

Mutant Gq/11 Promote Uveal Melanoma Tumorigenesis by Activating YAP

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SUMMARY

Uveal melanoma (UM) is the most common cancer in adult eyes. Approximately 80% of UMs harbor somatic activating mutations in *GNAQ* or *GNA11* (encoding Gq or G11, respectively). Herein, we show in both cell culture and human tumors that cancer-associated Gq/11 mutants activate YAP, a major effector of the Hippo tumor suppressor pathway that is also regulated by G protein-coupled receptor signaling. YAP mediates the oncogenic activity of mutant Gq/11 in UM development, and the YAP inhibitor verteporfin blocks tumor growth of UM cells containing Gq/11 mutations. This study reveals an essential role of the Hippo-YAP pathway in Gq/11-induced tumorigenesis and suggests YAP as a potential drug target for UM patients carrying mutations in *GNAQ* or *GNA11*.

INTRODUCTION

Uveal melanoma (UM) is the most common intraocular tumor in adults and accounts for ~5% of all melanomas (Singh et al., 2005). UM frequently metastasizes to the liver along a hematogenous route, as 90% of UM metastasis is found in the liver. Once metastasized, there is no effective therapy, with average survival of 2 to 8 months (Singh et al., 2005). Unlike cutaneous melanoma, UM originates from melanocytes of the choroid, ciliary

body, and iris (collectively known as the uvea) derived from the neural crest (Arnesen, 1985).

Molecular genetic analyses have shown that the mutational spectrum of UM is very different from that of cutaneous melanoma. Instead of the *BRAF* or *NRAS* mutations common in cutaneous melanoma, more than 80% of UMs carry activating mutations in either *GNAQ* or *GNA11* (Lamba et al., 2009; Van Raamsdonk et al., 2009, 2010). Only UM derived from the iris, a minor fraction (5%) of total UM cases, harbors *BRAF* mutations

Significance

UM is the most common type of adult eye cancer. Currently there is no effective treatment, especially for metastatic UM. Most UMs have activating mutations in one of two homologous G proteins, Gq or G11 (encoded by *GNAQ* or *GNA11*, respectively). We found that the Hippo pathway effector YAP is activated in UM containing mutant Gq/11, and inhibition of YAP by either genetic or pharmacological approaches blocks tumor growth of Gq/11-mutated UM cells in mouse models, suggesting a strategy for UM intervention by inhibiting YAP. This mechanism, whereby YAP activation mediates mutant Gq/11 signaling in tumorigenesis, may serve as a paradigm for general pathogenesis of human cancers with aberrant expression or mutations of G protein-coupled receptor or G proteins.

(Henriquez et al., 2007). Notably, the *GNAQ* mutation is frequent in benign blue nevi, whereas the *GNA11* mutation is frequent in malignant UM (Van Raamsdonk et al., 2010). The Gq and G11 proteins, encoded by the *GNAQ* and *GNA11* genes, respectively, are the alpha subunits of heterotrimeric G proteins that play an obligatory role in G protein-coupled receptor (GPCR) signaling. Interestingly, all mutations in Gq or G11 occur at either arginine 183 (R183) or glutamine 209 (Q209) in a mutually exclusive manner, suggesting that these mutations in Gq and G11 have similar functions in tumor promotion (Van Raamsdonk et al., 2010). R183 and Q209 are located in the switch I and switch II domains of Gq/11 proteins, respectively, and these mutations convert the G proteins into a constitutively active form by decreasing their guanosine triphosphatase activity. Therefore, the cancer-associated mutant Gq/11 would induce constitutive downstream signaling that presumably contributes to tumor development.

Previous work has shown that overexpression of active Gq/11 can induce transformation of normal melanocytes (Van Raamsdonk et al., 2009, 2010). Moreover, downregulation of mutant Gq/11 in UM cells abolished their ability to form tumors in immunocompromised mice, demonstrating a direct cancer-driving function of the active Gq/11 in tumorigenesis (Van Raamsdonk et al., 2009, 2010). Although it has been proposed that Gq/11 activates the MAP kinase, the precise molecular mechanism of these activating Gq/11 mutations in UM development remains to be defined.

The Hippo tumor suppressor pathway normally functions to control tissue homeostasis and limit organ size (Halder and Johnson, 2011; Pan, 2010; Tapon and Harvey, 2012; Yu and Guan, 2013). Core components of the Hippo pathway are represented by a kinase cascade consisting of MST1/2 and Lats1/2. The Lats1/2 kinases phosphorylate and inactivate YAP and TAZ, two homologous transcription coactivators with oncogenic potential. In fact, elevated expression or nuclear enrichment of YAP/TAZ has been observed in multiple types of human cancers (Chan et al., 2008; Steinhardt et al., 2008; Zhao et al., 2007). We recently reported that the Hippo pathway is strongly regulated by GPCR signaling (Miller et al., 2012; Mo et al., 2012; Yu et al., 2012). GPCR signaling can either activate or inhibit YAP activity in a manner dependent on the coupled G protein. For example, activation of G12/13 stimulates YAP by inducing YAP dephosphorylation, nuclear localization, and transcriptional activity, whereas activation of Gs inhibits YAP by increasing YAP phosphorylation. Interestingly, expression of active Gq/11 (containing the Q209L mutation), but not the wild-type, is able to stimulate YAP/TAZ dephosphorylation (Yu et al., 2012), indicating that YAP can be activated by Gq/11. These observations prompted us to investigate if the Hippo-YAP pathway might function as a mediator in active Gq/11-induced tumorigenesis, particularly in UM development.

