GNA11 Q209L Mouse Model Reveals RasGRP3 as an Essential Signaling Node in Uveal Melanoma

Highlights
- GNA11 Q209L mouse model induces uveal, cutaneous, and leptomeningeal melanoma
- Loss of Bap1 promotes aggressive melanomas
- RasGRP3 links GNA11/GNAQ activation to RAS activation
- RasGRP3 is required for GNA11/GNAQ-driven tumorigenesis

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In Brief
Moore et al. generate a preclinical mouse model of melanoma that recapitulates features of aggressive uveal melanoma. By comparing murine and human melanomas, they identify a dependency on RasGRP3 in uveal melanoma.

Data and Software Availability
GSE97225
GNA11 Q209L Mouse Model Reveals RasGRP3 as an Essential Signaling Node in Uveal Melanoma

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SUMMARY

Uveal melanoma (UM) is characterized by mutually exclusive activating mutations in GNAQ, GNA11, CYSLTR2, and PLCB4, four genes in a linear pathway to activation of PLCβ in almost all tumors and loss of BAP1 in the aggressive subset. We generated mice with melanocyte-specific expression of GNA11Q209L with and without homozygous Bap1 loss. The GNA11Q209L mice recapitulated human Gq-associated melanomas, and they developed pigmented neoplastic lesions from melanocytes of the skin and non-cutaneous organs, including the eye and leptomeninges, as well as at atypical sites, including the lymph nodes and lungs. The addition of Bap1 loss increased tumor proliferation and cutaneous melanoma size. Integrative transcriptome analysis of human and murine melanomas identified RasGRP3 to be specifically expressed in GNAQ/GNA11-driven melanomas. In human UM cell lines and murine models, RasGRP3 is specifically required for GNAQ/GNA11-driven Ras activation and tumorigenesis. This implicates RasGRP3 as a critical node and a potential target in UM.

INTRODUCTION

Uveal melanomas (UMs) arise from the melanocytes of the eye. While localized disease can be effectively treated surgically, half of all patients develop metastasis, and metastatic UM carries a dismal prognosis with an overall survival of only 6 months (Diener-West et al., 2005). Approximately half of the patients harbor metastases to multiple organs, with liver (93%), lung (24%), bone (16%), and lymph nodes (10%) representing the most common sites (Collaborative Ocular Melanoma Study Group, 2001). Over the past decade, we have gained considerable insight into the genetic basis of UM. This has not yet led to novel therapeutic options and there are still no proven systemic treatments for UM.

UM is highly distinct from cutaneous melanoma (CM) both clinically and molecularly. UM is not associated with sun exposure and has among the lowest mutation rates in cancer, whereas CM has among the highest mutation rates due to UV damage (Furney et al., 2013). A recent comparison of liver metastasis revealed that most CM metastases lacked gross melanin pigmentation while most UM metastases are hyperpigmented and express high levels of melanocyte lineage proteins, such as MART-1 (MLANA) and gp100 (PMEL) (Rothermel et al., 2016). Molecularly, CM is driven by recurrent somatic mutations that activate the mitogen-activated protein kinase (MAPK) pathway, including BRAF, NRAS, NF1, and KIT. Approximately 90% of UM harbor activating mutations in two homologous G-protein alpha (Gα) subunits, GNA11 (Gα11) and GNAQ (Gαq), at codons Gin209 or Arg183 (Robertson et al., 2017; Van Raamsdonk et al., 2009, 2010). Among the remaining 10% of UMs, most harbor activating mutations in a G-protein-coupled receptor (CYSLTR2) at the Leu129 codon activates Gα2 (PLCB4) or in phospholipase C β4 (PLCB4) (at the Asp630 codon, a direct downstream effector of Gα11) or in phospholipase C β4 (PLCB4) to produce the second messengers diacylglycerol [DAG] and inositol triphosphate [IP3] (Johansson et al., 2016; Moore et al., 2016). This indicates a requirement for Gα11/11-coupled signaling and, in particular, the phospholipase C β4 (PLCB4) effector pathway in the initiation of UMs.
While essentially all UMs harbor mutations in the CYSLTR2-G\(\alpha_{11/q}\)-PLC\(\beta\) pathway, the prognosis is largely determined by the presence of cooperative mutations. Monosomy 3 and an associated poor-prognosis gene expression pattern is the single most negative prognostic factor. Most of these tumors harbor inactivating mutations in \(BAP1\), located at 3q21, and essentially all of these tumors lose expression of the \(BAP1\) protein, implicating \(BAP1\) loss as a critical cooperating lesion driving poor prognosis in UM (Harbour et al., 2010; Robertson et al., 2017). Among tumors with disomy 3, there are mutually exclusive mutations in \(SF3B1\), associated with intermediate prognosis, and in \(EIF1AX\), associated with favorable prognosis (Martin et al., 2013; Robertson et al., 2017).

