Oncogenic G Protein GNAQ Induces Uveal Melanoma and Intravasation in Mice

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Abstract

GNAQ and GNA11 are heterotrimeric G protein alpha subunits, which are mutated in a mutually exclusive pattern in most cases of uveal melanoma, one of the most aggressive cancers. Here we introduce the first transgenic mouse model of uveal melanoma, which develops cancers induced by expression of oncogenic GNAQ<sup>Q209L</sup> under control of the Rosa26 promoter. Disease penetrance is 100% by 3 months of age, with 94% of mice also developing lung tumors. In this model, the Yap protein of the Hippo pathway is activated in the eyes, and blood vessels near the lesions in the head and lungs exhibit melanocytic invasion. While full transcription levels are not necessary for GNAQ<sup>Q209L</sup> to transform mouse melanocytes, we obtained suggestive evidence of a selective advantage for increased GNAQ<sup>Q209L</sup> expression in human tumors. Intriguingly, enforced expression of GNAQ<sup>Q209L</sup> progressively eliminated melanocytes from the interfollicular epidermis in adults, possibly explaining the near absence of GNAQ<sup>Q209L</sup> mutations in human epithelial melanomas. The mouse model also exhibited dermal nevi and melanocytic neoplasms of the central nervous system, accompanied by impaired hearing and balance, identifying a novel role for GNAQ in melanocyte-like cells of the inner ear. Overall, this model offers a new tool to dissect signaling by oncogenic GNAQ and to test potential therapeutics in an in vivo setting where GNAQ<sup>Q209L</sup> mutations contribute to both the initiation and metastatic progression of uveal melanoma.

Introduction

Uveal melanoma, a malignancy of melanocytes in the uveal tract of the eye, is a highly aggressive cancer without any effective treatment options once it metastasizes (1). Large-scale uveal melanoma sequencing projects have identified recurrent mutations in two G proteins (GNAQ and GNA11), BRCA1-associated protein-1 (BAP1), X-linked eukaryotic translation initiation factor 1A (EIF1AX), and Splicing factor 3B subunit 1 (SF3B1; refs. 2–8). Of these, mutations in GNAQ and GNA11 occur with a mutually exclusive, combined frequency of approximately 80% in both class I and the more aggressive class II uveal melanomas (2, 7). There are two oncogenic hotspots in GNAQ/11: glutamine(Q)209 and arginine(R)183 (2, 3).

GNAQ and GNA11 encode heterotrimeric G protein alpha subunits of the q class (Gaq/11; ref. 9). They are 90% identical at the amino acid level and play redundant roles; knocking out any three Gnaq and Gna11 alleles is lethal in mice (10). The function of Gaq/11 is to link various 7 transmembrane G protein–coupled receptors (GPCR) to downstream signaling effectors inside cells, canonically phospholipase C (9). Q209 and R183 mutations also activate the MAPK pathway and the Hippo tumor suppressor pathway (2, 3, 11–13). During signaling, GDP-bound alpha subunits are stimulated by ligand binding to GPCRs, which causes the alpha subunits to release GDP, bind GTP, and assume the active conformation that can interact with downstream effectors. Using an intrinsic Ras-like GTP hydrolysis (GTPase) domain, the alpha subunit cleaves the gamma phosphate from the GTP molecule to produce GDP and return the alpha subunit to its inactive conformation (14). Q209 and R183 lie in the GTPase domain and directly position the gamma phosphate for cleavage (15). Substitution mutations at these two highly conserved residues allow for GTP binding, but greatly reduce the rate of GTP hydrolysis, therefore generating constitutive active signaling (16, 17). In uveal melanoma, mutations at Q209 are found 13 times more frequently than mutations at R183 (2). Q209 mutations are also slightly more potent in tumorigenesis assays in nude mice injected with transformed cells (2).

Mutations in GNAQ and GNA11 are found in a striking distribution pattern in different types of melanocytic neoplasms. They are frequently found in uveal melanoma (83%), blue nevi (benign intradermal lesions, 63%), and melanocytomas of the central nervous system (~50%; refs. 2, 18–21). They are very rare among melanomas and nevi located in the epithelium. Initially, we reported a single GNAQ<sup>Q209L</sup> mutation among 27 cutaneous melanomas on chronically sun damaged (CSD) skin (3); however, we found no additional mutations in a much larger CSD sample set, nor in any other type of cutaneous melanomas (n = 164; ref. 2). The COSMIC database v72 reports four additional patients with GNAQ<sup>Q209L</sup> mutations among 1,696 entries for superficial spreading, lentigo maligna, nodular, and otherwise unspecified malignant melanomas of the skin (22–26). GNAQ and GNA11 mutations have not been found in acral or conjunctival melanoma (2, 3, 27, 28). A GNAQ<sup>Q209L</sup> mutation was recently reported in a single case of mucosal melanoma (29).