RESULTS

Activation of YAP by Mutant Gq/11 in UM

To test whether YAP can be activated by the cancer-associated mutant Gq/11, we first determined the effects of *GNAQ* and *GNA11* hot spot mutations found in UM on YAP activity. In human embryonic kidney 293A (HEK293A) cells, ectopic expres-

sion of mutant Gq/11 (Gq^{R183Q}, Gq^{Q209L}, or G11^{Q209L}), but not the wild-type Gq or G11, caused a dramatic dephosphorylation of cotransfected YAP, as indicated by faster migration of YAP on a phos-tag-containing gel (Figure 1A). Because phosphorylation inhibits YAP, these data suggest that mutant Gq/11 activates YAP. TAZ has two phosphodegrons and Lats-induced phosphorylation promotes TAZ ubiquitination and degradation (Huang et al., 2012; Liu et al., 2010). As expected, the endogenous TAZ protein levels were significantly increased in the presence of mutant Gq/11 (Figure 1A). Lats-induced phosphorylation inhibits YAP/TAZ by promoting YAP/TAZ cytoplasmic sequestration, while dephosphorylated YAP/TAZ translocate to the nucleus and stimulate gene expression. Consistently, overexpression of active Gq/11 mutants, but not wild-type Gq/11, induced nuclear localization of endogenous YAP/TAZ, as assessed by immunofluorescence staining with an antibody that recognizes both YAP and TAZ (Figures S1A–S1C available online). These results show that the mutant Gq/11 found in UM potently activates YAP/TAZ and suggest a model that activation of YAP/TAZ may contribute to mutant Gq/11-induced UM development, given the known oncogenic function of these two transcription coactivators.

We then investigated YAP/TAZ activation status in a panel of 13 cell lines established from primary or metastatic UM by different laboratories. We sequenced the genes of *GNAQ* and *GNA11*. Among these UM cell lines, seven (92.1, Mel202, Mel270, OMM1.3, OMM2.2, OMM2.3, and OMM2.5) contain the Gq^{Q209} mutation, one (OMM1) contains the G11^{Q209L} mutation, and the remaining five tumor lines (OCM1, OCM3, OCM8, Mel285, and Mel290) have no mutations in Gq/11 (Figure 1B). These data are consistent with other recently reported mutation analyses, and among these cell lines, three (OCM1, OCM3, and OCM8) contain BRAF^{V600E} mutations (Griewank et al., 2012). Next, we determined YAP/TAZ phosphorylation and subcellular localization for each of these UM cell lines. Interestingly, all UM cell lines with Gq/11 mutations displayed low or moderate YAP phosphorylation and strong nuclear YAP (or YAP/TAZ) localization (Figure 1B). On the other hand, YAP was highly phosphorylated and exhibited exclusive cytoplasmic localization in BRAF-mutant cells (Figure 1B). These observations demonstrate that YAP is activated in Gq/11-mutant UM cells but inactivated in BRAF-mutant cells.

YAP/TAZ are known to be activated by serum or lysophosphatidic acid (LPA) (Miller et al., 2012; Yu et al., 2012). Both serum and LPA activate YAP/TAZ by inducing rapid dephosphorylation and nuclear localization. In UM cells with wild-type Gq/11 and BRAF, serum and LPA induced a strong YAP dephosphorylation and concomitantly increased YAP nuclear localization (Figures 1B–1D; Figures S1D and S1E). In contrast, in UM cells containing mutated Gq/11, YAP was dephosphorylated and localized in the nucleus regardless of the serum or LPA conditions. Our findings show that YAP/TAZ are indeed more active in UM cell lines containing Gq/11 mutations and are no longer sensitive to serum or LPA. Notably, in UM cells with mutant BRAF, YAP was heavily phosphorylated, and the serum and LPA-induced YAP dephosphorylation and nuclear localization were blunted (Figures 1B–1D; Figures S1D and S1E). In support, Lats phosphorylation status, an indicator of kinase activity, was higher in BRAF-mutant cells than in Gq/11-mutant cells (Figure S1F). These data