In addition to UM, CYSLTR2-G\(\alpha_{11/q}\)-PLC\(\beta\) pathway mutations are found in most leptomeningeal melanocytic neoplasms (LMNs) and blue nevi (Möller et al., 2017; Van Raamsdonk et al., 2009). LMNs are rare neoplasms arising from melanocytes of the leptomeninges. Like UM, in addition to mutations in \(GNA11\) and \(GNAQ\), LMNs harbor mutually exclusive co-mutations in either \(EIF1AX\) or \(SF3B1\) in 33% of cases (Küsters-Vandevelde et al., 2016). Blue nevi are common benign neoplasms of dermal melanocytes, which are distinguished from CMs that arise from epidermal melanocytes. Rare malignant melanomas that either arise from blue nevi or show morphologic features of blue nevi are called malignant blue nevi. Recent genetic characterization of a large cohort of blue nevi showed both benign and malignant blue nevi harbored CYSLTR2-G\(\alpha_{11/q}\)-PLC\(\beta\) pathway mutations (Möller et al., 2017). \(EIF1AX\) mutations are found only in benign blue nevi, while \(SF3B1\) and \(BAP1\) mutations are found only in malignant blue nevi (Griewank et al., 2017). Therefore, UM, LMN, and blue nevi represent a molecularly similar spectrum of diseases that commonly harbor CYSLTR2-G\(\alpha_{11/q}\)-PLC\(\beta\) mutations and whose disease aggressiveness is defined by co-mutations, especially \(BAP1\).

Because the PLC\(\beta\) pathway is known to activate MAPK, the MEK inhibitor selumetinib has been clinically studied. While a phase 2 trial showed promising improvement in progression-free survival, the phase 3 trial failed to confirm the finding and neither trial showed an improvement in overall survival (Carvajal et al., 2014; KomatsuBara et al., 2016). As different MEK inhibitors have distinct properties (Lito et al., 2014), it is still currently unclear whether the MAPK pathway remains a viable therapeutic target in UM.

To identify molecular and lineage events downstream of \(G\alpha_{11}\) activation, the cooperative role of \(BAP1\) loss, and critical nodes required for \(G\alpha_{11}\)-mediated tumorigenesis, we generated a conditional Rosa26-LSL-GNA11\(^{Q209L}\) mouse model and crossed it with conditional \(Bap1\) knockout (KO) mice. These mice recapitulate the features of human \(G\alpha_{11}\)-driven melanomas. They developed neoplastic hyperpigmented melanocytic lesions in the uveal tract, skin, and leptomeninges. These mice developed lesions in the lung and lymph nodes. Deletion of \(Bap1\)accelerated skin tumor growth and mouse mortality. The GNA11\(^{Q209L}\) Bap1 loss tumors were resistant to the MEK inhibitor trametinib. To identify alternative therapeutic targets, we performed integrative analysis comparing \(BRAF\) mutant and \(G\alpha_{11}\) mutant human and murine cancers, and we identified a critical requirement of a Ras guanine exchange factor (GEF), RasGRP3, for \(G\alpha_{11}\)-mediated tumorigenesis.

