TARGETING EYA3 IN EWING SARCOMA RETARDS TUMOR GROWTH AND ANGIOGENESIS

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Abstract

*EWSR1/FLI1*, the most common fusion gene in Ewing sarcoma, upregulates expression of the Eyes Absent 3 (EYA3) transactivator–phosphatase protein. The purpose of this study was to investigate molecular and cellular mechanisms through which EYA3 might promote Ewing sarcoma tumor growth and to determine whether the EYA3 tyrosine phosphatase activity represents a viable therapeutic target. We used genetic and pharmacological modulation of EYA3 in cell-line based xenografts to examine how loss of EYA3 tyrosine phosphatase activity affects tumor growth and angiogenesis. Molecular mechanisms were evaluated in vivo and in vitro through analyses of tumor tissue and multi-cellular tumor spheroids. Our results show that both loss of EYA3 in Ewing sarcoma cells and pharmacological inhibition of the EYA3 tyrosine phosphatase activity inhibits tumor growth and tumor angiogenesis. EYA3 regulates levels of VEGFA in Ewing tumors, as well as promoting DNA damage repair and survival of Ewing sarcoma tumor cells. Target engagement is demonstrated in tumor tissue through elevated levels of the EYA3 substrate H2AX-pY142 upon loss of EYA3 or with Benzarone treatment. The efficacy of EYA3 tyrosine phosphatase inhibition in attenuating tumor growth and angiogenesis is corroborated in an Ewing sarcoma patient-derived tumor xenograft. Together, the results presented here validate EYA3 as a target for the development of novel Ewing sarcoma therapeutic strategies, and set the stage for evaluating the efficacy of combining the anti-angiogenic and anti-cell survival effects of EYA3 inhibition with cytotoxic chemotherapy.
INTRODUCTION.
Ewing sarcoma is an aggressive but rare tumor of the bone and surrounding soft tissue that predominantly afflicts children and young adults. While non-metastatic disease is usually responsive to chemotherapy, radiation, and surgery, recurrences are common[1]. Patients presenting with advanced, metastatic or relapsed disease have a very poor prognosis[2]. In over 85% of Ewing sarcomas a somatic mutation results in a translocation between the EWSR1 gene on chromosome 22 and the FLI1 gene on chromosome 11 (t(11;22)), creating the EWSR1/FLI1 fusion gene. The protein product EWS/FLI1 combines the DNA-binding properties of FLI1 and the transcriptional regulation function of EWSR1, and leads to dysregulated transcription of genes associated with cell proliferation and survival. Other than this driving translocation Ewing sarcoma is characterized by a low somatic mutational burden. From a therapeutic standpoint it is of note that mutations in genes involved in kinase signaling pathways have not been reported. A few targeted therapeutics are currently in clinical trials including insulin growth factor-1 receptor inhibitors (NCT02306161), the PARP inhibitor olaparib (NCT01858168), and LSD1 inhibitors (NCT03514497, NCT03600649). Immune checkpoint inhibitors are generally unsuccessful in treating Ewing sarcoma family tumors[3], most likely because of the absence of PD-L1 expression[4]. Although sarcomas are vascular and tumor cells secrete proangiogenic factors, monotherapy with anti-angiogenics has, at best, provided survival benefits on the order of weeks[5]. Clinical trials of anti-angiogenics in combination with cytotoxic chemotherapy are showing more promise[6, 7]. Overall, relative to other malignancies, few targeted therapeutics exist and there has been no major improvement in the treatment of relapsed or refractory Ewing sarcoma since the advent of multi-agent adjuvant chemotherapy. Hence the identification of new targetable pathways is of great interest.

Since the transcription factor fusion EWS/FLI1 is a master regulator of the tumorigenic phenotype in Ewing sarcoma, it is an obvious target for therapeutics. However, despite much effort no EWS/FLI1-targeted therapeutic has yet reached a clinical trial. The general difficulty in targeting transcription factors that lack a defined active site and the intrinsic disorder of the EWS/FLI1 protein[8, 9] contribute to the challenge. Approaches currently in favor include functional modulation of EWS/FLI1 through the disruption of either EWS/FLI1-protein interactions or EWS/FLI1 fusion-associated epigenomic or transcriptomic changes. Among these are micro-RNAs (miRs) that contribute to EWS/FLI1-initiated oncogenic pathways[10, 11]. miR-708 is an EWS/FLI1-downregulated miR that binds to the 3'-untranslated region of the EYA3 gene, thus repressing EYA3 expression (schematized in Fig. 6n). In Ewing sarcoma cells, down-regulation of miR-708 correlates with high levels of the EYA3 protein. EYA3 belongs to the Eyes Absent...
(EYA) family of proteins that are both protein tyrosine phosphatases (PTP) as well as transcriptional activators[12, 13]. It has previously been shown that loss of EYA3 in Ewing sarcoma cells confers chemo-sensitivity in vitro through an impaired DNA damage repair process[14]. In the present study we demonstrate that loss of EYA3 in the Ewing sarcoma cell-line A673 impairs tumor growth in vivo, inhibits cell survival and migration in vitro, and inhibits tumor angiogenesis through down-regulation of VEGFA levels. Furthermore, pharmacological inhibition of EYA3 PTP activity retards both cell-line derived xenograft tumor growth as well as the growth and vascularization of a patient-derived Ewing sarcoma xenograft. Together these observations demonstrate that EYA3 promotes Ewing sarcoma tumor growth and tumor angiogenesis, and that inhibiting EYA3-dependent molecular signaling could be beneficial in the treatment of Ewing sarcoma tumors.

**MATERIALS AND METHODS.**

**Antibodies and Reagents.** Anti-EYA3 (Abcam Cat#ab95876; RRID:AB_10681036), anti-Ki67 (Thermo-scientific Cat#MA5-14520; RRID:AB_10979488), pan anti-actin C4 (Seven Hills Bioreagents, Cincinnati OH [15]), anti-endomucin (Abcam Cat#ab106100 RRID:AB_10859306), anti-γ-H2AX (Millipore Cat#05-636 RRID:AB_309864), anti-H2AX (phospho-Y142) (Abcam Cat#ab94602, RRID:AB_10858263), anti-VEGFA (Thermo Scientific Cat#MA1-16629; RRID:AB_2212682), anti-cleaved caspase 3 (Cell Signaling Cat#9664S, RRID:AB_2070042), biotinylated goat anti rabbit IgG (H+L) (Vector Labs Cat#BA1000, RRID:AB_2313606), biotinylated goat anti rat IgG (H+L) (Vector Labs Cat#BA9400, RRID:AB_2336202), biotinylated goat anti mouse IgG (H+L) (Invitrogen Cat#62-6540, RRID:AB_2533949), Alexa Fluor 488 anti rat (H+L) (Invitrogen Cat#A21208, RRID:AB_2535794), Alexa Fluor 594 anti rabbit (H+L) (Invitrogen Cat#A21207, RRID:AB_141637).

