Inhibition of Eyes Absent Homolog 4 expression induces malignant peripheral nerve sheath tumor necrosis

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Abstract

Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive sarcomas without effective therapeutics. Bioinformatics was used to identify potential therapeutic targets. Paired Box (PAX), Eyes Absent (EYA), Dachsund (DACH), and Sine Oculis (SIX) genes, which form a regulatory interactive network in drosophila, were found to be dysregulated in human MPNST cell lines and solid tumors. We identified a decrease in DACH1 expression, and increases in expression of PAX6, EYA1, EYA2, EYA4, and SIX1-4. Consistent with the observation that half of MPNSTs develop in neurofibromatosis type 1 patients, subsequent to NF1 mutation, we found that exogenous expression of the NF1-GAP related domain (GRD) normalized DACH1 expression. EYA4 mRNA was elevated more than 100-fold as estimated by quantitative real time PCR in most MPNST cell lines. In vitro, suppression of EYA4 expression using shRNA reduced cell adhesion and migration and caused cellular necrosis without affecting cell proliferation or apoptotic cell death. MPNST cells expressing sh-EYA4 either failed to form tumors in nude mice or formed very small tumors, with extensive necrosis but similar levels of proliferation and apoptosis as control cells. Our findings identify a role for EYA4 and possibly interacting SIX and DACH proteins in MPNSTs and suggest the EYA4 pathway as a rational therapeutic target.
Introduction

Malignant peripheral nerve sheath tumors (MPNSTs) are highly aggressive soft tissue sarcomas with a poor prognosis. Half of MPNST cases arise in individuals with Neurofibromatosis type 1 (NF1), an autosomal dominant disorder affecting approximately 1 in 3500 individuals worldwide (Friedman et al. 1997; Rasmussen et al. 2000). While sporadic MPNSTs develop in the general population at the low incidence of 0.0001%, the lifetime risk of an NF1 patient developing MPNST is as high as 8–13% (Evans et al. 2002). There is evidence suggesting that cells within plexiform neurofibromas, benign tumors found in approximately 30% of NF1 patients, undergo malignant transformation and give rise to MPNST (Leroy et al. 2001; Ferner et al. 2002; Carroll et al. 2008). MPNST remains a major source of mortality for NF1 patients, because there is currently no effective therapy. Conventional radiation and chemotherapy are generally unsuccessful in controlling MPNST growth, especially in NF1 patients. Although early detection and excision of the tumor improve survival, complete resection is not always feasible and local recurrence is common (Carli et al. 2005). Metastasis to the lung, liver, and brain is also observed (Ferner et al. 2002). While the clinical course of NF1-associated MPNSTs is considerably worse than that of sporadic MPNSTs, their gene expression profiling is indistinguishable (Holtkamp et al. 2004; Watson et al. 2004; Miller et al. 2006).

A major pathway implicated in MPNST formation is the NF1-Ras pathway. The NF1 gene product, neurofibromin, is one of a family of GTPase activating proteins (GAPs) that accelerates the hydrolysis of active Ras-GTP to inactive Ras-GDP (McCormick 1995). Single missense mutations in the GAP-related domain (GRD) of NF1 have been detected in patients (Klose et al. 1998). Furthermore, MPNST cell lines and tumors have elevated basal Ras-GTP (Basu et al. 1992; DeClue et al. 1992; Kim et al. 1995; Sherman et al. 2000). Replacing the NF1-GRD rescued the cardiovascular defect responsible for embryonic lethality in Nf1-deficient mice but did not rescue abnormalities in neural crest development (Ismat et al. 2006), although several lines of evidence support the neural crest origin of NF1-associated MPNSTs (reviewed in Carroll et al. 2008; Miller and Jessen et al. 2009; Vogel et al., 1999), suggesting involvement of additional or alternative molecular pathways in NF1 tumorigenesis.

To identify dysregulated molecular pathways in MPNSTs that might guide novel therapeutic strategies, we recently conducted global gene expression analysis of NF1-associated tumors and tumor-derived Schwann cells, including sporadic and NF1-derived MPNSTs, on the whole-genome Affymetrix platform (Miller and Jessen et al. 2009). The mRNA encoding the SOX9 transcription factor was over-expressed in benign MPNST precursor lesions (neurofibroma), and expression of SOX9 was further elevated in MPNSTs. Computational promoter analysis identified EYA4, a gene highly expressed in MPNSTs, as a potential transcriptional target of SOX9. Supporting the in silico analysis, inhibition of SOX9
expression using shRNA in MPNST cells reduced EYA4 expression levels, and exogenous expression of SOX9 in normal human Schwann cells and neurofibroma cells increased EYA4 expression levels.