**RESULTS**

**Melanocyte-Specific GNA11\(^{Q209L}\) Expression Induces Skin, Uveal, and CNS Neoplasia**

To express GNA11\(^{Q209L}\) in the melanocyte lineage, we generated a genetically engineered mouse model (GEMM) with a conditional GNA11\(^{Q209L}\) allele (R26-LSL-GNA11\(^{Q209L}\)) under the control of the endogenous Rosa26 promoter (Figures S1A and S1B). To identify an active Cre-driver for uveal melanocytes, we crossed \(Tyr-CreERT2\) transgenic mice that express tamoxifen-inducible CreER\(^T2\) under the melanocyte-specific Tyrosinase (\(Tyr\)) promoter with the CAG-LSL-EYFP reporter. One week after tamoxifen injection, there was robust EYFP expression in both uveal and skin (hair follicle) melanocytes (Figure S1C). We thus generated \(Tyr-CreERT2;GNA11^{Q209L}\) mice for our studies.

To activate GNA11\(^{Q209L}\) expression, we treated 4-week-old mice with a single application of tamoxifen or vehicle by intraperitoneal injection. In tamoxifen-injected, but not vehicle-injected, mice, we observed hyperpigmentation of ears and tail within 2 weeks of treatment and bulging eyes within 1 month of treatment (Figure S2A). In vehicle-treated \(Tyr-CreERT2\)-positive or tamoxifen-treated \(Tyr-CreERT2\)-negative control mice, there was no discernible pathologic phenotype in the skin or the uveal tract up to 18 months (Figures 1A, 1B, S2B, and S2C). In \(Tyr-CreERT2;GNA11^{Q209L}\) mice, pathological analysis of the skin 3 months post-induction showed extensive follicular and dermal melanocytic proliferation (Figure 1C), which progressed to melanomas encompassing the dermis and subcutaneous tissues in 50% of mice by 6 months after injection (Figure 1D). These proliferating melanocytes stained positive for a melanocyte cocktail (Figure S2D). Tumor cells contained abundant pale amphiophilic cytoplasm and a small nuclear-to-cytoplasmic ratio (Figure S2D). For comparison, we also treated \(Tyr-CreERT2;Braf^{V600E}\) mice, which express the conditional \(Braf^{V600E}\) allele in melanocytes. We observed melanocytic hyperplasia without progression to melanoma up to 12 months post-induction, similar to previous observations (Figure S2E) (Dankort et al., 2009).

We next examined the oncogenic role of GNA11\(^{Q209L}\) and \(Braf^{V600E}\) expression in uveal melanocytes. Within the uveal tract, tamoxifen-injected \(Tyr-CreERT2;GNA11^{Q209L}\) mice displayed diffuse hyperplasia, thickening of the chorioid and ciliary body that progressed over time to overt UM with intraocular infiltration that distorted the normal architecture of the globe (Figures 1E and 1F). Uveal melanocyte proliferation was evident in mice as early 1 month post-tamoxifen (data not shown). In contrast, the uveal tracts of induced \(Tyr-CreERT2;Braf^{V600E}\) mice were indistinguishable from those of control mice (Figures S2F and S2G). Further examination revealed tumor cells with pathological characteristics similar to skin melanoma (Figure 1G, i and ii). These cells were positive for MITF and melanocyte cocktail staining (Figure 1G, iii and iv). We further observed perineural spread of malignant melanoma to the optic nerve (Figure 1H).