Intrigued by this pattern, we wondered whether melanocytes in the epithelium possess some natural resistance to the oncogenic...
effects of constitutively active GNAQ/GNA11, which could be harnessed for potential therapeutics. To test this, we engineered a floxed-stop GNAQlox2lox, conditional knock-in allele at the ubiquitous Rosa26 locus to force expression in these cells, as well as in other types of melanocytes. We report here that the expression of the Rosa26-floxed stop-GNAQlox2lox allele induced by the melanocyte driver, Mitf-cre, causes the rapid development of uveal melanoma, with local invasion of blood vessels and multiple tumors developing in the lungs.

Materials and Methods

Mouse husbandry

The research described in this article was conducted under the approval of the UBC Animal Care Committee. Strains were crossed to the C3HeB/FeJ genetic background for at least 6 generations before use. DNA from ear notches was isolated using DNeasy columns (Qiagen) and amplified using PCR. Mitf-cre (Tg (Mitf-cre)7114Gcb), Tyrosinase-creER (Tg(Tyr-cre/EERT2)13Bois/L), and Dct-LacZ (Tg(Dct-LacZ)A12Rn) mice were genotyped as previously described (30–32). For intraperitoneal injection, tamoxifen (Sigma T5648) was dissolved in a corn oil/ethanol (10:1) mixture at a concentration of 10 mg/mL. Mice were injected with 1 mg per dose. For topical treatment, tails were dipped in 25 mg/mL 4-hydroxytamoxifen (Sigma H6278) in DMSO.

Production of Rosa26-floxed stop-GNAQlox2lox mice

Using pROSA26-1, pSAβgeo, and pGKnneoAlox2 plasmids and a human GNAQcox2lox cDNA (UMR CDNA Resource Center), a construct was built that contains the minimal adenosivus type 2 major late splice acceptor, a loxP-flanked neo stop cassette, human GNAQcox2lox, and a bovine growth hormone polyadenylation signal, all flanked by 1.08-kb and 4.34-kb Rosa26 homology arms, upstream and downstream of the cassette, respectively. The targeting vector was linearized by ScaI and then transfected into ES cell clones identified by Southern blotting and PCR and were injected into C57BL/6 blastocysts to produce chimeras, which successfully transmitted the mutant allele to the germline. The PCR reaction components to genotype the Rosa26-floxed stop-GNAQlox2lox allele are 0.25 mM/L of each dNTP, 1 U Hotstar Taq (Qiagen), 1× Hotstar Taq buffer, and 0.5 μM/L of each primer in 25 μL total volume. The reaction consists of 40 cycles of 95°C (30 seconds), 58°C (1 minute), and 72°C (1 minute), using 5’-CCGAAACTCTGCTGGAAGTC and 5’-TGCCCTCATATGGCCCTCTGAG as primers, which amplify a product of 180 base pairs.

Histology

Sections of intact cranium were taken after a 3-day fixation in 10% formalin, followed by decalcification (10% formalin, 88% formic acid). For LacZ analysis, tissue samples were stained for 16 to 48 hours at room temperature in 100 mM/L sodium phosphate, 2 mM/L magnesium chloride, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM/L potassium ferricyanide, 5 mM/L potassium ferrocyanide, 1 mg/mL 5-bromo-4-chloro-3-indolyl-b-β-galactosyanoside, and 2.5% dimethylformamide. Some samples were also then split into dermis and epidermis, or embedded, sectioned, and counterstained with eosin. To separate the dermis and epidermis of whole mount skin samples, the skin was incubated in 2 mM/L sodium bromide for 30 minutes to 2 hours at 37°C and separated with forceps. These sheets were group photographed and the number of melanocytes, pixel intensity, and/or area of each sample was determined using ImageJ software. For all histologic examinations, at least three individuals of each genotype were examined.