The transcriptional network involving members of the EYA, SIX, DACH, and PAX gene families was originally characterized in fly eye development and is commonly referred to as the retinal determination (RD) pathway (Silver et al. 2005). While the epistatic relationships among the ey-so-eya-dac genes in flies is well characterized, the expression relationships among the vertebrate Pax-Six-Eya-Dach genes differ based on tissue types and stages of development. The SIX proteins are DNA-binding transcription factors, which variously activate (Xu et al. 1996) or repress (Kobayashi et al. 2001; Zhu et al. 2002) transcription. The DACH proteins can also activate (Ikeda et al. 2002) or repress (Wu et al. 2006; Wu et al. 2009) transcription in a context-dependent fashion. EYA proteins are both transcriptional activators (Xu et al. 1996) and tyrosine phosphatases (Rayapureddi et al. 2003; Tootle et al. 2003) and have recently been shown to dephosphorylate H2AX, promoting repair and cell survival in the response to DNA damage (Cook et al. 2009). EYA proteins (EYA1 - 4) are normally expressed early in development (Xu et al. 1996; Abdelhak et al. 1997; Borsani et al. 1999) and promote stem cell survival (Li et al. 2003). This is relevant, because mouse and human MPNST cells have characteristics of neural crest stem cells (Josephson et al. 1998; Vogel et al. 1999; Miller and Jessen et al. 2009).

Individual members of the RD pathway have been implicated in tumorigenesis (Christensen et al. 2008). Expression of several PAX family members has been associated with cancer cell line survival (Muratovska et al. 2003). Downregulation of DACH1 expression has been observed in breast (Wu et al. 2006) and prostate cancer (Wu et al. 2006; Wu et al. 2009). Upregulation of SIX1 expression has been observed in a variety of cancers (Reichenberger et al. 2005; Ng et al. 2006; Behbakhht et al. 2007; Kroemer et al. 2009), and overexpression of SIX1 is sufficient for malignant transformation of mammary cells (Coletta et al. 2008). Overexpression of EYA2 has been shown to promote ovarian tumor growth (Ramdas et al. 2001). Here, we describe the dysregulation of a transcriptional network consisting of the EYA, SIX, DACH and PAX genes in MPNSTs and provide evidence supporting a role for EYA4 in MPNST survival and tumorigenesis.

Materials and Methods

Microarray analyses

Collection of samples, RNA isolation, probe generation, Affymetrix HU133 Plus 2.0 microarray hybridization, and normalization of data was conducted as described for differential gene expression in neurofibroma and MPNST samples relative to normal human Schwann cells (Miller and Jessen et al., 2009). Using Genespring GX v7.3.1 (Agilent Technologies), hierarchical cluster analysis was conducted for all family members of the RD transcriptional network, and analysis of variance (ANOVA) with a Benjamini and Hochberg false discovery rate correction (FDR = 0.01) was used to determine statistically significant changes in gene expression between normal human Schwann cells, neurofibroma samples, and MPNST samples. For comparison of MPNST cells infected with adenovirus, gene expression was normalized to the average gene expression amongst three independent
normal human Schwann cell samples. Statistical analyses (ANOVA; FDR = 0.001) were used to identify significant gene expression changes between uninfected MPNST cells, MPNST cells infected with control (GFP) virus, and MPNST cells infected with NF1-GRD virus.

Cell Culture and Tissue Samples

Normal human Schwann cells (NHSCs) were cultured from trauma victims (Casella et al. 1996) and Schwann cells were cultured from dermal neurofibromas as described (Serra et al. 2000). MPNST cell lines were cultured as described (Miller et al. 2006; Mahller et al. 2007). NHSC = normal human Schwann cells; dNFSC = dermal neurofibroma Schwann cells; dNF = primary dermal neurofibromas; pNFSC = plexiform neurofibroma Schwann cells; pNF = plexiform neurofibromas; MPNST = malignant peripheral nerve sheath tumor; MPNST cell lines include: STS26T, ST8814, S462, S520, T265, 908-8, 88-3, and YST1.

Adenoviral infection

The NF1-GRD adenovirus was constructed by inserting a ~1.1kb Xba/HindIII fragment containing the coding sequence for the human NF1-GRD isoform I (GenBank NM_000267; (Viskochil et al. 1992) and a 5’-HA tag into the pAdTrack vector (He et al. 1998). The resulting vector, pAdTrack-NF1-GRD-HA, was linearized with Pmel and transformed into BJ5183 cells containing the pAdEasy vector. Recombinants were screened by restriction digestion, sequence-verified, and then transformed into DH5α cells for amplification. Amplified clones were verified by HA expression and ability to reduce Ras activity (see below). For microarray and QRT-PCR experiments, MPNST cells were infected with an adenoviral construct expressing the NF1-GRD or GFP, in triplicate, with cesium-purified adenovirus for 2 hours in serum-free medium at 200pfu/cell. Viruses were washed away and cells cultured in normal medium for another 30 hours, then RNA isolated as above was isolated for analysis of gene expression as described (Miller and Jessen et al. 2009).

