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Award Number: W81XWH-11-1-0424

TITLE: Combinatorial Therapy Approaches for NF2-Deficient Meningiomas

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REPORT DATE: June 2013

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2013	Final	01 June 2011-30 May 2013

4. TITLE AND SUBTITLE
Combinatorial Therapy Approaches for NF2-Deficient Meningiomas

5a. CONTRACT NUMBER
W81XWH-11-1-0424

5b. GRANT NUMBER
W81XWH-11-1-0424

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University

Baltimore, MD 21218-2680

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The aim of our research is to find novel targeted therapy for NF2 patients to provide a new therapeutic option for meningiomas that includes the standard of care radiotherapy. We have successfully selected four small molecule compounds that preferentially inhibit NF2 mutant meningioma cells *in vitro.* These are FDA approved drugs that are safe for human use. We validated these drugs by using human cell lines and tested their ability to synergize with radiation. We have optimized the use of a CT-guided conformal radiation that can be tested in a meningioma mouse model. The findings from this research project will be valuable for future clinical trials, for testing combinatorial approaches to treat meningioma patients.

15. SUBJECT TERMS
Meningioma, NF2 tumor suppressor gene, Neurofibromatosis Type 2, HDAC inhibitors

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Meningiomas are among the most common tumors of the central nervous system and are the second most prevalent tumors in Neurofibromatosis Type 2 patients (Louis, DN *et al* 2007). Despite their high frequency, currently there are no chemotherapeutic options for these tumors and treatment is limited to surgery and various forms of radiation therapy either as adjuvant or primary therapy. When treatment is recommended, these tumors almost uniformly receive some form of radiation therapy. Moreover, a significant proportion of these tumors present histopathological aggressive features (designated as WHO Grade II and III) with higher recurrence rates. In addition, a great number of tumors are either not surgically accessible, recur, or progress to malignant tumors, necessitating repeat surgery and sometimes radiation. Unfortunately for patients that develop the malignant meningiomas, there is no recognized effective chemotherapy and the patients either survive presenting significant morbidity or eventually die from this cancer. The aim of this study was to investigate new therapeutic opportunities for NF2-meningioma patients, possibly coupling them with radiation, given its common use with non-surgical tumors.

BODY

In this Final Report we present the research achievements of the first and second years of the current project. On Year 1 we evaluated a large library of FDA-approved inhibitors to their ability to inhibit NF2-meningioma cell growth *in vitro*. Since loss of the NF2 tumor suppressor gene is a major genetic alteration in meningiomas, the library compounds were tested against pairs of NF2 isogenic cells. Compounds that presented the greatest rates of cell proliferation inhibition were further tested and characterized in secondary screenings and also tested for *in vitro* radiation synergy. The best compound-candidates were tested in an orthotopical meningioma mouse model as a single treatment. In addition, in order to accomplish the research plan described on the research propososal, on Year 2 we investigated the implications of the Hippo signaling pathway on NF2-meningiomas. Research of our laboratory recently reported, identified

that YAP1, a component of the Hippo signaling cascade, is activated upon loss of *NF2* and functions as an oncogene promoting meningioma tumorigenesis (Baia, GS et al 2012). Therefore we evaluated large libraries of FDA-approved inhibitors for their ability to inhibit growth of meningioma cells overexpressing YAP1 *in vitro.* This drug screen showed that two Histone Deacetylase inhibitors (HDACi), Vorinostat and Romidepsin, had the highest specific anti-tumor activity on YAP1 expressing cells, compared to wild-type control. We further characterized the potent effects of these HDACi drugs in NF2-meningioma cells with a series of secondary and functional assays. Here we show evidence of potential beneficial utility of HDACi as therapeutic option for targeting NF2-meningioma. Given the scarcity of targeted therapies for meningioma patients in the clinic, the further clinical testing of HDACi is warranted.

-YEAR 1-

TASK 1 – Evaluate the in vitro efficacy of combinatorial therapy of small-molecule inhibitors for NF2 mutant meningiomas.

Primary drug screen of libraries of FDA-approved compounds. We used cell-based assays to search for small-molecule inhibitors that showed prevalent efficacy against NF2 mutant meningioma cells. Three pairs of NF2 isogenic meningioma cells: AC1, SF6717 and KT21MG1 (Baia, GS *et al*, 2006) were used to screen libraries of FDA approved compounds at a constant final concentration (2μM). Briefly, 750 cells/well were plated into 96-well plates and allowed to adhere for 16 hours. Next, 2μL of drugs plus 20μL of Alamar Blue reagent were added to individual wells. Alamar Blue fluorescence was measured daily over a period of 120h and it was plotted against time. Cells plated with DMSO were used as negative controls. The primary screen was performed using the KT21MG1 cells. We screened a total of 1,726 compounds from 2 libraries (Sigma/LOPAC library and NINDS library: National Institute of Neurological Disorders and Stroke). The top 5% compounds (86 compounds) showing preferential inhibition of NF2 mutant cells were selected for further testing. These compounds were then validated in a second cell proliferation assay that included all three NF2 isogenic

cells (3 replicate wells per each compound). Twelve compounds (~15%) showed prevalent inhibitory activity on these NF2 mutant cells. In Table 1 a list of selected compounds is shown. We next determined the half inhibitory concentration (IC_{50}) of the 12 compounds against the NF2 isogenic meningioma cells. A dose-response effect in NF2 mutant cells was observed for most of inhibitors. As seeing in Table 1, a prevalent inhibition of NF2 mutant cells was observed for most of the compounds, compared to the NF2+ cells, as demonstrated by their IC_{50} values (1.5 to 10-fold in difference).

Evaluation of drug-radiation synergy. NF2 isogenic cells (SF6717) were subjected to different doses of radiation (4, 8 and 12Gy) and plated in 96-well plates (1,000 cells/well) for Alamar Blue proliferation assay. Control cells (no radiation) were plated on each plate. Alamar Blue fluorescence data were normalized to the control (0Gy) and plotted against time. As observed in Figure 1, NF2 mutant cells were slightly more sensitive to radiation, compared to NF2 wild type cells. At a 120h time point NF2 mutant cells had a ~25% decrease in cell growth with a radiation dose of 12Gy. For further *in vitro* radiation experiments the dose of 12Gy was chosen to evaluate potential synergist effect with drug combinations. Briefly, for each of the 12 selected drugs, proliferation assays were carried out with cells treated with drugs, radiation (12Gy) or radiation+drugs. Control cells were plated with DMSO. The proliferation data was normalized to the control (DMSO) for each plate and plotted against time (0 to 120h). In Figure 2 the cell proliferation plots of individual drugs testing are shown. Of the tested compounds, an inhibitor targeting the mammalian target of rapamycin, mTOR (Everolimus), 2 inhibitors targeting the vascular endothelial growth factor receptor, VEGFR (Tandutinib, Sunitinib) and Gleevec presented the greatest synergistic effect of drugs in combination with radiation. Moreover, the cell proliferation inhibition was significant at 120h for all 4 compounds ($P \leq 0.05$). The other 8 inhibitors either presented either additive effect or no effect in combination with radiation.

Secondary drug screening. Next, we tested whether the combination of drug and radiation affected the anchorage-independent growth, a phenotype characteristic of tumor cells. Briefly, heavily irradiated (50Gy) IOMM-Lee cells were plated as feeder cells in 6-well plates (30,000 cells/well). Twenty four hours later, 600 experimental NF2 mutant cells were plated in triplicate wells. Plates were incubated for 7 to 10 days until visible colonies were observed. Cells were washed in PBS, fixed in 10% formalin and stained with crystal blue. Colonies of >50 cells were counted under a dissecting microscope. As shown on Figure 3, all combinations of drug and radiation treatment formed significantly fewer colonies compared to either controls (radiation or drug alone).

Together, these data demonstrate that in combination with radiation the inhibitors: Everolimus, Gleevec, Sunitinib and Tandutinib are promising for potential for therapeutic treatments of NF2 mutant meningioma cells.

TASK 2 – <u>Determine the potential survival benefit of radiation and combinatorial therapy of small-molecule inhibitors in an orthotopic meningioma mouse model.</u>

Preclinical testing of individual selected drugs. We employed an orthotopic meningioma mouse model to determine the potential efficacy of drugs in combination with radiation. The goal was to evaluate the potential survival benefit of combinatorial therapy *in vivo*. Briefly, mice intracranial implantations were performed in accordance with an animal protocol approved by the Johns Hopkins Institutional Animal Care and Use Committee. NF2 mutant cells were implanted orthotopically into athymic mice, as previously described (Baia, GS *et al*, 2008). Briefly, 6 week-old female athymic mice were anesthetized (Ketamine 80mg/Kg; Xylazine 10mg/Kg) and fixed in a stereotaxic frame. Cells (10^5 in 1µl) were implanted into the floor of temporal fossa by using the following stereotaxic coordinates relative to the bregma: 2 mm to the right, 2 mm posterior and 6 mm of depth. Figure 4A illustrates the tumor implantation site. Meningioma cells were tagged with a firefly Luciferase construct, under the control of the spleen focus forming virus promoter, via lentiviral transfection, as previously described (30, 31). Bioluminescence imaging (BLI) was used to monitor tumor growth

progression. Figure 4B shows a representative BLI image of a mouse at day 5 post-cell transplantation. After cell implantation, mice were appropriated monitored three times every week and euthanized if they exhibit neurological symptoms or had more than 15% of weight loss. NF2 mutant cells were tumorigenic in Athymic nude mice (Figure 4C).
The drugs Everolimus and Gleevec, selected on Taks#1 were chosen to begin the preclinical testing because they present very high *in vivo* tolerance at the equivalent maximum tolerated dose (MTD) in humans. NF2 mutant cells were implanted as described above and at day 3 animals were treated daily, for 5 days a week (Monday-Friday) with either Evelorimus (2mg/Kg) or Gleevec (100mg/Kg). Tumor bearing mice were used as controls (no treatment). Animal survival was plotted overtime (Figure 6). As observed, neither of the single agents was capable to prolong survival of NF2 mutant xenografts. We anticipate testing next, both combinations of drugs and radiation+drugs to evaluate potential beneficial survival of meningioma xenografts.

Small animal conformal radiation testing in NF2 meningiomas. To evaluate the synergistic effect of drugs and radiation we planned to employ the Small Animal Radiation Research Platform (SARRP) to precisely deliver localized radiation to xenografts. Briefly, NF2 mutant meningioma cells were implanted orthotopically into mice (10^5 cells/mouse). Three days after cells implantation mice were anesthetized and treated once with localized radiation (10, 12 or 20 Gy), using an in-house developed precision small animal radiation device (Wong, J *et al*, 2008). This technology is capable of delivery of high intensity and localized doses of radiation to the target tumor volume, while minimally affecting the surrounding normal tissue. Since radiosurgery is part of standard of care of meningioma patients, this CT-guided conformal radiation is pivotal in mimicking clinical radiotherapy. Treated mice showed longer survival times compared to control mice. Figure 6 shows a Kaplan-Meier survival plot of mice treated with 12Gy (n=6) compared to control group (n=6). Radiation treated mice had a slightly longer survival compared to control mice.

-YEAR 2-

Task 1 – *Characterization of the deregulation of the Hippo signaling pathway in NF2-deficient meningiomas.*

The Hippo signaling pathway is functionally conserved in *Drosophila melanogaster* and mammals, and its proposed function is to control tissue homeostasis by regulating cell proliferation and apoptosis. The core components are composed of a kinase cascade that culminates with the phosphorylation and inhibition of Yes-associated protein 1 (YAP1). Phospho-YAP is retained in the cytoplasm. In the absence of Hippo signaling, YAP translocates to the nucleus, associates with co-activators TEAD1-4, and functions as a transcriptional factor promoting the expression of key target genes. Components of the Hippo pathway are mutated in human cancers, and deregulation of this pathway plays a role in tumorigenesis. Loss of the NF2 tumor suppressor gene is the most common genetic alteration in meningiomas, and the NF2 gene product, Merlin, acts upstream of the Hippo pathway. We recently showed that primary meningioma tumors have high nuclear expression of YAP1. In meningioma cells, Merlin expression is associated with phosphorylation of YAP, as shown in Figure 8 (Baia, et al 2012). We further characterized the role of YAP1 by promoting YAP1 gene loss and gain of function in meningioma cells. Using siRNA transient knockdown of YAP1 in NF2-mutant meningioma cells, we showed that suppression of YAP1 impaired cell proliferation and migration (Figure 8B-C). Conversely, YAP1 overexpression led to a strong augment of cell proliferation and anchorage-independent growth and restriction of cisplatin-induced apoptosis (Figure 8E-G). In addition, expression of YAP1 in nontransformed arachnoidal cells led to the development of tumors in nude mice (Figure 8H).

Together, these findings suggest that in NF2-meningiomas, deregulation of the Hippo pathway is largely observed in primary tumors and that YAP1 functions as an oncogene promoting meningioma tumorigenesis.

Task 2 – *Primary drug screen of YAP1 isogenic cells shows that Histone Deacetylase inhibitors (HDACi) preferentially target meningioma cells with loss of NF2 gene.*

We screened compounds from 2 libraries (Approved Oncology Drug Set libraries, National Cancer Institute). Library compounds were initially tested against the SF1335 pair of *YAP1* isogenic cells. SF1335 cells are derived from a benign NF2-mutant meningioma (Baia, et al 2012). We used cell-based assays to search for small-molecule inhibitors that showed prevalent efficacy against *YAP1* overexpressing meningioma cells from libraries of FDA approved compounds at a constant final concentration (4μM). Briefly, 750 cells/well were plated into 96-well plates and allowed to adhere for 16 hours. Next, 2μL of drugs plus 20μL of Alamar Blue reagent were added to individual wells. Alamar Blue fluorescence was measured daily over a period of 120 hours and it was plotted against time. Cells plated with DMSO were used as negative controls (Figure 9A). Among several compounds tested, we identified two compounds that present the same mechanism of action: Vorinostat and Romidepsin (Figure 9B-C). These compounds belong to a class of drugs that target the acetylation of histones, which ultimately culminates with overall changes in chromatin structures and therefore affects the global cellular gene expression regulating essential cellular processes that include cell proliferation, cell survival and apoptosis, migration and immunity. Vorinostat and Romidepsin indeed showed preferential inhibition of YAP1 expressing cells and were selected for further testing and characterization.

Task 3 – *Secondary and validation drug screens reveal that NF2/YAP isogenic cells are preferentially inhibited by Histone Deacetylase inhibitors.*

Next, we validated the HDACi compounds in multiple NF2 and YAP1 isogenic meningioma cells. The NF2 isogenic cells were created by stably knocking down the NF2 gene. This was achieved by transfecting meningioma cells with specific short hairpin-RNA constructs (or empty vector). The YAP1 isogenic cells were created by transfection of meningioma cells with the pEGFP-N2-YAP1 expression construct, or empty vector as control (Baia, GS et al 2012). We determined the half inhibitory

concentration (IC_{50}) for the top hit compounds from the primary screen. Among those we determined the IC_{50} for the HDAC inhibitors Vorinostat and Romidepsin plus Crizotinib. In addition, we added to this assay the experimental HDACi drug Panobinostat. Panobinostat is a pan-deacetylase inhibitor that has been extensively characterized and is currently in advanced clinical testing. As observed in Figure 10A-10B, HDACi had a potent dose-response effect in both NF2 and YAP cells and interestingly HDACi showed indeed dramatic specificity to either NF2 or YAP cells. This effect is consistently observed across all HDAC inhibitors tested in all cell lines. Importantly, the micromolar IC_{50} differences between NF2 or YAP1 cells to their empty vector counterparts ranged from 3.5 to 133.5 for Vorinostat and from 6 to 35 fold for Romidepsin (Figure 10). However, this preferential inhibition of YAP or NF2 isogenic cells was not observed for Crizotinib (Figure 10C). Interestingly, the fold differences in IC_{50} values obtained with Panobinostat, except for one cell line, were above 100 fold and reached 1,713-fold difference in one of the NF2 isogenic cell lines. Although Panobinostat is not FDA-approved, it is currently in clinical development across multiple tumor types and it represents one of the most thoroughly studied HDACis to date. In all, these validation assays confirmed that NF2/YAP isogenic cells are preferentially inhibited by Histone Deacetylase inhibitors

Task 4 – *Functional characterization of HDACi effects on NF2-meningioma cells.*

We tested next whether the treatment with HDACi affected the anchorage-independent cell growth, a characteristic phenotype of cancer cells. Briefly, heavily irradiated (50Gy) feeder cells (IOMM-Lee) were plated in 6-well plates. Twenty-four hours later, 1,0

00 experimental AC1-NF2 shRNA or SF1335-YAP cells were plated in triplicate wells. Cells were treated with indicated concentrations of Vorinostat or DMSO (control). Plates were incubated for 10-15 days until visible colonies were observed. Cells were washed in PBS, fixed in 10% formalin and stained with crystal blue. Colonies of >50 cells were counted under a dissecting microscope. As shown in Figure 11, the anchorage-

independent growth of NF2/Yap meningioma cells was impaired by HDAC inhibitor (Vorinostat), in a dose-dependent fashion.

Several HDAC inhibitors have been reported to promote cell cycle arrest. To test whether cell cycle regulation was affected by treatment with HDACi, NF2/YAP meningioma cells were cultured without/with increasing concentrations of HDACi Vorinostat (2 and 4 μM) and Panobinostat (0.5 and 1 μM). Cells were sequentially fixed and stained with propidium iodide. Next, stained cells were analyzes by flow citometry for total DNA content. As shown in Figure 12, we observed a dramatic dose-dependent growth arrest in both cell lines treated with HDACi. Consistently, both HDACi significantly increased the percentage of cells in G_2/M, and decreased the cell population in S-phase.

To gain further insight into the effect of HDACi on YAP activity in meningioma cell lines, and as S*urvivin* was reported as a possible cell cycle-associated target for transcriptional suppression by YAP, we investigated *Survivin* expression after treatment with siRNA for YAP using quantitative RT- PCR and immunoblotting. Figure 13 shows a significant decrease in Survivin mRNA levels in both cell lines treated with either Vorinostat or Panobinostat. Moreover, Survivin protein levels were also decrease after Vorinostat treatment in both cell lines.

In summary, the drug screen showed dramatically that two Histone Deacetylase inhibitors (HDACi), Vorinostat and Romidepsin, had the highest specific anti-tumor activity on YAP1 cells, compared to wild-type control. The anti-tumor effects were mediated by increase apoptosis and G_2M arrest and increase in apoptosis rate. Moreover, cell proliferation was affected in a dose-dependent manner in NF2/Yap cells. Interestingly, the expression of anti-apoptotic regulator Survivin, a direct target of Yap is consistently affected by exposure of NF2/Yap meningioma cells to HDACi. In all, these observations reinforce the potential clinical utility of this class of drugs for the treament of meningiomas and other NF2 related tumors.