Immunofluorescence and immunohistochemistry

Whole eyes were fixed in 4% paraformaldehyde for 1 hour, washed in 8% 12%, 18%, and 20% sucrose (15 minutes each), incubated in 25% sucrose overnight, embedded in optimal cutting temperature, and sectioned at 8 to 12 μm. For immunofluorescence, sections were blocked with 1:25 mouse on mouse immunoglobulin blocking reagent (M.O.M.; Vector Labs) and BSA, incubated with mouse anti-RPE65 antibody (Abcam 13826, 1:250 dilution) overnight at 4°C, and then incubated for 1 hour at room temperature with Alexa594-conjugated goat anti-mouse antibody (1:500 dilution, Invitrogen). For immunohistochemistry, sections were bleached with 0.5% potassium permanganate for 20 minutes, 2% oxalic acid for 3 minutes, and then incubated in 0.3% hydrogen peroxide for 30 minutes. These sections were blocked with serum containing 1:25 M.O.M. and BSA, incubated with mouse anti-melanoma antibody cocktail (HM645 + DT101 + BC199; Abcam732, 1:50 dilution) overnight at 4°C, and then incubated 30 minutes with goat-anti-mouse-horseradish peroxidase secondary antibody (1:200, Life Technologies). Sections were washed and stained with DAB for 5 minutes.

For immunofluorescence of β-galactosidase in cultured melanocytes, cells were grown on coverslips, washed with 10 mM/L sodium phosphate (pH 7.3), fixed in 4% paraformaldehyde at 4°C for 10 minutes, blocked with 1:25 M.O.M. and BSA, incubated with mouse anti-β-galactosidase antibody (1:400, Promega, Z3781) overnight at 4°C, and then incubated for 1 hour at room temperature with Alexa594-conjugated goat anti-mouse antibody (1:500 dilution, Invitrogen).

Western blotting

Protein was extracted using the NE-PER Nuclear and Cytoplasmic Extraction kit and Halt Protease inhibitor cocktail (Thermo Scientific) and quantified using the Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich). After protein transfer to nitrocellulose, the membranes were blocked in 5% milk, divided into two sections, and either incubated with 1:1,000 anti-Yap (4912, Cell Signaling Technology) or 1:1,500 anti-GAPDH (14C10, Cell Signaling Technology) for 36 or 40 hours, respectively. Blots were washed two times in PBS for 5 minutes, then incubated with horseradish peroxidase-conjugated goat anti-rabbit (7074, Cell Signaling Technology) at either 1:1,000 or 1:2,000 dilution, respectively, for 3 hours at room temperature. Amersham ECL Prime (GE Healthcare) and a CCD-based imaging system were used to detect immunocomplexes. ImageJ was used to quantify the signal intensity, relative to a specific wild-type sample that was included in every experiment.

Pyrosequencing

Flash-frozen uveal melanomas from patients undergoing enucleation as a part of normal patient care were obtained under the approval of the institutional review boards at the University of British Columbia and the Vancouver General Hospital. Primary mouse melanocyte cultures were generated as previously described (33). DNA was extracted using DNeasy columns (Qiagen). RNA was extracted using RNAqueous-4PCR (Ambion), and reverse transcribed using random primers (Superscript VILO, Life
Technologies). For PCR, primers were designed to amplify Q209 in both human and mouse GNAQ/Gnaq alleles (Supplemental Materials and Methods). In the first round of PCR, Q209-containing products were amplified, while in the second round, biotin was tagged to the PCR products for subsequent immobilization. PCR reactions consisted of 0.25 mmol/L each dNTP, 1 U Hotstar Taq (Qiagen), 1 × Hotstar Taq buffer, and 0.5 μmol/L of each primer in 25 μL total volume, with roughly 100 ng of template. Reactions conditions were 95°C (30 seconds), 58.3°C (1 minute), and 72°C (1 minute) for 15 or 20 cycles (first and second rounds, respectively). Pyrosequencing was performed with the PyroMark Q96 MD pyrosequencer (Qiagen; ref. 34).

Auditory brainstem response

Auditory brainstem response tests were performed by the Mouse Biology Program at UC Davis (Davis, CA).

Statistical analysis

Data were analyzed with either ANOVA, Student's t test, or Welch two-sample t test.

Results

Creation of the Rosa26-flxed stop-GNAQO209L allele

We engineered a conditional allele in which GNAQO209L is expressed from the ubiquitous Rosa26 locus following the removal of a loxP flanked stop cassette that prevents transcription (35). We built a construct that contains a splice acceptor, a loxP-flanked stop cassette, human GNAQO209L, and a bovine growth hormone polyadenylation signal [Fig. 1]. This allele drives constitutive expression of a constitutive active GNAQ in cells that undergo Cre-mediated recombination and in all of the descendants of these cells. The resulting Rosa26-flxed stop-GNAQO209L mice were crossed to the CH3He/Fel genetic background for 6 generations before analysis. Rosa26-flxed stop-GNAQO209L mice are healthy, breed with normal efficiency, and are pigmented normally [Supplementary Fig. S6].