Lentiviral infection

For lentiviral shRNA infection, MPNST cells at 70 – 90% confluence were infected with lentiviral particles containing shRNAs targeting EYA4 (Open Biosystems; TRC library) or GFP (Addgene). The CCHMC Viral Vector Core produced virus using a 4-plasmid packaging system (http://www.cincinnatichildrens.org/research/div/exp-hematology/translational/vpf/vvc/default.htm). Lentiviral particles were incubated with MPNST cells (MOI ~ 10) in the presence of polybrene (8 µg/mL; Sigma) for 24 hours followed by selection in puromycin at a concentration (2 µg/mL) that killed uninfected cells within three days.

Quantitative real time PCR (QRT)-PCR

Total RNA was isolated from cells using the RNeasy kit (Qiagen) and used as a template for cDNA synthesis (Invitrogen Superscript II) and QRT-PCR (ABI 7500 Sequence Detection System) as described (Miller et al. 2006).
Western Analysis

Cell lysates were created and Western blotting conducted as described (Miller et al. 2006), membranes were probed with anti-EYA4 antibodies (Santa Cruz Biotechnology, Inc.; EYA4 (E-20): sc-15107), then, stripped and re-probed with anti-β-actin antibodies (Cell Signaling Technology, Inc. #4967) as a loading control. Signals were detected using horseradish peroxidase-conjugated secondary antibodies (BioRad; Hercules, CA) and the ECL Plus developing system (Amersham Biosciences; Piscataway, NJ). ImageJ 1.33u software (http://rsb.info.nih.gov/ij/) was used to quantify EYA4 from scanned autoradiographs.

Ras activation assay

Ras activity was measured in MPNST cells as described (Mahller et al. 2006). MPNST cell lines were infected with the NF1-GRD adenovirus or a vector encoding green fluorescent protein. Two days later lysates were prepared and blotted directly for total canonical Ras proteins (H,N,K-Ras), active ERK, or active MEK. Ras-GTP was evaluated by centrifuging beads conjugated with GST-Raf-RBD from cell lysates, and probing by Western blots (Ras Activation Assay; Upstate Biotechnology).

Bromodeoxyuridine (BrdU) Incorporation

Twenty-four hours post-plating 3x10^4 MPNST cells onto glass coverslips, cells were labeled for one hour with BrdU labeling reagent, fixed in ethanol, and incubated with anti-BrdU antibodies followed by detection with fluorescein-conjugated secondary antibodies according to the manufacturer’s protocol (5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II; Roche Applied Science). All cell nuclei were labeled with propidium iodide (PI; 50 µg/mL). Total number of cells (PI+) and number of BrdU positive cells were counted in five fields per sample and averaged.

Detection of apoptotic cells

MPNST cells were plated in quadruplicate wells (2 x 10^4 cells/well) of LabTek chamber slides (Nalge-Nunce International), then infected with shEYA4 or shGFP lentiviral particles. After 3 days in the presence or absence of puromycin, cells were fixed for detection of DNA damage in apoptotic cells (Apo ssDNA kit; Cell Technology, Inc.). Briefly, DNA was denatured, and single-stranded DNA (ssDNA) was detected with a primary anti-ssDNA antibody, followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies. Five random fields in triplicate wells were counted, and TRITC-positive apoptotic cells were expressed as a percentage of total cells visualized by bisbenzimide staining of nuclei.

Detection of necrotic cells

Live MPNST cells were incubated with propidium iodide (PI; Sigma; 50 µg/mL) for 2 hours at room temperature, 24 hours after plating at a density of 50,000 cells per well in 35mm dishes. Cells were photographed in 7 randomly chosen fields per dish, in each of 2 dishes per condition. PI-positive cells (necrotic cells) were expressed as a percentage of total cells.
**Migration assay**

The migratory response of MPNST cells was measured using a modified Boyden chamber assay as described (Miller et al. 2006). MPNST cells \(4 \times 10^4\) expressing shGFP or shEYA4 were plated in serum-free DMEM on the upper chamber of a transwell with 8 µm pores (Costar); The lower chamber contained 800 µl DMEM plus serum. Cells were incubated for 16 h at 37°C in 10% CO\(_2\). Nonmigrating cells were removed from the upper surface of the membrane with cotton swabs. Membranes were stained with bisbenzimide and mounted onto glass slides. Migration was quantified by counting cells in four fields. Each condition was performed in triplicate, and the number of migrated cells was normalized to the total number of cells on an unscraped filter to validate the total number of cells plated. Data shown are representative of three independent experiments; values presented are the mean±s.d. Statistical significance was determined by t-test using Microsoft Excel software.