Benzarone (2-Ethyl-1-benzofuran-3-yl)-(4-hydroxyphenyl)methanone, obtained from Toronto Research Chemicals, Canada), DMSO (D2650, Sigma-Aldrich, St. Louis, MO), CCK-8 kit (Dojindo Molecular Technologies, Rockville, MD USA). A673 and RD-ES cells were obtained from ATCC (A673 ATCC Crl-1598 obtained January 2016, RD-ES ATCC HTB-166 obtained February 2016) tested for mycoplasma contamination (ATCC Mycoplasma detection kit, 30-1012K), maintained in DMEM containing 1% v/v penicillin (100 IU ml⁻¹), streptomycin 100 mg ml⁻¹, 10% v/v Fetal Bovine Serum (FBS), and used within 10 passages. CRISPR/Cas9 was used to disrupt Eya3 expression by causing a double-strand break within the gene using the Eya3 CRISPR/Cas9 KO
system (SC-406221 and SC-406221-HDR, Santa Cruz Biotechnology, Dallas TX) consisting of a pool of 3 plasmids encoding the Cas9 nuclease and one of three target-specific guide RNAs:

- Guide RNA 1: TCGCTCATCCAATGATTATA
- Guide RNA 2: AACGTATGGACTACCTCCT
- Guide RNA 3: GAAATACTTACTACCTCCT

Lentiviral short hairpin constructs targeting EYA3 (shEya3#3 and shEya3#5) and control shRNA (scramble) were obtained from Sigma-Aldrich and Addgene.

- pLKO.1 puro shEYA3#3 (TRC000005163):
  CCGGCCCTTTCTAGAAGTGACCTTTCTCGAGAAAGATGGACTTGTATAAGGGTTTTTG
- pLKO.1 puro shEYA3 #5 (TRC0000051605)
  CCGGCCGAAAGTGAGAGAAATCTACTCGAGTAGATTCTTCTACCTTTCGGTTTTTG
- pLKO.1 puro non target shRNA control (scramble)
  CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTTT

Puromycin selection was used in all instances. In addition, several CRISPR/Cas9 generated Eya3-ko colonies were selected and amplified over about 30 days.

For experiments using conditioned medium one million cells were seeded in triplicate and cultured in DMEM with 10% FBS for 24 hours. Cells were washed with PBS and then cultured in 1ml of EBM-2 (endothelial cell basal medium, Lonza) with 5% FBS for 24 hours. Medium was collected and centrifuged for the collection of cell free supernatant (conditioned medium CM).

For primary Ewing sarcoma tumor cells tissue from a recently diagnosed 11-year-old patient with aggressive, untreated Ewing sarcoma with a EWSR1-FLI1 (type 2) fusion was used in an initial xenograft in NSG mice (IRB 2008-0021). Xenografts were established and serially transplanted by intra-muscular injection of minced tumor fragments in a 50% Matrigel (Corning) suspension. Tumors obtained after the third intra-muscular implantation of tumor tissue (F3) were used for experiments. Tumor tissue was dissociated using 1 mg/ml collagenase (17018-029, Gibco) and PDX cells were cultured in RPMI-1640 medium (Gibco Lifetechnology) with 5% FBS, 0.005mg/mL Insulin (Sigma-Aldrich), 0.01mg/mL Transferrin (Sigma-Aldrich), 30nM Sodium Selenite(Sigma-Aldrich), 10nM Hydrocortisone (Sigma-Aldrich), 10nM β-estradiol (Sigma-Aldrich) and 10mM HEPES.

Cell survival in two-dimensional culture was measured using the CCK-8 kit as previously described [16, 17] and following the manufacturer's protocol.

**Xenografts.** Animal experiments were performed in accordance with the recommendation of the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center.
IACUC2016-0019. The use of de-identified patient tumor tissue was approved by the Institutional Review Board at Cincinnati Children’s Hospital Medical Center IRB2008-0021.

A total of 5 x 10^6 A673 wild type or Eya3-ko cells mixed with 30% Matrigel in 100 µL PBS were injected subcutaneously into the flank of NSG mice (6-week-old, female), and tumor size was monitored by caliper measurements (volume (mm^3) = (length x width^2) / 2). At the indicated times, mice were randomly divided into vehicle and treatment groups and were administered either vehicle (5% DMSO in phosphate-buffered saline PBS) or Benzarone (BZ) in 5% DMSO/PBS (25 µg/g body weight) via intraperitoneal injection once every 2 days.

For patient-derived xenografts (PDX) 1X10^6 PDX cells were mixed with 30% Matrigel in 100 µL PBS and injected intramuscularly into the right thigh of NSG mice (6-week-old, male). Mice body weight was monitored every two days. After one month, mice were randomly divided into vehicle and treatment groups and treated as for the A673 xenografts.

All drug studies were blinded. Animal welfare and weight was monitored every 2 days. At the end of the experiment, tumors were harvested and weighed.

**Immunostaining.** Xenograft tumor tissue specimens were fixed in 4% PFA and embedded in paraffin. Five micrometer sections were deparaffinized in xylene and treated with a graded series of alcohol and rehydrated in PBS. Antigen retrieval was done using 10 mM sodium citrate buffer. Samples were incubated with 3% H_2O_2 for 10 mins followed by blocking in 2% bovine serum albumin in PBS for 1 hour. The slides were incubated with primary antibody overnight at 4°C. For immunohistochemistry sections were incubated with secondary antibody followed by amplification using the ABC kit (Vector laboratories), then developed with dianinobenzidine (Vector Laboratories) reagent and counterstained with fast red nuclear stain. For immunofluorescence, sections were incubated with secondary antibody conjugated with Alexa Fluor (Invitrogen) for 1 hour at room temperature, and DAPI was used as counterstain.

**Colony formation assays.** 500 A673 cells were seeded with 2ml DMEM containing 10% FBS in each well of a six well plate and cultured for 24 hours. The existing medium was replaced with fresh DMEM containing 5% FBS and 0.1% DMSO or the indicated concentrations of Benzarone (BZ) once every three days and cultured for 14 days. Colonies were fixed with 100% methanol for 15 mins at room temperature and stained with 0.05% of crystal violet for 30 mins. Colonies were counted to investigate the effect of BZ.

**Trans-well Migration Assays.** 2000 cells were seeded per trans-well (Cat # P18P01250, Millipore, MA) in 100µl DMEM containing 5% FBS and different concentrations of BZ in 0.1% DMSO, or conditioned medium as indicated. Both chambers of the trans-well contained the same medium. Trans-wells were incubated for 18 hours. Cells from upper well were cleaned with a
cotton swab, migrated cells fixed in 100% methanol at room temperature for 30 mins and then stained with giemsa for 30 mins. The number of migrated cells were counted after three washes. All experiments were performed three times.

