

# High Throughput Mass Spectrometry-Based Mutation Profiling of Primary Uveal Melanoma

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**PURPOSE.** We assessed for mutations in a large number of oncogenes and tumor suppressor genes in primary uveal melanomas using a high-throughput profiling system.

**METHODS.** DNA was extracted and purified from 134 tissue samples from fresh-frozen tissues ( $n = 87$ ) or formalin-fixed, paraffin-embedded tissues ( $n = 47$ ) from 124 large uveal melanomas that underwent primary treatment by enucleation. DNA was subjected to whole genome amplification and MALDI-TOF mass spectrometry-based mutation profiling (>1000 mutations tested across 120 oncogenes and tumor suppressor genes) using the OncoMap3 platform. All candidate mutations, as well as commonly occurring mutations in *GNAQ* and *GNA11*, were validated using homogeneous mass extension (hME) technology.

**RESULTS.** Of 123 samples, 97 (79%, representing 89 unique tumors) were amplified successfully, passed all quality control steps, and were assayed with the OncoMap platform. A total of 58 mutation calls was made for 49 different mutations across 26 different genes in 34/98 (35%) samples. Of 91 tumors that

underwent hME validation, 83 (91%) harbored mutations in the *GNAQ* (47%) or *GNA11* (44%) genes, while hME validation revealed two tumors with mutations in *EGFR*. These additional mutations occurred in tumors that also had mutations in *GNAQ* or *GNA11*.

**CONCLUSIONS.** The vast majority of primary large uveal melanomas harbor mutually-exclusive mutations in *GNAQ* or *GNA11*, but very rarely have the oncogenic mutations that are reported commonly in other cancers. When present, these other mutations were found in conjunction with *GNAQ*/*GNA11* mutations, suggesting that these other mutations likely are not the primary drivers of oncogenesis in uveal melanoma. (*Invest Ophthalmol Vis Sci.* 2012;53:6991-6996) DOI: 10.1167/iovs.12-10427

Uveal melanoma is the most common primary intraocular malignancy in adults, with an incidence of approximately 6 cases per million per year in the United States.<sup>1</sup> Local treatment, either by plaque radiotherapy or proton beam irradiation, is highly effective at eradicating the primary tumor,<sup>2,3</sup> with rates of local tumor control as high as 95% to 98%.<sup>2</sup> Despite local control, up to 50% of patients develop metastases, with average survival of less than 1 year after diagnosis of metastatic disease.<sup>4-6</sup> Unlike cutaneous melanomas, which metastasize locally via lymphatics, uveal melanomas spread hematogenously, with the most common site of metastasis being the liver.<sup>7</sup> The risk of metastasis appears to be correlated highly with specific genetic mutations in the primary tumor. Chromosomal alterations, such as monosomy 3 or gain of chromosome 8q, are associated with decreased survival,<sup>8,9</sup> and recent work has demonstrated specific gene expression profiles that are very strong predictors of survival (class 1) or metastasis (class 2).<sup>10-13</sup>

Not only do the routes of metastasis differ between cutaneous and uveal melanomas, but they appear to be distinct entities on a molecular level as well. The profile of early driver mutations in cutaneous melanomas is quite well known, involving predominantly mutations in *BRAF* and *RAS* family genes,<sup>14</sup> leading to activation of the MAPK/MEK/ERK pathway.<sup>15</sup> In contrast, *RAF* and *RAS* mutations almost never are found in primary uveal melanomas.<sup>15</sup> Recent work has begun to identify some common mutations in uveal melanoma, including in *GNAQ*<sup>16</sup> and *GNA11*.<sup>17</sup> Mutations in one or the other of these genes are present in most uveal melanomas and blue nevi.<sup>16,17</sup> However, these appear to be early mutational events in melanomagenesis, since they are present in all stages of disease, and in nonmalignant nevi as well.<sup>18</sup> More recently, *BAP1* mutations have been described in a large proportion of uveal melanomas, and appear to correlate strongly with metastasis.<sup>19</sup>

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Recently, we described high-throughput methods of onco-gene screening in other tumors, which permit the rapid interrogation of known oncogenic mutations in fresh frozen and paraffin-embedded samples.<sup>20</sup> This has been most useful in cancers for which samples are rare, that had not been studied previously in a systematic manner, or that displayed heterogeneity of oncogenic activation or tumor suppressor inactivation. For example, we showed that a small but significant proportion (11%) of pediatric low grade astrocytomas and the majority of Langerhans cell histiocytosis samples harbored activating mutations in *BRAF*.<sup>21,22</sup> Therefore, we set out to perform a comprehensive mutational profile of known oncogene and tumor suppressor genes using our OncoMap3 platform in a large collection of uveal melanomas, in an effort to identify potential targets for treatment or prevention of metastatic disease.