**Mouse xenograft**

Mouse xenografts of MPNST cells were conducted as described (Mahller et al. 2008). 1.2 \(\times 10^6\) one million S462TY MPNST cells expressing shControl (n=10), shEYA4.3 (n=10), shEYA4.5 (n=10), or shEYA4.7 (n=10) were injected subcutaneously into 6-to 8-week-old female athymic nude (nu/nu) mice (Harlan, Indianapolis, IN). Left and right flanks were injected with control or shEYA cells in each animal. In accordance with CCHMC rodent tumor guidelines, tumors may not exceed 10% of body weight. Tumors formed in shControl cells in approximately 30 days, reaching ~2000 mm\(^3\) in approximately 60 days. Therefore, 59 days post-injection, we measured tumor volume and euthanized all shControl mice and shEYA mice. Tumors were dissected and submitted to the CCHMC Pathology Lab for histological characterization, including H&E staining, Mib-1 detection of proliferating cells, TUNEL, and detection of blood vessels using anti-CD31.

**Adhesion assay**

MPNST cells expressing shGFP or shEYA4 \(10^4\) cells per well on LabTek slides) were plated and incubated in standard growth conditions for 2 hours. The wells were gently washed with PBS to remove non-adherent cells, and remaining adherent cells were counted. Adhesion was quantified by counting cells in six fields per well.

**FACS analysis**

Approximately 100,000 MPNST-shControl, MPNST-shEYA4.3, or MPNST-shEYA4.7 cells were fixed in 0.5% paraformaldehyde, permeabilized 0.1% Triton X100 and incubated for one hour at 4°C in PBS containing RNaseA (2 mg/mL) and propidium iodide (50 µg/mL). Flow cytometry analysis was performed in a FACSCanto (Becton Dickinson, San Jose, CA). Analysis of forward scatter (FSC) and side scatter (SSC) in linear amplification was performed along cell cycle analysis. Identification of the A\(_0\) (apoptotic), G\(_0)/G, S, and G2/M peaks were performed by ModFit (Verity, Topsham, ME) analysis.
**Results**

**Expression of genes comprising the Retinal Determination pathway is dysregulated in MPNST**

Global gene expression profiling in NF1-associated tumors and Schwann cells revealed a cluster of genes significantly over-expressed in MPNSTs relative to neurofibromas and normal Schwann cells (Miller and Jessen *et al.* 2009). A striking observation within this cluster is an up-regulation in expression of *EYA4* and one of its potential binding partners, *SIX1*. Expression profiles of *EYA1, EYA2, EYA4, and SIX1-4* genes show differential expression of multiple family members in more than one MPNST sample relative to normal Schwann cells. Expression of *PAX6* was also upregulated in all primary MPNSTs and 11 of 13 MPNST cell lines (Figure 1a), and *PAX6* is transcriptionally upstream of *SIX* and *EYA* in the RD pathway. Furthermore, expression of *DACH1* was downregulated in MPNSTs (Figure 1a). Down-regulation of *DACH1* and up-regulation in *EYA1, EYA2, EYA4* and *SIX1-4*. *DACH1* expression is lower than normal in most benign neurofibroma samples as well as MPNST samples. Expression levels of *EYA4* and *SIX1* are statistically different (ANOVA; FDR = 0.01) between benign neurofibromas and MPNSTs and up-regulated at least three-fold in all MPNST samples (Figure 1a).

We confirmed the gene expression microarray data revealing differential expression of *PAX6, EYA, SIX1*, and *DACH1* in MPNSTs relative to NHSCs using quantitative real-time PCR (QRT-PCR) (Figure 1b – e). The magnitude of differential expression obtained using QRT-PCR was higher as compared to the microarray results yet displayed the same trend. The QRT-PCR data corroborated the most robust overexpression in *EYA4* (Figure 1c), ranging from 50-fold to over 800-fold above NHSCs (13-fold to 67-fold for microarray data). The microarray expression data for *DACH1* was somewhat variable in NHSCs (ranging from −3.7 to 3.2-fold relative to the mean), and one MPNST cell line, YST1, displayed overexpression of *DACH1* (Figure 1a). QRT-PCR results validated the microarray data, confirming reduced *DACH1* expression in all MPNST cell lines, with the exception of YST1, relative to 3 independent NHSC samples (Figure 1d).