Skin pigmentation changes driven by GNAQO209L induced by Mitf-cre

To examine the effects of GNAQO209L expression initiated in melanoblasts (immature melanocytes) duringembryogenesis, we obtained Mitf-cre transgenic mice, which express Cre recombinase under the control of the melanocyte-specific promoter of the Microphthalmia gene (31). Mitf-cre is the earliest Cre driver expressed specifically in melanocytes. Mitf-cre has an efficiency of 25% at E15.5 (36) and 68% at P40 (Supplementary Table S1). On the CH3He/Fel genetic background, Mitf-cre animals are smaller than nontransgenic animals [Supplementary Fig. S1 and 60% exhibit microphthalmia (n = 35; Supplementary Fig. S4)]. The cause of these phenotypes is unknown (31). Mitf-cre is expressed in epidermal, dermal, and follicular melanocytes of the tail and trunk skin (31, 36). Expression in uveal, otic, and leptomeningeal melanocytes of the central nervous system (CNS) and spinal cord has not been previously reported.

We crossed Rosa26-flxed stop-GNAQO209L/+ animals to Mitf-cre/+ animals and obtained the expected percentage of double heterozygous progeny. Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ animals can be distinguished from their littermates 5 days after birth, because they exhibit early stages of tail darkening [Supplementary Fig. S2A]. Separation of the dermis and the epidermis at 3 weeks of age indicated that the dermis is hyperpigmented [Supplementary Fig. S2B]. Dermal hyperpigmentation is progressive, leading to extremely dark skin by 3 months, with visibly thickened ears [Fig. 2A]. Hyperpigmentation can be observed extending into the hypodermis in histologic sections [Fig. 2B]. The trunk dermis also contains abundant melanin, even though this area is usually sparsely pigmented [Fig. 2A and C].

In addition, we observed a melanocytic lesion on the trunk of two Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ animals [Fig. 2D]. Another lesion was found on the head of one Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ mouse [Supplementary Fig. S2C]. Although rare, these lesions were never observed in any other mice in our colony. We note that the localization of these lesions on the trunk and head is different from transgenic mice overexpressing the G protein–coupled Glutamate 1 receptor, Gm1, which might activate Gnaq/11 (37). Instead, Dct-Grm1 mice exhibit lesions on the tail and ears.

We next examined the effect of GNAQO209L expression in melanocytes located in the epidermis. In young Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ animals, the amount of melanin in the tail epidermis appeared to be normal [Supplementary Fig. S2B]. To quantify the number of melanocytes in the epidermis, we obtained the melanocytic reporter line, Dopachrome tautomerase (Dct)-LacZ, and crossed it to Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ mice. (Dct)-LacZ labels melanocytes beginning at E10.5 (38). In the epidermis, (Dct)-LacZ labels melanocytes in both the tail scales and hair follicles (38). At 3 weeks of age, we found no significant difference in the number of LacZ-positive cells in Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ tail scales as compared with Mitf-cre/+, supporting our observations of the melanin content at this time point (Fig. 3A).

However, in older animals, melanin content in the tail scales was significantly reduced [Fig. 3C]. We used the Rosa26-flxed stop-LacZ reporter allele to identify cells in which Cre had been expressed in sufficient levels to mediate recombination of loxP sites (35). At 5 weeks of age, LacZ-positive cells were present, but there were 39% fewer in the Rosa26-flxed stop-LacZ/Rosa26-flxed stop-GNAQO209L; Mitf-cre/+ tail scales as compared with the Rosa26-flxed stop-LacZ/+; Mitf-cre/+ controls (P = 0.018; Student's t test; Fig. 3B). This indicates that the expression of GNAQO209L negatively impacts the ability of melanocytes to persist in the interfollicular epidermal environment.

Hair follicles are epidermal appendages. Melanocytes actively producing pigment are located at the base of hair follicles, where they transfer pigment producing organelles called melanosomes to specialized keratinocytes of the growing hair. We found that in hair follicles, melanocytes respond to GNAQO209L by overgrowth. The hair follicle bulbs of Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ trunk [Fig. 2C] and tail [Fig. 3C] develop dark, ectopic pigmentation. Despite this, the coat starts to gray by 5 months, suggesting that GNAQO209L expression directly or indirectly disrupts normal melanosome transfer to keratinocytes [Fig. 3D, Supplementary Fig. S3].