Task 5 – *Modulation of expression of Cancer/Testis genes upon treatment of cells with HDACi.*

Given the scarcity of therapeutic options for meningioma patients, there is a definite need for better and more efficient therapeutic options in particular for higher grade and recurrent tumors, including combinations involving immunotherapeutic approaches. No therapeutic cancer vaccine has been proposed for meningioma patients and only a few immunogenic tumor antigens have been previously identified in meningioma. However, a comprehensive analysis of the expression and spontaneous immune response to Cancer Testis (CT) proteins, which are the basis of therapeutic approaches that are reaching encouraging successes in recent phase II/III clinical trials has not previously been reported.

We have systematically analyzed the expression of 37 testis-restricted CT genes in a discovery set of 18 meningiomas by reverse transcription PCR. The overall frequency of expression of CT genes ranged from 5.6 to 27.8%. The most frequently expressed was NY-ESO-1, in 5 patients (27.8%). We subsequently analyzed NY-ESO-1 protein expression in a larger set of meningiomas by immunohistochemistry and found expression in 108/110 cases (data not shown). In some cases NY-ESO-1 expression was diffuse and homogenous, but in most instances was heterogeneous (Figure 13). Importantly, NY-ESO-1 expression positively correlated with higher grade and patients presenting with higher levels of NY-ESO-1 staining had significantly worse disease-free and overall survival (data not shown). Considering the limited treatment options for meningioma patients, the potential of CT-based immunotherapy should be explored as a complement for standard therapy, particularly to avoid relapse of the disease of clinically aggressive tumors.

The cancer/testis (CT) antigens represent a unique class of cancer antigens, which are expressed by germ cells, normally silenced in somatic cells, but activated in a wide variety of cancer types. Although the mechanism promoting their derepression is not entirely clear, it has been demonstrated that DNA methylation is one of the central mechanisms responsible for gene silencing. Consistent with these findings, several studies have shown that inhibiting DNA methyltransferase (DNMT) activity with 5 aza-

deoxycytidine (5-AZA) results in robust somatic expression of a set of CTAs both *in vitro* and *in vivo.* Importantly, in some cases, CT-specific T-cell responses have been observed following 5-AZA exposure. The combination of demethylating agents with HDACi has been shown to work synergistically to induce CTs expression and immunogenicity. However, the ability of HDACi agents alone to induce CT expression was not evaluated previously. We sought to investigate in the meningioma cell lines treated with the different HDACi in the experiments described above if the expression of CT genes was induced. This would potentially allow for the combination of HDACi with immunotherapeutic approaches targeting the CT antigens. To this end, we analyzed the expression of CT genes in SF1335-YAP cells treated with vehicle or 5 μM Vorinostat for 72 hours using quantitative RT-PCR. Expression of six CT genes *CT7, NY-ESO-1 and MAGEA3* were analyzed. The expression of *MAGEA3* and *NY-ESO-1* was not significantly changed between the untreated and treated cells. However, the expression of eight CT gene *CT7(MAGEC1)* was dramatically upregulated by Vorinostat treatment (Figure 15). To verify if the increase in CT mRNA was accompanied by increase in protein levels, we analyzed the expression of MAGEC1 by Western blotting. MAGEC1 mRNA levels were found to be upregulated 213-fold in the cells exposed to 5 μM Vorinostat, compared to the untreated cells. Immunoblotting probed with a monoclonal antibody specific to MAGEC1 (CT7.33, Sigma) of both SF1335-YAP and AC1-NF2 showed a dramatic upregulation of MAGEC1, consistent with the upregulation observed at mRNA level (Figure 16).

In summary, we have evidence that in addition to the important anti-tumor effects of HDACi in meningioma cells, this treatment can upregulate the expression of CTs, at mRNA and protein levels and this upregulation may be useful for combining immunotherapy approaches and HDACi.

KEY RESEARCH ACCOMPLISHMENTS

1- We have screened large libraries of FDA approved compounds searching for small-molecule inhibitors that preferentially inhibit NF2 mutant meningiomas. We further characterized and validated a set of selected compounds by screening these drugs in 2 additional pairs of cells and employing a series of secondary assays.
2- We tested the set of compounds for synergy effect with radiation. Four compounds showing the greatest synergy with radiation were selected for further testing. The colony forming efficiency assay was performed as a secondary screening to test the inhibitory efficiency of selected drugs in combination with radiation.
3- We tested the *in vivo* efficacy of Everolimus and Gleevec in a meningioma mouse model.
4- We characterized the deregulation of the Hippo signaling pathway in NF2 deficient meningiomas and identified YAP as a candidate oncoprotein, that drives the tumorigenesis on these tumors.
5- Identified a new class of FDA compounds, the histone deacetylase inhibitors, that preferentially inhibited NF2/YAP meningioma cells.
6- We characterized the cellular effects of HDACi in NF2/YAP meningioma cells.
7- We discovered that the expression of a subset of Cancer/Testis genes are upregulated in meningioma cells upon HDACi treatment. This may lead to further investigation on the effectiveness of combining immunotherapy approaches and HDACi.

REPORTABLE OUTCOMES

- Research data presented at the **103rd American Association for Cancer Research Annual Meeting**, Chicago, IL, USA, 2012 (Poster format, Figure 7).
- Optimized the use of CT-guided conformal radiation applied to the meningioma mouse model.
- Journal Publication: Baia, GS, Caballero OL, Orr BA, Lal A, Ho JS, Cowdrey C, Tihan T, Mawrin C, Riggins GJ. **Yes-associated protein 1 is activated and functions as an oncogene in meningiomas**. Mol Cancer Res. 10(7):904-13, 2012
- Journal Publication: - Baia, GS, Caballero, OL, Riggins GJ **The Hippo signaling pathway and translational opportunities for brain cancers. CNS Oncology**, Vol. 1, No. 2, Pages 113-115, 2012
- Scientific Conference Presentation: 11th Annual International Neuro-Oncology Updates (Invited Oral Presentation), Johns Hopkins University, Baltimore, MD, USA. 2012. **Hippo Signaling Pathway and Translational Opportunities in Meningioma Research**

CONCLUCIONS

We identified and characterized YAP protein as a candidate oncoprotein in NF2-meningiomas. Using cell-based assays we have successfully selected small molecule compounds that preferentially inhibit NF2 mutant meningioma cells *in vitro*, affecting the function and activity of YAP. These selected FDA approved drugs are safe for human use. We validated these drugs by using human cell lines and tested their ability to synergize with radiation. We extensively characterized the cellular effects of these FDA compounds in meningioma cells. We anticipate that future clinical trials using HDACi for meningioma patients might provide insights on the efficacy of this class of drugs in these CNS tumors driven by NF2 tumor suppressor gene.

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APPENDICES

Table 1 – IC_{50} values (μM) determined for NF2 isogenic cells. Drugs were selected for preferential inhibition of NF2 mutant cells from primary drug screening of 1,726 compounds.

	KT21MG1		SF6717		AC1	
	NF2-	**NF2+**	**NF2-**	**NF2+**	**NF2-**	**NF2+**
VEGFR/PDGFR						
Sorafenib (Nexavar)	3.7μM	5.7μM	0.9μM	3.2μM	1μM	3μM
Sunitinib (Sutent)	2.3μM	2.8μM	2.7μM	4.7μM	3.9μM	3.1μM
Tandutinnib (MLN518)	15.3μM	20.4μM	47μM	45.8μM	41μM	41μM
EGF Receptors						
Lapatinib (Tyrkeb)	3.8μM	5.2μM	3μM	4μM	2.5μM	4μM
Gefitinib (Iressa)	18μM	22μM	22μM	24μM	17μM	22μM
BCR-ABL inhibitors						
Imatinib (Gleevec)	16μM	23.3μM	13.7μM	39.7μM	13.8μM	18.3μM
mTOR/PI3K						
BEZ235	0.6nM	2.8nM	0.6nM	1.2nM	0.9nM	1.8nM
Rapamycin	1μM	2.2μM	0.6μM	1.3μM	1μM	3.5μM
Everolimus (Afinitor)	23μM	26.5μM	17.4μM	20.6μM	18μM	20μM
Anti-parasitic						
Emetine	0.04μM	0.06μM	0.02μM	0.04μM	0.02μM	1μM
Topo Inhibitors						
Idarubicin	8.5nM	85.5nM	0.3μM	0.1μM	x	x
Mitoxantrone	7μM	8.2μM	5μM	10μM	x	x

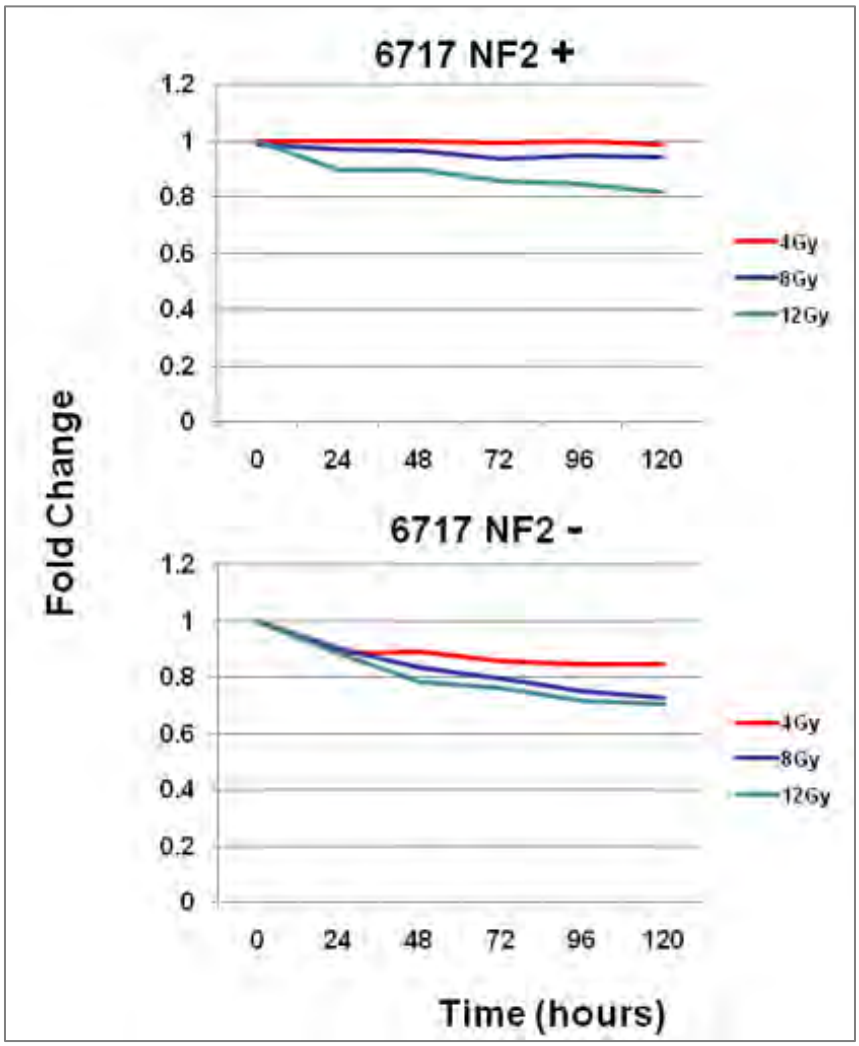

Figure 1 – *In vitro* Alamar Blue proliferation assay of wild type and mutant NF2 cells treated with different doses of radiation. Cell proliferation was normalized to the control cells (0Gy) and is plotted over time.

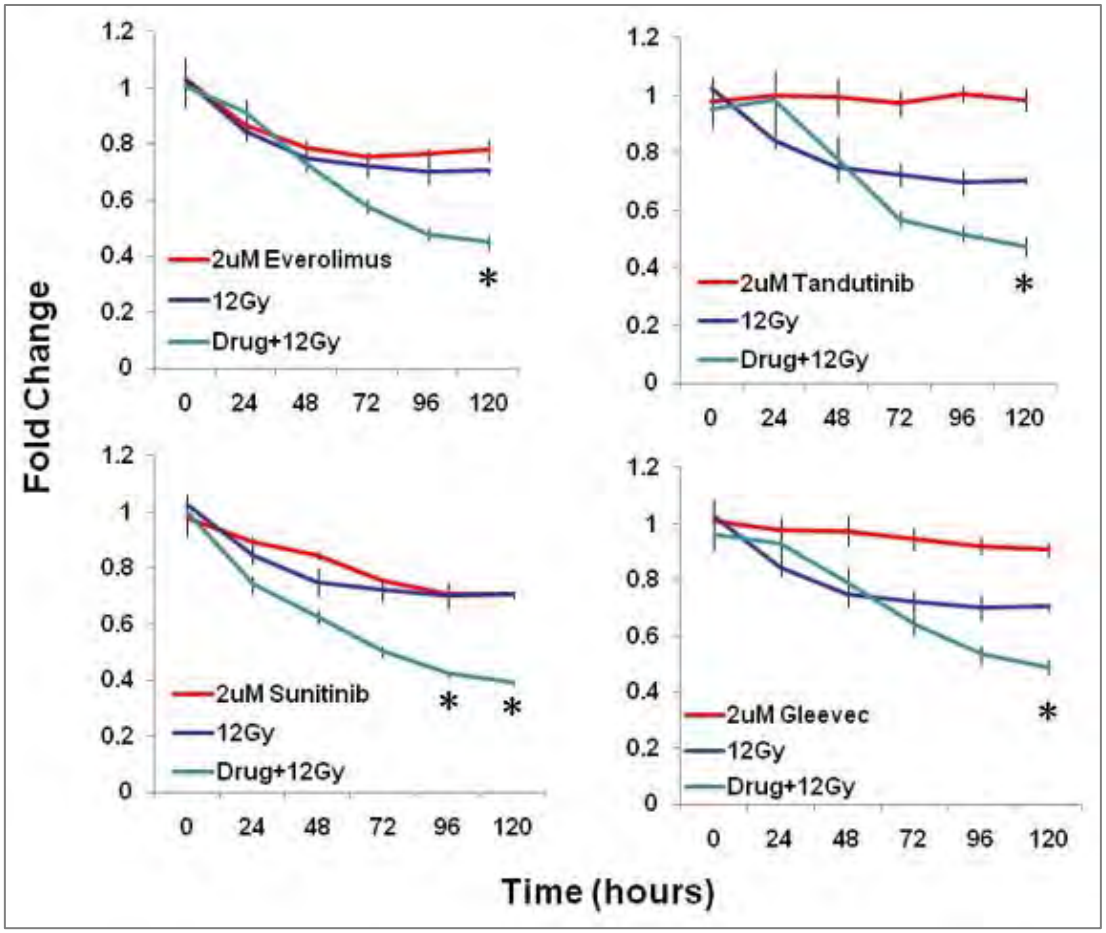

Figure 2 – Synergistic inhibition of drugs with radiation (12Gy) in KT21MG1 mutant cells. Proliferation assays were carried out over 120h with cells. Proliferation assays were carried out over 120h with cells treated with drugs (2μM), radiation (12Gy) or drugs+radiation. Synergistic effect of drugs in combination with radiation was observed for Everolimus, Tandutinib, Sunitinib and Gleevec (* $P \leq 0.05$).

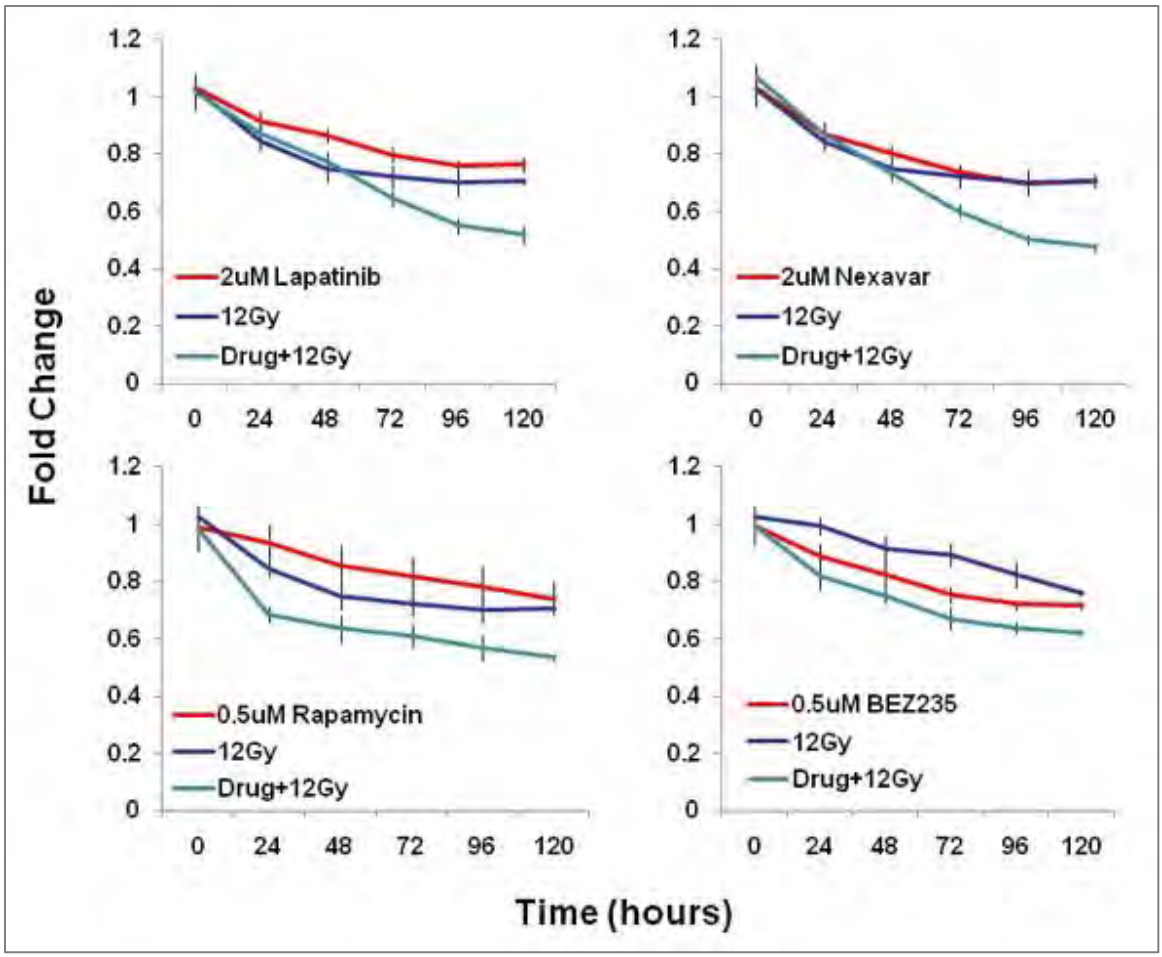

Figure 2 – Synergistic inhibition of drugs with radiation (12Gy) in KT21MG1 mutant cells. (Continued).