**The NF1-GAP related domain (GRD) regulates expression of DACH1 in MPNST cells**

The NF1-GRD is the best characterized domain of NF1, converting activated Ras to its inactivated form. To observe transcriptional changes downstream of the NF1-GRD, we conducted gene expression microarray analysis on MPNST cells 32 hours after infection with an adenovirus encoding the NF1-GRD (relative to a GFP control) (Figure 2a). This timepoint was chosen because it represents a significant reduction in Ras-GTP yet is prior to cell death induced by exogenous expression of the NF1-GRD (data not shown). Forty-five genes were differentially expressed (≥3-fold) in the MPNST 8814 cell line in response to the NF1-GRD expression (data not shown), including *DACH1*. Three of six *DACH1* probe sets confirmed the decrease in expression in ST8814 MPNST cells relative to NHSCs that was not significantly changed by infection with a control adenoviral vector but increased significantly subsequent to infection with the NF1-GRD adenovirus. Failure to show this expression pattern with three *DACH1* probe sets was likely due to probe set unreliability; note that data in Figure 1 is shown with these unreliable probe sets removed as described...
Expression of PAX, EYA, and SIX genes was not significantly altered by the NF1-GRD (Figure 2a), but DACH1 expression was increased to a level comparable to NHSCs (Figure 2b).

Importantly, we confirmed that MPNST cells infected with the NF1-GRD adenovirus have significant reductions in Ras-GTP and downstream phospho-MEK and phospho-ERK protein levels at the 32 hour time point (Figure 2c). In addition, the significant increase in DACH1 expression by the NF1-GRD adenovirus was not observed with a GFP adenovirus control (Figure 2d). These results were confirmed by a time-course QRT-PCR analysis, showing a progressive increase in DACH1 expression levels from 18 – 32 hours in MPNST cells infected with the NF1-GRD relative to the GFP control (Figure 2e).

Reducing EYA4 expression inhibits MPNST cell adhesion and migration

To study the functional role for EYA4 in MPNST tumorigenesis, we first validated the microarray and QRT-PCR data by confirming elevated levels of EYA4 protein in MPNST cell lines relative to NHSCs at the protein level by western blotting. Two bands were observed in MPNST cell lines, likely representing the products of EYA4 transcript variant 1 and transcript variant 2 (Borsani et al. 1999), but not in NHSCs (Figure 3a).

To observe the effects of reducing EYA4 expression in MPNST cells, S462TY MPNST cell lines stably expressing EYA4 shRNAs were created. We observed a marked decrease in EYA4 expression at the RNA level, as quantified by real time PCR (Figure 3b), and at the protein (Figure 3c) level using four independent EYA4 shRNAs compared to the shRNA control. Similar results were obtained in two additional independent MPNST cell lines (data not shown). The extent of EYA4 knockdown varied from 2- to 9-fold and was stable, as the pattern of expression across clones was retained through several cell passages (passage 1 – passage 6).

To test the effect of reducing EYA4 on specific cellular mechanisms that regulate MPNST cell behavior, we conducted functional assays in vitro. The rounded appearance of cells expressing EYA4 shRNA several hours after plating and the lower number of cells remaining on plates after 4 days (Figure 3d) suggested a defect in cell adhesion that was confirmed in a cell adhesion assay (Figure 3e). When EYA4 expression was reduced in MPNST cells, a 60 – 95% reduction in migration was evident, and the extent of inhibition of migration correlated with extent of reduction in EYA4 expression (Figure 3f). Thus, MPNST cell adhesion and motility are dependent on EYA4.

Reducing EYA4 expression induces MPNST cell death by necrosis

An apparently slower growth rate was observed in culturing the MPNST-shEYA4 cells. A modest decrease in cell accumulation was evident, yet not significant, in a nine day MTS assay (data not shown). Further investigation by FACS analysis confirmed no significant change in cell proliferation, apoptosis, or cell cycle progression. (Figure 4a–f). BrdU incorporation was used to confirm similar rates of proliferation in MPNST-shEYA4 and MPNST-shControl cells (data not shown). A similar small percentage of apoptotic cells...
were detected in both MPNST cells expressing shEYA4 and shControl (Figure 4g) as confirmed by analysis of DNA fragmentation (data not shown).

