expect EYA-targeted therapeutics to have significant side effects. As developmentally important proteins, EYAs are highly expressed in embryonic tissues and their expression is lost in most adult tissues (48–50). However, EYA3 is

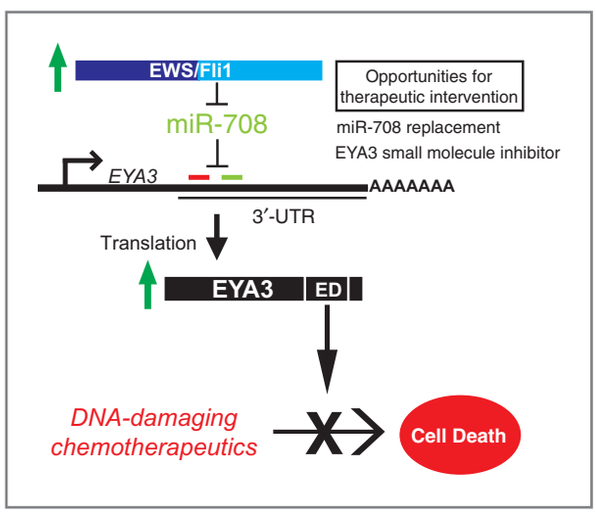

Figure 7. EWS/Fli1 repression of miR-708 leads to EYA3 upregulation and chemoresistance in Ewing sarcoma. EWS/FLI1 inhibits a miRNA that targets the *EYA3* 3′-UTR, miR-708, resulting in overexpression of EYA3 in Ewing sarcoma. Overexpression of EYA3 in Ewing sarcoma then renders Ewing sarcoma cells resistant to DNA-damaging chemotherapeutics used in the treatment of Ewing sarcoma, including etoposide and doxorubicin, via increasing DNA repair after treatment. Chemoresistance in Ewing sarcoma via EWS/FLI1 repression of miR-708 and thus EYA3 overexpression, highlights 2 therapeutic opportunities for targeting chemoresistance in Ewing sarcoma: (i) small molecule inhibitors of EYA3 and (ii) synthetic replacement of miR-708.

expressed in more adult tissues than the other EYA family members (50). Despite the presence of EYA3 in some adult tissues, EYA3 knockout mice have very few significant phenotypes (51). Thus, we anticipate that EYA inhibitors will be highly specific to cancer cells, while conferring few side effects. Furthermore, because EYA3 knockdown sensitizes cells to DNA-damaging chemotherapies via decreased DNA repair, it is possible that EYA inhibitors may be promising potential agents to sensitize cells to highly targeted radiation therapy as well.

In conclusion, Ewing sarcoma is an aggressive pediatric malignancy with poor outcomes. Chemoresistance is an important negative predictor of prognosis in Ewing sarcoma patients, and we have identified the DNA repair protein, EYA3, as an indirect target of EWS/FLI1 through its regulation of miRNAs. Furthermore, we have shown that EYA3 mediates chemoresistance and cell survival in this cancer, and this is the first report to show that EYA mediates chemoresistance in any cancer. We propose that EYA3 is a novel therapeutic target in Ewing sarcoma, that if inhibited, has the potential to synergize with standard chemotherapeutic treatment regimens, thus improving outcomes for patients with chemoresistant disease, and minimizing acute toxicities and life-long side effects in Ewing sarcoma survivors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T.P. Robin, L. Reaves, H.L. Ford, P. Jedlicka
Development of methodology: T.P. Robin, H.L. Ford
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.P. Robin, A. Smith, E. McKinsey, L. Reaves, H.L. Ford
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.P. Robin, A. Smith, L. Reaves, H.L. Ford
Writing, review, and/or revision of the manuscript: T.P. Robin, E. McKinsey, P. Jedlicka, H.L. Ford
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.P. Robin
Study supervision: P. Jedlicka, H.L. Ford

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