We next examined the effect of GNA11\(^{Q209L}\) in resident melanocytes of other organs, including the heart, harderian gland,
and brain (Aoki et al., 2009). Gross examination of the brain revealed focal pigmentation of the leptomeninges in 80% of mice (Figure S3A). Pathological evaluation of the CNS of the Tyr-CreER<sup>T2</sup>;GNA11<sup>Q209L</sup> mice showed melanocytic proliferation in the leptomeninges at the base of the brain, around cranial nerve roots, and within the longitudinal fissure (Figure 2A, ii–iv). There was prominent melanocytic hyperplasia within the third ventricle (Figure 2A, i and iv). Clinically, primary melanocytomas occasionally occur within the ventricular system of the CNS (Tandon et al., 2008). There was invasion of melanocytes to the

Figure 1. Melanocyte-Specific GNA11<sup>Q209L</sup> Expression Induces Cutaneous and UM

(A and B) H&E of skin (A) and eye (B) from Tyr-CreER<sup>T2</sup>-negative control mice 3 months post-induction.
(C and D) H&E of skin from GNA11<sup>Q209L</sup> mice 3 months (C) or 6 months (D) post-induction.
(E and F) H&E of eyes from GNA11<sup>Q209L</sup> mice 3 months (E) or 6 months (F) post-induction.
(G) H&E (i), H&E with melanin bleaching (ii), MITF immunohistochemistry (IHC) using diaminobenzidine (DAB) (brown) (iii), and melanoma cocktail IHC using red chromogen (red) (iv) from a GNA11<sup>Q209L</sup> mouse 6 months post-induction. High-magnification insets of top panels are shown in bottom panel.
(H) H&E of a selected eye (i) with melanocytic perineural invasion of the optic nerve (ii).

See also Figures S1 and S2.
periventricular space, and one mouse exhibited invasion to the olfactory bulb (Figure 2A, iv and v). We observed robust proliferation of resident melanocytes in the harderian gland (Figure 2A, arrow).

Examining the melanocytes of the heart in GNA11 Q209L mice, we observed invasive neoplasms that infiltrated and thickened the tricuspid valve and infiltrated the myocardium of the right atrium and interventricular septum (Figures 2B and 2C). We suspect these lesions were primary tumors of the resident melanocytes of the heart. Melanocytic lesions were not evident in Tyr-CreERT2;BRafCA/+ mice in these areas (Figures S3C and S3D).

In GNA11 Q209L mice, we observed multi-focal lesions in the lungs that may represent metastases, although we cannot rule out transformation of rare resident lung melanocytes (Figures 2B, 2D, and 2E). However, the morphology of these lesions resembled the primary tumors (Figures 1D, 1G, ii, 2B, and 2C). These lesions occurred early and were observed in mice with less advanced skin and uveal lesions (Figure S3E). We observed melanocytes infiltrating the lymphatic system, as visualized by MITF staining in the axillary lymph nodes (Figures 2F and 2G).

In contrast, we did not observe melanocytic lesions in the CNS, heart, lungs, or lymph nodes in Tyr-CreERT2;BRafCA/+ mice.

Figure 2. Melanocyte-Specific GNA11Q209L Expression Induces Leptomeningeal Melanocytic Neoplasia and Possible Metastasis

(A) H&E of coronal skull sections (left), magnified images of regions showing melanocytic neoplasia in the (i) choroid plexus of the third ventricle (i), leptomeninges of brain base surrounding cranial nerve roots (ii and iii), longitudinal fissure and ependyma of the third ventricle olfactory bulb (iv and v). Arrow indicates the harderian gland.

(B) H&E of heart and lung. Arrows indicate lesions.

(C) Magnification of heart in (B) showing melanoma in the tricuspid valve, right atrial wall, and interventricular septum indicated by box and gray arrow in (B).

(D and E) Magnification of a selected melanocytic lesion (D) in the lung indicated by box and arrow in (B) and with melanin bleaching (E).

(F and G) H&E (F) and MITF IHC (G) of axillary lymph node melanocytic lesion (G, ii).