**COMET assays.** 10,000 cells were suspended in 1% low melting temperature agarose diluted in TBE (90mM Tris, 90mM Boric Acid, 2mM EDTA, ph8.5) and spread on coverslips previously coated in 1% low melting temperature agarose and allowed to solidify for 10 minutes at 4°C. Cells were lysed overnight at 4°C in 2.5M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% w/v sarkosyl, 1% Triton X-100. Lysed cells were neutralized with TBE. Lysates were incubated in alkaline buffer incubation (300mM NaOH, 1mM EDTA, pH 12.3) and then electrophoresed at 25V for 40 minutes at room temperature. Slides were dehydrated in 70% ethanol and stained with propidium iodide (1 mg/ml). Slides were imaged on a Zeiss confocal microscope. Data were analyzed using OpenCOMET [18].

**Multicellular tumor spheroids (MCTS)** were formed using the liquid overlay method [19], collected and fixed in 4% paraformaldehyde/PBS. For cryopreservation fixed MCTS were dehydrated with sucrose and snap-frozen with Optimal Cutting Temperature media (Tissue Tek, Torrance, CA). 5 μm cryo-sections were blocked in PBS pH 7.4, 0.15% triton-X 100 vv⁻¹, 10% FBS vv⁻¹, and 2% BSA vv⁻¹ in a humidified chamber, incubated with the indicated antibodies. DNA was labeled with Hoechst dye. Sections were mounted using Fluorogel with DABCO (17985-04; Electron Microscopy Science, Hatfield, PA). Fluorescently stained MCTS sections were imaged on a Zeiss confocal microscope. Nuclei were counted as Hoechst-positive cells by using watershed separation and quantification with particle analysis in ImageJ software (ImageJ, RRID:SCR_003070) followed by manual counting of the stained nuclei.

4 x 10⁴ PDX cells were seed in 96 wells plate pre-coated with 1% agarose in 100 μL DMEM with 10% FBS. Spheroids were monitored and measured. After treated with BZ for 96 hours, spheroids were fixed in 4% PFA for 30 min and cryoprotected in 15%, 30% sucrose and embedded in O.C.T. and 5 μm cryostat sections were cut.

**Sprout formation assays.** Sprout formation and cell migration from endothelial spheroids were assessed as previously described[20] with minor modifications. Briefly, 8x10⁴ HUVEC cells (between passages 5 – 7; Cat #D2650, Sigma obtained 1/20/2016) were resuspended in 4.8ml EGM2. 200μl of methyl cellulose was added to the suspension. The entire mixture was pipetted in 25μl hanging droplets on the lid of a non-tissue culture treated petri-dish and incubated for 24 hours for spheroid formation. The spheroids were collected in PBS and washed twice with PBS.
and resuspended in 500µl FBS, 1.5ml of EBM-2 and 500µl 2.5% methyl cellulose. 10X M199 was diluted in freshly prepared rat tail collagen (prepared as described in[20]) and titrated on ice to pH 7.4 using 0.1N NaOH to a final 1X M199 concentration. 2.5ml of this mixture was added to the spheroid suspension, mixed and immediately pipetted into a 48-well plate, incubated at 37°C/5% CO₂ for two hours and 100µl of the indicated conditioned medium were overlaid on top of the solidified collagen. The plates were incubated for a further 24 hours and imaged on an EVOS FL microscope with a 10X objective. The length of individual sprouts was measured from the spheroid margin to sprout apex using ImageJ and the number of sprouts were manually counted after image acquisition.

**RT-PCR.** The mRNA levels of *Eya1, Eya2, Eya3, Eya4* in Ewing sarcoma tumor cells were analyzed by RT-PCR. Total RNA was purified via PureLink™ RNA Mini Kit (Life technologies, USA). cDNA was synthesized with PrimeScript™ reagent Kit (Takara, Japan). The sequences of primers used are:

**EYA1**
- Sense: ATGGAAATGCAGGATCT
- Anti-sense: GGTAGCTGTATGGTG

**EYA2**
- Sense: GGACAATGAGATTGAGCGTGT
- Anti-sense: ATGTCCCCGTGAGGTAAGGAGT

**EYA3**
- Sense: ATGGAAGAAGAGCAAGA
- Anti-sense: GTTTGGGTGCTGAGG

**EYA4**
- Sense: GGAGGTGCTTTCCCCATTGA
- Anti sense: CAGAAGGGCATGTTGTGCTTT

**GAPDH**
- Sense: TTCATTGACCTCAACTAC
- Anti-sense: CATGGACTGTGGTCATGAG

**Statistics.** Results presented as the mean ± SD (standard deviation). Statistical analyses were performed using Graphpad PRISM version 9.0 for Mac OSX, [www.graphpad.com](http://www.graphpad.com) (GraphPad Prism, RRID:SCR_002798). A t-test was used when two samples/conditions were compared and ANOVA for more than two groups.
RESULTS.

Loss of EYA3 or pharmacological inhibition of the EYA3 PTP activity with Benzarone (BZ) retards growth of A673 tumor xenografts.

EYA3 is present at elevated levels in Ewing sarcoma cells[14]. RT-PCR performed with the A673 cell line shows Eya3 as the predominant Eya transcript, with a significantly fainter band for Eya4 (Supp. Fig. 1a). We used CRISPR/Cas9 editing to generate Eya3-ko A673 cells (Eya3-ko; Supp. Fig. 1b). Subcutaneous implantations of wild-type A673 and Eya3-ko cells were conducted on the two flanks of immune-deficient NSG mice to compare tumor growth in the same host. When tumors reached an average volume of 400mm³ they were administered either vehicle (once every 2 days intra-peritoneally) or the previously characterized EYA3 PTP inhibitor Benzarone [16, 17, 21-23] (Supp. Fig. 1c, BZ; 25 mg/kg once every 2 days intra-peritoneally). Tumor growth was monitored by caliper measurement and animal welfare monitored through observation and body weight measurements. Experiments were terminated when the vehicle-treated tumors approached 10% of body weight. Both Eya3-ko and BZ-treated tumors grew at a slower rate than control tumors and were 62% and 43% smaller respectively at the end of the experiments (Fig. 1a, b). Notably, BZ-treatment has no effect on the growth of Eya3-ko tumors (Supp. Fig. 1d). Histologic analysis of tumor sections showed increased areas of necrosis in the Eya3-ko and BZ-treated tumors relative to vehicle-treated controls (Fig. 1c). The proliferation index, estimated by quantifying the percentage of Ki67 positive cells, showed that loss or inhibition of EYA3 negatively impacts tumor cell proliferation (Fig. 1d). A modest increase in apoptosis as measured by cleaved caspase 3 (CC3) staining was seen in Eya3-ko tumors, while a much more robust increase in CC3-staining was present in BZ-treated tumors (Fig. 1e). Since BZ treatment affects both tumor and stromal cells, this difference could be a consequence of EYA3-inhibition in both tumor and non-tumor cells. Together these observations are consistent with a role for the EYA3 PTP activity in the survival and proliferation of Ewing sarcoma tumor cells.