## METHODS

### Patients and Tumor Tissue Collection

Tissue samples ( $n = 134$ ) were obtained from 124 tumors from patients with large melanomas (Collaborative Ocular Melanoma Study definition: basal diameter  $>16.0$  mm or an apical height  $>10.0$  mm),<sup>23</sup> who underwent primary enucleation. Samples from tumors were either snap-frozen in liquid nitrogen in the operating room at the time of enucleation and stored at  $-80^{\circ}\text{C}$  ( $n = 87$ ), or were taken from formalin-fixed paraffin-embedded (FFPE) specimens ( $n = 47$ ). Patients were treated either at the Massachusetts Eye and Ear Infirmary or at the Ocular Oncology Service at Washington University in St. Louis, MO. Informed consent was obtained from all patients. This study was approved by the Massachusetts Eye and Ear Infirmary Institutional Review Board. All work reported herein is compliant with the Health Insurance Privacy and Accountability Act, and the protocol was consistent with the Declaration of Helsinki.

### Isolation and Purification of DNA

For fresh-frozen samples, DNA isolation and purification was performed according to the manufacturer's protocols using the QIAamp DNA micro or QIAamp All prep DNA/RNA mini kit (Qiagen Inc., Valencia, CA). Briefly, tissues were disrupted in lysis buffer using a pellet pestle motor and were homogenized using a QIAshredder homogenizer. The DNA then was purified via sequential column centrifugation.

For samples harvested from formalin-fixed, paraffin-embedded archival tumors, 10  $\mu\text{m}$  thick sections were cut from the paraffin blocks. All slides were re-examined microscopically to confirm the diagnosis, and to ensure that the sample was representative of the tumor and that normal surrounding tissues were not included. Five to 15 slides were used for tissue collection per tumor, depending on the tumor size on the slides. DNA purification then was carried out using the QIAamp DNA FFPE Tissue kit (Qiagen Inc.), according to manufacturer's protocols. Briefly, scraped tissues were treated with xylene to dissolve and remove paraffin. Proteinase K then was added and the tubes were incubated at  $90^{\circ}\text{C}$  to reverse formalin crosslinking. Afterwards, elution of DNA was achieved via sequential centrifugation through membrane spin columns.

For samples in which eluted DNA was rich in melanin pigments, OneStep PCR inhibitor removal kit (Zymo Research Corporation, Irvine, CA) was used to remove melanin pigments from the samples for downstream analysis, according to the manufacturer's protocols.

### Rationale of OncoMap Platform Design

To select genes and mutations for use in the OncoMap Platform, we queried the following databases for known somatic oncogene and tumor suppressor gene mutations: the Sanger Institute COSMIC

database (available online at <http://www.sanger.ac.uk/genetics/CGP/cosmic/>), PubMed (available online at <http://www.ncbi.nlm.nih.gov/pubmed/>), and The Cancer Genome Atlas (TCGA) datasets (available online at [http://tcga-data.nci.nih.gov/docs/somatic\\_mutations/tcga\\_mutations.htm](http://tcga-data.nci.nih.gov/docs/somatic_mutations/tcga_mutations.htm)) for non-synonymous coding mutations that have been reported previously to occur as somatic mutations in human cancers. We rank ordered mutations for inclusion based on frequency of mutation in cancers and across cancer subtypes, as well as existence of approved inhibitors or those in clinical development. Most genes with single-instance mutations were excluded; the exceptions being if the gene in question was highly relevant to cancer and/or druggable (e.g., *AKT1* mutation in multiple cancer types). "Hotspot" mutations from selected well-known tumor suppressor genes were included based on the number of documented occurrences, with higher weight given to genes commonly deleted or genetically inactivated across cancer types.