See also Figure S3.
Figure 3. Bap1 Loss Promotes Aggressive Melanomas
(A) Photographs of GNA11Q209L (left) and GNA11Q209L Bap1KO (right) mice 3 months post-induction.
(B and C) H&E of eyes from GNA11Q209L (B) and GNA11Q209L Bap1KO (C) mice 12 months post-induction.
(D) Kaplan-Meier curve comparing the survival percentage of GNA11Q209L (blue) or GNA11Q209L Bap1lox/+ (green) to GNA11Q209L Bap1KO mice (red). p = 0.0013.
(E and F) Photograph of large invasive melanoma from the tail dermis (E) and H&E (F) from a GNA11Q209L Bap1KO mouse 12 months post-induction.
(G) H&E of an invasive melanoma from the dermis of the ear from a GNA11Q209L Bap1KO mouse 12 months post-induction.
(H) Ki-67 IHC in GNA11Q209L (top) and GNA11Q209L Bap1KO (bottom) cutaneous tumors.
(I) Quantification of Ki-67-positive cells. Scatter-dot plot: each dot represents the quantification of one field. Error bars represent means ± SEM. p < 0.0001.
(J) GSEA plot using a gene set comprised of genes upregulated in mice (Mouse_Bap1KO_UP) on a profile of genes ranked by correlation to BAP1 expression in the UM TCGA dataset.

(legend continued on next page)
mice (Figures S3B–S3D). Our data are consistent with the clinical absence of BRAF mutations in UMs and LMNs and the sporadic occurrence of GNAQ/11 mutations in CMs.

Recently, a mouse model harboring GNAQQ209L was characterized (Huang et al., 2015). When activated in melanoblasts during embryogenesis with Mif-Cre, the mice exhibited UM, LMN, neoplastic melanocytic growth in the hardier glands, and rare lesions in the skin, as well as lymph nodes and lung (Huang et al., 2015). When activated in 8-week-old adult mice using Tyr-CreERT², GNAQQ209L drove melanocyte overgrowth without progression to melanoma. The phenotype of our Tyr-CreERT²; GNA11Q209L mouse model, activated at 4 weeks, appears to be an intermediate between Mitf-Cre activated before birth and Tyr-CreERT² activated at 8 weeks. The Mitf-Cre;GNAQQ209L mice developed earlier invasive UM and more diffuse LMN, likely due to earlier expression at mid-gestation and prolonged Cre activation (Alizadeh et al., 2008).

**Loss of Bap1 Accelerates Skin Melanomas in the Presence of GNA11Q209L**

We sought to examine the combinatorial effect of GNA11Q209L and the loss of the tumor suppressor Bap1 in the development of UM. To achieve Bap1 deletion, we crossed Bap1lox/lox mice (LaFave et al., 2015) to the Tyr-CreERT²;GNA11Q209L line. Tamoxifen-treated Tyr-CreERT²;Bap1KO mice had no discernible phenotype and were histologically normal over ~20 months, indicating Bap1 loss alone was insufficient to initiate melanoma (n = 35; Figures S4A and S4B).

We compared the Tyr-CreERT²;GNA11Q209L;Bap1lox/lox mice to the Tyr-CreERT²;GNA11Q209L mice. We observed a stronger ocular phenotype in GNA11Q209L than GNA11Q209L Bap1KO mice (Figures 3A–3C). However, the GNA11Q209L Bap1KO mice succumbed to disease at an accelerated rate compared to GNA11Q209L or GNA11Q209L Bap1LOX/+ mice (Figure 3D; p < 0.05), due to increased skin melanoma burden (Figures 3E and 3F). The loss of Bap1 in these mice did not appreciably alter the size or incidence of uveal lesions, but it contributed to an increased progression to skin melanomas originating from the tail and ears (Figures 3E–3G). We confirmed Bap1 KO in uveal melanocytes using PCR (Figure S4C). In human UM patients, no pigmented liver lesions were observed (Figures S4D and S4E). We observed no significant increase in the size or incidence of lung lesions in the absence of Bap1 (Figures S3E and S4F–S4H). Histologically, GNA11Q209L skin melanomas exhibited slender oval nuclei while GNA11Q209L;Bap1KO had larger euchromatic nuclei (Figure 3H). GNA11Q209L;Bap1KO skin melanomas exhibited a higher proliferation index (Figures 3H and 3I; p < 0.0001).