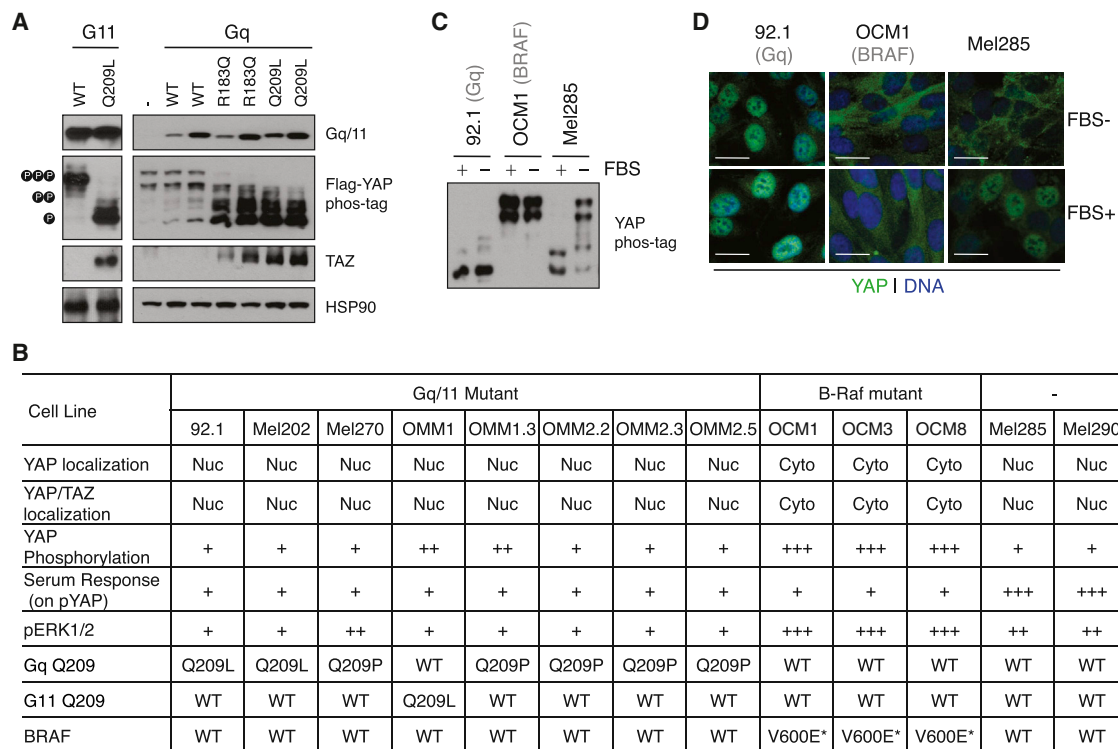


Figure 1. Activation of YAP by Mutant Gq/11 in UM Cell Lines

(A) Effects of UM-associated mutant Gq/11 on YAP/TAZ activity. HEK293A cells were transfected with different Gq/11 plasmids together with FLAG-YAP, and following 12 hr serum starvation, cells were harvested and YAP/TAZ activation status was analyzed by immunoblotting using the indicated antibodies. YAP phosphorylation was assessed using gels containing phos-tag, which slows down migration of phosphorylated YAP during electrophoresis. Endogenous TAZ protein levels were determined with a TAZ-specific antibody. For the Gq plasmids, two concentrations of plasmids were used in transfection, and the expression levels of transfected Gq were determined by immunoblotting.

(B) Table summarizing Gq/11 or BRAF mutation status, YAP phosphorylation, YAP localization, and responses to serum of multiple UM cells. Cyto, cytoplasm; Nuc, nucleus; WT, wild-type. The number of plus signs indicates the strength. *Information from Griewank et al. (2012).

(C) YAP phosphorylation and response to serum in representative UM cell lines. UM cells were cultured with or without 10% FBS for 16 hr.

(D) YAP localization and response to serum in representative UM cell lines. Cells were maintained in the presence or absence of 10% FBS for 16 hr, and after fixation, YAP localization was determined by immunostaining. The green and blue colors represent YAP and DNA staining, respectively. The scale bars represent 5 μ m.

See also Figure S1.

suggest that YAP activation is not needed for tumor growth of BRAF-mutant cells, and moreover, active BRAF might suppress YAP activation.

Previously, Gq/11 mutant-induced activation of the extracellular-signal-regulated kinases (ERKs), also known as MAP kinase, was proposed as a potential mechanism for mutant Gq/11 in UM development (Van Raamsdonk et al., 2009, 2010). Indeed, overexpression of Gq/11 mutants moderately induces phosphorylation of ERK (Van Raamsdonk et al., 2009, 2010). On the basis of western blot analyses of the 13 UM cell lines, ERK phosphorylation in Gq/11-mutant UM cell lines was evident, however it was much lower than that of BRAF-mutant UM cells (Figure 1B; Figure S1D). Moreover, ERK phosphorylation in the Gq/11-mutant UM cells was no higher than that in UM cells (Mel285 and Mel290) without Gq/11 mutations, suggesting that the ERK pathway is unlikely a major mediator for UM driven by mutant Gq/11. On the basis of the above data, we speculate that YAP/TAZ activation may play an important role in UM tumorigenesis.

Activation of YAP in UMs with Gq/11 Mutations

To determine YAP/TAZ activation status in UM, we examined formalin-fixed, paraffin-embedded sections of enucleated tumors. We collected 23 UM samples and performed genomic DNA sequencing for exon 4 (R183) and exon 5 (Q209) of Gq/11. Thirteen UM samples had Q209 mutations, whereas none had R183 mutation in Gq/11 (Table S1). The same clinical samples were immunostained using a YAP or YAP/TAZ antibody (Figure 2A; Table S1). The subcellular localization of YAP was assessed and scored from 1 to 5, with 1 representing exclusive nuclear localization and 5 representing exclusive cytoplasmic localization (Table S1). When YAP localization data were compared with the mutation status of Gq/11, we observed a strong correlation between mutated Gq/11 and YAP nuclear localization (Figure 2B). On the basis of these observations, we conclude that mutated Gq/11 is associated with YAP activation in UM, supporting a possible pathological role of YAP in Gq/11 mutation-induced tumorigenesis.

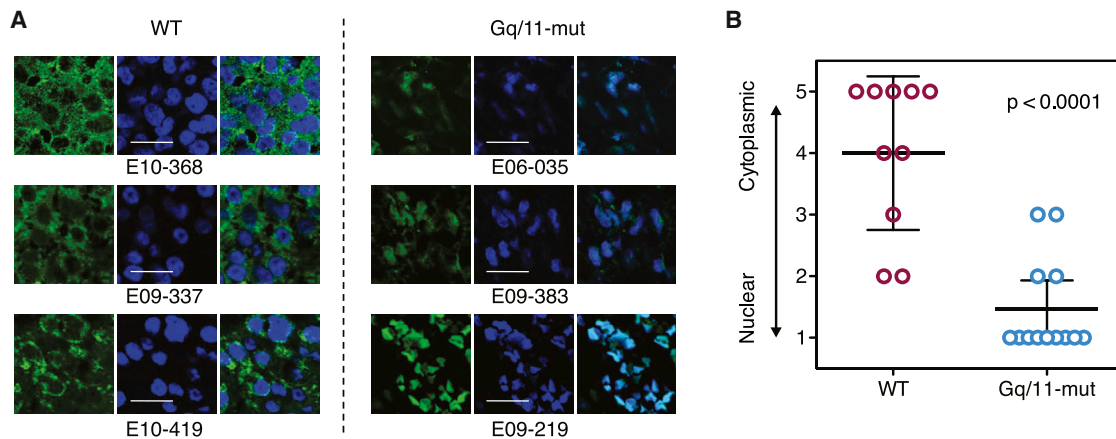


Figure 2. YAP Nuclear Localization Correlates with Gq/11 Mutations in UM Specimens

(A) Representative images for YAP localization in UM specimens. Immunofluorescence staining was performed for YAP (green) and DNA (blue, 4',6-diamidino-2-phenylindole). Shown are three representative samples of wild-type (left) and Gq/11 mutant (right). The scale bars represent 10 μ m.

