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

Tyler P. Robin1,2, Anna Smith1, Erin McKinsey3, Lisa Reaves6, Paul Jedlicka2,3,4, and Heide L. Ford1,2,3,5

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.
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'-TGATGACCTTCTTCCCATGAA-3', reverse primer: 5'-AAATGATGGGAAGATGATGGTT-3', and probe: 5'-FAM-AGAACGGA-TAATTCCTGTGGACATTGAT-GT-3'. For EYA3 and β-actin (Applied Biosystems; #Hs01018503_m1). Samples were normalized to GAPDH (Applied Biosystems; #Hs99999905_m1) or cyclophilin (Applied Biosystems; #Hs01018503_m1). For miRNA detection, RNA was isolated with QiAzo1 and miRNAeasy 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 was 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 Biosoystems). 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 error 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′-cctccagga- ggaaaagctgctcaaggagggagc-3′) were used, and both miR-145 seed sequence sites were mutated consecutively in the same plasmid using primers (5′-gcctgtatttctcaagcttgagcagctctccccctggg-3′ and 5′-ccaa-gagggaggttcctggatcagctctccccctggg-3′ and 5′-actctgtagcagttccctcgactg-3′ and 5′-ttctagctgctgtctggacttggactcctcgagggagt-3′). For these assays representative data are shown.

Annexin V assays

A total of 10⁶ 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 error 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 assay

A673-sh scramble, A673-sh EYA3#1, and A673-sh EYA3#5 cells were grown to about 30% to 40% confluence 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 CASP Lab 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-sh scramble, A673-sh EYA3#1, and A673-sh EYA3#5 cells were grown to about 40% to 60% confluent in 6-cm dishes and then treated with 10 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 FlowCelect 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 activity 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 Biosoystems) 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

![Graph showing EYA3 expression in Ewing sarcoma tumor samples](image-url)
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/propiodum 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 A673 EYA3 knockdown cell lines was in fact increased DNA damage. Thus, we asked whether inhibition of EYA3 could sensitize Ewing sarcoma cells to DNA-damaging chemotherapeutics. Because EYA3 is important for Ewing sarcoma cell survival and because EYA3 contributes to DNA repair, we examined whether inhibition of EYA3 could sensitize Ewing sarcoma cells to DNA-damaging chemotherapeutics. Because EYA3 is important for Ewing sarcoma cell survival and because EYA3 contributes to DNA repair, we examined whether inhibition of EYA3 could sensitize Ewing sarcoma cells to DNA-damaging chemotherapeutics.
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,
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.
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 EYA3 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

**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.
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 functional consequence is of having increased EYA3 levels in Ewing sarcoma. Notably, Saini and colleagues showed that 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.

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
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.

References


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