To determine the extent that Bap1 KO molecularly recapitulates human melanoma and to understand the Bap1-regulated transcriptional programs, we performed transcriptome analysis of mouse and human melanomas. RNA sequencing (RNA-seq) revealed the R26-GNA11Q209L transcript level was 4- to 8-fold lower than endogenous murine Gnaq and Gna11 transcript levels, indicating modest expression of mutant GNA11 is required for tumorigenesis (Figure S5A). Analysis of RNA-seq of GNA11Q209L and GNA11Q209L;Bap1KO skin melanomas confirmed deletion of Bap1 (Figure S5B), using a gene set comprised of genes upregulated in GNA11Q209L;Bap1KO versus GNA11Q209L skin melanomas (Mouse_Bap1KO_UP), we performed gene set enrichment analysis (GSEA) using The Cancer Genome Atlas (TCGA) UM dataset (Robertson et al., 2017). This showed Mouse_Bap1KO_UP genes are significantly enriched among genes negatively correlated with BAP1 expression in UM, suggesting BAP1 deletion in skin melanomas of mice results in the upregulation of similar genes to human UM (Figure 3J; Table S1). To explore the function of the shared genes, we performed functional annotation of the leading edge genes (Figure 3J, red) that drove the GSEA enrichment. We found the most enriched gene ontology (GO) and Swiss-Prot (SP) pathways all involved cell cycle and mitosis (Figure 3K).

In a complementary approach, we identified a UM primary tumor line, UPMM3, contained a frameshift deletion of BAP1 (Figure S5C) (Griewank et al., 2012). We restored wild-type BAP1 (Figure S5D) and generated a gene expression profile using RNA-seq. As controls, we expressed BAP1 with mutations in the deubiquitinase domain (p.Cys91Trp, p.Ala95Pro) found in cancer (Harbour et al., 2010) as well as EGFP. Wild-type BAP1 restoration significantly changed gene expression while the presumably non-functional mutants did not, observed by gene hierarchical clustering (Figure S5E). We next performed GSEA, and we found the Mouse_Bap1KO_UP gene set was significantly enriched among genes downregulated in UPMM3 cells by BAP1 (wild-type [WT] restoration (Figure 3L). Functional analysis of leading edge genes showed cell cycle pathways were enriched (Figure 3M). GSEA on three BAP1 datasets (mouse model, TCGA, and UPMM3) using the >8,300 gene sets from the Molecular Signatures Database (MSigDB) showed cell cycle and melanoma metastasis signatures are highly enriched in each (Table S1; Figures S5F and S5G). Therefore, in UM, the loss of Bap1 can promote aggressive disease with a propensity to proliferate and metastasize, consistent with clinical data implicating BAP1 loss as a poor prognostic biomarker.

**Gz11/q-Driven Cells Have Reduced Sensitivity to MEK Inhibition**

Activation of the Gz11/q-PLCβ pathway leads to downstream activation of the MAPK pathway. While preclinical data using UM cell lines suggest MEK inhibition may be a therapeutic strategy (Ambrosini et al., 2012), a recent phase 3 study comparing selumetinib and chemotherapy failed to show significant improvement in progression-free or overall survival (Komatsubara et al., 2016).
Prolonged selumetinib treatment induces RAF-MEK dimer formation, leading to reactivation of MAPK signaling, particularly in non-BRAFV600E-driven tumors. The newer MEK inhibitor trametinib uniquely decreases RAF-MEK interaction and MAPK reactivation (Lito et al., 2014).