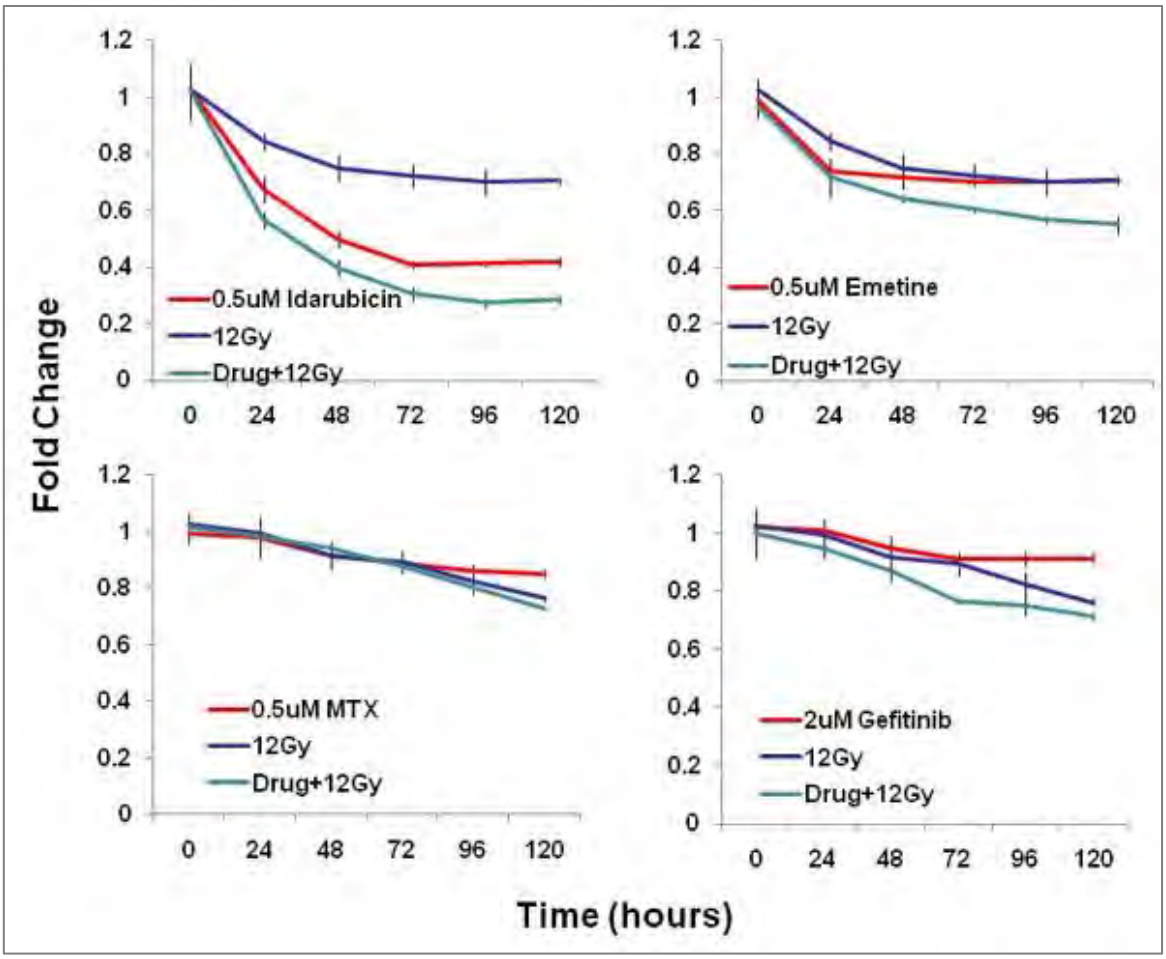

Figure 2 – Synergistic inhibition of drugs with radiation (12Gy) in KT21MG1 mutant cells. (Continued).

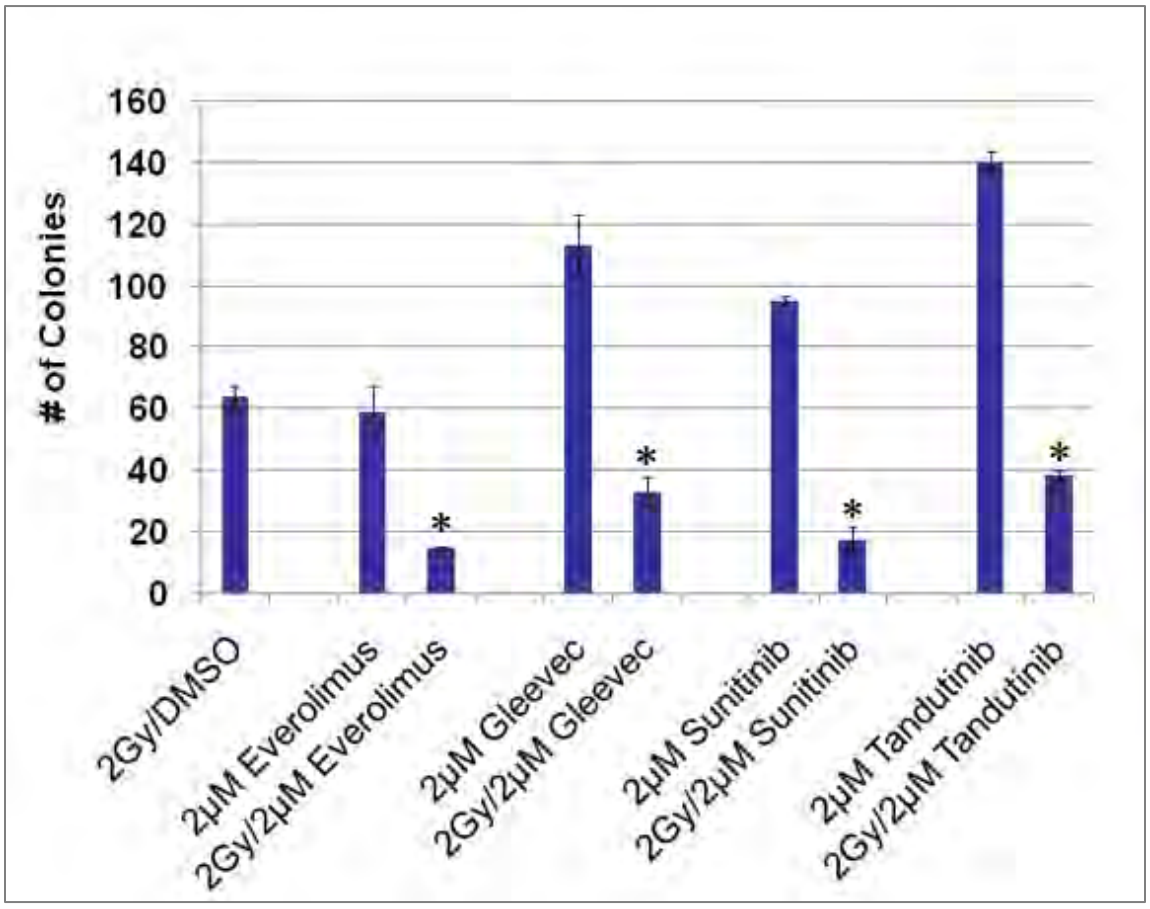

Figure 3 – Secondary drug/radiation screening – Colony-forming efficiency assay was performed with NF2 mutant cells treated with drugs and radiation. Cells treated with both drug and radiation together formed significantly fewer colonies compared to the controls with either radiation or drugs alone ($P \leq 0.05$).

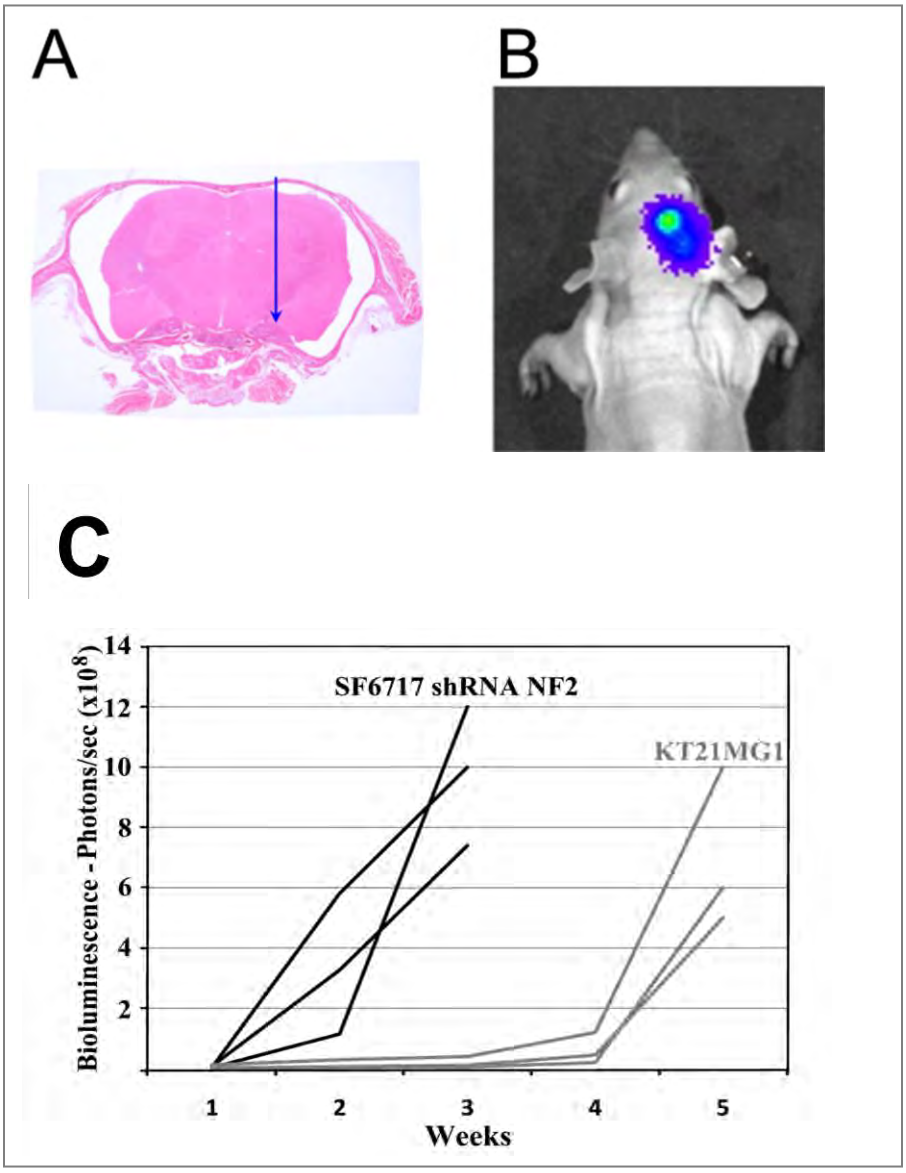

Figure 4 – NF2 mutant cells are tumorigenic *in vivo* in Athymic nude mice. A) Tumor implantation site (arrow) shown on a coronal section of a mouse brain. Meningioma cells were implanted into the floor of temporal fossa by using the following stereotaxic coordinates relative to the bregma: 2 mm to the right, 2 mm posterior and 6 mm of depth. B) Bioluminescence (BLI) of Firefly Luciferase tagged cells was used to monitor tumor growth. A BLI image of a representative animal is shown at day 5 after cell implantation. C) Meningioma cells were orthotopically implanted into nude mice. NF2 mutant cells were tumorigenic in nude mice as shows by the BLI measurements over time (weeks).

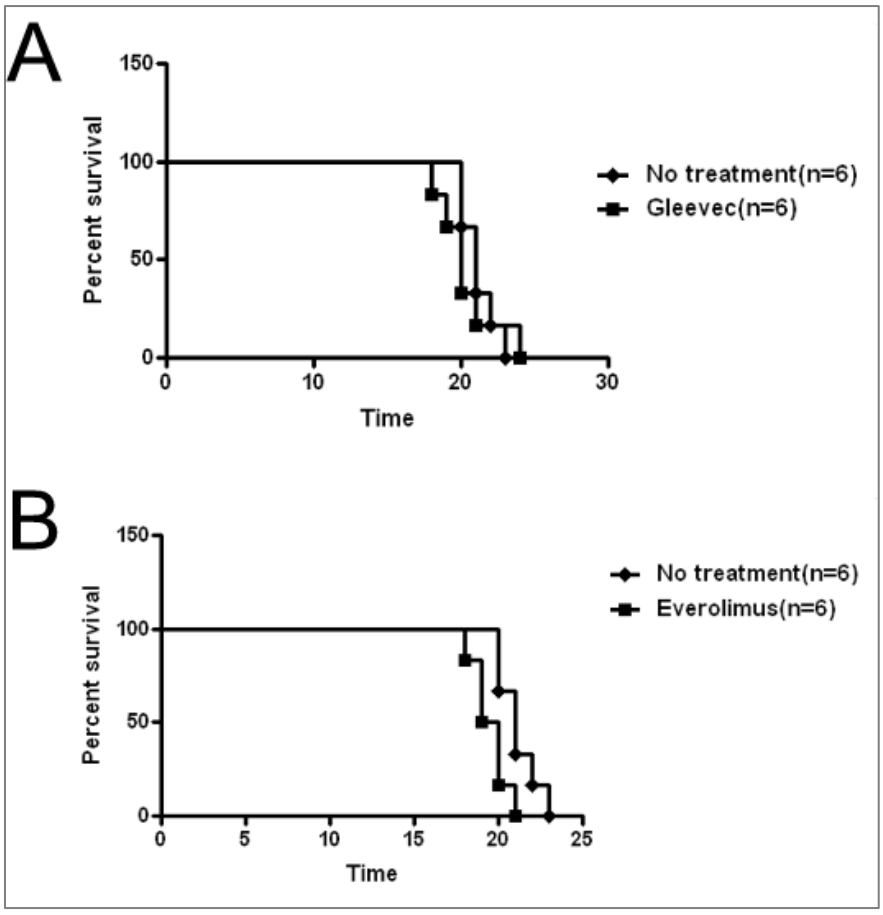

Figure 5 – Kaplan-meier survival plots of NF2 mutant meningioma xenografts. Mice were treated with either Everolimus (A) or Gleevec (B) compared to control animals (no treatment). (Time in days).

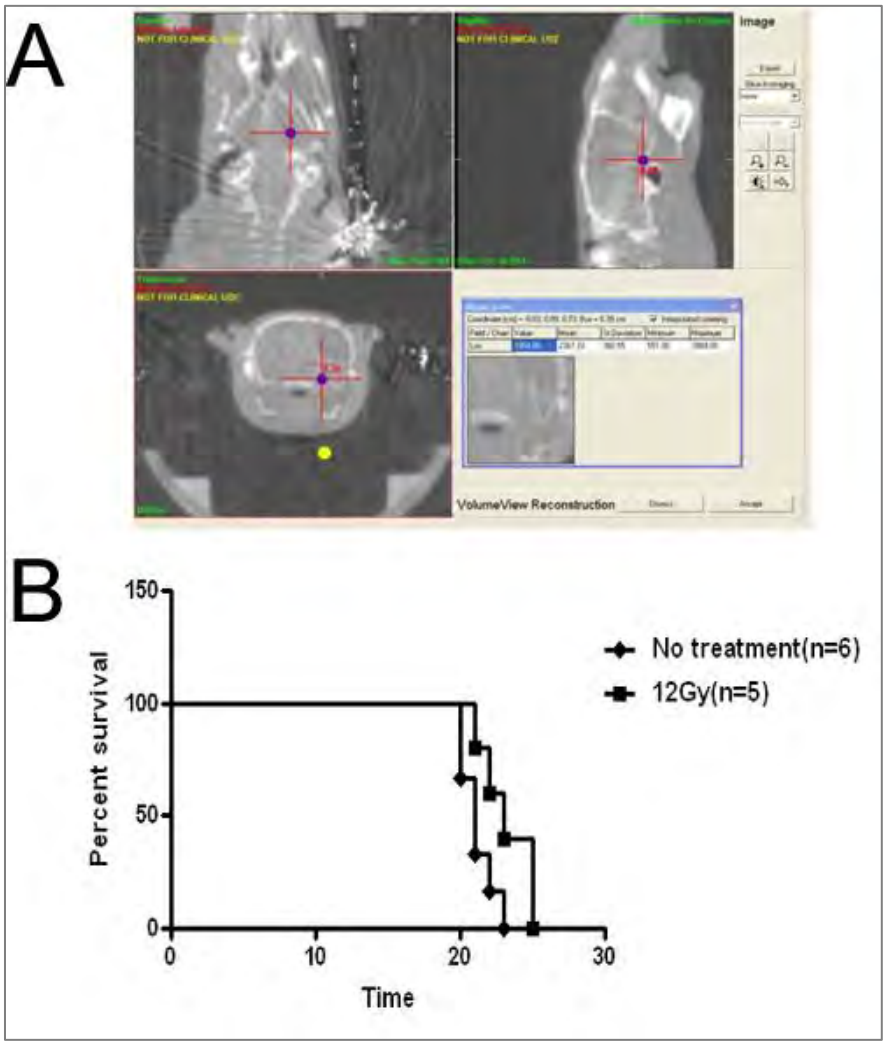

Figure 6 – Stereotaxic radiosurgery device. NF2 mutant meningioma cells were orthotopically implanted into mice (10^5 cells/mouse). Three days after cell implantation mice were anesthetized and treated once with conformal radiation (12Gy), using an in-house developed precision small animal radiation device. This technology is capable of delivering high intensity and localized doses of radiation to the target tumor volume, while minimally affecting the surrounding normal tissue. Since radiosurgery is part of the standard of care of meningioma patients, this CT-guided conformal radiation is pivotal in mimicking clinical radiotherapy (A). Mice survival plots (B) of animals treated with radiation (12Gy) compared to the non-treated controls. (Time in days).

Figure 7 – Poster presented at the 103rd American Association for Cancer Research Annual Meeting, Chicago, IL, USA, 2012.