### OncoMap Mutation Profiling

Purified DNA from tumor samples was subjected to whole-genome amplification (WGA) as described previously.<sup>20,24</sup> Fresh frozen tissues were amplified using the phi29-mediated WGA (Repli-g; Qiagen Inc.) and FFPE DNA was amplified with the Sigma GenomePlex Complete WGA kit (Sigma, Monticello, IA), for better performance. After quantification and dilution of amplified DNA, multiplexed PCR was performed as described previously.<sup>20</sup> After residual salts were removed from the reactions with the addition of cation exchange resin, the purified primer extension reaction was loaded onto a matrix pad of a SpectroCHIP (Sequenom Inc., San Diego, CA). SpectroCHIPS were analyzed using a Bruker Biflex III matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (SpectroREADER; Sequenom Inc.). Specific genes and mutations assayed are described in the Table and Supplementary Table S1 (see Supplementary Material and Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10427/-/DCSupplemental>), and included the previously described OncoMap Core panel<sup>21</sup> as well as an OncoMap extended panel, together termed OncoMap3.

### Analytical Methods

Mutation calls for each sample were determined using the techniques and methods described previously<sup>20</sup> using the Sequenom MassArray Analyzer and software, except that a higher cutoff of 80% was used for successful genotyping, rather than the previously reported 75%, for conservative calls.

### Homogenous Mass Extension (hME) Validation

The hME genotyping for validation of candidate mutations was performed on 100 (80 frozen, 20 FFPE) of the original 134 tumor samples (representing 91 unique tumors) using unamplified purified tumor source DNA, as described previously.<sup>25</sup> The hME genotyping consisted of 69 assays for mutations (across 30 different genes) initially detected in OncoMap3 and 11 assays for previously reported<sup>16-18,26,27</sup> mutations in *GNAQ* and *GNAI1*, which were not included in OncoMap3. The entire set of validation assays was performed on 71 of the samples. For the other remaining 29 samples with less available DNA, validation was performed only for a more limited set of mutations (i.e., those most likely to be validated based on the number and strength of calls in the initial screen), including *CTNBN1*, *EGFR*, *NOTCH1*, *GNAQ*, and *GNAI1*. The list of validation assays is provided as Supplementary Table S2 (see Supplementary Material and Supplementary Table S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10427/-/DCSupplemental>). Only mutations that were confirmed at this validation step were considered positive for the purposes of further analysis.

TABLE. Genes Tested in OncoMap3 Core + Extended

Gene	# of Mutations Assayed	Gene	# of Mutations Assayed	Gene	# of Mutations Assayed
ABL1	21	ERBB2	21	NTRK2	1
ABL2	1	FBXW7	8	NTRK3	6
ADAMTSL3	4	FES	4	PAK7	1
AKT1	1	FGFR1	2	PALB2	3
AKT2	2	FGFR2	16	PDGFRA	34
ALK	10	FGFR3	21	PDGFRB	3
AML1/RUNX1	8	FGFR4	3	PDPK1	2
APC	15	FLNB	5	PIK3CA	48
AR	2	FLT3	24	PIK3R1	13
ATM	3	FLT4	1	PKHD1	5
ATP8B1	4	FMS	3	PTCH	6
AURKA	3	FYN	3	PTEN	27
AURKB	1	GATA1	16	PTPN11	14
AURKC	3	GNAS	4	RAF1	2
AXL	1	GUCY1A2	3	RB1	14
BMX	1	HRAS	20	RET	30
BRAF	53	IGF1R	5	ROBO1	2
BRCA1	3	JAK2	5	ROBO2	4
BRCA2	6	JAK3	4	ROS1	4
BUB1	2	KIT	47	64	4
C14orf155	3	KRAS	57	SMAD2	3
CDH1	5	LRP1B	12	SMAD4	4
CDK4	2	LYN	1	SMARCB1	9
CDKN2A	21	MADH4	7	SMO	3
CEBPA	18	MAP2K4	13	SPTAN1	4
CREBBP	2	MEN1	6	SRC	1
CSF1R	7	MET	11	STK11	19
CTNNB1	58	MLH1	1	SUFU	3
CUBN	3	MLL3	5	TBX22	3
DBN1	2	MPL	3	TCF1	3
DDR1	2	MSH2	2	TEC	1
DDR2	1	MSH6	3	TFDP1	2
EGFR	88	MYC	22	TIAM1	3
EPHA1	2	MYH1	3	TIF1	3
EPHA3	18	NF1	5	TP53	22
EPHA4	4	NF2	10	TRIM33	4
EPHA5	6	NOTCH1	11	TSC1	2
EPHA8	1	NPM1	7	TSHR	5
EPHB1	10	NRAS	38	VHL	16
EPHB6	6	NTRK1	2	WT1	2