Inhibition of EYA3 PTP activity inhibits tumor cell survival, proliferation and migration.

To better understand how EYA3 and its tyrosine phosphatase activity affect A673 cell proliferation and survival in vitro we used the tetrazolium salt WST-8 and colony formation assays. Upon either loss of EYA3 or when EYA3 PTP activity was pharmacologically inhibited there was a substantial reduction in colony formation (Fig. 2a, b). Interestingly, loss of EYA3 does not have a significant and consistent negative effect on cell proliferation in 2D culture, while BZ-treatment impairs proliferation in a dose-dependent manner as measured by the WST-8 reagent (IC50 of 7 µM) (Fig. 2c). In line with previous reports showing that EYA3 PTP activity promotes cell migration
trans-well assays showed that both Eya3-ko cells and BZ-treated A673 cells displayed reduced cell migration (Fig. 2d). Similar results as those reported for these CRISPR/Cas9-generated Eya3-ko A673 cells were obtained when Eya expression was silenced using two different shEya3 (Supp. Fig. 1b, e, f). Furthermore, an additional A673 Eya3-ko clone (Eya3-ko#2; Supp. Fig. 2a, b, d, f) as well as the effect of Eya3 silencing in another Ewing sarcoma cell line RD-ES (Supp. Fig. 2a, c, e, g) were analyzed with similar results in terms of cell proliferation, migration and colony formation.

Tumor cell proliferation and survival in a 3D context was examined using multicellular tumor spheroids (MCTS) and previously optimized protocols[19, 25]. BZ treatment resulted in a dose-dependent inhibition of MCTS growth (Fig. 3a) commensurate with its ability to inhibit cell proliferation. MCTS treated with 5µM BZ for 96 hours were visually damaged, while 2.5 µM BZ-treated MCTS were physically intact but displayed a larger dark center typical of a developing hypoxic, necrotic core [26] (Fig. 3b). Interestingly, Eya3-ko did not impede MCTS growth, rather Eya3-ko MCTS consistently expanded in diameter and became more necrotic and fragile (Fig. 3c, d). MCTS were sectioned and stained with either H&E, Ki-67 or cleaved caspase-3 (CC3) to better understand how cell proliferation and survival were impacted by loss of EYA3 or its PTP activity. BZ treatment resulted in a dose-dependent reduction in the number of Ki67-positive cells (Fig. 3e) and a large increase in apoptotic cells (Fig. 3f). EYA3-ko modestly reduced proliferation (Fig. 3e) in this 3D context and increased apoptosis (Fig. 3f). Notably, BZ had negligible effect on the growth of Eya3-ko MCTS (Supp. Fig. 3a). The consequences of EYA3 loss on MCTS growth, proliferation and apoptosis in a 3D context was corroborated using an independent Eya3-ko clone (Eya3ko#2) in A673 cells, and using RD-ES cells (Supp. Fig. 3b – g).

**EYA3-PTP inhibition retards growth of a patient-derived ES xenograft.** To extend observations made using the Ewing sarcoma cell lines into a more clinically relevant model we used a patient-derived xenograft (PDX). Tissue from a recently diagnosed 11-year-old patient with aggressive, untreated Ewing sarcoma was used in an initial xenograft in immune-compromised NSG mice. The patient had a large pelvic tumor with metastases to bone (but not lung). On H&E stains the primary intraosseous tumor (Fig. 4a) and its soft tissue extensions (Fig. 4b) displayed the typical small round cell morphology of Ewing sarcomas. Tumor cells were relatively uniform with round nuclei, delicate chromatin, inconspicuous nucleoli, and scant cytoplasm. Immunohistochemical staining for CD99 showed a classic sharp, crisp membranous staining pattern (Fig. 4c). FISH demonstrated rearrangement of the *EWSR1* gene supportive of the diagnosis of Ewing’s sarcoma. P1 (first-generation) tumors were excised 3 months after the

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initial implantation, characterized by H&E staining and re-implanted into NSG mice to expand the tumor tissue. A third intra-muscular implantation of tumor tissue (P3) was used for experiments (Fig. 4d). Treatment with either vehicle or BZ was initiated 30 days after xeno-transplantation. Intra-peritoneal vehicle or 25 mg/kg BZ was administered on alternate days for a total of 18 doses. Animal well-being and weight was monitored, but because of the site of implantation tumor size could not be readily monitored during the experiment. No significant difference in weight gain over the treatment period was observed between vehicle-treated and BZ-treated animals. The experiment was terminated when vehicle-treated mice began showing signs of distress. Tumors were excised and weighed; tumors from BZ-treated animals were on average 50% smaller than from vehicle-treated controls (Fig. 4e). Tumor sections were stained for the proliferation marker Ki67 and showed that BZ-treatment resulted in a large reduction in proliferation index (Fig. 4f, g). Staining for the apoptosis marker cleaved caspase 3 showed increased apoptosis in BZ-treated tissue (Fig. 4h) as was observed in A673 xenografts.

In vitro, using both WST-8 and trypan blue assays in 2D culture the IC50 with BZ for these PDX cells was 6.7 µM. We analyzed the effect of BZ-treatment on PDX cells in 3D culture. MCTS were generated and treated with either vehicle or BZ when they reached an average diameter of 400 µm. Growth rate was then followed until control MCTS stopped growing further. Treatment of PDX cell MCTS with BZ resulted in dose-dependent growth inhibition (Fig. 4i). MCTS were then fixed, sectioned and stained with markers of proliferation and apoptosis; BZ-treatment led to reduced Ki67 staining and increased apoptosis (Fig. 4j, k, l). Together these results corroborate the relevance of the EYA3 PTP activity in progression of a patient-derived Ewing sarcoma tumor.

**γH2AX-positive cells in tumor tissue are reduced upon loss of EYA3 PTP activity.**

The EYA3 PTP activity has previously been shown to promote DNA damage repair[27, 28]. In various pathologies, loss of EYA3-PTP activity results in impaired DNA damage repair as evidenced by smaller percentages of cells that stain positively for the DNA damage and repair marker γH2AX[21-23]. In order to evaluate the effect of EYA3 PTP inhibition on DNA damage repair in Ewing sarcoma we stained both A673 and PDX tumor tissue for γH2AX. Positivity for γH2AX (Ser139 phosphorylated form of H2AX) has been ascribed with prognostic value in a variety of cancers including breast, endometrial, lung, cervical and ovarian cancer[29, 30]. DNA damage and γH2AX-positivity is commonly induced by genotoxic chemotherapy, but is also present in untreated tumors as a result of replicative and oxidative stress. We examined γH2AX staining to evaluate the levels of constitutive DNA damage and ongoing repair. A673 tumors show
high levels and broadly distributed $\gamma$H2AX-positivity (Fig. 5a). In contrast, PDX tumors have less $\gamma$H2AX staining overall and the staining was often present in distinct patches (Fig. 5e, f). In both A673-Eya3-ko tumors (Fig. 5c, d) and in BZ-treated A673 tumors (Fig. 5b, d) $\gamma$H2AX staining was substantially reduced. BZ treatment did not reduce the percentage of $\gamma$H2AX-positive cells in Eya3-ko tumors (Supp. Fig. 4b) attesting to target-specificity of BZ.