(B) Correlation between YAP nuclear localization and Gq/11 mutation in UM specimens. The subcellular localization of YAP were scored from 1 to 5, with 1 representing exclusive nuclear localization and 5 representing for exclusive cytoplasmic localization. Student's t test (two tailed, 95% confidence intervals) was used for statistical analysis, and error bars represent SD.

See also Table S1.

Downregulation of Gq in UM Cells Inactivates YAP

In a subcutaneous xenograft mouse model, 92.1 (Gq^{Q209L}) cells transfected with small hairpin RNA (shRNA) targeting Gq failed to develop tumor (Figure 3A), confirming an essential role for mutant Gq in tumorigenicity of 92.1 cells. We tested whether the mutant Gq is required for high YAP activity in UM cells. In both 92.1 and Mel270 (Gq^{Q209P}) cells, we established stable lines expressing control shRNA or Gq shRNAs (#1 and #2 target different regions). Knockdown of Gq was confirmed by western blotting (Figure 3B). We observed that YAP phosphorylation (as indicated by the phosphorylated YAP western blot) was increased in cells expressing Gq shRNA (Figure 3B). When dephosphorylated, YAP localizes in the nucleus and interacts with the TEAD family of transcription factors to stimulate gene transcription (Cao et al., 2008; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008). We examined the interaction of YAP with TEAD. In Gq-knockdown cells, the interaction of YAP and TEAD was decreased, whereas Gq knockdown had no effect on TEAD1 expression (Figure 3C). In addition, YAP nuclear localization was decreased in Gq-knockdown cells (Figures 3D and 3E), consistent with YAP inactivation. We have recently shown that Gs-protein kinase A (PKA) signaling stimulates YAP/TAZ phosphorylation, an effect opposite to Gq/11 activation (Kim et al., 2013; Yu et al., 2013; Yu et al., 2012). As expected, YAP phosphorylation was increased in 92.1 cells when treated with forskolin and 3-isobutyl-1-methylxanthine (IBMX), which increase cellular cyclic AMP and activate PKA (Figure 3F). Notably, forskolin and IBMX induced stronger YAP phosphorylation in the Gq-knockdown 92.1 cells (Figure 3F), indicating that the active mutant Gq in 92.1 cells functions antagonistically to PKA. The above data support a function for mutant Gq in maintaining YAP in a dephosphorylated and activated status in UM cells.

YAP Is Required for Mutant Gq/11, but Not Mutant BRAF, Driving Tumorigenesis

Expression of active Gq/11 (Q209L) in immortalized melanocytes (melan-a cells) is sufficient to induce cell transformation (Van

Raamsdonk et al., 2009, 2010). This offers a well-defined and cleaner system for functional studies than UM-derived cell lines, which certainly contain mutations besides Gq/11. In melan-a cells expressing Gq^{Q209L} or G11^{Q209L}, YAP phosphorylation was reduced (Figures S2A and S2B), and the YAP-TEAD interaction was increased (Figure S2C), indicating higher YAP activity. To test the role of YAP/TAZ in Gq^{Q209L}-induced cell transformation, we generated Gq^{Q209L}-stable melan-a cells expressing control, YAP, and/or TAZ shRNAs (Figure 4A). As an indicator of transformation, Gq^{Q209L}-stable melan-a cells, but not control melan-a cells expressing GFP, could support anchorage-independent growth in soft agar. Importantly, Gq^{Q209L}-stable melan-a cells expressing YAP and/or TAZ shRNAs failed to form colonies (Figures 4B; Figure S2D). In addition, when subcutaneously grafted into nude mice, Gq^{Q209L}-stable melan-a cells with YAP knockdown exhibited a significant reduction in tumor growth (Figures 4C; Figure S2H). These results indicate that YAP/TAZ are important for Gq-induced neoplastic transformation.

To investigate the role of YAP in the tumorigenesis of UM-derived cell lines, we attempted to knock down YAP in 92.1 cells (Gq^{Q209L}), which have high YAP activity, and OCM1 (BRAF^{V600E}) cells, which have low YAP activity. Although it was easy to knock down YAP in OCM1 cells, we failed to establish an efficient YAP knockdown in 92.1 cells (data not shown). These observations suggest a critical role for YAP in 92.1 cell proliferation, which may be addicted to high YAP activity. We then made an inducible shRNA (pTRIPz system) containing the same YAP targeting sequences used in the conventional vector (pLKO.1) and successfully established both 92.1 and OCM1 stable cells. YAP expression in these cell lines was effectively reduced upon doxycycline (Dox) treatment that induced expression of the shRNAs (Figure 4D). In vitro, the proliferation of 92.1 cells was slightly reduced upon Dox treatment, whereas knockdown of YAP in OCM1 cells showed no significant effect on cell proliferation (Figures S2E and S2F). We also assessed the cell-migratory