## RESULTS

### OncoMap Genotyping Performance

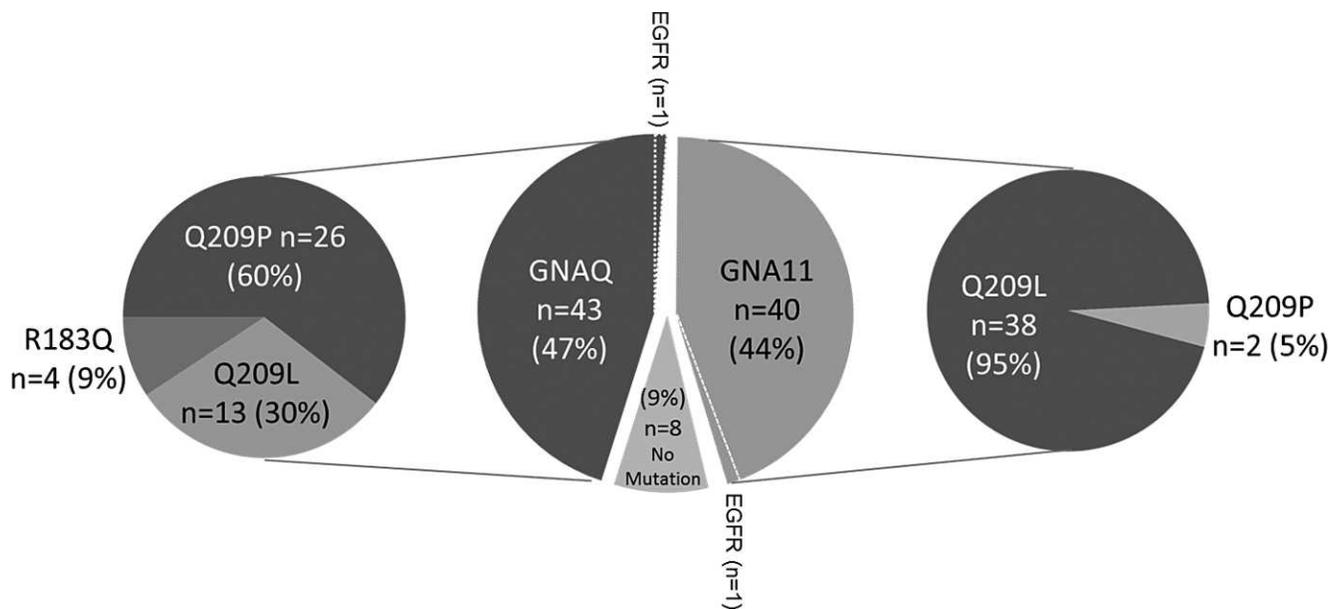
In an effort to maximize true call rates, strict quality control measures were applied to our samples before mutation profiling on OncoMap3, as detailed in the Methods section above. Of 134 tumor tissue samples (from 124 tumors) that underwent WGA, 97 samples (from 89 tumors, representing 73% of samples and 72% of tumors) passed initial fingerprinting and had a sufficiently high genotyping percentage, and were included in the analyses for the OncoMap Core panel. For the subsequent OncoMap Extended panel, 91 samples (representing 83 tumors [68% of samples, 67% of tumors]) were included in analyses (see the Table for all genes and mutations assayed by OncoMap3). This stringency allowed us to achieve a very high genotyping success rate (>90%); a reliable mutation determination (either wild type or mutant) could be made for 97% of mutations tested.

For OncoMap Core and Extended combined, 58 mutation calls were made for 49 different candidate mutations across 26

genes in 34/97 (35%) of samples. For this initial genotyping step, mutation calling was set to achieve the highest sensitivity, allowing false positive mutation calls. Therefore, all candidate mutations were validated with the more specific hME chemistry on unamplified DNA.