Targeting NF2 deficient meningiomas with combinations of small molecule inhibitors and radiation. Gilson S. Baia, Graeme Woodworth, Eric Ford, Gregory Riggins. Johns Hopkins, Baltimore, MD

Poster Session - PO.ET01.01. Combination Therapy 1 -Mon, Apr 2, 1:00 - 5:00 PM

(Figure on next Page)

5893

Targeting NF2 Deficient Meningiomas with Combinations of Small Molecule Inhibitors and Radiation

Gilson S. Baia, PhD[1]; Graeme Woodworth MD[1]; Eric Ford PhD[2]; Gregory J. Riggins MD PhD[1]

[1]Department of Neurosurgery and [2]Radiation Oncology Department

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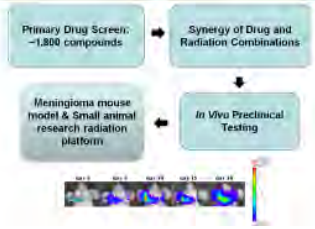
JOHNS HOPKINS MEDICINE

BACKGROUND

Meningiomas are common tumors of the central nervous system and are the second most prevalent tumors in Neurofibromatosis type 2 patients. Despite their high frequency, currently there are no chemotherapeutic options for these tumors and treatment is limited to surgery and various forms of radiation therapy either as adjuvant or primary therapy. Some tumors are unresectable due to location and have histopathological aggressive features (designated as WHO grade II and III) with higher recurrence rates. When treatment is recommended, these tumors almost uniformly receive some form of radiation therapy. The aim of this study was to find new therapeutic options to couple with radiation, given its common use with non-surgical meningiomas. We are interested in targeting oncogenic pathways in the context of loss of the NF2 gene, which represents the most common genetic alteration in meningiomas, present is 50-70% of sporadic tumors and all of NF2 cases. We used cell-based assays searching for small molecule inhibitors that showed prevalent efficacy against NF2-deficient cells. Cell proliferation and clonogenic assays were employed to investigate the potential synergy effect of drugs and radiation combinations. Three pairs of NF2 isogenic meningioma cells (AC1, SF6717 and KT21MG1) were used to screen libraries of FDA approved compounds. The primary screen was performed using KT21MG1 cells. The top 5% compounds (86 compounds) showing preferential inhibition of NF2-deficient cells were selected for further validation. The secondary screen was used to validate useful targets with AC1 and SF6717 cells. Twelve compounds (~15%) showed prevalent inhibitory activity on these cells. Small molecules targeting mammalian target of rapamycin (mTOR) and vascular endothelial growth factor receptor (VEGFR) showed preferential synergistic inhibition of NF2 cells with radiation, compared to either radiation or drug treatment alone. Currently, preclinical testing is in progress to investigate the efficacy of these inhibitors in combination with radiation in a meningioma mouse model.

STUDY DESIGN



RESULTS

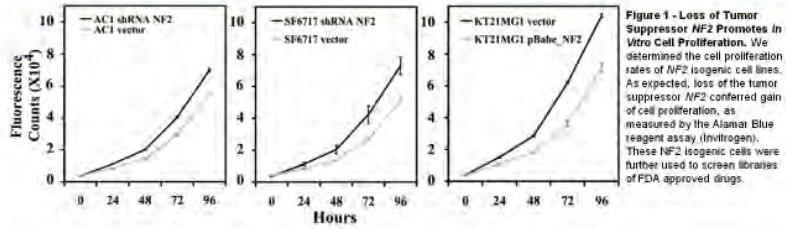


Figure 1 - Loss of Tumor Suppressor *NF2* Promotes *In Vitro* Cell Proliferation. We determined the cell proliferation rates of *NF2* isogenic cell lines. As expected, loss of the tumor suppressor *NF2* conferred gain of cell proliferation, as measured by the Alamar Blue reagent assay (Invitrogen). These NF2 isogenic cells were further used to screen libraries of FDA approved drugs.

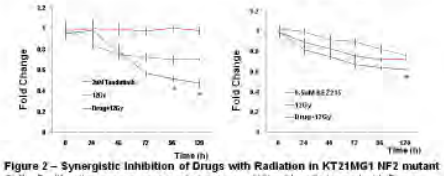


Figure 2 – Synergistic Inhibition of Drugs with Radiation in KT21MG1 NF2 mutant Cells. Proliferation assays were carried out over 120h with cells treated with Drugs, Radiation or Drugs+Radiation. Synergistic effect of drugs in combination with radiation was observed for BEZ235 and Tandutinib ($P = 0.05$).

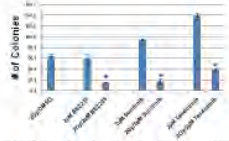


Figure 3 – Secondary Drug/Radiation Screening. Colony-forming efficiency assay was performed with KT21MG1 NF2 mutant cells treated with drugs and radiation. Cells treated with both drug and radiation together formed significantly fewer colonies compared to the controls with either radiation or drugs alone ($P = 0.05$).

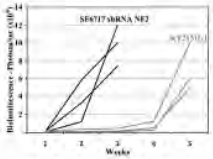


Figure 4 - NF2 Deficient Cells Are Tumorigenic *In Vivo*. Meningioma cells were orthotopically implanted into nude mice and bioluminescence was used to monitor tumor progression. NF2 mutant cells were tumorigenic in nude mice.

Figure 5 - Stereotaxic Radiosurgery Device. NF2 mutant meningioma cells were implanted orthotopically into mice (250,000cells/mouse).Three days after cells implantation mice were anesthetized and treated once with localized radiation (20Gy), using an in-house developed precision small animal radiation device. This technology is capable of delivery of high intensity and localized doses of radiation to the target tumor volume, while minimally affecting the surrounding normal tissue. Since radiosurgery is part of standard of care of meningioma patients, this CT-guided conformal radiation is pivotal in mimicking clinical radiotherapy.

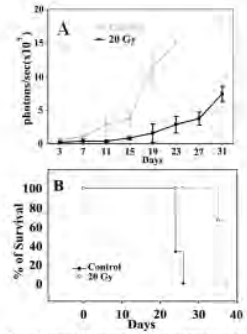


Figure 6 - Radiotherapy of meningioma implanted mice. A) Bioluminescence plot (BLI of 3 mice/group averaged for each time point) associated with monitoring of intracranial tumor growth and B) survival times of radiation treated mice and control group ($p<0.0735$). Treated mice showed much lower bioluminescence reads, compared to control groups (Fig. A), demonstrating that the radiation treatment is capable of slowing down tumor growth. In addition, treated mice had longer survival times compared to control mice (Fig. B)

Table 1 – IC50 Values Determined for NF2 Isogenic Cells. Drugs were selected for preferential inhibition of NF2 mutant cells.

	NF2-	NF2+
Sorafenib (Nexavar)	3.78um	6uM
Sunitinib (Sutent)	2.3uM	3uM
Vandetanib (Zactima)	5.2uM	4uM
Pazopanib (Votrient)	3uM	3uM
Tandutinib (MLN518)	15.3uM	20.4uM
Gefitinib (Iressa)	13.5uM	10uM
Erlotinib (Tarceva)	120uM	120uM
Nilotinib (Tasigna)	2uM	1.7uM
Dasatinib (BMS-354825)	23uM	17uM
NVP-BEZ235	0.5nM	3nM

CONCLUSIONS

-- Small molecule inhibitors preferentially affecting NF2 mutant cells were selected.

-- Compounds synergizing with radiation were selected for further pre-clinical testing in nude mice.

-- CT-guided conformal radiation was optimized for NF2 mutant cells.

FUTURE DIRECTIONS

-- Preclinical testing is in progress to investigate the efficacy of selected small molecule inhibitors in combination with conformal radiation in a NF2 mutant meningioma mouse model.

ACKOWLEDGEMENTS

This research is supported by the Congressionally Directed Medical Research Programs contract W81XWH-11-1-0424 to G.S.B. and by generous donations from Leonard and Phyllis Altman and from the Meningioma Mommas Foundation.

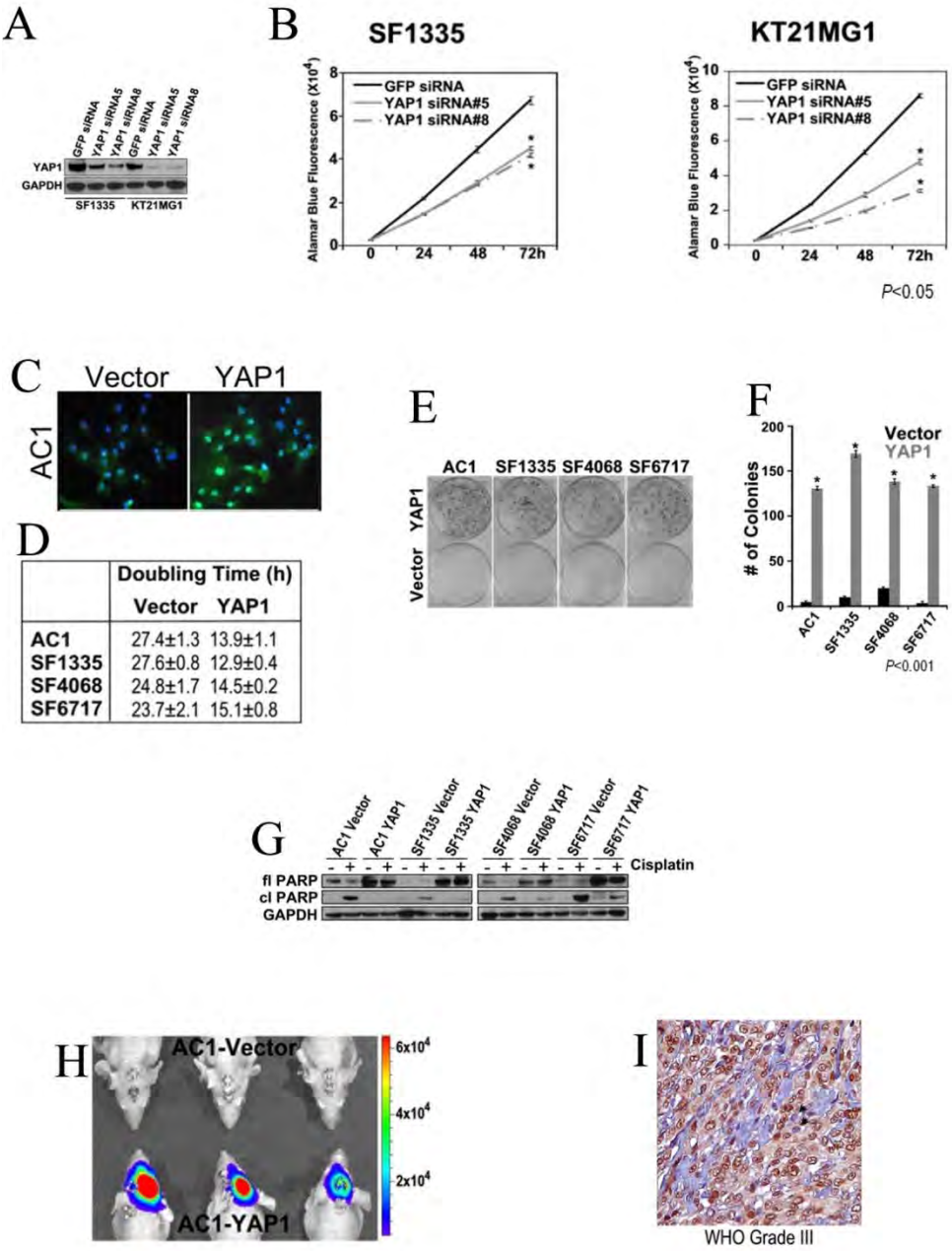


Figure 8 – Yes-Associated Protein is activated and functions as an oncogene in meningioma. A- YAP1 and GAPDH immunoblotting of SF1335 and KT21MG1 transiently transfected with siRNA oligos targeting YAP1 or GFP, as a nontargeting control. Following YAP1 and GFP siRNA transient transfections, cells were plated for alamarBlue proliferation assay. **B-** Growth curve plots conducted for SF1335 and KT21MG1 cells are shown. *, $P \leq 0.05$. The P value is for YAP siRNAs versus GFP siRNA. **C-** YAP1 immunofluorescence staining using the YAP1 polyclonal antibody to demonstrate the nuclear localization of YAP1 in meningioma cells. Nuclei were counterstained with 4,6'-diamidino-2-phenylindole (DAPI). 100X magnification. **D-** Growth rates of meningioma cells associated with YAP1 expression indicated as doubling time of cells. **E-** Representative pictures of wells of colony-forming efficiency assay for cells transfected with either YAP1 or empty vector. **F-** Quantification of colony-forming efficiency assay expressed in average of the total number of colonies per cell. *, $P \leq 0.001$. The P value is for YAP1 versus empty vector cells. **G-** Immunoblotting for full-length PARP (fl PARP), cleaved PARP (cl PARP), and GAPDH of cells incubated either with vehicle (DMSO) or 30 µmol/L of cisplatin for 72 hours. **H-** YAP1 overexpression promotes *in vivo* tumor growth of non-neoplastic meningeal cells. Bioluminescence of AC1 xenografts. Representative images of control and YAP1 mice groups at day 15 after implantation are shown. **I-** YAP1 was found to be highly expressed in all meningiomas regardless of histologic subtype and grade. The distribution of staining was consistently high in the nucleus with more variable staining in the cytoplasm. Vascular elements showed some YAP1 positivity, albeit reduced compared with the tumor. A minor percentage of cells have nuclei that are negative for YAP1 expression (black arrows).

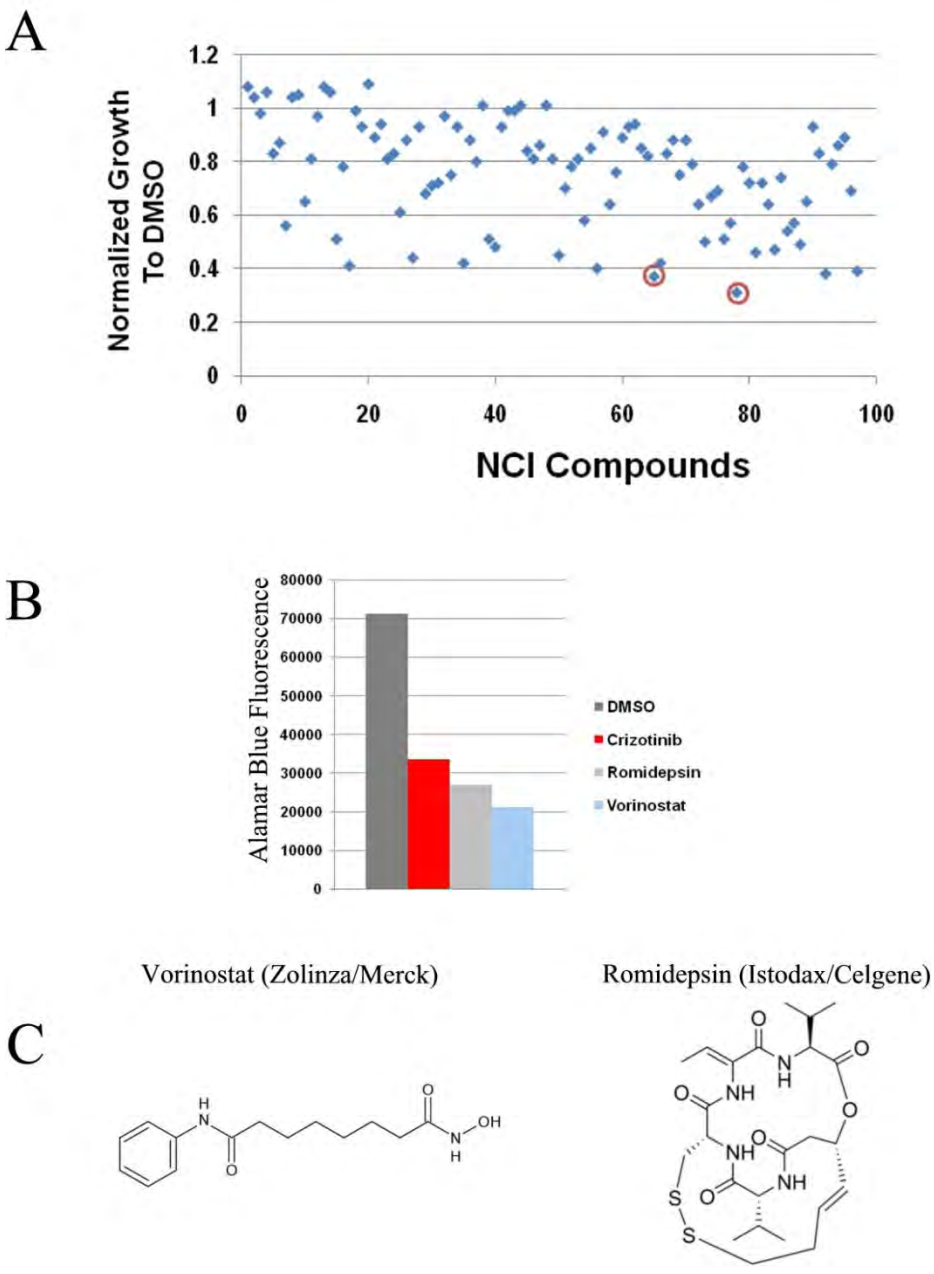
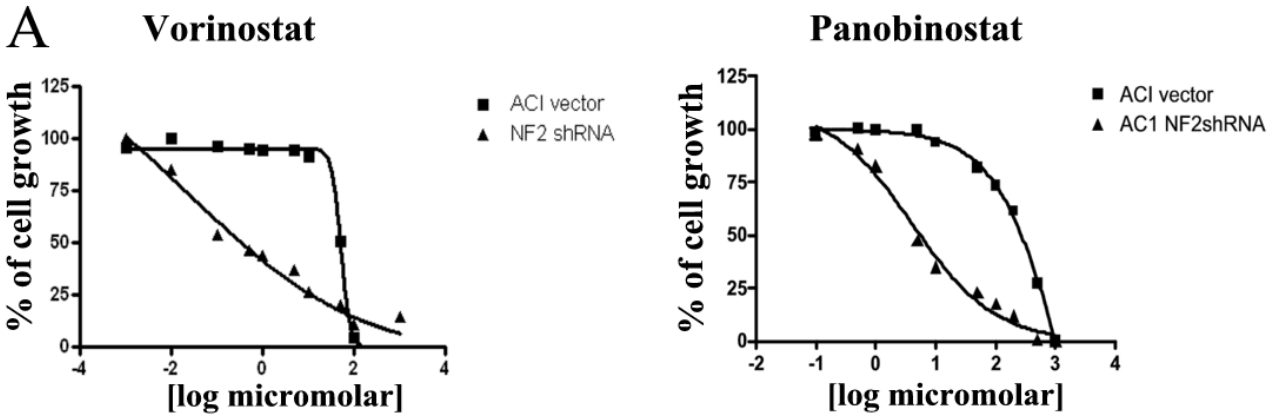


Figure 9 – Primary drug screen shows that YAP cells are sensitive to Histone Deacetylase (HDAC) inhibitors. A- Cell-based screening of NCI drug libraries performed in SF1335 YAP cells. Each dot represents the cell proliferation change of individual compounds at 96h after adding drugs to wells. Data represented as fold change vs. control group (DMSO). **B-** Alamar Blue cell proliferation at 96h time-point comparing the effect of HDAC inhibitors and Crizotinib as a single agents, compared to DMSO. **C-** Chemical structure of HDAC inhibitors Vorinostat and Romidepsin.