Because fewer cells were present in growth assays with undetectable differences in proliferation or apoptosis, we tested if cell death by necrosis might account for the decrease in cell number. Necrotic cell death is associated with morphological changes including cell shrinkage, cytosolic condensation, membrane morphology changes, loss of anchorage and finally, cellular degradation (Krysko et al. 2008). Evidence of cell death by necrosis was observed in live cultures of MPNST-shEYA4 cells by the robust uptake of propidium iodide due to extensive membrane damage. Only 4.3% of S462TY cells expressing a scramble shRNA were PI+, whereas 15.0% of cells expressing EYA4.7 were PI+ (Krysko et al. 2008). To validate this observation we conducted flow cytometry. By flow cytometry, induction of cell death generally results in a shift of low side light scatter population to higher side light scatter population (Dive et al. 1992; Weston et al. 1994; Waring et al. 1999). To prevent analysis confusions with possible artifacts from cell aggregates and debris in suspension, our analysis by flow cytometry focused on cell singlets from adherent cells. Despite performing the flow cytometry analysis in the adherent cell fraction, which is enriched in surviving cells, there was a ~10% increase in light side scatter mean channel in cells expressing shEYA4 (Figure 4h).

Reducing EYA4 expression inhibits MPNST formation in vivo in xenografts

To determine if EYA4 expression is critical for MPNST tumorigenesis, we tested the MPNST-shEYA4 cells in a mouse xenograft model using the S462TY cell line. By 60 days, MPNST cells expressing three independent EYA4 shRNAs displayed significant reduction in tumor volumes (Figure 5a – b). As in the in vitro cell migration studies above (Figure 3f), we observed a correlation with levels of EYA4 and effects on tumor size. Expression levels of EYA4 in MPNST-shEYA4.5 and MPNST-shEYA4.7 were reduced greater than 6-fold, and these cells did not develop detectable tumors; expression levels of EYA4 in MPNST-shEYA4.3 were reduced only 2-fold, and these cells developed small tumors by 60 days. Reduced expression of EYA4 was maintained in the MPNST-shEYA4.3 tumors relative to control tumors, as confirmed by QRT-PCR (Figure 5c).

Histological analyses revealed significant areas of necrosis within the shEYA4-expressing tumors (Figure 5e, g). Similar degrees of proliferation and apoptosis in control and shEYA tumors were confirmed with Ki67 staining (Figure 5h, i) and TUNEL assay (Figure 5j, k), respectively. CD31 staining revealed similar vascularity (Figure 5l, m).

Discussion

Microarray gene expression analysis revealed differential expression of members of the RD transcriptional network, PAX6, EYA4, SIX1, and DACH1, in MPNSTs relative to normal human Schwann cells. The NF1-GRD induced DACH1 expression, implicating Ras signaling in the decreased DACH1 expression seen in MPNSTs. Expression of EYA4 was dramatically upregulated in MPNST cells and primary tumors, and inhibition of EYA4 expression using shRNA reduced MPNST cell adhesion and migration and induced cell necrosis. Furthermore, tumorigenesis was profoundly inhibited when MPNST cells stably
expressing shEYA4 RNA were injected into athymic nude mice, again correlating with necrosis. Our previous study (Miller et al., 2009) provided evidence that overexpression of SOX9 is sufficient to induce EYA4 expression. We propose a model in which overexpression of SOX9 and loss of NF1 functions independently lead to dysregulation of the EYA4-SIX1-DACH1 transcription in MPNST.

The most dramatic differential expression was observed for EYA4, which was robustly overexpressed in all but one MPNST cell line and all primary MPNSTs, suggesting it and/or its family members, may represent an MPNST oncogene. Its activity may be context-specific, given other reports implicating EYA4 as a tumor suppressor gene in gastrointestinal tumors (Zou et al. 2005; Osborn et al. 2006). The expression pattern of PAX6, SIX1, EYA4, and DACH1 in MPNST cells suggests a scenario whereby expression of multiple members of a potential transcriptional network is dysregulated in a manner that promotes tumorigenesis. High levels of PAX6, EYA4, and SIX1 expression in combination with low levels of DACH1 expression are consistent with previous studies supporting a tumor promoting role for PAX (Maris et al. 1997; Muratovska et al. 2003), EYA (Ramdas et al. 2001), and SIX (Christensen et al. 2008) family members, and a tumor suppressor role for DACH1 (Wu et al. 2006; Wu et al. 2008; Wu et al. 2009). However, the existence of a transcriptional network involving these genes has not been demonstrated here and has not been definitively identified in mammalian cells.

This study and our previous studies used expression differences between cultured Schwann cells and MPNST cells to identify genes relevant to tumorigenesis (Miller et al., 2006, 2009). Gene expression changes are induced by cell isolation and tissue culture, and can interfere with the identification of relevant genes. In spite of these potential problems, our approach is supported by in vivo validation of the microarray data in solid tumors for EYA4, coupled with functional validation in xenografts for EYA4.