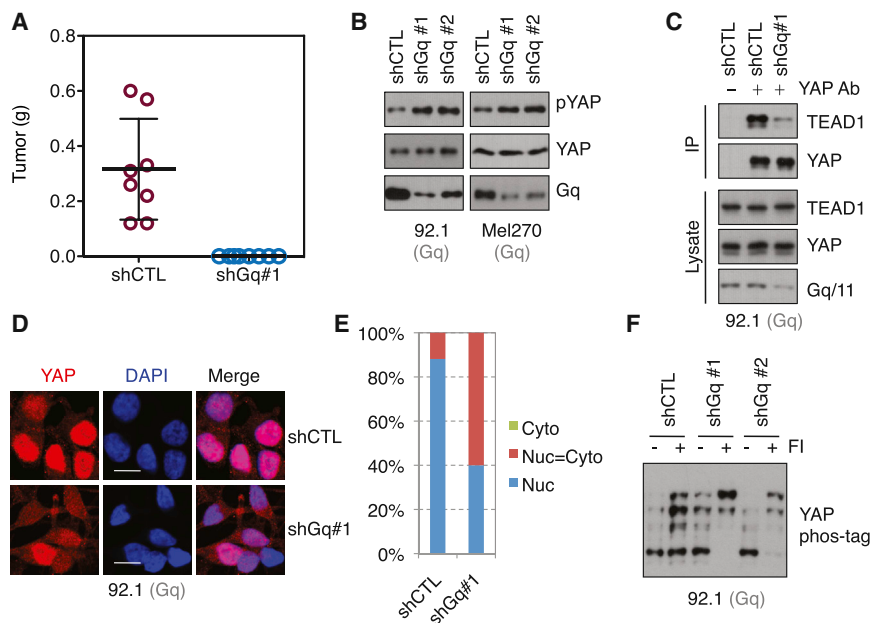


Figure 3. Downregulation of Mutant Gq in UM Cells Inactivates YAP

(A) Tumor formation ability of 92.1 (Gq^{Q209L}) cells with or without Gq knockdown. Control or Gq-knockdown 92.1 cells were grafted into nude mice subcutaneously, and tumor formation was monitored. Knockdown efficiency is shown in (B). Error bars represent SD.

(B) Effect of Gq knockdown on YAP phosphorylation. Stable 92.1 cells expressing control shRNA (shCTL) or two Gq-targeting shRNAs (shGq#1 and shGq#2) were established. Gq knockdown efficiency and YAP phosphorylation in these cells were assessed by western blotting. pYAP indicates western blotting with an antibody that specially recognizes the S127 phosphorylated YAP. The same experiment was performed in Mel270 (Gq^{Q209P}) cells.

(C) Effect of Gq knockdown on YAP-TEAD1 interaction. Immunoprecipitation (IP) of YAP from control or Gq-knockdown cell lysates was performed, and TEAD1 coprecipitated was determined by immunoblotting.

(D and E) Effect of Gq knockdown on YAP sub-cellular localization. Control or Gq-knockdown

92.1 cells were serum starved for 16 hr and fixed, and YAP localization was determined. The scale bars represent 5 μ m.

(F) Effect of Gq knockdown on PKA-induced YAP phosphorylation (inactivation). Control or Gq-knockdown cells were treated with forskolin (10 μ M) and IBMX (100 μ M) for 1 hr, and then YAP phosphorylation was determined. Both forskolin and IBMX increase cAMP and activate PKA, which stimulates YAP phosphorylation.

potential of these cells. Again, knockdown of YAP in 92.1 but not OCM1 cells reduced cell migration in a transwell assay (Figures 4E; Figure S2G). Furthermore, knockdown of YAP greatly impaired tumor formation of 92.1 cells (Figure 4F) but not OCM1 (Figure 4G) or OCM8 (Figure 4H) cells in nude mice (Figure S2H). Together, these observations suggest that YAP plays a pivotal role in tumorigenesis of Gq/11^{Q209L}-induced, but not BRAF^{V600E}-induced, UM.

The YAP-Inhibitory Drug Verteporfin Selectively Suppresses Gq/11-Mutant UM Tumorigenesis

The strong correlation between the Gq/11 mutation and YAP activation in UM specimens and UM cell lines and the effectiveness of YAP knockdown in preventing tumor growth of Gq/11-mutated UM cells in a mouse xenograft model prompted us to test the effect of pharmacological inhibition of YAP on the tumorigenesis of UM cells. It has recently been reported that the porphyrin-family compounds, such as verteporfin, disrupt the YAP-TEAD interaction and therefore inhibit the function of YAP in liver size control (Liu-Chittenden et al., 2012). Verteporfin is a drug with Food and Drug Administration (FDA) approval for photodynamic therapy to eliminate abnormal blood vessels in the eye (Bressler and Bressler, 2000). Interestingly, when treated with verteporfin, the UM cells with Gq/11 mutations were effectively killed, as indicated by the cleavage of poly (ADP-ribose) polymerase-1 (PARP1, an apoptosis marker; Figure 5A; Figure S3A). Similarly, the Gq/11-mutant UM cells were sensitive to growth inhibition by verteporfin (Figure S3B). In comparison, the BRAF-mutant cells were more resistant to both growth inhibition and apoptosis in response to verteporfin treatment (Figure 5A; Figures S3A and S3B). On the other hand, the BRAF-mutant UM cells were more

easily killed by U0126 (an inhibitor for MAP kinase kinase [MEK], the ERK-activating kinase), while the Gq-mutant cells were resistant to U0126 (Figure 5B). These results suggest a model in which YAP activation is more important for Gq/11-mutant tumor cells, whereas ERK activation is more important for BRAF-mutant tumor cells. Our data indicate that verteporfin may be used to selectively kill tumor cells with elevated YAP activity, such as UM containing mutations in Gq/11.