### hME Validation Results

Mutations in *GNAQ* and *GNA11* are not interrogated as part of the OncoMap3 platform. Therefore, specific assays for known mutations in these genes were designed as part of the hME genotyping step. Of 91 tumors that underwent hME genotyping, 83 (91%, comprising 92/100 samples) harbored mutations in *GNAQ* or *GNA11*. *GNAQ* mutations were found in 43/91 tumors tested (47%, see Fig.). Of those 43 tumors with *GNAQ* mutations, 26 (60%) had the Q209P mutation, 13 (30%) had the Q209L mutation, and 4 (9%) had the R183Q mutation (see Fig.). *GNA11* mutations were found in 40/91 tumors (44% [46/102 samples], see Fig.). The Q209L mutation was found in 38 of these 40 tumors (95%), while Q209P was found in 2 tumors (5%, see Fig.). In total, 87% of all tumors harbored mutations in



**FIGURE.** Distribution of mutations among the primary uveal melanoma tumors. *EGFR* mutations were found only in tumors that also harbored *GNAQ* or *GNA11* mutations. The breakdown of the specific *GNAQ* and *GNA11* mutations found in primary uveal melanoma tumors is shown in the side graphs.

either *GNAQ* or *GNA11* at the Q209 residue (see Fig.). *GNAQ* and *GNA11* mutations were mutually exclusive; no tumor was found to have mutations in both genes.

hME validation confirmed mutations in *EGFR* (E746\_A750 > IP) in 2 FFPE tumor samples. These mutations occurred in tumors that also harbored *GNAQ* or *GNA11* mutations (see Fig.).

There were 9 tumors for which both FFPE and frozen samples were examined. *GNAQ* or *GNA11* mutation status was consistent for all pairs of samples derived from the same tumor. No other mutations were found in any of these duplicate samples.

## DISCUSSION

To our knowledge, our study represents the first application of the OncoMap platform to the study of uveal melanoma and demonstrates a very low frequency of oncogenic mutations reported commonly in other cancers, while corroborating a high prevalence of mutations in *GNAQ* and *GNA11*. We chose to focus our search on known oncogenes because many of these oncogenes already have known inhibitors and, therefore, if a recurring mutation were to be detected, a clinical trial of targeted therapy might easily ensue. This approach, using earlier versions of the OncoMap platform, has proven effective in other rare cancers with no effective therapies. For example, 11% of pediatric low grade gliomas were shown to harbor a particular activating mutation in *BRAF*,<sup>21</sup> and 15% of pediatric patients with diffuse intrinsic pontine gliomas who underwent stereotactic biopsies had *PI3KCA* mutations using the OncoMap platform.<sup>28</sup> Similarly, 20% of high grade serous ovarian cancers<sup>29</sup> and 13% of gallbladder carcinomas<sup>30</sup> were shown recently to harbor mutations in *PIK3CA* using this platform.

Strengths of our study include the large number of tumors analyzed (124 unique tumors) and the large number of mutations (>1000) assayed across a large number of oncogenes (120). In addition, the high sensitivity of the OncoMap platform allows for significant confidence regarding the absence of these common oncogenic mutations in uveal

melanoma. The sensitivity of this platform is higher than that of Sanger sequencing, because it can detect mutations in a minor subset of cells.<sup>21</sup> Based on previous studies in other cancers, the sensitivity was found to be 93.8% in fresh frozen tissue and 89.3% in FFPE samples, and the specificity was found to be 100% in fresh frozen tissue and 99.4% in FFPE samples.<sup>21</sup>

Weaknesses of this study include the limitation that only known oncogenes and tumor suppressor genes were investigated, and thus mutations in novel oncogenes could not be discovered. Similarly, not every gene that has been found to be mutated in cancer was included; rather our assays were limited to only 120 of the most commonly mutated oncogenes and tumor suppressor genes. Furthermore, only commonly found mutations in these 120 genes were assayed, so although over 1000 mutations in total were included, it is possible that other rare mutations in these genes were missed.

*BAP1* was not included in this survey, because it had not yet been described as a tumor suppressor gene for uveal melanoma at the time our studies were performed.<sup>19</sup> Additionally, a variety of *BAP1* mutations have been described across the gene, with no "hotspot" of common occurrence.<sup>19,31-35</sup> Therefore, *BAP1* mutation screening would require direct sequencing of the gene and would not be feasible with this type of platform.

In ocular melanoma samples, we found very low rates of mutations in known oncogenes. Two percent of tumors harbored mutations in exon 19 of the *EGFR* gene, which is known to confer sensitivity to protein tyrosine kinase inhibitors. However, these rare mutations occurred in tumors that also harbored mutations in either the *GNAQ* or *GNA11* genes, and never occurred in isolation (see Fig.). Thus, the clinical importance of these less prevalent mutations in two samples should not be overstated.