To address whether improved MEK inhibition can lead to therapeutic efficacy in UM, we utilized the GEMMs to perform in vivo trametinib treatment. We needed relevant control tumors that formed nodules of a similar size and were responsive to trametinib treatment. We observed Bap1KO also accelerated BRafV600E-driven tumors to form nodules amenable for treatment (unpublished data). We subcutaneously grafted skin melanomas, isolated from BrafV600E;Bap1KO and GNA11Q209L;Bap1KO mice, into severe combined immunodeficiency (SCID) mice. The grafts retained features of the in situ tumors, where GNA11Q209L;Bap1KO tumors retained hyperpigmentation whereas BRafV600E;Bap1KO tumors were hypopigmented and exhibited elevated MAPK output (Figures 4A and 4B). In BrafV600E;Bap1KO tumors, short-term trametinib treatment decreased proliferation and MAPK output, whereas in GNA11Q209L;Bap1KO tumors, the effects were modest (Figures 4A–4C). Long-term treatment resulted in initial tumor shrinkage followed by stabilization in BRafV600E Bap1KO mice, into severe combined immunodeficiency (SCID) mice. The grafts retained features of the in situ tumors, where GNA11Q209L;Bap1KO tumors retained hyperpigmentation whereas BRafV600E;Bap1KO tumors were hypopigmented and exhibited elevated MAPK output (Figures 4A and 4B). In BrafV600E;Bap1KO tumors, short-term trametinib treatment decreased proliferation and MAPK output, whereas in GNA11Q209L;Bap1KO tumors, the effects were modest (Figures 4A–4C). In BRafV600E Bap1KO tumors, short-term trametinib treatment decreased proliferation and MAPK output, whereas in GNA11Q209L Bap1KO tumors, the effects were modest (Figures 4A–4C).

To determine if relative resistance to trametinib treatment was more generalized, we treated human BRAFV600E CM and Gx11/q14 mutant UM cell lines with a clinically achievable concentration of trametinib (10 nM) (Infante et al., 2012). Trametinib sustainably inhibited MAPK in CM cells. In UM cells, MEK phosphorylation was stable or increased over time and ERK phosphorylation was variably but re-bounded by 24 hr (Figure 4E). This is consistent with known hyper-persensitivity of BRAFV600E melanoma to MEK inhibition (Soill et al., 2006). Together, activating mutations in the Gx11/q-mediated tumorigenesis, we screened a panel of UM and CM cells, and we found RasGRP3 to be expressed exclusively in UM cells (Figure 5G).

**Cross-Species Analysis Shows Gx11/q-Driven Tumors Enforce a Melanocyte Lineage Program and Express High Levels of RasGRP3**

To identify critical nodes in Gx11/q-mediated tumorigenesis, we utilized a cross-species transcriptome analysis approach of human and murine melanoma. We sought to generate a transcriptional signature of Gx11-q-driven GEMM melanoma. We generated GEMM Gx11 and Braf signatures with differentially expressed genes between GNA11Q209L Bap1KO and BrafV600E Bap1KO melanomas (>3-fold, false discovery rate [FDR] < 0.01). In human melanoma, we combined and curated TCGA skin CM (SKCM) (Cancer Genome Atlas, 2015) and UM datasets, and we compared tumors with hotspot mutations in Gx11/q (74/80 UM and 5/333 SKCM) with BRAFV600E (0/80 UM and 121/333 SKCM). GSEA using the GEMM signatures on the TCGA transcriptomes showed significant enrichment of the Gx11 and Braf signatures in human Gx11/q-mutated and BRAFV600E tumors, respectively (Figure 5A). Therefore, the oncogenic signaling driver, in addition to the location of the tumor, contributes to the oncogenic transcriptome. Functional annotation of the leading edge Gx11/q signature genes showed upregulation of pigmentation and melanocyte differentiation pathways (Figures 5B–5D), consistent with the observation of highly pigmented melanomas in the GNA11Q209L GEMM (Figures 1, 2, and 4). This is also consistent with the clinicopathological observations that metastatic UMs retain greater pigmentation than CMs (Rothermel et al., 2016) and with our previous observation that CYSLTR2<sup>1290</sup> enforces a melanocyte lineage (Moore et al., 2016).