Similarly, BZ treatment reduced $\gamma$H2AX staining in PDX tumors (Fig. 5g, h). Along with the evidence of increased apoptosis presented in Figures 1-4 these observations can be interpreted as a failure to induce an adequate DNA damage repair response thus tipping the balance towards cell death.

Both A673 and PDX cell MCTS were evaluated for the effect of EYA3 loss or BZ treatment on $\gamma$H2AX levels. As in the case of tumor tissue, either loss of EYA3 or BZ treatment reduces $\gamma$H2AX levels in A673 MCTS (Fig. 5i, j), and BZ-treatment reduced $\gamma$H2AX levels in PDX cell MCTS (Fig. k, l). An alkaline COMET assay was used to evaluate DNA damage and showed that both loss of EYA3 and BZ-treatment resulted in increased DNA damage (Fig. 5m, Supp. Fig. 4a).

**Loss or inhibition of EYA3 PTP activity elevates levels of the substrate H2AX-pY$_{142}$ in tumor tissue.**

H2AX-pY$_{142}$ is an established substrate of the EYA3 PTP activity[27]. To assess target-engagement we stained tumor tissue with anti-H2AX-pY$_{142}$. Both A673 and PDX tumor sections showed low levels of constitutive H2AX-Tyr142 phosphorylation (Fig. 5n, r). Interestingly, this was not uniform but rather concentrated in areas with smaller more dense nuclei suggestive of cell death. Either loss of EYA3 (Fig. 5o, q) or inhibition of EYA3-PTP activity with BZ (Fig. 5p, q, r, s, t) led to a substantial increase in positivity for H2AX-pY$_{142}$. This observation provides evidence that EYA3 dephosphorylates H2AX-pY$_{142}$ in Ewing sarcoma tumor cells and that BZ directly impairs this de-phosphorylation reaction in vivo.

**Loss of EYA3 in tumor cells and pharmacological inhibition of the EYA3-PTP activity reduces tumor vascularity.**

We evaluated vascular hotspots within the tumors (maximal vascular density MVD), a widely used method for evaluating vascular density. In both A673(Eya3-ko) xenografts and in A673 xenografts treated with BZ we see a significant reduction in MVD (Fig. 6a, b), while BZ did not impact the vascularity of Eya3-ko tumors (Supp. Fig. 4c). BZ treatment also reduced vascularity in PDX tumors (Fig. 6e, f). Inhibition of tumor angiogenesis upon BZ-treatment is consistent with our previous studies showing that endothelial EYA3-PTP activity promotes angiogenesis[17] and that
loss of endothelial *Eya3* inhibits tumor angiogenesis in Lewis lung carcinoma (LLC) xenografts[21]. However, the anti-angiogenic effect upon loss of Ewing sarcoma tumor cell EYA3 differs from our results with LLC tumors and suggests the presence of an EYA3-dependent pro-angiogenic property in Ewing sarcoma cells.

Previous studies of the angiogenic growth factor expression profile in Ewing sarcoma cells revealed that vascular endothelial growth factor (VEGFA) expression is most significantly associated with the development of micro-vessels [31]. Both Eya3-ko tumors and BZ-treated A673 tumors had lower levels of VEGF as detected by immunohistochemistry (Fig. 6c, d). Analysis of PDX tumor tissue corroborated both the anti-angiogenic effect of BZ as measured by endomucin staining (Fig. 6e, f) and the reduction in VEGF levels in tumor tissue (Fig. 6g).

We then investigated whether EYA3 in A673 tumor cells contributes to the release of pro-angiogenic factors by testing the effect of tumor cell conditioned medium (CM) on endothelial cell proliferation and migration. Significantly reduced endothelial cell migration and proliferation was recorded in experiments conducted with CM from A673 cells lacking EYA3 relative to CM from control A673 cells (Fig. 6h, i). Similar results were obtained upon using CM from RD-ES cells (Supp. Fig. 4d, e). To further explore the effect of tumor cell EYA3 on angiogenesis we used an endothelial spheroid sprouting assay. HUVEC spheroids were embedded in collagen and maintained in either A673 conditioned basal medium or A673 Eya3-ko conditioned medium. Sprouting angiogenesis was observed with A673 CM, but negligible sprouting was observed with Eya3-ko CM (Fig. 6j - l). Similar results were obtained when EYA3 levels in A673 were reduced using shEya3 (Fig. 6k, l). Together these observations support a role for EYA3 in Ewing sarcoma cells as a positive regulator of a secreted angiogenic factor, and our in vivo data suggests that VEGFA is regulated by EYA3 in Ewing sarcoma cells. The molecular mechanism(s) through which EYA3 regulates VEGF levels in Ewing sarcoma cells remains to be determined. Because there is evidence, in other contexts, that EYA1 could regulate VEGFA through the HIF1α-PI3k-Akt pathway[32] we evaluated the effect of either EYA3 loss or BZ-treatment on activated Akt (pS474). In our studies we do not see a significant impact on either HIF1α or Akt activation (Fig. 6m). The mechanism of VEGFA regulation by EYA3 is the subject of ongoing investigation.

**DISCUSSION.**

In this study we show that EYA3 in Ewing sarcoma tumor cells promotes both tumor growth and tumor angiogenesis. Further, pharmacological targeting of the EYA3 PTP activity inhibits Ewing
sarcoma tumor growth and vascularization. Hence EYA3 PTP activity can contribute to Ewing sarcoma progression through multiple cellular mechanisms. While the retardation of tumor growth by EYA3-PTP inhibition or EYA3 loss is consistent with our previous observations using a murine lung carcinoma model (LLC in C57/Bl6 mice)[21], this is the first report of a role for tumor cell EYA3 in Ewing sarcoma tumor angiogenesis.