To assess the role of verteporfin in inhibiting tumorigenesis of UM cells, we used an orthotopic mouse model. Tumor cells were injected into the suprachoroidal space of the eye of the severe combined immunodeficient (SCID) mice. Tumor formation was monitored by noninvasive fundus examinations and optical coherent tomography (OCT) in live animals and histological analyses after mice were euthanized (Figures S3C and S3D). Aggressive endophytic growth was seen in 92.1 cells, resulting in filling of the inside of the eye with tumor cells (Figures S3C and S3D). We investigated the effect of verteporfin on the inhibition of tumor growth in vivo. Clinical-grade verteporfin (40 μ g/injection) was packaged into nanoparticles and mixed with 92.1, Mel270, or OCM1 cells prior to injection into the eye. As negative controls, UM cells were coinjected with empty nanoparticles. For the verteporfin-treated group, mice were also administered 100 mg/kg verteporfin along an intraperitoneal route every other day over a period of 14 days, whereas control mice were injected with PBS. After 6 weeks, compared with the control group, verteporfin treatment significantly reduced tumor growth of the Gq mutant 92.1 and Mel270 cells (Figures 5C and 5D; Figures S3E and S3F). In contrast, verteporfin treatment had little effect on tumor growth of the BRAF-mutant OCM1 cells (Figures 5E and 5F). Therefore, these mouse model studies demonstrate that

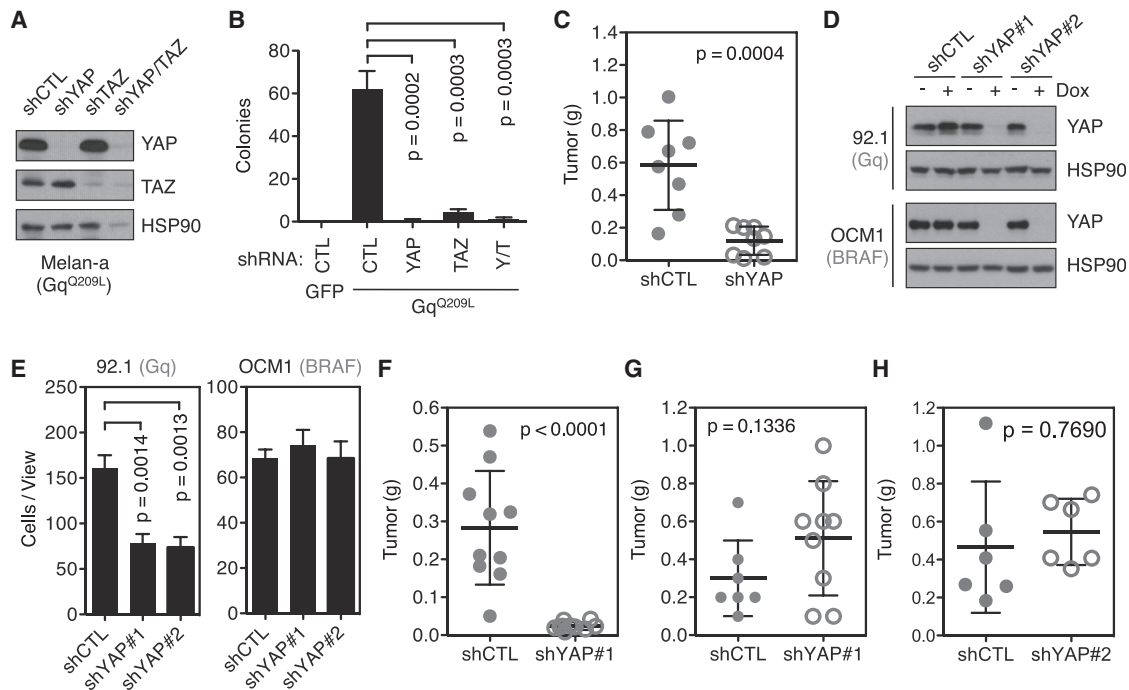


Figure 4. YAP Is Required for Mutant Gq/11-Induced Tumorigenesis

(A) Knockdown of YAP and/or TAZ in melan-a cells expressing Gq^{Q209L}.

(B) Effect of YAP and/or TAZ knockdown on anchorage-independent growth of melan-a cells expressing Gq^{Q209L}. Different melan-a cell lines were cultured in soft agar, and colony formation was assessed. GFP and Gq^{Q209L} denote melan-a cells stably expressing GFP control and Gq^{Q209L}, respectively. shRNA knockdown of YAP and/or TAZ is indicated; Y/T stands for combination of YAP and TAZ shRNAs.

(C) Tumorigenicity of the Gq^{Q209L} expressing melan-a cells following YAP knockdown. The same cell lines used in (B) were grafted into nude mice subcutaneously, and tumor formation was monitored.

(D) Inducible knockdown of YAP in 92.1 or OCM1 cells. Cells were treated with Dox (2.5 μg/ml) for 3 days, and YAP knockdown was assessed by immunoblotting.

(E) The effect of YAP knockdown on cell migration. Migration potential of 92.1 or OCM1 cells following YAP knockdown was assessed using a transwell assay.

(F–H) Tumorigenicity of Gq (92.1) mutant and BRAF (OCM1 and OCM8) mutant UM cells following YAP knockdown. Control or YAP knockdown 92.1 (F), OCM1 (G), or OCM8 (H) cells were grafted into nude mice subcutaneously, and tumor formation was monitored. Student's t test (two tailed, 95% confidence intervals) was used for statistical analysis, and error bars represent SD.

See also Figure S2.

verteporfin is effective in inhibiting tumor cell growth and might be considered for targeted treatment of UM with Gq/11 mutations and elevated YAP activity.

DISCUSSION

UM is the most common intraocular tumor in adults and frequently metastasizes to the liver. Early-stage UM can be treated by radiation or enucleation (removal of the eye), but there is no effective treatment for metastatic UM, which is the most feared complication and the main cause of death (Singh et al., 2005). Enucleation has been the last resort to prevent metastasis and has a long-lasting adverse and psychological impact on patients, even though enucleation does not significantly improve the outcome of survival (Collaborative Ocular Melanoma Study Group, 1998). Therefore, a systemic treatment of metastasis is urgently needed. The high penetrance of Gq/11-activating mutations and the essential role of mutant Gq/11 in UM oncogenesis warrants the need for an in-depth mechanistic understanding of Gq/11 in tumorigenesis. Moreover, the establishment of mutant Gq/11 as a cancer driver suggests a potential of developing tar-

geted therapies for UM treatment. Unfortunately, a drug that targets constitutively active Gq/11 is currently not available. It is therefore important to identify downstream effectors essential for Gq/11-induced tumorigenesis, and these effectors may provide opportunities to develop molecular-targeted drugs for UM management.