Uveal melanoma driven by GNAQO209L induced by Mitf-cre

We selected Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ mice without severe microphthalmia for histologic analysis at various time points. At 3 months of age, obvious melanocytic neoplasms were present with 100% penetrance in Rosa26-flxed stop-GNAQO209L/+; Mitf-cre/+ mice.
Figure 1.
A conditional GNAQ<sup>Q209L</sup> allele. A, the targeted allele contains a splice acceptor, loxP-flanked stop cassette (PGKneo3xpAlox2), GNAQ<sup>Q209L</sup>, and polyadenylation signal. After loxP recombination, GNAQ<sup>Q209L</sup> is expressed (activated allele). PCR genotyping amplicon (green). B and C, Southern blot analysis using EcoRI and probing with PB3/4 (B), and EcoRV and probing with AT1/AT2 (C). ES cell clones 114, 132, 133, 242, and 263 are positive. H, HindIII; pA, polyadenylation signal; R, EcoRI; RV, EcoRV; SA, splice acceptor; triangles, loxP sites.
stop-GNAQQ209L/þ; Mitf-cre/þ animals (n = 15), but not in +/+; Mitf-cre/+ controls. In younger animals, the uveal tract (composed of the iris, ciliary body, and choroid) was thickened, with a mass forming at the anterior of the eye (Fig. 4A). Older animals exhibited bulging eyes (Supplementary Fig. S4) and bigger tumors that largely filled the vitreous space (Fig. 4C). We performed immunofluorescence using an antibody specific to the retinal pigmented epithelium (RPE; Fig. 4B), and immunohistochemistry with an anti-melanoma cocktail that stains human uveal melanomas (refs. 39, 40; Fig. 4C). The tumors were positive for the anti-melanoma antibody and negative for the RPE antibody. Furthermore, the RPE antibody labeled a single layer of cells above the lesion, with the sclera, the outermost layer of the eye, lying below the lesion. This indicates that the lesions are derived from the uveal tract. By 3 months of age, Rosa26-flxed stop-GNAQQ209L/þ; Mitf-cre/+ animals weighed significantly less than...
Mitf-cre/+ controls, as their overall health was impacted (Supplementary Fig. S1). We conclude that Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mice. A, no significant difference in the number of LacZ-positive cells in Rosa26-flxed stop-GNAQQ209L/+; Dct-LacZ/+; Mitf-cre/+ mice at P21 (mean ± SEM; P = 0.702 by t test). n, number of animals examined. Representative whole mount stained scales are shown (right). B, fewer LacZ-positive cells in Rosa26-flxed stop-GNAQQ209L/+; Rosa26-flxed stop-LacZ; Mitf-cre/+ animals at P40 (mean ± SEM; P = 0.018 by t test). C, extensive depigmentation in epidermal sheet of a 5-month-old Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mouse (right). Alterations in hair follicles are visible (compare red arrowheads). D, lighter colored coat of a 5-month-old Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mouse (right).