The EYA proteins are known to contribute to cell cycle progression and DNA damage repair [27, 28], both of which impact tumor growth. DNA damage repair pathways are commonly upregulated in cancers, but in Ewing sarcoma cell lines and primary tumors several DNA-damage repair genes, including BRCA1, GEN1 and ATM, are down-regulated [33]. In contrast, EYA3 levels are elevated via EWS/FLI1-mediated downregulation of miR-708[14] (Fig. 6n). In growing tumors repair of replicative and oxidative stress-induced DNA damage permits unregulated tumor cell proliferation and genomic instability. The EYA PTP activity promotes DNA damage repair through dephosphorylation of the C-terminal Y142 residue of H2AX[27] (Fig. 6n). H2AX is a mediator of the DNA damage response acting as a scaffold for either repair-mediating or apoptosis-mediating factors. The phosphorylation state of H2AX-Tyr142 acts as a switch in this repair versus apoptosis decision, with the EYA-PTP tipping the balance towards repair[27] (Fig. 6n). Upon loss of EYA3 or its PTP activity we consistently see less staining for γH2AX (a marker for DNA damage and repair), and increased apoptosis. Interestingly loss of EYA3 does not significantly impact tumor cell proliferation in 2D cell culture or the growth of multicellular tumor spheroids derived from either A673, RD-ES, or patient-derived primary tumor cells. But there is a significant increase in the size of the necrotic zone and the percentage of apoptotic cells in MCTS lacking EYA3. Together these observations are consistent with a role for EYA3 in the survival of tumor cells under hypoxic stress. Loss or inhibition of EYA3-PTP activity also inhibited cell motility consistent with previous studies showing a role for the EYA3 PTP activity in cell migration[24]. We were unable to evaluate the effect of EYA3 inhibition on metastasis here since neither the A673 nor the patient-derived xenografts yielded metastatic foci.

Benzarone (BZ) has been extensively characterized in its role as an EYA PTP inhibitor[13, 16, 17, 21-23]. But BZ and the related compound Benzbromarone have a long and checkered history as therapeutics[34, 35]. Both compounds are uricosurics with exceptional efficacy in the treatment of gout. They were withdrawn by the manufacturer from European markets after over 20 years of use due to some reports of hepatotoxicity. However, because of their effectiveness as anti-gout agents, there remains debate about their clinical utility[34, 35]. Relevant to this report, with the dosage regimen used in this study we saw no detectable toxicity[22], and there is a case to be
made that acceptable toxicity for an anti-cancer agent is different than that for the chronic treatment of gout. The issues regarding the safety profile of Benzaron notwithstanding, the results presented here provide strong validation of the EYA3-PTP activity as a target for the development of Ewing sarcoma therapeutics, supported by the use of Benzaron as a pharmacological tool. In addition to the efficacy readout obtained through monitoring tumor growth and angiogenesis, we also examined a pharmacodynamic readout through target-engagement assays. We measured changes in the level of the substrate H2AX-pY142 to evaluate the molecular events triggered by BZ treatment, and the assembly of DNA damage repair complexes (γH2AX staining) as a functional consequence (Fig. 5). Strong upregulation of the EYA3-PTP substrate H2AX-pY142 was seen upon BZ treatment. Importantly, these results phenocopied observations made using a genetic strategy (loss of EYA3 in tumor cells, Eya3-ko). While off-target effects cannot be ruled out for BZ, or indeed any chemical compound, our results are strongly supportive of EYA3 as a biologically relevant target of BZ in vitro and in vivo.

We anticipated a reduction in tumor vascularity upon treatment with BZ, based upon our previous studies showing that loss of EYA3-PTP activity in host endothelial cells can inhibit tumor angiogenesis[21]. Unexpectedly loss of EYA3 in A673 cells also reduced tumor vascularity and reduced VEGF levels in tumor tissue (Fig. 6). Previous studies have shown that VEGFA is the single most important regulator of angiogenesis in Ewing sarcoma [31]. Positive regulation of VEGFA (and HIF1α) by EYA1 has previously been reported to occur via activation of the PI3K-Akt pathway in colorectal tumor cells [32]. A more recent report demonstrates EYA1-dependent regulation of VEGFA levels in adipose-derived stem cells[36]. This is the first evidence that EYA3 also regulates VEGFA levels. However, our studies do not support a role for the PI3k-Akt pathway in EYA3-mediated VEGFA regulation in the present context. The precise mechanism(s) through which EYA3 regulates the VEGFA pathway in Ewing sarcoma tumor cells, and the possibility that other angiogenic cytokines or growth factors are regulated by EYA3 remain the subject of ongoing investigations. Furthermore, we have previously shown that DNA damage repair promoted by EYA3 can play a role in pathological angiogenesis[23]. Hence loss of EYA3 PTP activity could modulate tumor vascularization through multiple mechanisms.

In Ewing sarcoma, micro-vessel density is a prognostic marker that is significantly correlated with the expression of VEGF-A [31, 37-39]. Accordingly, anti-VEGF agents inhibit the growth of Ewing sarcoma family tumors[39]. While only modest success has been reported for VEGF-targeting anti-angiogenics as monotherapy in the treatment of Ewing sarcoma, there is evidence that combination of conventional chemotherapy with antiangiogenic agents may be more effective[7].
EYA3 could represent a unique target in this respect. Previous studies demonstrated chemosensitization in vitro upon loss of EYA3 in Ewing sarcoma cell lines[14], consistent with the ability of EYA3 to promote DNA damage repair. Exploiting the ability of EYA3-PTP inhibitors to act as anti-angiogenics as well as chemo-sensitizers would allow the use of lower doses of cytotoxic chemotherapy in such combination regimens. Evaluation of the efficacy and safety of such treatment strategies are ongoing. The present report provides proof-of-principle that EYA3 PTP in Ewing sarcoma tumor cells contributes to tumor growth and angiogenesis (Fig. 6n) and is pharmacologically targetable in a patient-derived tumor model.

Author contributions:
YW and RNP performed all of the experiments described here except the HUVEC sprouting experiments that were performed by KR. DM and TVK implanted patient tumors into NSG mice to generate the initial patient-derived xenograft. JGP provided patient tumor tissue. SS was the clinical pathologist who characterized the patient tumor. RSH conceived the project, designed the experiments, oversaw the performance and analysis of all experiments and wrote the manuscript. All authors have read and approved the data, analysis and conclusions in this manuscript.
REFERENCES.


FIGURES AND LEGENDS.

Figure 1. Loss or inhibition of the EYA3-PTP inhibits A673 tumor growth.

a) Tumor growth curve of A673 and A673 Eya3-ko tumors treated with either vehicle or the EYA-PTP inhibitor Benzarone (BZ). The arrow indicates the time when treatment was initiated. Data shown as mean ± SD, two-way ANOVA with Bonferroni’s post-test was used for analysis (n = 6, * P<0.05, ** P<0.01, *** P<0.001).

b) Weights of tumors at the termination of the experiment. Data is represented as the mean ± SD, and one-way ANOVA was used; n = 6.

c) Representative H&E staining of tumor tissue.

d) Quantitation of Ki-67 positive cells. Six representative images were analyzed from each tumor. Data represents mean ± SD. One-way ANOVA was used.

e) Quantitation of cleaved caspase 3 (CC3) staining. Six representative images were analyzed from each tumor. Data represents mean ± SD. Unpaired t-tests were used to compare the indicated pairs.