In this report, we reveal a strong correlation between Gq/11 mutations and YAP activation in UM. We have established a causal relationship between Gq/11 mutation and YAP activation, and we show that YAP is essential in transducing the oncogenic activity of mutant Gq/11 to induce UM. Thus, YAP may serve as a drug target for pharmaceutical intervention of UM. Indeed, this concept is strongly supported by our data showing that down-regulation of YAP selectively inhibits tumor growth of UM cells containing mutated Gq/11. Furthermore, verteporfin inhibits the proliferation of UM cells with Gq/11 mutations in vitro and is effective in suppressing their growth in a mouse model. Verteporfin is already an FDA-approved drug for eye disease indications such as macular degeneration, so it would be relatively easy to adapt this drug for UM treatment. Hence, the result of UM inhibition by verteporfin offers exciting possibilities not

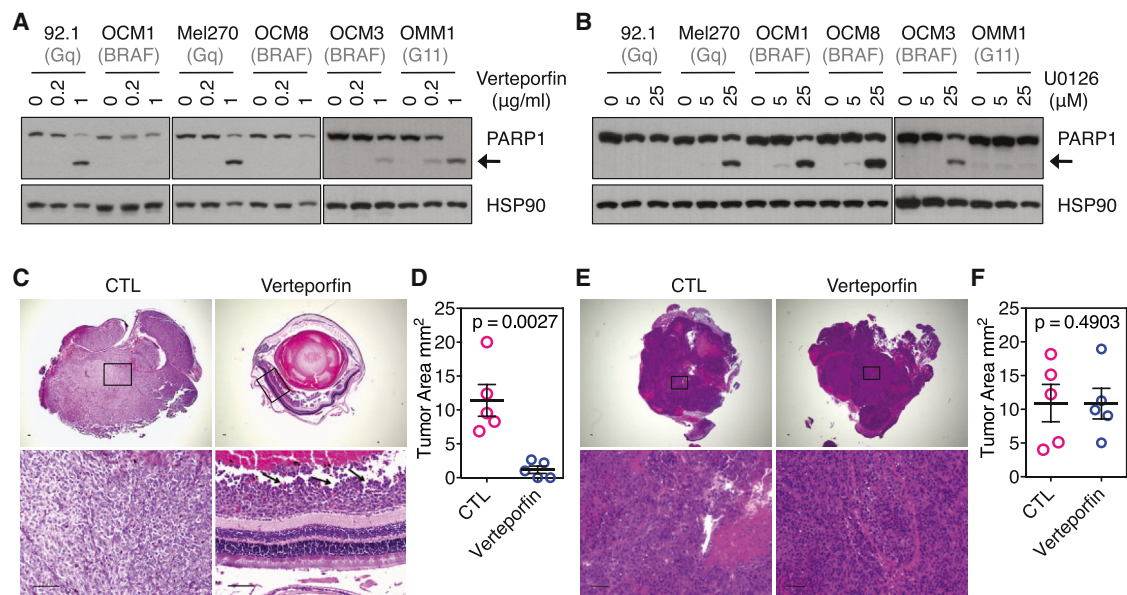


Figure 5. YAP Inhibitor Suppresses Tumor Growth of Gq/11-Mutated UM Cells

(A) Sensitivity of Gq/11-mutant and BRAF-mutant UM cells to verteporfin, a YAP inhibitor. Gq-mutant cells or BRAF-mutant cells were treated with 0.2 or 1 $\mu\text{g/ml}$ of verteporfin for 48 hr, and cell lysates were assessed for PARP1 cleavage (the black arrow indicates the position of cleaved PARP1, an indicator of cell death). (B) Sensitivity of Gq/11-mutant and BRAF-mutant UM cells to U0126, a MEK inhibitor. UM cells were treated with 5 and 25 μM of U0126 for 36 hr, and then cell apoptosis was assessed by PARP1 cleavage.

(C and D) Effects of verteporfin treatment on tumor growth of 92.1 cells in an orthotopic UM mouse model. Before injection into the suprachoroidal space of the eye, 92.1 cells were mixed with nanoparticles containing verteporfin or buffer (control [CTL]). After injection, verteporfin was delivered systematically to mice (treated) via intraperitoneal injection. Tumor formation was monitored by OCT, and tumors were harvested and sectioned for histological analysis. Representative sections of the eye showed the presence of large pigmented melanoma xenografts filling the eyes of all vehicle-treated animals (C, left), whereas tumors in the verteporfin treatment group (C, right) were smaller, as indicated by black arrows. The tumor areas from five injected eyes were quantified and are shown in (D). (E and F) Effect of verteporfin treatment on tumor growth of OCM1 cells. Similar experiments were performed as in (C) and (D) using OCM1 cells. Student's t test (two tailed, 95% confidence intervals) was used for statistical analysis, and error bars represent SD. The scale bars represent 100 μm .

See also Figure S3.

only for the treatment of local intraocular UM but also for deadly metastasis. Notably, the FDA-approved application of verteporfin is based on photodynamic therapy to eliminate neovascularization of blood vessels. Light activation of the drug is required for verteporfin to inhibit neovascular angiogenesis but is not required to disrupt the interaction between YAP and TEAD (Liu-Chittenden et al., 2012). Therefore, verteporfin could have a dual function, inhibiting angiogenesis and YAP activity, both of which can positively contribute to inhibiting UM. Our data indicate that verteporfin should be considered for UM treatment. It is equally important to note that verteporfin is ineffective toward the BRAF-mutant UM cells, which are sensitive to MEK inhibitors. These observations suggest that the therapeutic effects of verteporfin observed on the Gq/11 mutant UM cells are not due to general toxicity but rather target specific and mechanism-based inhibition of the Gq/11-mutant UM cells.