Expression of GNAQQ209L in mice and humans
To estimate the expression level of Rosa26-flxed stop-GNAQQ209L compared with endogenous Gnaq, we cultured melanocytes from 8.5 day-old mouse tails. We developed primary cultures from the dermis of three different Rosa26-flxed stop-GNAQQ209L; Mitf-cre/+ mice. A, no significant difference in the number of LacZ-positive cells in Rosa26-flxed stop-GNAQQ209L/+; Dct-LacZ/+; Mitf-cre/+ mice at P21 (mean ± SEM; P = 0.702 by t test). n, number of animals examined. Representative whole mount stained scales are shown (right). B, fewer LacZ-positive cells in Rosa26-flxed stop-GNAQQ209L/+; Rosa26-flxed stop-LacZ; Mitf-cre/+ animals at P40 (mean ± SEM; P = 0.018 by t test). C, extensive depigmentation in epidermal sheet of a 5-month-old Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mouse (right). Alterations in hair follicles are visible (compare red arrowheads). D, lighter colored coat of a 5-month-old Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mouse (right).
Figure 4.
Uveal melanoma in Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/++ mice. A, progression of uveal melanoma in 3-week-old (left bottom) and 5-week-old (right bottom) Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/++ mice. B, structures of the eye (top). Anti-RPE (red) stains a single layer of retinal pigmented epithelial cells above the developing melanoma in a 3-week-old Rosa26-flxed stop-GNAQ<sup>Q209L</sup>/++; Mitf-cre/++ mouse (right subpanel; bottom). Corresponding light microscopy images (left and middle). Red box, area of enlargement. C, a uveal melanoma from a 5-month-old mouse stains positive for the antimelanoma antibody cocktail (DAB, brown; top right). Melanoma before bleaching (left) and counterstained with hematoxylin (middle). Red arrow, extraocular extension of the melanoma. Hg, harderian gland; Nc, nasal cavity; red asterisks, uveal melanomas.
Figure 5.
Yap activation and enhanced GNAQ\textsuperscript{Q209L} allele expression in uveal melanomas. A, representative Western blot analysis against Yap and Gapdh using nuclear protein extracted from Rosa26-floxed stop-GNAQ\textsuperscript{Q209L}/+; Mitf-cre/+ and +/+ or Rosa26-floxed stop-GNAQ\textsuperscript{Q209L}/+; +/+ eyes (“wild-type”). B, increased nuclear Yap is detected in Rosa26-floxed stop-GNAQ\textsuperscript{Q209L}/+; Mitf-cre/+ eyes (P = 0.02, Welch two-sample t test). Signal was normalized to Gapdh and a wild-type sample included in each experiment. C, four of five human uveal melanoma samples contain more GNAQ\textsuperscript{Q209L} transcript than wild-type transcript, measured by pyrosequencing. Signal is corrected for the amount of each allele in the DNA of each sample. D, bilateral eye bulging (red arrowheads, left) and skin hyperpigmentation (red arrowheads, right) in a 4-month-old tamoxifen-treated Rosa26-floxed stop fs-GNAQ\textsuperscript{Q209L}/+; Tyr-creER/+ mouse, compared with treated control littermate. E, melanocytic hyperplasia (red arrowhead), but not uveal melanoma, in the eye of the same mouse.
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introduction, we were interested in forcing GNAQQ209L expression in melanocytes of the epidermis. We next asked what relative level of GNAQQ209L to wild-type transcripts is present in human uveal melanomas with a somatic GNAQQ209L mutation. We obtained flash-frozen uveal melanoma samples removed as a part of normal patient care, extracted DNA and RNA from each sample, reverse transcribed the RNA, and used pyrosequencing to measure the ratio of the GNAQQ209L allele to the wild-type allele in each cDNA sample, relative to the ratio in the corresponding genomic DNA sample. In 4 of 5 uveal melanomas, the GNAQQ209L allele was expressed at a higher level than the wild-type allele, as much as 1.7-fold more (Fig. 5C). Thus, greater levels of GNAQQ209L transcription may provide a selective advantage.

**Tyr-creER is not sufficient to drive uveal melanoma in mice when initiated in adulthood**

To determine whether Rosa26-flxed stop-GNAQQ209L drives uveal melanoma when induced in adulthood, we obtained Tyrosinase(Tyr)-creER mice (32). In this transgene, Cre recombinase is fused to the estrogen receptor and requires tamoxifen for the Cre protein to be transported to the nucleus for expression (Supplementary Fig. S2). When initiated by Tyr-creER introduction, we were interested in forcing GNAQQ209L expression in melanocytes of the epidermis. When initiated by Tyr-creER, Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mice (n = 28). This was due to melanocytic overgrowth within the leptomeninges (Fig. 6B).

We observed invasion of pigmented cells within the orbital–frontal cortex (Fig. 6A) and cerebellum and medulla oblongata (Fig. 6B). In one animal, a large darkly pigmented lesion was also recovered from the brain surface (Fig. 6C). Spinal cord meninges were heavily pigmented in all animals (Supplementary Fig. S7). These findings are consistent with the previously reported presence of GNAQ and GNA11 mutations in human melanocytomas of the CNS (18–21).

The stria vascularis of the cochlea of the inner ear is composed of basal cells, intermediate cells, and marginal cells. Intermediate cells are similar to melanocytes and arise from the neural crest, requiring Pax3, Sox10, c-Ki67, and Mitf (43–47). Through Na+/K+/2Cl– ion transportation, the stria vascularis produces an endocochlear potential in the endolymph. Changes in the potential in response to sound triggers hair cells to release neurotransmitters that excite afferent nerves. Because we noticed that some older Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mice failed to startle in response to loud noises, we tested the auditory brainstem response (ABR) of 5 Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ animals and 5 +/+; Mitf-cre/+ control littermates at two time points, P34 and P76. We found that at P76, there is a significant increase in the threshold needed to produce a brainstem response in Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mice compared with +/+; Mitf-cre/+ mice over a broad range of frequencies (ABR click test, Fig. 6D), with individual Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mice worsening in their ability to respond to auditory stimulus with age (Supplementary Table S2).