A



B

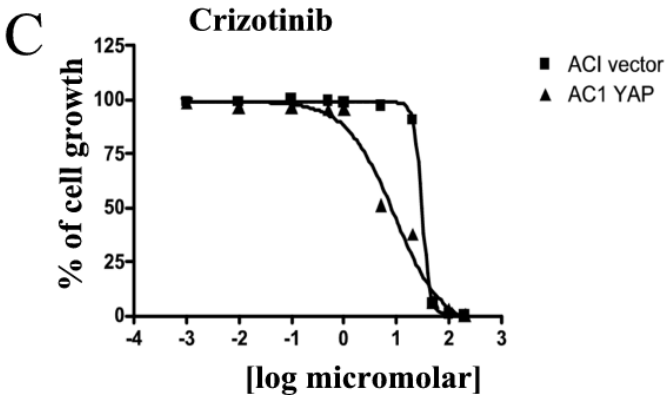
YAP1 isogenic cells

	AC1 Vector	AC1 YAP1	SF1335 Vector	SF1335 YAP1	6717 Vector	6717 YAP1
Vorinostat (VN)	640	34.8	267.4	6.9	274.4	33.3
Panobinostat (PN)	52.8	0.06	50.3	0.06	58.5	0.56
Romidepsin (RD)	98.7	16	106.8	5.1		

NF2 isogenic cells

AC1 Vector	AC1 NF2shRNA	6717 Vector	6717 NF2shRNA
975	7.3	268.2	75
51.4	0.03	62.8	12
326.9	9.2		

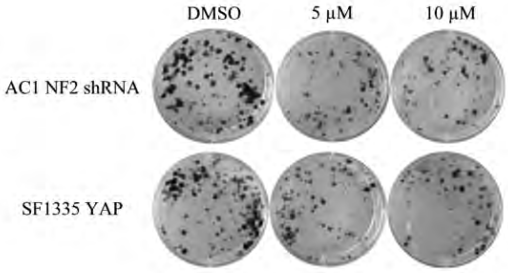
C



	IC_{50}
AC1 Vector	30.4
AC1 YAP	9.1
SF1335 Vector	25
SF1335 YAP	8.7

Figure 10 – Secondary drug screen reveals that NF2/YAP isogenic cells are preferentially inhibited by HDAC inhibitors. A- IC_{50} plots for NF2 isogenic cells (AC1) for HDAC inhibitors, Vorinostat and Panobinostat. **B-** IC_{50} values of HDAC inhibitors (Vorinostat, Panobinostat and Romidepsin) at 72 hours against NF2 and YAP isogenic cells. C- Crizotinib IC_{50} plot and values for YAP isogenic meningioma cells (for all IC_{50} calculations $R^2 \geq 0.95$).

A



B

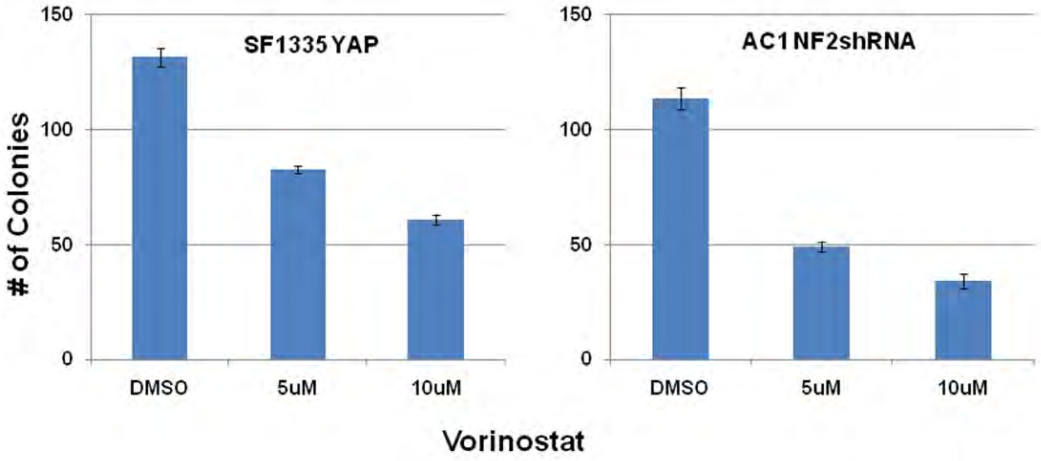
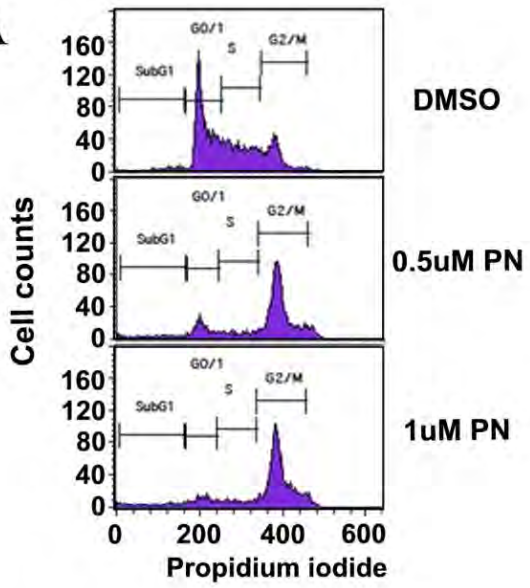


Figure 11– Anchorage-independent growth of NF2/YAP meningioma cells is impaired by the HDAC inhibitor, Vorinostat. A- Representative pictures of wells of colony-forming efficiency assay for NF2/YAP cells treated with the HDAC inhibitor, Vorinostat. **B-** Quantification of colony-forming efficiency assay expressed in average of the total number of colonies per cell.

A



B

VORINOSTAT (VN)

	AC1 NF2shRNA		SF1355 YAP	
DMSO	SubG1	5.3	SubG1	4.7
	G0/G1	53.3	G0/G1	55.5
	S	28.2	S	23.4
	G2/M	13.3	G2/M	16.3
2μM VN	SubG1	14.5	SubG1	8.0
	G0/G1	49.9	G0/G1	58.6
	S	13.7	S	12.4
	G2/M	20.7	G2/M	20.4
4μM VN	SubG1	15.0	SubG1	8.5
	G0/G1	36.2	G0/G1	32.7
	S	15.4	S	11.3
	G2/M	31.0	G2/M	46.0

PANOBINOSTAT (PN)

	AC1 NF2shRNA		SF1355 YAP	
DMSO	SubG1	2.7	SubG1	2.5
	G0/G1	50.8	G0/G1	49.5
	S	27.9	S	28.0
	G2/M	18.0	G2/M	20.4
0.5μM PN	SubG1	5.3	SubG1	4.9
	G0/G1	14.2	G0/G1	10.7
	S	10.9	S	9.6
	G2/M	66.0	G2/M	74.0
1μM PN	SubG1	5.6	SubG1	5.4
	G0/G1	10.7	G0/G1	8.7
	S	11.2	S	8.6
	G2/M	70.3	G2/M	76.3

Figure 12 – HDAC inhibitors promote cell cycle arrest in G2/M in NF2/YAP cells. NF2 and YAP cells were treated with HDAC inhibitors (Vorinostat or Panobinostat) or vehicle (DMSO). Forty eight hours later, total DNA content was stained with propidium iodide and analyzed by flow cytometry. **A-** Representative plots of AC1 NF2shRNA cells treated with Panobinostat and stained with propidium iodide. **B-** Cell cycle distribution of NF2/YAP cells treated with HDAC inhibitors, Vorinostat and Panobinostat.

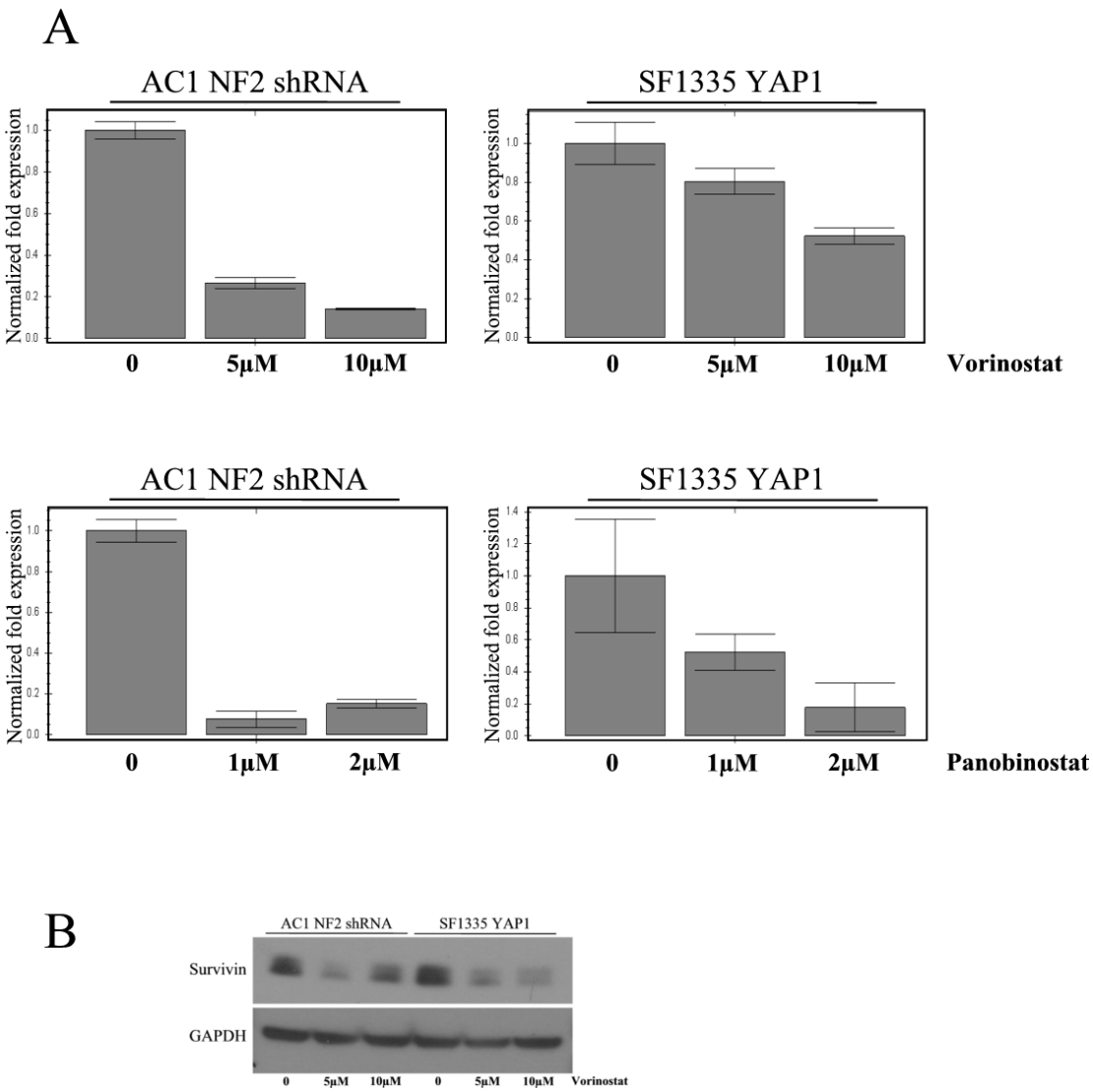


Figure 13 – Expression of Survivin, a YAP target gene is impaired by HDAC inhibitors. A- Quantitative real time-PCR analysis of Survivin transcript in AC1 NF2shRNA and SF1335YAP cells. B- Immunoblotting of lysates of meningioma cells treated with the HDAC inhibitor, Vorinostat showing levels of expression of Survivin protein. GAPDH blotting is shown as protein loading control.

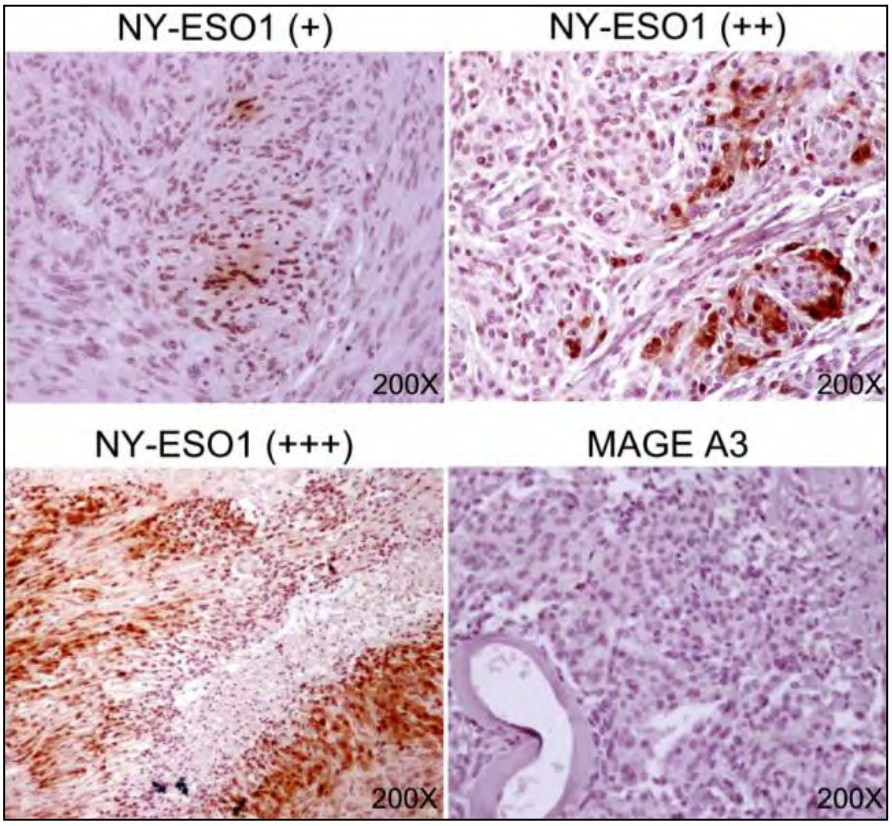


Figure 14: Staining of meningioma samples using monoclonal antibodies specific to NY-ESO-1 and MAGEA (clones E978 and MAGE6C1, respectively) (shown in brown). E978 stains the cytoplasm and nucleus, showing diffuse positivity in >90% of tumor cells (+++) or only scattered positive cells and many tumor cells were negative (+) while MAGEA3 staining was negative or only focal in the samples tested. (Magnifications:200x)

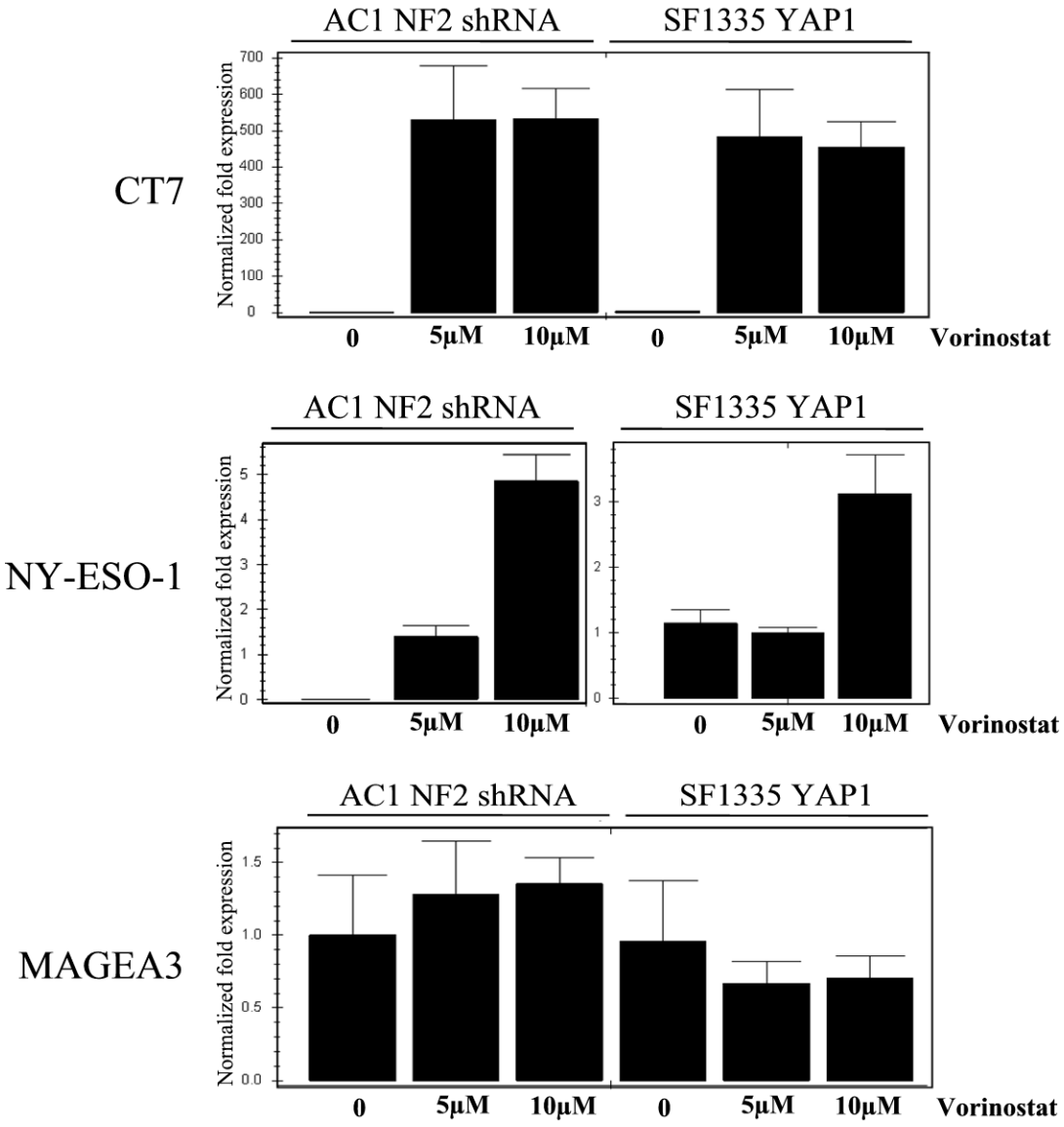


Figure 15 – Up-regulated expression of Cancer/Testis genes, CT7 and NY-ESO-1 in cells treated with the HDAC inhibitor, Vorinostat. Quantitative real time-PCR analysis of Cancer/Testis genes (CT7, NY-ESO-1 and MAGEA3) transcripts in AC1 NF2shRNA and SF1335YAP cells treated with Vorinostat.

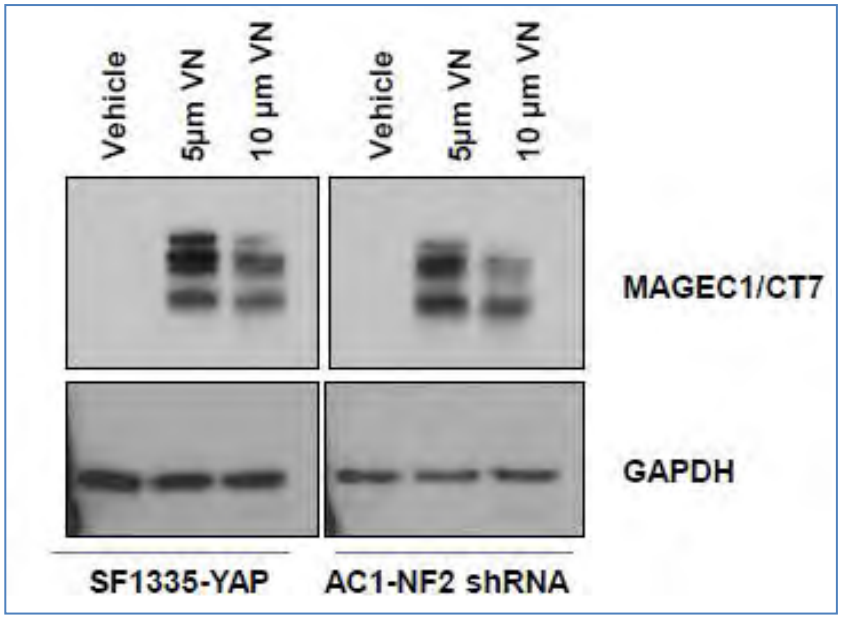


Figure 16: Expression of the CT protein MAGEC1 is dramatically increased by treatment with the HDAC inhibitors Vorinostat (VN). Immunoblotting of whole cell lysates of meningioma cells treated with the HDAC inhibitor, Vorinostat showing high levels of expression of MAGEC1 protein. GAPDH blotting is shown as protein loading control.