Figure 2. Loss or inhibition of EYA3-PTP inhibits A673 tumor cell survival and migration.

a) Loss of EYA3 or inhibition with BZ inhibits colony formation. Representative images are shown.

b) Quantification of colony formation assays. Data represent mean ± SD of 3 assays and significance was evaluated by one-way ANOVA.

c) Loss of EYA3 does not affect A673 cell proliferation as measured by WST-8 assays. Treatment with BZ has a dose-dependent effect on cell survival. Data shown as the mean ± SD of 3 independent assays with 2 readings per assay. Significance was evaluated by one-way ANOVA.

d) Loss or inhibition of EYA3 inhibits cell migration in a trans-well assay. Data represents the mean ± SD of 3 independent assays with 2 readings per assay and significance was evaluated by one-way ANOVA.

Figure 3. Effect of EYA3-PTP loss or inhibition on A673 tumor spheroids (MCTS).

a) A673 MCTS were treated with either vehicle or the indicated concentrations of BZ when they were between 425 – 475 µm in diameter. Medium was changed every 3 days and the growth of MCTS monitored. Data represent mean ± SD, two-way ANOVA with Bonferroni’s post-test was used for analysis (n = 9, * P<0.05, ** P<0.01, *** P<0.001).
b) Brightfield and H&E-stained images of vehicle and 2.5 μM BZ-treated MCTS showing the growing necrotic core even when the diameter of the MCTS is not substantially reduced.

c) Growth curves of A673 and A673 Eya3-ko MCTS. Data represent mean ± SD, two-way ANOVA with Bonferroni’s post-test was used for analysis (n = 15).

d) Brightfield and H&E-stained images of A673 and A673 Eya3-ko MCTS showing the growing necrotic core even when the diameter of the MCTS is not substantially reduced.

e) Quantitation of Ki67-positive cells in MCTS sections. Data represent mean ± SD. One-way ANOVA used for significance assessment.

f) Quantitation of cleaved caspase 3 (CC3)-positive cells in MCTS sections. Data represent mean ± SD. One-way ANOVA used for significance assessment.

Figure 4. Inhibition of the EYA3-PTP activity retards growth of a patient-derived Ewing sarcoma xenograft (PDX).

a) H&E-stained tumor tissue from an image-guided core biopsy. Blue areas are cellular tumor with small round cell morphology permeating through bone. Tumor fills marrow spaces between bone spicules which are being secondarily remodeled and weakened.

b) Soft tissue extension of patient tumor. Cellular tumor islands, sheets and cords are seen in a collagenous/desmoplastic stromal background. Tumor cells are relatively uniform, of small to moderate size, with round nuclei, delicate chromatin and inconspicuous nucleoli. Nuclei are closely spaced to each other given their scant amount of cytoplasm. Scattered mitotic figures and spotty karyorrhectic nuclei are present.

c) Immunohistochemical stain for CD99 shows classic membranous staining patterns in well-preserved portions of the patient tumor tissue sample.

d) H&E staining of the first-generation xenograft from patient tumor tissue.

e) Final weights of PDX tumors after 18 doses of vehicle or BZ (25 mg/kg, intra-peritoneal administered every other day). Two-tailed t-test was used and data is represented as the mean and SD.

f) Representative images of vehicle- and BZ-treated tumor tissue stained for the proliferation marker Ki67 (brown).

g) Quantitation of Ki67-positive cells in vehicle- and BZ-treated tumor tissue. Two-tailed t-test was used and data is represented as the mean and SD.

h) Quantitation of CC3-positive cells in vehicle- and BZ-treated tumor tissue. Two-tailed t-test was used and data is represented as the mean and SD.
i) Growth curves of MCTS generated with PDX-derived tumor cells. Data represent mean ± SD, two-way ANOVA with Bonferroni’s post-test was used for analysis (n = 6, * P<0.05, ** P<0.01, *** P<0.001).

j) Representative images of Ki67 (red) and CC3 (green) stained PDX-MCTS sections treated with either vehicle or the indicated concentrations of BZ for 12 hours. Sections are counter-stained with DAPI (blue).

k) Quantitation of Ki67-positive cells in vehicle- and BZ-treated MCTS. Data represented as mean and SD. One-way ANOVA was used.

l) Quantitation of CC3-positive cells in vehicle- and BZ-treated MCTS. Data represented as mean and SD. One-way ANOVA was used.

Figure 5. Loss or pharmacological inhibition of EYA3-PTP reduces the percentage of γH2AX-positive cells and increases the percentage of EYA3 substrate H2AX-pY142 cells in tumor tissue.

a) Representative tumor section from A673 tumors treated with vehicle stained for γH2AX (brown).

b) Representative tumor section from A673 tumors treated with BZ stained for γH2AX (brown).

c) Representative tumor section from A673 Eya3-ko tumors stained for γH2AX (brown).

d) Either loss of EYA3 or treatment with BZ reduces the percentage of γH2AX-positive cells in A673 tumor xenografts. Data is shown as the mean and SD, n = 4 mice with 4 random sections per mouse analyzed. One-way ANOVA was used to assess significance.

e) Representative tumor section from PDX tumor treated with vehicle and stained for γH2AX (brown). Vehicle treated tumor tissue shows less γH2AX-positive cells than in the case of A673 xenografts.

f) Another representative tumor section from PDX tumors treated with vehicle stained for γH2AX (brown) showing that discrete regions were strongly γH2AX-positive.

g) Representative tumor section from PDX tumors treated with BZ and stained for γH2AX (brown) showing an overall reduction in γH2AX positivity.

h) Treatment with BZ (using treatment protocols described in Fig. 4) reduces the percentage of γH2AX-positive cells in PDX tumor xenografts. Data is shown as the mean and SD, n = 4 mice with 4 random sections per mouse analyzed. Two-tailed t-test was used to assess significance.
i) A673 MCTS were sectioned and stained for the DNA damage and repair marker γH2AX (red) and counter-stained with DAPI (blue). Both Eya3-ko and treatment with the EYA3-PTP inhibitor BZ results in fewer γH2AX-positive cells.

j) Quantification of the percentage of γH2AX-positive cells in MCTS. Data represent mean ± SD. One-way ANOVA was used to assess significance.

k) MCTS generated from patient-derived tumor cells (PDX) were sectioned and stained for the DNA damage and repair marker γH2AX (red) and counter-stained with DAPI (blue). Treatment with the EYA3-PTP inhibitor BZ results in fewer γH2AX-positive cells.

l) Quantification of the percentage of γH2AX-positive cells in PDX MCTS. Treatment with BZ significantly reduced the percentage of γH2AX-positive cells. Data represent mean ± SD. One-way ANOVA was used to assess significance.

m) OpenComet[18] quantification of percentage of DNA in tails in an alkaline COMET assay performed on the indicated cells. Increased DNA in tails upon loss or inhibition of EYA3 indicates elevated levels of DNA damage. The median is indicated by the line in these scatter dot plots. One-way ANOVA was used to assess significance.