Replacement of the NF1-GRD in MPNST cells rescued DACH1 expression but did not alter transcription of EYA or SIX at 18 – 32 hours, although we cannot exclude the possibility that at other time points or levels of GRD expression transcription of EYA or SIX might be changed. It is possible that a primary effect of loss of NF1 on DACH1 secondarily affects transcription of the other members of the complex. Indeed, several lines of evidence support feedback activation of the network members on each other (O’Neill et al. 1994).

Furthermore, while only some neurofibroma samples have elevated SIX1 or EYA4 expression, DACH1 expression was low in many neurofibroma Schwann cell cultures and primary neurofibromas. Thus, our data support the idea that decreased DACH1 expression is an early event downstream of NF1 loss.

EYA4 was up-regulated in NF1-related and in sporadic MPNST cell lines, although Ras-GTP is not elevated in the same sporadic MPNST cells (Mahller et al. 2006). In addition, EYA4 expression was not affected by replacing the NF1-GRD in NF1-deficient MPNST cells, suggesting NF1-Ras signaling does not regulate EYA4 expression. In Drosophila it has been shown that Ras signaling phosphorylates EYA proteins, increasing its transcriptional activity (Hsiao et al. 2001; Silver et al. 2003). Thus, post-transcriptional regulation of EYA4 by the NF1-Ras pathway warrants future investigation. The mechanism(s) underlying high
EYA4 expression in MPNST remain to be definitively determined but is likely to involve elevated SOX9 expression. SOX9 expression is elevated in both NF1-related and sporadic MPNST cell lines, and increasing SOX9 in wild type Schwann cells is sufficient to increase EYA4 mRNA expression, while decreasing SOX9 mRNA levels in MPNST cells decreases EYA4 expression (Miller and Jessen et al. 2009).

Our results provide several lines of evidence supporting a role for EYA4 in cell survival, specifically in inhibition of cell death by necrosis. Cell death is generally classified as either apoptosis or necrosis, a major distinction being the early breakdown of the plasma membrane in necrotic cells (Kroemer et al. 2009). Molecular mechanisms regulating necrosis are not well understood, however overactivation of poly (ADP-ribose) polymerase (PARP) in response to extensive DNA damage results in depletion of ATP which can convert apoptosis to necrosis (Ha et al. 1999). The absence of changes in proliferation and apoptosis along with an increase in propidium iodide uptake suggested that necrosis may be the cause of decreased cell numbers in MPNST cells with reduced EYA4 expression.

Further investigation in vitro supported necrotic cell death as increased side scatter was observed in FACS analysis. In addition, tumors that developed in xenografts of MPNST cells with low levels of EYA4 showed significant areas of necrosis. In other systems, loss of EYA induces apoptosis (Cook et al. 2009), suggesting the mechanism of cell death resulting from decreased EYA may depend upon co-regulatory factors. Our data also support a role for EYA4 in MPNST cell adhesion and migration both of which are inhibited in response to reducing EYA4 expression. A reduction in cell adhesion can prompt anoikis, however, anoikis is usually associated with apoptosis rather than necrosis. Cellular changes downstream of EYA4, including cell adhesion, migration, and survival, may reflect the metastatic behavior of MPNSTs (Klymkowsky et al. 2009) and provoke further investigation.

We conclude that the NF1-Ras pathway can regulate expression of DACH1, directly or indirectly, and EYA4 expression controls adhesion, migration, and blocks necrosis in MPNST cells. Inhibition of EYA4 reduced tumorigenic properties of MPNST cells in vitro and resulted in MPNST cell death ex vivo, suggesting that developing therapeutics aimed at diminishing EYA4 expression or EYA4 transcriptional targets represents a strategy for killing MPNST cells.

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Figure 1. Expression of the PAX/EYA/SIX/DACH transcriptional complex is dysregulated in MPNST
(a) Hierarchical clustering of gene expression microarray data of PAX, EYA, SIX, and DACH genes family members. *EYA4, SIX1, and PAX6* expression in MPNST are statistically (ANOVA; FDR=.01) different from normal Schwann cells or neurofibroma samples. MPNST samples expressed, on average, at least three-fold higher levels of each mRNA relative to neurofibroma samples or normal Schwann cells. MPNST samples (n=6 primary MPNSTs; n=13 MPNST cell lines). Yellow = normal; Red = overexpression; Blue =