It has been previously shown that the active Gq/11 mutant also stimulates the ERK pathway (Van Raamsdonk et al., 2009, 2010). This is not surprising, because G proteins are known to initiate multiple downstream signaling events. However, activation of the ERK pathway by active Gq/11 is less potent compared with that of the BRAF^{V600E} mutation, which is frequently found in cutaneous melanoma (Figure 1B; Figure S1D). BRAF inhibition is an effective target-based therapy commonly used to treat BRAF^{V600E} mutant melanoma. Our study indicates that YAP activation

is more important than ERK activation in UM. Consistent with this notion, downregulation of YAP or verteporfin treatment induces more cell death and tumor inhibition in Gq/11-mutant UM cells than in BRAF-mutant UM cells (Figures 4F–4H and 5). Further studies are needed to determine the effect of a combined treatment inhibiting both MEK and YAP in treating UM with the Gq/11 mutation.

YAP/TAZ are frequently activated in human cancers (Harvey et al., 2013). However, the underlying mechanisms leading to YAP/TAZ activation in cancers are largely unknown. Mutations in the Hippo pathway components are rare in human cancers. One notable example is the neurofibromin 2 (NF2) tumor suppressor. Mutation in NF2 activates YAP, and YAP activation is required for NF2-induced tumorigenesis (Zhang et al., 2010). Our report not only reveals a mechanism of YAP activation in Gq/11-induced tumorigenesis but also suggests that YAP activation plays a critical role in cancer development with altered GPCR signaling. In addition to UM, other types of neoplastic lesions, such as blue nevi (Van Raamsdonk et al., 2009) and leptomeningeal melanocytic lesions (Kusters-Vandeveldel et al., 2010) also contain prevalent Gq/11 mutations. More recently, the GqR183Q mutation has been identified in 80% to 90% of Sturge-Weber syndrome and port-wine stain patients (Shirley et al., 2013). We also showed that GqR183Q also potently activated YAP (Figure 1A; Figures S1A–S1C). Interestingly, most of

these tumors or overgrowths, including those caused by NF2 loss-of-function mutations, are derived from the neuroectoderm. Activation of YAP/TAZ by Gq/11 or NF2 mutations in these tumors suggests that the Hippo pathway plays an important role in the development and differentiation of the neuroectoderm, consistent with a role of the Hippo pathway in regulating neural progenitor cells (Cao et al., 2008).

GPCRs constitute the largest family of cell surface receptors encoded by the human genome. Although cancer-associated mutations in GPCR signaling are less frequent than receptor tyrosine kinases, extensive cancer genome sequencing has revealed that approximately 20% of all human cancers may have altered GPCR signaling (O'Hayre et al., 2013). For example, mutation of metabotropic glutamate receptor occurs at an appreciable frequency in cutaneous melanoma (Prickett et al., 2011). Besides genetic alterations (mutation or amplification), GPCRs are stimulated by their cognate ligands, and therefore altered ligand levels can also lead to abnormal GPCR signaling. For example, LPA is a potent mitogen and strongly activates YAP/TAZ (Miller et al., 2012; Yu et al., 2012). In fact, LPA is elevated and defined as a biomarker for ovarian cancer (Mills and Mooleenaar, 2003). We have recently shown that YAP is broadly regulated by a large number of GPCRs (Yu et al., 2012). Collectively, these observations suggest a potential paradigm that YAP activation plays a broad role in cancers driven by altered GPCR signaling, and thus YAP inhibitors, such as verteporfin, represent a potential therapeutic for cancers with altered GPCR signaling.

EXPERIMENTAL PROCEDURES

More experimental procedures are shown in the [Supplemental Information](#).

Cell Culture

UM cell lines, provided by Dr. Martine Jager (Leiden University), were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). Melan-a cells, a gift from Dr. Dorothy Bennett (St. George University), were cultured in RPMI medium with 10% FBS and 200 nM 12-O-tetradecanoylphorbol-13-acetate. HEK293A, human embryonic kidney 293T, and human embryonic kidney 293P cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS. All media were supplemented with 50 µg/ml penicillin/streptomycin. Cells were maintained at 37°C with 5% CO₂.

Animal Work

All animal procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. For subcutaneous xenograft experiments, 12-week-old male nude mice were used. Cells (melan-a, 92.1, OCM1, or OCM8) with manipulations of YAP or Gq expression were grafted subcutaneously into both flanks of mice, and tumor growth was monitored three times a week. Mice were euthanized after 10 weeks of cell injection or until the tumor size reached 1 cm³ to document the formation of primary tumors. For the orthotopic UM mouse model, male 4-week-old SCID mice were used. Mice were anesthetized, and 50,000 cells of 92.1, Mel270, or OCM1 were injected into the suprachoroidal space in the right eye using a 33-gauge needle. Tumor formation was monitored every week by fundus examinations and OCT (Spectralis; Heiderberg Engineering). Mice were euthanized after 6 weeks because of the development of very large masses in the eyes of several mice in the control group. Eyes were enucleated and fixed in 4% paraformaldehyde and subjected to histological analysis.

Human Clinical Samples

Patients were diagnosed with UM by clinical history, complete ophthalmic examination, ultrasound, and ancillary studies. Twenty-three enucleated eyes

due to large UM lesions were collected with patients' consent and approval of the Institutional Review Board of West China Hospital. UM specimens were paraffin embedded and sectioned for histology and immunofluorescence staining. The use of human tissue samples was in accordance with the protocols approved by the Institutional Review Boards at West China Hospital and the University of California, San Diego.

Other methods are described in [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2014.04.017>.

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