Melanocyte-like cells are also present in the vestibular system of the inner ear, which regulates balance. Between 1 and 3 months of age, Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mice develop abnormal behaviors that are suggestive of balance deficiencies (e.g., see Supplementary Movie S1). These behaviors include head tossing, head tilting, and flipping onto the back. We performed histologic analysis of the ears at 3 months of age and discovered an extensive overgrowth of pigmented cells, which filled the spaces in the cochlea and vestibular system (Fig. 6E). We note that there were no behavioral abnormalities observed in Rosa26-flxed stop-GNAQQ209L/+; Tyr-creER/+ animals treated with tamoxifen. These experiments indicate that Mitf-cre drives Cre expression in the melanocyte-like cells of the inner ear and that unregulated activity of GNAQ significantly impacts normal inner ear function.

**Evidence of GNAQQ209L-driven metastasis**

Ninety-four percent of the Rosa26-flxed stop-GNAQQ209L/+; Mitf-cre/+ mice (n = 19) exhibited at least four pigmented lesions within the lungs at 3 months of age (Fig. 7A). The size of the lung neoplasms ranged from 200 μm to 6 mm in diameter, with 4 to 18 tumors per affected mouse. These tumors could be primary, or be the result of metastasis. While examining the vasculature around the eyes, we found tracks of pigmented cells extending toward blood vessels (Fig. 7C) and invading vessel walls to grow within the lumen (Fig. 7D). An extension of a uveal lesion outside the eye can be seen in Fig. 4C. The lung lesions themselves exhibited blood vessel invasation (Fig. 7E). In addition, heavily pigmented and enlarged lymph nodes were present in every animal in the neck and trunk.
Figure 6.
Melanocytic hyperplasia in the CNS and inner ears. A, hyperpigmentation on the brain surface of a 3-month-old Rosa26-floxed stop-GNAQ<sup>Q209L</sup>/; Mitf-cre/+ mouse (middle). Invasion of pigmented cells into the orbital frontal cortex (red asterisk), visible in coronal section (right bottom). B, thickened and hyperpigmented leptomeninges (white arrows in enlargement, top right). Invasion of pigmented cells into the cerebellum and medulla oblongata (white arrowheads in enlargement, right bottom). C, lesion found on the surface of the brain (right). Deep indentation (red arrowhead) left on brain after the lesion was removed. D, hearing loss in Rosa26-floxed stop-GNAQ<sup>Q209L</sup>/; Mitf-cre/+ animals at P76 (right), but not P34 (left; mean ± SEM; P = 0.249 at P34; P = 0.044 at P76; ABR click test). E, melanocytic hyperplasia in the cochlea and vestibular system of a Rosa26-floxed stop-GNAQ<sup>Q209L</sup>/; Mitf-cre/+ animal (hematoxylin and eosin). CB, cerebellum; Co, cochlea; dB SPL, decibels of sound pressure level; LM, leptomeninges; MO, medulla oblongata; OB, olfactory bulb; OFC, orbital–frontal cortex; V, vestibular system.
Figure 7.
Melanocytic lesions in the lungs and blood vessel intravasation. A, multiple pigmented lesions in the lungs of a 3-month-old Rosa26-flxed stop-GNAQ foam/þ; Mitf-cre/þ mouse (red arrowheads). Front and back of lungs are shown. B, enlarged and darkly pigmented lymph nodes from a 3-month-old Rosa26-flxed stop*-GNAQ foam/þ; Mitf-cre/þ mouse (left). C and D, tracks of pigmented cells extending from eye lesion toward blood vessels (open arrowheads in C), invading vessel walls (black arrow in D), or growing within the lumen (white arrow in D) in 3-month-old Rosa26-flxed stop*-GNAQ foam/þ; Mitf-cre/þ mice (hematoxylin and eosin). Enlarged areas on the right are indicated by white boxes on left. E, lung lesion closely associated with blood vessels showing signs of intravasation (black arrow) in a 3-month-old Rosa26-flxed stop*-GNAQ foam/þ; Mitf-cre/þ mouse (hematoxylin and eosin). BV, blood vessel.
Rosa26
Gnaq
and
Hyperactive, but not constitutively active, germline mutations in GNAQ have been frequently found in melanoma and nevi located in the dermis, but are rare in human melanoma and nevi that are located in the epithelium (2). It is not known why lesions located in the epidermis and dermis exhibit different mutational signatures, although there are several possibilities. First, there could be intrinsic or developmental differences among melanocytes colonizing different parts of the skin (36, 50). Second, there could be different paracrine signals emanating from keratinocytes versus fibroblasts, surrounding melanocytes in the epidermis and dermis, respectively. Third, melanocytes in the dermis and epidermis could be exposed to different types and/or intensities of environmental mutagens, such as UV light.