- Journal Publication: Baia, GS, Caballero OL, Orr BA, Lal A, Ho JS, Cowdrey C, Tihan T, Mawrin C, Riggins GJ. **Yes-associated protein 1 is activated and functions as an oncogene in meningiomas**. Mol Cancer Res. 10(7):904-13, 2012

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

Cancer Genes and Genomics

Molecular Cancer Research

Yes-Associated Protein 1 Is Activated and Functions as an Oncogene in Meningiomas

Gilson S. Baia[1], Otavia L. Caballero[1], Brent A. Orr[2], Anita Lal[3], Janelle S. Y. Ho[1], Cynthia Cowdrey[4], Tarik Tihan[5], Christian Mawrin[6], and Gregory J. Riggins[1]

Abstract

The Hippo signaling pathway is functionally conserved in *Drosophila melanogaster* and mammals, and its proposed function is to control tissue homeostasis by regulating cell proliferation and apoptosis. The core components are composed of a kinase cascade that culminates with the phosphorylation and inhibition of Yes-associated protein 1 (YAP1). Phospho-YAP1 is retained in the cytoplasm. In the absence of Hippo signaling, YAP1 translocates to the nucleus, associates with co-activators TEAD1-4, and functions as a transcriptional factor promoting the expression of key target genes. Components of the Hippo pathway are mutated in human cancers, and deregulation of this pathway plays a role in tumorigenesis. Loss of the *NF2* tumor suppressor gene is the most common genetic alteration in meningiomas, and the *NF2* gene product, Merlin, acts upstream of the Hippo pathway. Here, we show that primary meningioma tumors have high nuclear expression of YAP1. In meningioma cells, Merlin expression is associated with phosphorylation of YAP1. Using an siRNA transient knockdown of YAP1 in *NF2*-mutant meningioma cells, we show that suppression of YAP1 impaired cell proliferation and migration. Conversely, YAP1 overexpression led to a strong augment of cell proliferation and anchorage-independent growth and restriction of cisplatin-induced apoptosis. In addition, expression of YAP1 in nontransformed arachnoidal cells led to the development of tumors in nude mice. Together, these findings suggest that in meningiomas, deregulation of the Hippo pathway is largely observed in primary tumors and that YAP1 functions as an oncogene promoting meningioma tumorigenesis. *Mol Cancer Res; 10(7); 904–13. ©2012 AACR.*

Introduction

Biallelic inactivation of the *NF2* gene is observed in patients with neurofibromatosis type 2 (NF2) resulting in the development of tumors of the central nervous system (CNS), including meningiomas (1). Loss of the *NF2* gene is observed in the majority of sporadic meningiomas of all histopathologic grades and it is thought to be an early event in the tumorigenesis of these tumors (1, 2). In addition, genetic mouse model based on leptomeningeal knockout of the *Nf2* gene led to the development of meningiomas (3, 4). Taken together, these observations corroborate the association of the *NF2* tumor suppressor gene as an initiating mechanism in meningioma tumorigenesis (3, 5, 6). The *NF2* gene product, Merlin, is a FERM (four-point-one protein, ezrin, radixin, and moesin) domain protein associated with the membrane cytoskeleton and capable of interactions with numerous proteins, including CD44, reviewed in the work of Okada and colleagues (7). Upon phosphorylation at serine-518 residue by p21-activated kinase (PAK1), Merlin alternates to an open conformation. It is the closed and unphosphorylated form of Merlin that shows activity as a tumor suppressor (8).

The Hippo cascade, initially identified in *Drosophila*, is one of the signaling pathways downstream of Merlin. Mammalian counterparts of this pathway have been identified and characterized as pivotal for normal cellular development (9, 10) and organ size control (11, 12). Moreover, overexpression of Yes-associated protein 1 (YAP1), the central effector of the pathway, in human nontransformed mammary epithelial cells generated altered phenotype cells that exhibited prominent tumorigenic characteristics (13). Recently, Zhang and colleagues (11) showed that inactivation of *Nf2* in mouse hepatocytes and biliary epithelial cells was accompanied with YAP1 activation and led to the formation of hepatocellular carcinoma and bile duct hamartoma, strongly suggesting a role for the Hippo pathway in carcinogenesis. The core of the Hippo pathway is composed of a phosphorylation cascade of events that culminates with the phosphorylation and inhibition of YAP1 (and/or its homolog TAZ, transcriptional co-activator with PDZ-binding motif; refs. 14, 15). Upon

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-12-0116

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

release of inhibition, YAP1 translocates to the nucleus where it associates with transcriptional co-activators TEAD1–4, to promote expression of target genes (16, 17).

Importantly, genetic alterations of Hippo pathway components have been associated with human cancers. Deletion of *LATS2* in a subset of human mesotheliomas has been identified, implicating *LATS2* as a tumor suppressor gene (18). Other significant genetic alterations of components of the pathway include: homozygous deletion of *WW45* in renal carcinoma cells (19); *NF2* mutation in sporadic Schwannoma (20) and mesothelioma (21); hypermethylation of *MST* in soft tissue sarcoma (22); and overexpression of *TAZ* in breast cancer (15). In contrast, deletion of 11q22 locus, the *YAP1* chromosomal location, is frequent in breast cancer, and in these cancers, YAP1 has been shown to associate with the p73 protein in the nucleus and regulate DNA repair and apoptosis (23). Thus, under certain cellular context, YAP1 appears to function as a tumor suppressor.

In meningiomas, it has been reported that *NF2* loss confers a proliferation advantage to tumor cells. Moreover, *YAP1* knockdown in *NF2*-deficient cells rescues the effects of Merlin loss on cell proliferation and restores normal proliferation rates (24). However, the functional contribution of *YAP1* expression in meningiomas has not been fully explored. Using human cells lines and *in vivo* mouse models, we investigated the role of YAP1 in meningiomas and its effects on cell proliferation, migration, apoptosis, and tumorigenesis. Here, we present strong evidence that YAP1 is activated upon loss of *NF2* gene and functions as an oncogene promoting meningioma tumorigenesis.

Materials and Methods

Human cell lines

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. The non-neoplastic meningeal cells, AC1, and meningioma cells SF4068 and SF6717 were immortalized with human telomerase and E6/E7 oncogenes, as described earlier (24, 25). The KT21MG1 cell line was established from a human malignant meningioma and is *NF2*-mutant, as previously described (26). The SF1335 is an *NF2*-mutant cell line established from a benign meningioma (27) and was kindly provided by Dr. Marco Giovannini (House Ear Institute, Los Angeles, CA).

YAP1 construct and cell transfections

The pEGFP-N2-YAP1 expression construct was a gift from Dr. Yoshitaka Sekido (Nagoya University, Nagoya, Japan; ref. 28). YAP1 expression construct and empty vector (pEGFP-N2) were transfected into meningioma cells using Lipofectamine 2000 (Life Technologies). Nontransfected cells were used as a negative control for selection. Cells were cultured for 5 days with G418 at 500 μg/mL before assays were conducted.

siRNA transient knockdown

siRNA duplexes (27-mer) YAP1#5 (AAAUAAAGCCAUUUCUGGUUUGCUCCU) and YAP1#8 (ACUGGCAAAUUAUAGGCACUCCUUCCA) were purchased from IDT DNA Technologies. Transient transfections of duplex oligos (final concentration of 10 nmol/L) were carried out by using Lipofectamine 2000 (Life Technologies), following the manufacturer's instructions. Nontargeting GFP siRNA (IDT DNA Technologies) was used as a control. alamarBlue reagent (Life Technologies) was added to the wells, and its fluorescence was read daily over the indicated period of time in a Victor-3 plate reader (Perkin-Elmer). For Western blot analysis, cells were collected 72 hours after siRNA transfection.

Quantitative PCR

Total RNA was purified using the RNeasy Mini Kit (Qiagen), and RNA amounts were estimated by NanoDrop Spectophotometric analysis (Thermo Scientific). One microgram of RNA for each sample was reversed-transcribed to cDNA by using iScript DNA Synthesis Kit (BioRad). Quantitative PCR was carried out in duplicate on cDNA templates using SsoFast Probes Supermix Kit (BioRad) in a BioRad cycler. TaqMan Gene Expression Assays (Life Technologies) for *NF2* (Hs00966302_m1), *YAP1* (Hs00902712_g1), and transferrin receptor (Hs00951091_m1) were used. The expression of transferrin receptor was used for assay normalization. The PCR conditions were 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Duplicate threshold cycles (C_t) were averaged for each sample. Relative quantification of gene expression (relative amount of target RNA) was determined using the equation $2^{-\Delta\Delta C_t}$.

Antibodies

Commercial antibodies were purchased and used at the dilutions stated as follows: rabbit polyclonal anti-NF2 (1:500) from Sigma Prestige Antibodies; rabbit polyclonal anti-YAP (1:500), rabbit polyclonal anti-phospho-YAP (S127) (1:500), and rabbit polyclonal full-length and cleaved PARP (1:1,000) from Cell Signaling; and rabbit polyclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 1:1,000) from Santa Cruz.

Cell proliferation assay

Cells in log-phase were trypsinized, counted, and 10^3 cells per well were plated in a 96-well black flat-bottom plates, in a total volume of 200 μL of growth medium. Twenty microliters of alamarBlue reagent (Life Technologies) was added. Plates were incubated in 5% CO_2 and 37°C. Fluorescence was measured at indicated time points at 540-nm excitation/590-nm emission wavelengths using a Victor-3 automated plate reader (Perkin-Elmer). Growth curves were analyzed by using GraphPad Prism software. To determine the doubling time cell population, 10^4 cells were plated in triplicate in 6-well plates, and cells were counted daily using trypan blue. Averages of individual growth data were used to calculate the cell population doubling times by using the Doubling-Time Software v1.0.10 (http://www.doubling-time.com).

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

Wound-healing migration assay

Experimental cells transfected with siRNA oligos were plated in triplicate in 6-well plates to near 100% confluence. Cells were allowed to adhere for 12 hours. Next, at 48-hour time point after siRNA transfection, cells were incubated with 10 μmol/L mitomycin C (Sigma-Aldrich) for 4 hours to prevent proliferation effect. Then, the wells were washed twice with FBS, fresh media were replaced, and the bottom of wells was diametrically scratched with a pipette tip. Migration of cells was observed every 10 hours under the microscope and photographed at indicated times. A diagram of the migration assay time points corresponding to the siRNA transfection is shown in Supplementary Fig. S2B.

Colony-forming efficiency assay

Colony-forming efficiency assays were conducted as described earlier (29). Briefly, heavily irradiated (50 Gy) IOMM-Lee cells were plated as feeder cells in 6-well plates (30,000 cells per well). Twenty-four hours later, 600 experimental cells were plated in triplicate wells. Plates were incubated for 7 to 10 days until visible colonies were observed. Cells were washed in PBS, fixed in 10% formalin, and stained with crystal blue. Colonies of more than 50 cells were counted under a dissecting microscope.

IC_{50} assay

Cells in log-phase were counted and plated (10^3 cells per well) in triplicate in 96-well black flat-bottom plates in a total of 200 μL of medium, and 20 μL of alamarBlue reagent was added. Two microliters of cisplatin in increasing concentrations, ranging from 1 nmol/L to 500 μmol/L, was added. alamarBlue fluorescence was measured after 72 hours. The half maximum inhibitory concentration (IC_{50}) of cisplatin was calculated using GraphPad Prism software.

Western blot analysis

Protein expression levels were analyzed by Western blotting standard protocols. Briefly, 50 μg of total protein extracts was resolved by electrophoresis in denaturing SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked and incubated with commercially available antibodies, followed by incubation of secondary horseradish peroxidase (HRP)-conjugated antibodies. Bound antibodies were visualized by chemiluminescence by using SuperSignal West Pico Substrate (Pierce).

Immunofluorescence

Indirect immunofluorescence of YAP1 was conducted as previously described (25). Briefly, cells were grown in glass chamber slides for 24 hours, fixed, and sequentially blocked and incubated with YAP1 primary antibody followed by fluorescent-conjugated secondary antibody (Alexa 488 goat anti-rabbit IgG). Staining and protein subcellular localization were assessed by confocal microscopy.

Tissue microarrays and immunohistochemistry

Human meningioma tissue microarrays were used to study the expression of YAP1. Specimens were obtained upon appropriate Institutional Review Board approval. Tissue microarrays included tissue cores from 47 benign, 14 atypical, and 9 anaplastic meningiomas plus cores for control tissues, including the dura mater. A summary of all meningioma tumor samples is presented in Supplementary Table S1. For immunohistochemistry *in situ*, paraffin-embedded tissue sections (5 μm) were sequentially deparaffinized, rehydrated, and antigen unmasking was conducted by boiling the sections in antigen retrieval citrate solution (Biogenex) for 1 hour in a pressure cooker. Next, sections were blocked and incubated with primary antibody, followed by incubation with biotinylated secondary antibody and streptavidin-conjugated HRP (Super Sensitive Detection System, Biogenex). Sections were stained with 3,3′-diaminobenzidine (DAB) chromogen and counterstained with hematoxylin. Images of stained sections were visualized under a light microscope and photographed. A pathologist (B.A. Orr) blinded to the data scored the YAP1 immunostaining. Each tissue core was scored based on the percentage of positive nuclear labeling for YAP1. For each meningioma tumor sample, the average of YAP1 staining of duplicate tissue cores was calculated.

Xenograft mouse model

Mice intracranial implantations were conducted in accordance with an animal protocol approved by The Johns Hopkins Institutional Animal Care and Use Committee (Baltimore, MD). Meningioma cells were tagged with a firefly luciferase construct, under the control of the spleen focus forming virus promoter, via lentiviral transfection, as previously described (30, 31). Bioluminescence imaging (BLI) was used to monitor tumor growth progression. AC1 cells were implanted orthotopically into athymic mice (6 mice per group), as previously described (24, 32). Briefly, 6-week-old female athymic mice were anesthetized (ketamine, 80 mg/kg; xylazine, 10 mg/kg) and fixed in a stereotaxic frame. Cells (10^5 in 1 μL) were implanted into the floor of temporal fossa by using the following stereotaxic coordinates relative to the bregma: 2 mm to the right, 2 mm posterior, and 6 mm of depth. Animals were monitored 3 times every week and euthanized if they exhibit neurologic symptoms or had more than 15% of weight loss. After euthanasia, mice brains were formalin-fixed and processed for immunohistochemistry.

Bioluminescence imaging

Intracranial tumor growth was monitored by using the Xenogen IVIS 200 System (Caliper Life Sciences). Animals were anesthetized (ketamine, 80 mg/kg; xylazine, 10 mg/kg) twice a week, and BLI was conducted after intraperitoneal (i.p.) administration of 150 mg/kg of luciferin (Gold Biotechnologies).

Statistical analysis

The GraphPad Prism software version 5 was used for data plotting and statistical analysis. In summary, the Student *t* test was conducted to evaluate significant differences of *in vitro* cell growth following transfections. Quantitative data

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

were analyzed as mean ± SD. A statistical significance was considered at $P < 0.05$.

Results

YAP1 is highly expressed in human meningiomas and localizes to the nucleus

Immunohistochemistry *in situ* was used to investigate YAP1 expression and nuclear localization in clinical samples of meningiomas. We surveyed the YAP1 expression in a total of 188 tissue cores from 70 patients with meningiomas. The 188 tissue cores represented samples of all 3 WHO histopathologic grades of meningiomas, including normal tissue as control. Given the importance of YAP1 subcellular localization to its function, immunolabeling was scored on the basis of the total percentage of positive nuclear staining. Cytoplasmic staining was not scored. Meningiomas of all grades were positive for YAP1. Moreover, nuclear YAP1 labeling was abundant and markedly strong (Fig. 1). No substantial differences in YAP1 immunoreactivity were observed with regard to grade, sex, or histologic subtypes. Among all samples, 92% of nuclei in average presented YAP1 immunoreactivity (Supplementary Table S1). YAP1 immunostaining of normal tissue cores is shown in Supplementary Fig. S1.

YAP1 expression in meningioma cell lines

The phosphorylation status of YAP1 (S127) was investigated in a set of 4 meningioma cell lines and in the immortalized non-neoplastic arachnoidal cells (AC1) and correlated with the *NF2* status of the cell line. An antibody that recognizes the phosphorylation of serine 127 of YAP1 was used. The phosphorylation of the S127 site has been reported to be implicated with YAP1 cytoplasmic localization leading to its further degradation (28). We determined the *NF2* gene expression level by quantitative PCR followed by the immunoblotting to confirm the endogenous expression of Merlin, the *NF2* protein product, in the same cells. *NF2* transcript levels in SF1335 and KT21MG1 meningioma cells were more than 10-fold lower than the one in AC1 cells. In contrast, meningioma cell lines SF4068 and SF6717 showed levels of *NF2* transcript that were comparable with the levels in AC1 (Fig. 2A). Consistent with the quantitative PCR data, endogenous Merlin protein levels were absent or nearly undetectable in SF1335 and KT21MG1 cells, compared with SF4068, SF6717, and AC1 cells (Fig. 2B). YAP1 protein levels were detected by immunoblotting in all cell lines. Because phosphorylation of YAP1 (S127) is a key regulatory mechanism of the Hippo pathway, we investigated the levels of phospho-YAP1 in the same cells. Phospho-YAP1(S127) (inactivated form of YAP1) was only detected in Merlin-expressing cells (Fig. 2B). These results suggest that Merlin expression is associated with phosphorylation of serine 127 of YAP1. Thus, *NF2* status might be a critical mechanism for control of the Hippo pathway in meningiomas.