In contrast to uveal melanoma, other cancer types have significantly higher rates of mutations detected with the OncoMap platform. In a heterogeneous population of 903 samples comprising 11 different types of cancers, at least one mutation was found in 37% of all samples.<sup>21</sup> Even in the initial version of OncoMap, which only queried 238 different known

oncogene mutations within 17 commonly mutated oncogenes, 30% of the 1000 samples assayed contained at least one mutation.<sup>20</sup> In that initial study, cutaneous melanoma was the cancer type that was found most frequently to contain an OncoMap mutation (78% of all melanoma samples), with *BRAF* being the most frequently mutated oncogene among melanoma samples. Subsequent studies of a later version of OncoMap (396 mutations in 33 cancer genes) demonstrated mutation prevalences ranging from 3.8% in renal cell carcinoma to 39.6% in breast, 57.7% in lung, 64.2% in colon, and 78.3% in endometrial cancers.<sup>21</sup>

These findings demonstrate the unique mutation pattern in uveal melanoma compared to cutaneous melanoma and most other human cancers, with the paucity of mutations in common oncogenes in uveal melanoma quite striking. It is yet to be determined whether fewer mutational “hits” are necessary to achieve full malignant potential in uveal melanoma, or whether there are frequent mutations in currently unidentified genes. Our study also underscores the fact that a candidate gene approach of oncogenes found in other cancers is unlikely to yield much new fruit in future studies of uveal melanoma, even if they were to evaluate genes not assayed by our platform. Given that the OncoMap platform was designed to interrogate mutations for which approved or “in development” drugs exist, clinically, these results suggest that currently available targeted therapies are unlikely to be effective in uveal melanoma.

Our results also corroborated those of other investigators in finding a very large proportion of uveal melanomas with mutations in *GNAQ* and *GNA11*, especially at the conserved Q209 residue.<sup>26</sup> Overall, we found that 91% of tumors harbored mutations in either *GNAQ* or *GNA11*, similar to the prevalence (83%) reported previously.<sup>17</sup> Specifically, the prevalence of the *GNAQ* mutation in the tumors in our study (47%) is comparable to that reported in previous studies. Van Raamsdonk et al. have reported prevalences of *GNAQ* mutations in 46 to 48% of uveal melanomas,<sup>16,17</sup> and Bauer et al. found *GNAQ* mutations in 53% of uveal melanomas.<sup>36</sup> Similarly, Onken et al. found mutations in the *GNAQ* gene in 49% of all uveal melanomas, with a higher rate (54%) among posterior segment choroidal melanomas and a much lower rate (22%) among iris melanomas.<sup>18</sup> Rates of *GNA11* mutation in our study (45%) were slightly higher than that reported initially (34%). As with all previous reports, the vast majority of mutations in *GNAQ* and *GNA11* occurred at the Q209 residue, with only a small fraction occurring at R183 in *GNAQ*. The high prevalence of *GNAQ* and *GNA11* mutations in uveal melanoma provides a unique genetic signature for this tumor, which could be used in clinical situations where tumor identity may be ambiguous.

Our study also supports certain notions regarding the process of oncogenesis in general. First, the mutual exclusivity of mutations in *GNAQ* and *GNA11* in uveal melanoma would suggest that activation of the MAPK/MEK/ERK pathway, and perhaps other important pathways, can occur via mutation of any one of several upstream proteins. Not only can pathway activation occur via different proteins, but the particular protein mutated may be a tissue-specific event. For example, it is interesting to note that melanocytes of the eye and brain derive from cranial neural crest, and only uveal melanomas, and central nervous system melanocytomas and melanomas harbor mutations in *GNAQ* and *GNA11*. On the other hand, cutaneous melanocytes, which tend to have *BRAF* and *NRAS* mutation, and not *GNAQ* or *GNA11* mutations, derive from thoracic neural crest. Therefore, it is possible that there are developmental differences between cranial and thoracic neural crest-derived cells that make them differentially sensitive to mutation of one set of genes versus another. This suggests a

direction for future studies that seek to identify genes involved in the pathogenesis of ocular melanoma and other rare cancers. Since certain pathways are altered commonly in cancer (e.g., Wnt pathway activation, apoptosis pathway downregulation), a pathway-based approach, focusing on relevant components not already known to be drivers of oncogenesis could prove fruitful.

In conclusion, extensive profiling of a large collection of uveal melanomas for mutations in known oncogenes and tumor suppressor genes confirmed a high prevalence of mutations in *GNAQ* and *GNA11*, but a very low frequency of the commonly-reported mutations in other genes often associated with different types of cancer. These findings underscore the unique molecular signature of this rare tumor.

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