We were curious as to whether the absence of GNAQ expression in lesions located in the epithelium was a result of a lack of expression of GNAQ or a differential response downstream of it. To test this, we used the ubiquitous Rosa26 promoter to force expression of GNAQQ209L in all melanocytes. This normalized GNAQQ209L expression levels among melanocytes and prevented built-in feedback mechanisms that could suppress its expression. We found that GNAQQ209L expression appears to be incompatible for melanocyte residence in the interfollicular epidermis. Furthermore, GNAQQ209L is extremely oncogenic for uveal melanocytes, but is far less so for dermal melanocytes. We conclude from these observations that the downstream effects of GNAQQ209L vary greatly depending upon the location of the melanocyte. As it is reasonable to assume that cultured melanocytes receive no positional cues in a typical in vitro environment, their response to GNAQ and GNA11 may be different than that of uveal melanocytes in the eye. Going forward, Rosa26-floxed stop-GNAQQ209L/+; Mitf-cre/+ mice could serve as a valuable tool to validate the pathways activated downstream of GNAQQ209L in the eye, as we have done here for the Hippo pathway.

The role of GNAQQ209L appears to be highly conserved in that there are similar effects in the same populations of melanocytes in both mice and humans; however, this is not always the case. Just considering the skin, melanocytic hyperploration occurs exclusively in the dermis in Hgf, Grm1, and Edn3 mouse models, which is expected, but also in Braf mutant mice, which is not, given that BRAF mutations are abundant in nevi and melanoma located in the epidermis (37, 41, 51–53). The dermal location of Braf600E melanocytic lesions has been suggested to be the result of a rarity of interfollicular epidermal melanocytes in mice; however, these cells are numerous in the mouse tail and could respond to a Braf mutation if there was not some unknown difference between them and human interfollicular epidermal melanocytes. To define these differences, a genome-wide characterization of the genes and pathways that are active in melanocytes in different locations would be very helpful. In humans, this could also immediately help underpin the different frequencies of oncogenic and tumor suppressor mutations found in different types of melanoma.

The Rosa26-floxed stop-GNAQQ209L mice are the first genetically modified mice to develop uveal melanoma. We note that the expression of Rosa26-floxed stop-GNAQQ209L induced by Mitf-cre is more potent than Rosa26-floxed stop-GNAQQ209L induced by Tyr-creER. This might either be due to differences in the target population of melanocytes that express these two transgenes, or maybe to the difference in the time of initiation of expression. The transcription factor, Mitf, is a critical determinant of melanocyte cell fate (54), while Tyrosinase, a melanogenic enzyme, is expressed only in differentiated melanocytes (55). Therefore, Mitf might be expressed in cells with a higher potential for transformation. Recently, the promoter of another melanogenic enzyme, Dopachrome tautomerase (Dct), was used to drive transgenic GNAQQ209L expression in a tetracycline-inducible mouse model (13). Despite expression beginning during embryogenesis, no ocular or skin lesions developed. Pigmentation could not be assessed because the mice were on the FVB genetic background, which is albino. When the tumor suppressors, p16 and p19, were also knocked out, 50% of these mice developed lesions on the trunk by 9 months of age, but there were still no reported effects on the eye. Thus, Mitf-cre may better target uveal melanocytes or melanocytes with a higher melanomagenic capacity than Tyr-creER or Dct-cre. Another intriguing possibility that remains to be tested is whether the Mitf-cre BAC transgene somehow alters Mitf protein levels, which could theoretically contribute to melanogenesis (56).

In summary, we have developed the first mouse model of uveal melanoma driven by oncogenic GNAQ. The three known human lesions driven by GNAQ/11 mutations are recapitulated in the mice (blue nevi, uveal melanoma, and melanocytomas of the CNS). We have established that GNAQQ209L has multiple effects depending upon the location of the melanocytes. The rapid localized invasion of blood vessels and multiple lung tumors by 3 months of age suggests that GNAQQ209L mutations may not only help initiate uveal melanoma, as previously hypothesized, but also contribute to disease progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: J.L.-Y. Huang, C.D. Van Raamsdonk
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Oncogenic G Protein GNAQ Induces Uveal Melanoma and Intravasation in Mice

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