YAP1 transient knockdown restrains cell proliferation and migration in *NF2*-mutant cells

Next, we investigated whether YAP1 has a role in meningioma tumorigenesis. First, we knocked down YAP1 in SF1335 and KT21MG1 cells, by transient transfection of 2 siRNA oligos targeting *YAP1*. The GFP siRNA was used as negative control. Transfection of YAP1 siRNA oligos resulted in considerable suppression of endogenous YAP1 expression in both SF1335 and KT21MG1 cells, compared with the nontargeting GFP siRNA transfection control at both mRNA and protein levels (Fig. 2C; Supplementary Fig. S2A). Suppression of YAP1 mediated by siRNA transfection in both cells led to a significant decrease of cell proliferation ($P \leq 0.05$), as shown by the alamarBlue assay (Fig. 2D and E, top). Similar results were observed when YAP1 siRNA transfections were done on AC1 cells (data not shown). In addition to analyzing cell proliferation, we tested whether suppression of YAP1 affected meningioma cell motility. We conducted a wound-healing assay to compare cells transiently transfected with YAP1 siRNAs and GFP siRNA. Cells were incubated with 10 μmol/L of the cell proliferation inhibitor mitomycin C for 4 hours before the assay. In the

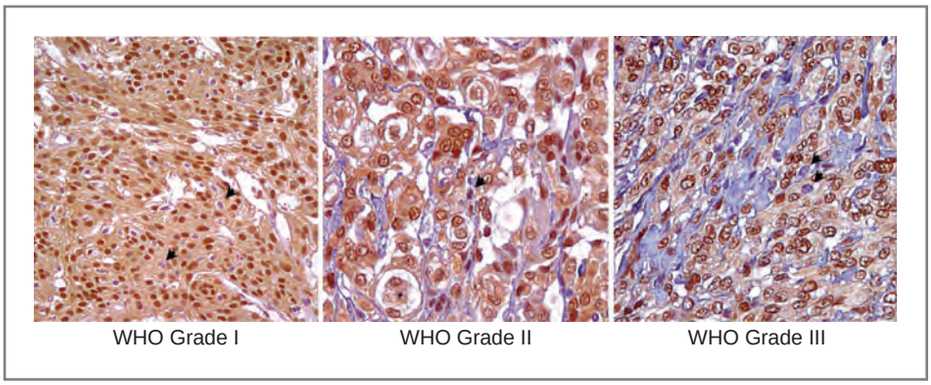

Figure 1. Immunohistochemical survey of YAP1 expression in benign, atypical, and anaplastic meningioma tumors. YAP1 was found to be highly expressed in all meningiomas regardless of histologic subtype and grade. The distribution of staining was consistently high in the nucleus with more variable staining in the cytoplasm. Vascular elements showed some YAP1 positivity, albeit reduced compared with the tumor. A minor percentage of cells have nuclei that are negative for YAP1 expression (black arrows). Original magnification, ×200.

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

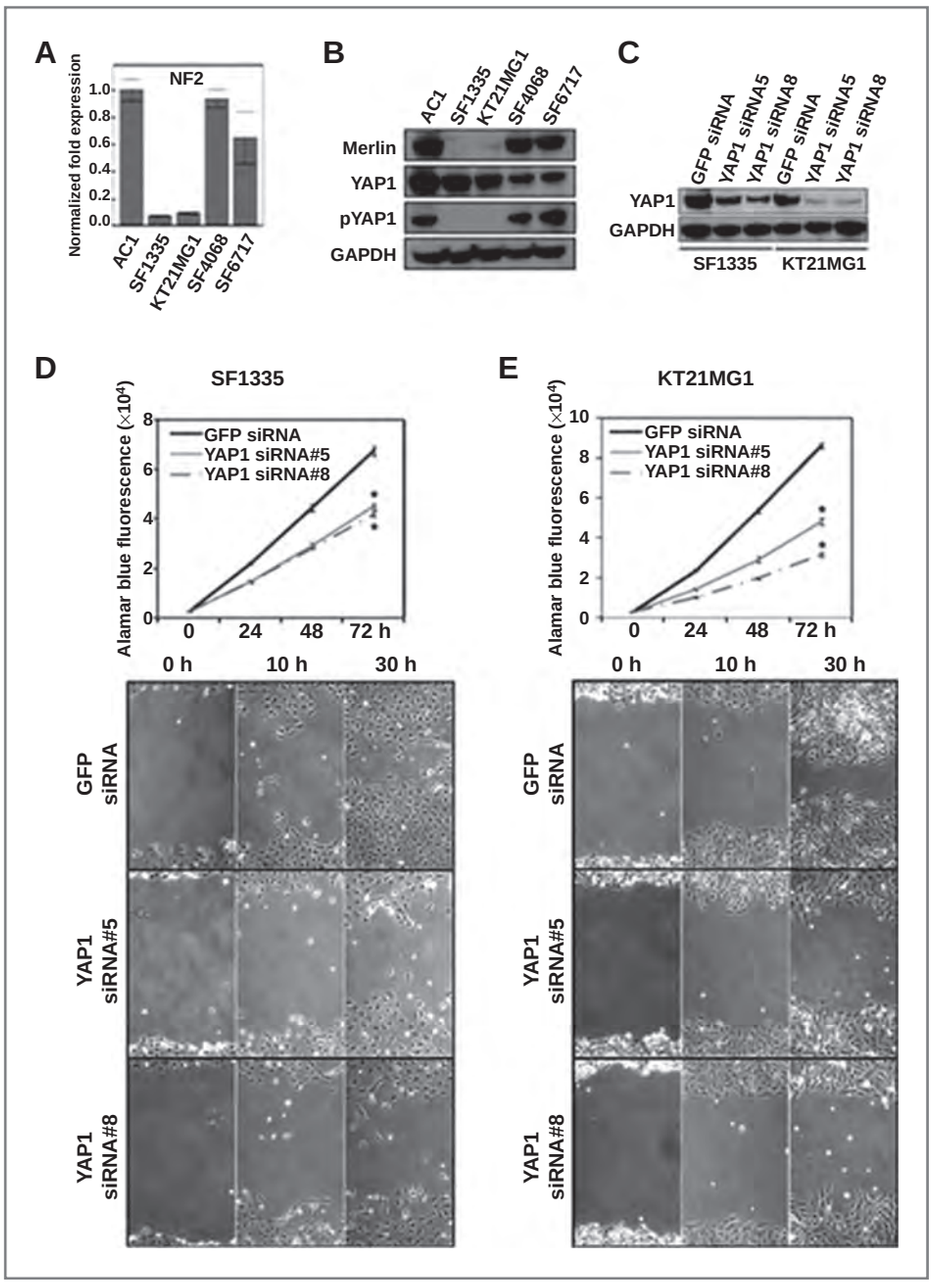

Figure 2. *YAP1* knockdown suppresses *in vitro* cell proliferation and migration. A, quantitative real time-PCR analysis of *NF2* transcript in non-neoplastic cells (AC1) and meningioma cells (SF1335, KT21MG1, SF4068, and SF6717). B, immunoblotting of lysates of meningioma cells showing endogenous levels of proteins: Merlin, YAP1, and phospho-YAP1. GAPDH blotting is shown as protein loading control. C, YAP1 and GAPDH immunoblotting of SF1335 and KT21MG1 transiently transfected with siRNA oligos targeting YAP1 or GFP, as a nontargeting control. Following YAP1 and GFP siRNA transient transfections, cells were plated for alamarBlue proliferation assay and wound-healing migration assay. Growth curve plots (top) and microscopic images representative of migration assays (bottom) conducted for SF1335 (D) and KT21MG1 (E) cells are shown (×40 magnification). *, $P \leq 0.05$. The *P* value is for YAP siRNAs versus GFP siRNA.

presence of mitomycin C, suppression of YAP1 in SF1335 and KT21MG1 cells disrupted cell migration within 30 hours of the assay (Fig. 2D and E, bottom). In all, these results suggest that YAP1 expression might play a role in meningioma tumor growth by enhancing cell proliferation and motility.

Ectopic expression of YAP1 promotes *in vitro* cell proliferation and anchorage-independent growth in arachnoidal and meningioma cells

To further verify the function of YAP1, we investigated the consequences of overexpression of YAP1 in meningioma cells. Here, we included the non-neoplastic cells (AC1), both NF2/Merlin-expressing cells (SF4068 and SF6717) and the *NF2*-mutant SF1335 cells. Meningioma cells were either transfected with the YAP1 construct or the empty vector (pEGF-N2), and G418 was used to select transfected cells. Nontransfected cells were used as negative control for selection. Five days after selection with G418, the GFP fluorescence was visually checked under a microscope (Supplementary Fig. S3A). In both YAP1- and empty vector–transfected cells, the frequency of GFP fluorescent cells was greater than 70%. In addition, immunofluorescence was used to examine YAP1 subcellular localization upon YAP1

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

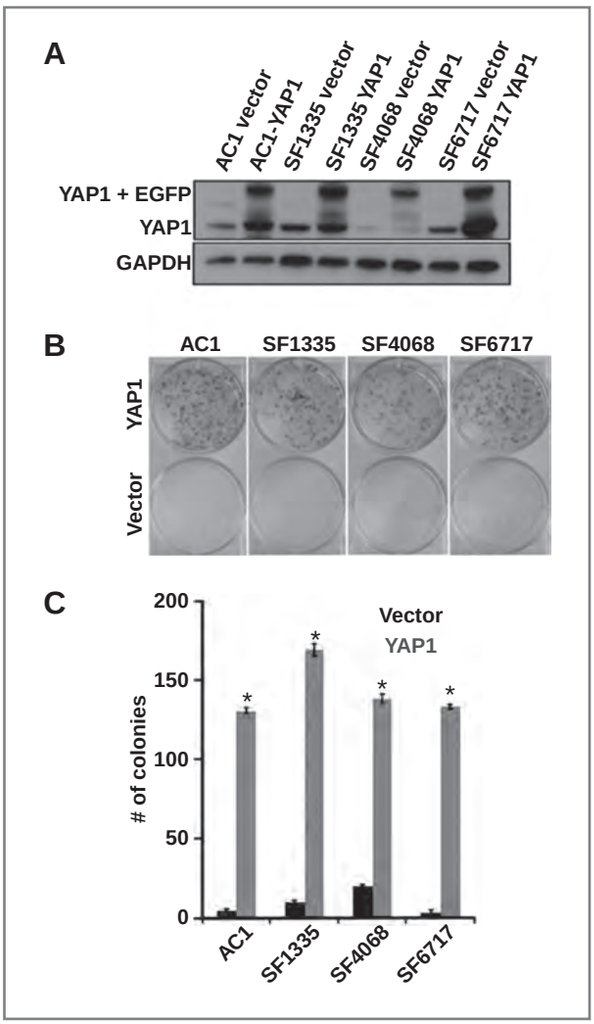

Figure 3. YAP1 overexpression promotes *in vitro* cell proliferation and anchorage-independent growth. A, YAP1 and GAPDH immunoblotting of meningioma cells transfected with either YAP1 construct or empty vector. Cells transfected with YAP1 show both endogenous YAP1 and YAP1 + EGFP. B, representative pictures of wells of colony-forming efficiency assay for cells transfected with either YAP1 or empty vector. C, quantification of colony-forming efficiency assay expressed in average of the total number of colonies per cell. *, $P \leq 0.001$. The *P* value is for YAP1 versus empty vector cells.

(or empty vector) transfections. As observed in Supplementary Fig. S3B, nuclear YAP1 is seen in the majority of the YAP1-transfected cells, although YAP1 cytoplasmic localization is also observed. Immunoblotting of transfected cells confirms overexpression of YAP1 cells, compared with control cells (empty vector; Fig. 3A). Next, YAP1 and control (empty vector) stably transfected cells were analyzed by conducting cell proliferation and colony-forming efficiency assays. A total of 10^4 cells were plated in triplicate in 6-well plates and counted daily over a 120-hour period. The average number of cells was calculated for each time point, and the cell growth data were plotted against time. Transduction of YAP1 in meningioma cells promoted *in vitro* cell proliferation, compared with the control cells (Supplementary Fig. S4). In addition, the doubling time population of cells growing from 0 to 72 hours was calculated for each cell type. Compared with the control cells (empty vector), YAP1-expressing cells were more proliferative, showing a lower doubling time population that ranged from 1.7- to 2-fold in difference (Table 1). Next, we tested whether YAP1 expression affected the anchorage-independent growth, a phenotype characteristic of tumor cells. IOMM-Lee feeder cells were first plated, and 24 hours later, experimental cells were plated in triplicate in 6-well plates. Cells transfected with empty vector were used as a control. Consistent with the cell proliferation data, YAP1-expressing cells exhibited an enhanced anchorage-independent growth in all cell lines tested (Fig. 3B and C). Moreover, the difference in number of YAP1 cells forming colonies was statistically significant, compared with the control cells ($P \leq 0.001$). Thus, YAP1 overexpression in Merlin-expressing and -deficient meningioma cells increased cell proliferation and anchorage-independent growth, suggesting an oncogenic role for YAP1 in meningioma tumor formation.

Overexpression of YAP1 inhibits the effect of proapoptotic cisplatin treatment

In addition to promoting cell proliferation and migration, we sought to investigate whether YAP1 expression also conferred resistance to apoptosis. G418-resistant cells, AC1, SF1335, SF4068, and SF6717, transfected either with YAP1 or empty vector (pEGFP-N2) were plated in 96-well plates in triplicate (10^3 cells per well). Cisplatin was added to wells in increasing concentrations and controls were plated with the vehicle (dimethyl sulfoxide; DMSO). IC_{50} of the chemotherapeutic agent cisplatin was calculated at 72 hours after exposure to the drug. YAP1-expressing cells presented much higher IC_{50} values, compared with control cells, indicating that YAP1 might in fact confer resistance to cisplatin-induced apoptosis. A comparison between the IC_{50} values of YAP1 cells and controls is shown in Table 2. A representative plot of the IC_{50} assay is shown for AC1 cells that illustrates a more than 50-fold

Table 1. Growth rates of meningioma cells associated with YAP1 expression indicated as doubling time of cells

	Doubling time, h	
	Vector	YAP1
AC1	27.4 ± 1.3	13.9 ± 1.1
SF1335	27.6 ± 0.8	12.9 ± 0.4
SF4068	24.8 ± 1.7	14.5 ± 0.2
SF6717	23.7 ± 2.1	15.1 ± 0.8

NOTE: Doubling times were calculated for cells in exponential growth from 0 to 72 hours of culture. Cells transfected with empty vector were used as controls.

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

Table 2. Cisplatin IC_{50} values at 72 hours for AC1, SF1335, SF4068, and SF6717 cells

	Vector	YAP1
AC1	1.4 μmol/L ($R^2 = 0.94$)	76.9 μmol/L ($R^2 = 0.94$)
SF1335	64.5 μmol/L ($R^2 = 0.91$)	297.4 μmol/L ($R^2 = 0.93$)
SF4068	218.9 μmol/L ($R^2 = 0.93$)	571.8 μmol/L ($R^2 = 0.92$)
SF6717	75.9 μmol/L ($R^2 = 0.97$)	113.7 μmol/L ($R^2 = 0.94$)

resistance to cisplatin of YAP1 cells, compared with the control (Fig. 4A). To further confirm that YAP1 expression protects cells from apoptosis, we conducted Western blot analysis of full-length and cleaved nuclear PARP, a major caspase-3 cleavage target. Both YAP1 and controls cells were incubated for 72 hours with either 30 μmol/L of cisplatin or vehicle (DMSO). After incubation, cell lysates were subjected to Western blot analysis. Consistent with the IC_{50} data, immunoblotting analysis of full-length and cleaved PARP indicated that indeed YAP1 expression confers resistance to cisplatin-induced apoptosis. Compared with control cells, YAP1-expressing cells showed considerable lower to undetectable PARP cleavage activity (Fig. 4B). Thus, in addition to the effects of YAP1 on cell proliferation, it confers resistance to apoptosis in meningiomas.

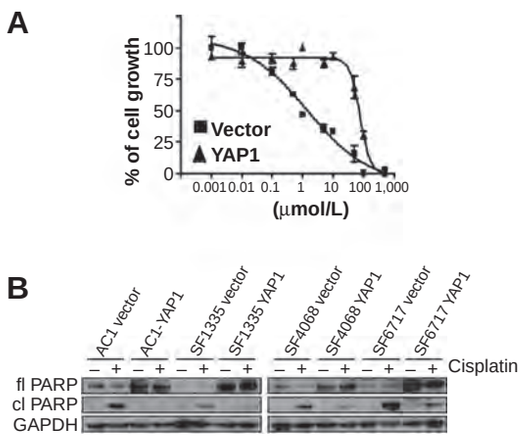

Figure 4. YAP1 overexpression promotes apoptosis resistance to cisplatin. IC_{50} assays were conducted using indicated concentrations of cisplatin on cells stably transfected with either YAP1 or empty vector. Treatments were done in triplicate, and alamarBlue fluorescence was measured after 72 hours of incubation. A, the IC_{50} plot for AC1 cells is shown and illustrates a more than 50-fold resistance to cisplatin of YAP1 cells, compared with the control. B, immunoblotting for full-length PARP (fl PARP), cleaved PARP (cl PARP), and GAPDH of cells incubated either with vehicle (DMSO) or 30 μmol/L of cisplatin for 72 hours.

YAP1 expression promotes *in vivo* tumor growth of immortalized non-neoplastic arachnoidal cells

As observed above, the non-neoplastic AC1 cells stably expressing YAP1 were markedly more proliferative than the control cells (empty vector), as shown by their *in vitro* growth rates. To investigate whether YAP1 promotes *in vivo* tumor formation, we implanted AC1-YAP1 cells orthotopically into athymic nude mice. Cells transfected with the empty vector were implanted as controls. AC1-YAP1-Luc and AC1-vector-Luc cells were implanted into the floor of temporal fossa site of 6-week-old female athymic mice. Bioluminescence activity was detected as early as day 3 after cell implantation. All 6 mice injected with YAP1-expressing AC1 cells developed tumors whereas the control mice did not. Figure 5A shows representative BLI images of mice at day 15 after xenograft implantation. Mice implanted with YAP1 cells had a median survival time of 22 days. Meningioma xenografts developed as well-circumscribed tumors, mostly localized to the site of cell implantation, as observed by the hematoxylin and eosin (H&E) stain of coronal brain sections (Fig. 5B). Control mice appeared to be healthy up to 90 days after cell implantation, when they were sacrificed and the brains were formalin-fixed and processed for immunostaining. Observation of H&E stain of coronal sections of brains showed no evidence of xenograft growth in control mice (Fig. 5B). AC1-YAP1 xenografts were immunostained for YAP1, human vimentin (clone Vim 3B4), and for human Ki67 (clone MIB1). As shown in Fig. 5C, AC1-YAP1 xenografts were strongly positive for YAP1. Moreover, xenografts were vimentin-positive, presenting antigen immunoreactivity typically observed in human meningiomas. The Ki67 stain showed highly proliferative activity on tumor cells, with markedly intense labeling at the sharp borders of the xenograft with mice brains. Similar to our findings in the brain, AC1 and SF1335 cells (YAP1 construct and control vector) implanted subcutaneously into athymic nude mice only showed tumor growth when overexpressing YAP1 (data not shown).

Discussion

The deregulation of the Hippo pathway has been implicated in several human cancers and seems to alter the adequate balance of cell proliferation and apoptosis, promoting tumorigenesis (11). In particular, the oncogenic contribution of YAP1, the main effector of Hippo, has been described and evidence points to a major role in tumorigenesis (14, 33, 34). To the best of our knowledge, thus far, our study is the most extensive functional characterization of YAP1 in an NF2-associated brain tumor. We investigated the functional role of YAP1 in meningiomas, assessing its contribution to cell proliferation, migration, apoptosis, and tumorigenesis of these CNS tumors.