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underexpression. *DACH1* expression is down-regulated in MPNST cell lines and the majority of benign neurofibroma samples. (b) Confirmation of microarray gene expression data for *PAX6, SIX1, EYA4*, and *DACH1* using quantitative real time PCR (QRT-PCR). Black bars = microarray data; White bars = QRT-PCR data. QRT-PCR values shown are fold-change in expression relative to normal human Schwann cells (N). Samples 1 – 8 are MPNST cell lines: 1 = STS26T; 2 = ST8814; 3 = S462; 4 = T265; 5 = S520; 6 = 90-8; 7 = 88-3; 8 = YST1.
Figure 2. The NF1-GRD normalizes $DACH1$ expression
(a) Gene expression microarray data of normal human Schwann cells (NHSC), and ST8814 NF1 patient-derived MPNST cells (control) infected with an adenoviral vector expressing GFP (Vector) or the NF1-GRD (GRD). Samples were analyzed in triplicate, and expression is shown relative to the mean of the NHSC samples. Yellow = normal; Red = overexpression; Blue = underexpression. (b) The microarray expression data for the three $DACH1$ probesets designated by the * in (a) is expressed as fold-change relative to NHSC, showing normalization of $DACH1$ expression by the NF1-GRD. (c) The NF1-GRD
adenovirus blocks Ras/Map-kinase signaling in ST8814 MPNST cells. Ras-GTP was measured in cell lysates after control (Vector) or GRD infection. Ras-GTP, phospo-MEK and phospo-ERK protein levels were each diminished in the presence of the NF1-GRD. (d) Quantification of NF1-GRD effects on DACH1 expression in ST8814 MPNST cells in the gene expression microarray. (e) Confirmation of increased levels of DACH1 expression in response to the NF1-GRD at 18, 24, and 32 hours post-infection using QRT-PCR. Values are fold-change in expression relative to adenoviral vector control.
Figure 3. Reducing EYA4 expression with shRNA inhibits cell adhesion and migration
(a) EYA4 protein is expressed in MPNST cell lines (STS26T, ST8814, S462, T265) but not normal human Schwann cells (NHSC), assessed by western blotting. Staining with anti-actin confirmed equal amounts of protein across samples. (b, c) EYA4 expression in S462TY MPNST cells is reduced subsequent to introduction of shEYA4 RNAs (4.3, 4.4, 4.5, 4.7). Data are shown for level of RNA using QRT-PCR (b) and protein by western blotting with anti-EYA4 or an anti-AKT loading control (c). (d) Phase contrast micrographs of living S462TY cells at day 0 (d0) and day 4 (d4). Fewer S462TY cells containing shEYA4 shRNAs attach to dishes, correlating with decreased cell numbers at day 4. (e) Adhesion assay of S462TY cells containing control or shEYA4 cells. Significantly fewer cells remain attached (*p<0.05) in the presence of EYA4 shRNAs (4.3, 4.7) relative to control shRNA.
(f) Migration of S462TY MPNST cells through transwell filters is significantly reduced (*p<0.05; **p<0.01) in the presence of EYA4 shRNAs (4.3, 4.4, 4.5). Values are expressed as percent migration relative to control shRNA.
Figure 4. Reducing EYA4 expression causes MPNST cell death by necrosis

shControl (a, b), MPNST-shEYA4.7 (c, d), or MPNST-shEYA4.3 (e, f) cells were fixed and stained with propidium iodide. Flow cytometry analysis of forward scatter (FSC) and side scatter (SSC) in linear amplification was performed along cell cycle analysis are shown in a, c, and e. Identification of the A₀ (apoptotic), G₀/G₁, S, and G2/M peaks are shown in b, d, and f. (g) Quantification of cell cycle shows no differences in control or shEYA4.7 or 4.3 cells. (h) A 10% increase in light side scatter mean channel is shown in cells expressing shEYA4.3 or 4.7 as compared to control.
Figure 5. Reducing EYA4 expression inhibits tumorigenesis
(a) Gross photographs of nude mice tumors. Black arrows point to flank tumors in mice injected with MPNST cells expressing sh-scramble or shEYA4.3, 4.5, or 4.7. (b) Quantification of tumor volume over time. (c) QRT-PCR showing persistent EYA4 downregulation in small tumors dissected from two mice 59 days after implantation with shEYA4.3, to control for possible loss of shEYA4 expression. (d, f, h, j, l) are photomicrographs of control, and (e, g, i, k, m) are of shEya4.3 tumors. (d, e) Low power views of hematoxylin-eosin stain of control (d) tumor and the smaller shEya (e) tumors.
The shEya tumor exhibits disproportionate necrosis. (f, g) Higher power views of each tumor. Larger amounts of necrosis in shEya tumor (f) compared to control (scramble) tumor (g) is apparent (100×). Ki67 stains of control (h) and shEya (i) tumors exhibiting similar degree of proliferation. (400×). Tunel assay of control (j) and shEya (k) tumors exhibiting similar degrees of apoptosis (400×). CD31 stain of control (l) and ShEya (m) tumors exhibiting similar vascularity (400×).