n) Representative vehicle-treated A673 tumor section stained for the EYA3-PTP substrate H2AX-pY142 (red).

o) Representative vehicle-treated A673 Eya3-ko tumor section stained for the EYA3-PTP substrate H2AX-pY142 (red). Increased staining is seen in discrete areas with nuclear morphology indicative of early stages of cell death.

p) Representative section from a BZ-treated A673 tumor (using the treatment protocol described in Fig. 1) stained for the EYA3-PTP substrate H2AX-pY142 (red). Increased staining is seen in discrete areas with nuclear morphology indicative of early stages of cell death.

q) Quantification of H2AX-pY142 staining in A673 xenograft tissue. Data represent mean ± SD; four sections per tumor were averaged for each data-point. One-way ANOVA was used to assess significance.

r) Representative vehicle-treated PDX tumor section stained for the EYA3-PTP substrate H2AX-pY142 (red).

s) Representative section from a BZ-treated PDX tumor (using the treatment protocol described in Fig. 4) stained for the EYA3-PTP substrate H2AX-pY142 (red). Increased staining is seen in discrete areas with nuclear morphology indicative of early stages of cell death.
t) Quantification of H2AX-pY_{142} staining in PDX xenograft tissue. Data represent mean ± SD; four sections per tumor were averaged for each data-point. Two-tailed t-test was used to assess significance.

**Figure 6. Loss of EYA3-PTP activity reduces the vascularity of both A673 and patient-derived Ewing sarcoma xenografts.**

a) Representative sections of A673 vehicle or BZ-treated tumors and A673-Eya3-ko tumors stained with the endothelial marker endomucin (brown).

b) Quantification of vascularity (endomucin-positive cells) in A673 xenografts. Mean and SD is shown with 4 random sections per mouse analyzed. One-way ANOVA was used to assess significance.

c) Representative A673 tumor sections stained for VEGFA (brown).

d) Quantification of VEGFA-positive cells in A673 xenograft tissue (using the xenograft protocol described in Fig. 1). Mean and SD is shown with 4 random sections per mouse analyzed. One-way ANOVA was used to assess significance.

e) BZ-treatment reduces vascularity in PDX tumor tissue as shown by the reduction in endomucin staining (brown).

f) Quantification of vascularity (endomucin-positive cells) in PDX xenografts (using the protocols described in Fig. 4). Mean and SD is shown with 4 random sections per mouse analyzed. Two-tailed t-test was used to assess significance.

g) Quantification of VEGFA-positive cells in PDX xenografts (generated using protocols described in Fig. 4). Mean and SD is shown with 4 random sections per mouse analyzed. Two-tailed t-test was used to assess significance.

h) Endothelial cells (HUVECs) were cultured in conditioned medium from A673, A673 Eya3-ko, A673-scramble, or A673-shEya3 cells. HUVEC proliferation was measured after 72 hours using the WST-8 reagent. The experiment was conducted 3 times and representative data is shown here as the mean and SD. Two-tailed t-test was used to assess significance.

i) Trans-well migration of HUVECs in conditioned medium from A673, A673 Eya3-ko, A673-scramble, or A673-shEya3 cells was measured. The experiment was conducted 3 times and representative data is shown here as the mean and SD. Two-tailed t-test was used to assess significance.
H) HUVEC spheroids were cultured in A673, A673 Eya3-ko, A673-scramble, or A673-shEya3 conditioned medium. Loss of EYA3 reduced the number (k) and average length (l) of sprouts. One-way ANOVA was used to assess significance.

m) Western blot showing no significant EYA3-dependent changes in the levels of HIF1α, AKT, AKT-pS473 either under normal tissue culture conditions or upon the induction of HIF1 alpha (hypoxia) by treatment with the hypoxia-mimic CoCl₂.

n) Schematic depiction of the proposed model (integrating both data from this study and published reports) through which the EYA3-PTP activity contributes to tumor growth and angiogenesis, and is attenuated by the EYA3-PTP inhibitor BZ (shown in red). Tumor hypoxia can induce both DNA damage and upregulation of the pro-angiogenic factor VEGFA. DNA damage results in the upregulation of ATM/ATR kinases that phosphorylate the histone protein H2AX on Ser139 (forming γH2AX). H2AX is constitutively phosphorylated on Tyr142 by the WSTF kinase[40]. The Tyr142 phosphorylated form of H2AX promotes apoptosis. EYA3-PTP dephosphorylates H2AX at Tyr142. The Y142-dephosphorylated form of γH2AX promotes assembly of DNA damage repair complexes[27], that in turn can promote cell survival and tumor growth. Our data show that EYA3 also upregulates VEGFA levels promoting tumor angiogenesis, and thus tumor growth. In Ewing sarcoma EWS/FLI1 suppresses miR-708 which is a negative regulator of EYA3 (inset box)[14]. Hence in Ewing sarcoma cells EYA3 levels are elevated and can contribute to tumor cell survival and angiogenesis. BZ inhibits the EYA3-PTP activity and can thus inhibit both tumor angiogenesis and tumor cell survival.
Figure 1

(a) Tumor volume (mm³) over time for A673-Veh, A673-BZ, and Eya3-Ko - Veh groups. 
(b) Tumor weight (g) at different time points for A673, Eya3-ko, and BZ groups. 
(c) Representative images of tumor sections stained with H&E. 
(d) % Ki-67/DAPI for A673, Eya3-ko, and BZ groups. 
(e) % CG3/DAPI for A673, Eya3-ko, and BZ groups.

Figure 2

(a) Representative images of colony formation assay for A673, A673-Eya3ko, Veh, BZ 2.5 μM, and BZ 5 μM groups. 
(b) Number of colonies > 50 cells for A673, Eya3-ko, BZ 2.5 μM, and BZ 5 μM groups. 
(c) % fold change in colony formation for A673, Eya3-ko, BZ 2.5 μM, and BZ 5 μM groups. 
(d) % migration for A673, Eya3-ko, BZ 2.5 μM, and BZ 5 μM groups.
Figure 5
Figure 6

(a) A673-Veh, A673-BZ, A673, Eye3-ko-Veh.
(b) % vascularization.
(c) A673-Veh, A673-BZ, A673 Eye3-ko-Veh.
(d) % VEGF positive.
(e) PDX-vehicle, PDX - BZ.
(f) Endomucin.
(g) % VEGF positive.
(h) Fold change.
(i) CTL, Eye3-ko acr, shEye3.
(j) A673, Eys-ko, Eys, BZ, shEys.
(k) # cells migrated.
(l) CTL, Eye3-ko acr, shEye3.
(m) A673 Conditional Medium.
(n) H2AX-pS139, H2AX-pS139, DNA DAMAGE.

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TARGETING EYA3 IN EWING SARCOMA RETARDS TUMOR GROWTH AND ANGIOGENESIS

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