The most prominent finding was the broad activation of YAP1, evidenced by its high expression and nuclear localization in the set of clinical samples analyzed, suggesting that the Hippo signaling pathway is reduced or inactive in these tumors. Although we did not analyze whole paraffin

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

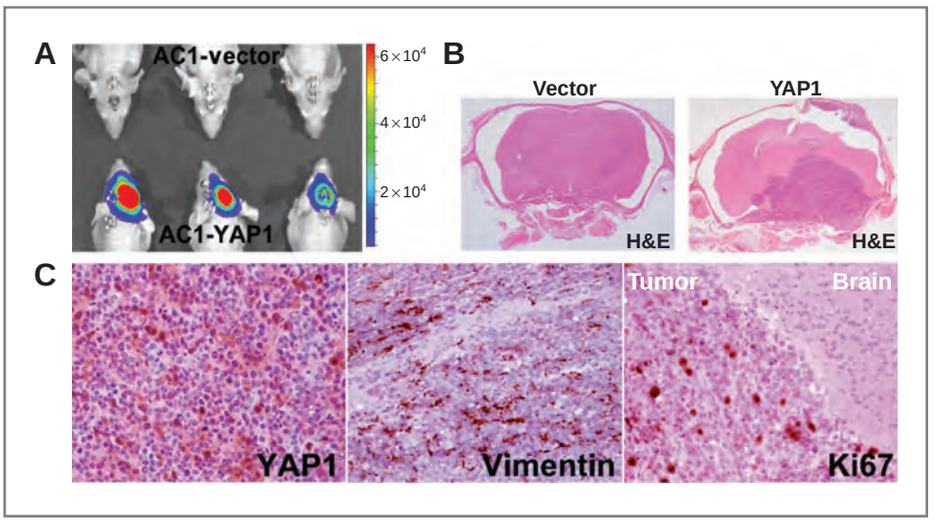

Figure 5. YAP1 overexpression promotes *in vivo* tumor growth of non-neoplastic meningeal cells. A, BLI of AC1 xenografts. Representative images of control and YAP1 mice groups at day 15 after implantation are shown. Three animals per group were randomly selected and shown. A bar scale is shown for total photon counts comparison. B, H&E staining of coronal sections of mice brains at the site of implantation of AC1 xenografts (×0.5 magnification). C, immunohistochemical staining of AC1-YAP1 xenograft tissue sections for YAP1, for human vimentin intermediate filament, a characteristic cytoskeleton component marker for meningiomas, and for Ki67/MIB1 antigen (×200 magnification).

sections, it is clear that YAP1 is highly expressed at least within subsections of these tumors. This finding may support the hypothesis that YAP1 plays an important role in meningioma tumor growth. Loss of *NF2* gene is the most common genetic alteration in meningiomas and it is found in up to two thirds of the tumors (20). Although we did not correlate *NF2* loss and YAP1 activation, we did appreciate that in this set of samples, the level of YAP1 activation seems much higher than the expected. If confirmed, it can be speculated that additional activating mechanisms of Hippo pathway, other than loss of the *NF2* tumor suppressor gene, might potentially drive meningioma tumorigenesis. Further investigation is warranted to clarify whether other genetic or nongenetic alterations are responsible for YAP1 activation in meningiomas. In addition, we did not observe any evident correlation between nuclear YAP1 and histopathologic subtypes in the set of human meningioma analyzed.

Our data are in concordance with previous reports that YAP1 overexpression is frequently observed in various cancers, highlighting its potential role as an oncogene. Kim and colleagues (14) reported high levels of YAP1 expression and that nuclear YAP1 might contribute to non–small cell lung carcinoma growth. Ge and colleagues (35) and Zhang and colleagues (36) described elevated YAP1 expression in head and neck squamous cell carcinoma. Lam-Himlin and colleagues (37) observed increased YAP1 expression in a large cohort of samples of gastric carcinoma and adenocarcinoma of the esophagus. Orr and colleagues (38) described high levels of nuclear YAP1 in brain tumors, including glioblastoma multiforme and medulloblastoma. Fernandez and colleagues (39) reported amplification of YAP1 in human hedgehog-associated medulloblastomas. In addition to reporting the levels of YAP1 expression, several groups have described a positive correlation of YAP1 expression and poor clinical outcome in esophageal squamous cell carcinoma (40), non–small cell lung cancer (41), and ovarian cancer (36, 42).

Depending on the cellular context, YAP1 has been reported functioning as either an oncogene or a tumor suppressor (23). Our data indeed support that function of YAP1 is critical for the regulation of cell proliferation in meningiomas. siRNA-mediated knockdown of YAP1 impaired *in vitro* cell proliferation and migration of meningiomas cells. Conversely, overexpression of YAP1 enhanced cell proliferation and promoted anchorage-independent growth in meningioma cells, including in the immortalized non-neoplastic arachnoidal cells (AC1). Our data are in agreement with mounting evidence of the oncogenic function of YAP1 in different contexts, reviewed in the works of Pan (43) and Halder and Johnson (44).

Furthermore, YAP1 seems to regulate apoptosis in meningiomas. When challenged with the chemotherapeutic agent cisplatin, YAP1-expressing cells were nearly unsusceptible to apoptosis induced by cisplatin, compared with control cells, shown by the higher cisplatin IC_{50} values of the first. The resistance to apoptosis in YAP1 cells was further evidenced by the absence of cleavage of PARP, a major target of the caspase cascade. Our data are also supported by previous reports of apoptosis suppression by YAP1 in human nontransformed mammary epithelial cells (13) and in ovarian cancer cell lines (36, 42). Because YAP1 confers apoptosis resistance to meningioma cells, we also speculate that targeting the Hippo pathway might be useful for successful therapeutic options for certain cancers.

To further characterize the functional role of YAP1 *in vivo*, we used an immortalized non-neoplastic arachnoidal cell (AC1) to assess the transforming properties of YAP1 in this cell type. Overexpression of YAP1 in AC1 cells promoted

Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

in vivo tumorigenesis in nude mice. This might reflect the importance of YAP1 activation for tumor formation and development in tumors derived of leptomeningeal origin. In line with this evidence, Orr and colleagues (38) reported the detection of YAP1 immunoreactivity in leptomeninges, a solid indication supporting the importance of YAP1 expression in the meningioma cell of origin.

In summary, our data strongly suggest that deregulation of the Hippo pathway is largely observed in meningiomas, represented by the intense nuclear localization of YAP1 expression. In addition, in this cell-type context, YAP1 exhibits transforming properties characterized by the regulation of cell proliferation, migration, and apoptosis. Further characterization of the downstream target genes of the deregulated Hippo pathway might reveal useful therapeutic targets for these tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Authors' Contributions

Conception and design: G.S. Baia, A. Lal
Development of methodology: G.S. Baia, O.L. Caballero
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.S. Baia, C. Cowdrey, C. Mawrin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.S. Baia, B.A. Orr, J.S.Y. Ho
Writing, review, and/or revision of the manuscript: G.S. Baia, O.L. Caballero, B.A. Orr, A. Lal, J.S.Y. Ho, T. Tihan, C. Mawrin, G.J. Riggins
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.S.Y. Ho, T. Tihan
Study supervision: G.J. Riggins

Acknowledgments

The authors thank Dr. Yoshitaka Sekido for the YAP1 construct, Dr. Marco Giovannini for the SF1335 cells, Drs. Marie-Odile Parat and Filipe Carvalho for fruitful discussions during this project, and the technical expertise of Sandra Hartmann (Neuropathology Magdeburg) for the construction of the meningioma tissue microarray.

Grant Support

This work was supported by the generous donations from Leonard and Phyllis Attman and from the Meningioma Mommas Foundation that made this research possible.

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Received February 25, 2012; revised April 25, 2012; accepted May 14, 2012; published OnlineFirst May 22, 2012.

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Published OnlineFirst May 22, 2012; DOI: 10.1158/1541-7786.MCR-12-0116

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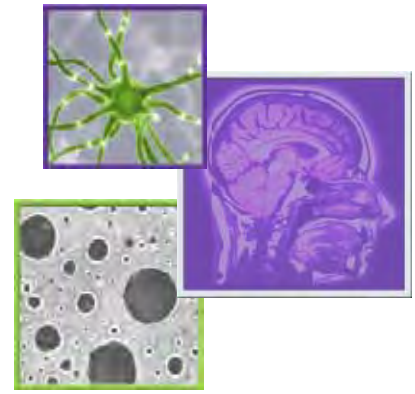
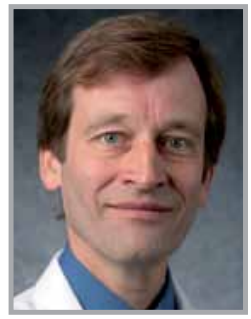
- Journal Publication: - Baia, GS, Caballero, OL, Riggins GJ **The Hippo signaling pathway and translational opportunities for brain cancers. CNS Oncology**, Vol. 1, No. 2, Pages 113-115, 2012

The Hippo signaling pathway and translational opportunities for brain cancers





Gilson S Baia*[1] Otavia L Caballero[1] Gregory J Riggins[1]

"...the evidence points to the Hippo pathway having an important role in driving tumor formation in multiple types of tumors."

The Hippo signaling pathway is a critical regulator of tissue homeostasis, organ size control and stem cell renewal [1]. The pathway exerts its tumor suppression activity by restraining cell proliferation and promoting apoptosis. Hippo and core pathway components were first identified in *Drosophila melanogaster* and are functionally conserved in mammals [1,2]. The mammalian Hippo pathway is comprised of a sequential phosphorylation cascade of MST1/2 and LATS1/2 that culminates with the phosphorylation and inactivation of YAP1 and TAZ. Cytoplasmic phospho-YAP1 and TAZ are then ubiquitinated and further degraded or bound to 14-3-3 binding leading to YAP1/TAZ cytoplasmic retention [3]. When the pathway is inactivated the transcription unphosphorylated co-activators YAP1/TAZ translocate to the nucleus, bind to TEAD1/4 transcription factors and promote the expression of key target genes that increase cell proliferation and prevent apoptosis [4].

Merlin/NF2 tumor suppressor protein is a cytoskeleton-binding protein that modulates the activity of the Hippo pathway [5]. Investigating the activity of YAP1 in the mouse liver, Zhang and colleagues defined a functional role between Merlin/NF2 tumor suppressor and the Hippo pathway. The inactivation of *Nf2* in the mouse liver led to YAP1 activation and to the formation of hepatocellular carcinoma [6]. Loss of *NF2* tumor suppressor gene is a well-characterized genetic alteration in several cancers, largely in cancers of the CNS [7], prompting the attention on the importance of the Hippo pathway in the development of these cancers. In meningiomas, loss of Merlin activity is also associated with nuclear localization and YAP1 activation, and leads to the development of cells with a characteristic transformed phenotype [8]. In addition, deregulation of the Hippo pathway seems to be associated with several human cancers not associated with *NF2* loss. Genetic and epigenetic mechanisms have been reported to drive the deregulation of the Hippo pathway [9,10]. Amplification and overexpression of YAP1 have been identified and are thought to be relevant oncogenic mechanisms [11,12]. Overall, the evidence points to the Hippo pathway having an important role in driving tumor formation in multiple types of tumors.

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Hippo signaling pathway in brain cancers

The Hippo pathway, in particular the expression and activity of YAP1, has been investigated in adult and pediatric brain tumors. Orr *et al.* analyzed YAP1 expression and subcellular localization in 264 brain tumors [13]. Nuclear YAP1 expression was found to be heterogeneous in medulloblastomas and markedly prominent in high-grade gliomas. In addition, analysis of YAP1 expression in fetal and adult brain autopsy specimens showed high levels of YAP1 expression in the subventricular zone and in the external granular cell layer of the cerebellum. These findings suggest that YAP1 expression and activity might be essential for maintenance and expansion of neural stem cells. This also supports the hypothesis that the Hippo pathway plays an important role in controlling cell fate and survival in certain tumors of neural crest origin [13]. Furthermore, recent studies in medulloblastomas revealed high YAP1 expression in these tumors, in particular in sonic hedgehog-associated subtypes [14]. Importantly, Fernandez and colleagues demonstrated that YAP1 expression is upregulated by the sonic hedgehog signaling in proliferating cerebellar granule neural precursors (CGNPs), the proposed cells of origin for some medulloblastoma subtypes [14]. In meningiomas, YAP1 was found highly expressed and localized to the nucleus [15]. Moreover, it has been demonstrated that *NF2* loss confers proliferative advantage to meningioma cells, and that *YAP1* knockdown in NF2-mutant cells rescues, in part, the effect of Merlin loss on cell proliferation [8]. Data generated in our laboratory demonstrated that YAP1 expression induces a transformed phenotype in meningiomas cells by modulating cell proliferation and motility, and by restraining cisplatin-induced apoptosis. Moreover, overexpression of YAP1 in non-neoplastic arachnoidal cells led to the development of tumor-like growth in athymic nude mice [15]. In summary, these studies demonstrate an oncogenic role for YAP1 in brain tumors.

"...better understanding of the complex oncogenic functions of the pathway, cell-type specificities and tissue-specific transcriptional programs might reveal better insight into the biological functions and novel cancer therapeutic targets within the Hippo pathway."

Hippo signaling pathway & cancer stem cells

The stem cell concept has been developed over the past years and it is defined by the existence of a small subpopulation of undifferentiated progenitor cells, confined to organ-specific compartments and accountable for cell fate decisions and renewal, as well as organ size control [16,17]. Evidence supports the hypothesis that the Hippo cascade is one of the pathways responsible for modulating tissue homeostasis by controlling stem cell proliferation and expansion [2]. For instance, it has been observed that YAP1 expression is markedly intense in precursor cells from small intestine crypts, and that YAP1 expression induces a loss of differentiation and expansion of these progenitor cells [2]. Similarly, YAP1 expression was observed in mouse single-layered basal epidermal progenitors and seemed to be responsible for the undifferentiated state of these epithelial progenitors [18]. Importantly, YAP1 expression and activity has also been observed in cancer stem cell precursors, including brain cancers [14]. Considering the fact that cancer stem cells are thought to be the cells with greater capacity for tumor repopulation and expansion and are involved in drug-resistance [16], it is reasonable to speculate that targeting the Hippo pathway will be relevant for treating certain cancers.

Targeting the Hippo signaling pathway for cancer treatment

Recent studies have demonstrated that strategies targeting YAP1 activity might indeed prove to be useful for therapeutic purposes. Using cell-based assays, Bao *et al.* found that dobutamine, a β-adrenergic receptor agonist, induces cytoplasmic retention of YAP1 by a mechanism that is yet to be determined [19]. Additionally, *in vitro* treatment with dobutamine was shown to increase phospho-S127 of YAP1 and to inhibit YAP1-dependent TEAD reporter activity [19]. This might suggest the use of dobutamine to inactivate YAP1, although further characterization and *in vivo* testing will be necessary to clarify the efficacy of dobutamine in targeting YAP1. More recently, Liu-Chittenden *et al.* identified compounds of the porphyrin family that potently inhibited YAP1–TEAD interaction, therefore inhibiting the transcriptional activity of this complex [20]. One of the porphyrin compounds, verteporfin, inhibited YAP1 oncoprotein activity in mouse models, generated by either inducing YAP1 overexpression or by deletion of the *Nf2* gene in the liver. These studies showed that verteporfin was efficacious in inhibiting YAP1 transcriptional activity, therefore preventing tissue overgrowth in both models, but with no evident effects on liver homeostasis [20]. If the efficacy of verteporfin in other tumor models is confirmed, especially in shrinking pre-established tumors, therapeutic strategies using porphyrin

compounds might be very attractive for certain tumors driven by YAP1 oncogenic activity.

In conclusion, the Hippo tumor suppressor signaling pathway is deregulated in a variety of human cancers. Progress has been made in identifying the major players in the pathway and its functionality, although key points are still to be elucidated. For instance, better understanding of the complex oncogenic functions of the pathway, cell-type specificities and tissue-specific transcriptional programs might reveal better insight into the biological functions and novel cancer therapeutic targets within the Hippo pathway.

Financial & competing interests disclosure

The authors' research is funded by donations from Leonard and Phyllis Attman, from the Meningioma Mommas Foundation, by the US Department of Defense (W81XWH-11-1-0424 to GSB and W81XWH-10-1-0387 to GJ Riggins) and by The Ludwig Institute for Cancer Research. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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- Scientific Conference Presentation: 11th Annual International Neuro-Oncology Updates (Invited Oral Presentation), Johns Hopkins University, Baltimore, MD, USA. 2012. **Hippo Signaling Pathway and Translational Opportunities in Meningioma Research**

Hippo Signaling Pathway and Translational Opportunities in Meningioma Research

11th Annual International Neuro-Oncology Updates, Johns Hopkins University, Baltimore, MD, USA. 2012.

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The Hippo signaling pathway has been characterized as a critical regulator of tissue homeostasis, organ size control and stem cell renewal. Importantly, the deregulation of the Hippo pathway has been implicated in several human cancers and seems to alter the adequate balance of cell proliferation and apoptosis, promoting tumorigenesis. The mammalian Hippo pathway is comprised of a sequential phosphorylation cascade that culminates with the phosphorylation and inactivation of Yes-associated protein 1 (YAP1). Phospho-YAP1 is retained in the cytoplasm. In the absence of Hippo signaling, YAP1 translocates to the nucleus, associates with co-activators TEAD1-4 and functions as a transcriptional factor promoting the expression of key target genes. Loss of the *NF2* tumor suppressor gene is the most common genetic alteration in meningiomas and the *NF2* gene product, Merlin acts upstream of the Hippo pathway. In this study we show that primary meningioma tumors have high nuclear expression of YAP1. In meningioma cells, Merlin expression is associated with phosphorylation of YAP1. Using a siRNA transient knockdown of YAP1 in *NF2* mutant meningioma cells we show that suppression of YAP1 impaired cell proliferation and migration. Conversely, YAP1 overexpression led to a strong augment of cell proliferation and anchorage-independent growth, and restriction of cisplatin-induced apoptosis. In addition, expression of YAP1 in nontransformed arachnoidal cells led to the development of tumors in nude mice. Together, these findings suggest that in meningiomas deregulation of the Hippo pathway is largely observed in primary tumors and that YAP1 functions as an oncogene promoting meningioma tumorigenesis.

Recent studies have demonstrated that strategies targeting YAP1 activity might prove to be useful for therapeutic purposes. Liu-Chittenden and colleagues have identified compounds of the porphyrin family, which drastically inhibited YAP1-TEAD interaction, therefore inhibiting the transcriptional activity of the complex.

In summary, the Hippo tumor suppressor signaling pathway is deregulated in meningiomas. Better understanding of the complexity of this pathway, including its cell-specific target genes might lead to the discovery of novel therapeutic targets.

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