Further examination of top-ranked genes identified RasGRP3 as highly expressed in both Gx11/q-mutated human and GEMM melanomas (Figures 5E and 5F). Pan-cancer analysis of RNA-seq datasets from TCGA (Figure 5E) and Affymetrix U133Plus2 datasets curated by gene expression across normal and tumor tissue (GENT) (Figure 5E) showed RasGRP3 is expressed at significantly higher levels in UMs than in CMs and other cancer types. Notably, all TCGA UMs expressed high RasGRP3, and the five TCGA SKCMs with the highest RasGRP3 expression harbored either GNAQ or GNA11 mutations and either BAP1 loss (mutation or monosomy 3) or SF3B1 mutations, suggesting they may be malignant blue nevi (Griewank et al., 2017) (Figure 5E, circled). RasGRP3 encodes Ras guanyl-releasing protein 3 (RasGRP3), a GEF that promotes the release of GDP-bound Ras in order to bind GTP, yielding active Ras (Ras-GTP) (Rebhun et al., 2000). RasGRP3 activation is dependent on both DAG binding and phosphorylation on Thr 133 by protein kinase C (PKC) (Aiba et al., 2004; Zheng et al., 2005). Together, these signaling events place the activation of RasGRP3 downstream of UM-activating mutations (CYSLTR2, GNAQ, GNA11, and PLCB4), and they suggest RasGRP3 may be a key signaling node that can integrate the UM-activating mutations into the MAPK pathway. To determine if RasGRP3 expression is retained in cell lines, we screened a panel of UM and CM cells, and we found RasGRP3 to be expressed exclusively in UM cells (Figure 5G).

**RasGRP3 Is Required for Ras-MAPK Activation and Growth in UM Cells**

To determine if RasGRP3 is required for Gx11/q-mediated activation of the MAPK pathway and for growth in UM, we generated two short hairpin RNAs (shRNAs) to mediate knockdown of RasGRP3 (shRasGRP3-1 and shRasGRP3-2) and a control (shSCR). Depletion of RasGRP3 significantly reduced cell proliferation in GNA11 or GNAQ mutant cells (Figures 6A and S7A). In contrast, knockdown of RasGRP3 in CM cells did not (Figures 6B and S7A).

We next sought to characterize the response of RasGRP3 depletion in the context of Ras activation and subsequent downstream MAPK signaling. We stably expressed two doxycycline (dox)-inducible RasGRP3 shRNAs (dox-shRasGRP3-1 and dox-shRasGRP3-2) and a dox-inducible control (dox-shSCR) in the panel of UM and CM cells. Depletion of RasGRP3 with dox significantly reduced the proportion of Ras-GTP and phosphorylation of ERK1/2 and P90 RSK in UM cells (Figure 6C). Consistent with the cellular growth data, there was no change in Ras activation or MAPK signaling upon depletion of RasGRP3 in CM cells (Figure 6C).

To elucidate the requirement of the Ras GEF activity of RasGRP3, we examined if ectopic expression of KRAS<sup>G12V</sup> could rescue the proliferation and MAPK signaling in three UM cell lines depleted of RasGRP3. We performed growth
competition assays in which we first generated cells where
20%-50% expressed KRAS G12V-IRES-GFP and empty vector
at low MOI. We next infected these cells with shSCR or
shRasGRP3, and we tracked the percentage of GFP-positive
cells over time using fluorescence-activated cell sorting
(FACS). The percentage of empty vector-expressing GFP-posi-
tive cells remained stable over time regardless of RasGRP3
depletion in both CM and UM cells (Figure S7B). In contrast,
the percentage of KRAS G12V-expressing GFP-positive cells
increased after RasGRP3 depletion compared to shSCR in
all three UM lines (Figure 6D), indicating KRAS G12V conveys a
growth advantage specifically after RasGRP3 depletion. Expres-
sion of KRAS G12V rescued the reduction in ERK phosphorylation
observed upon depletion of RasGRP3 (Figure 6 E). Ectopic
expression of KRAS G12V in a CM cell line (A375) provided no
changes in proliferation or ERK phosphorylation (Figures 6 D
GNA11Q209L Bap1KO Tram (n= 8)
GNA11Q209L Bap1KO (n= 8)
BRafV600E Bap1KO Tram (n= 24)
BRafV600E Bap1KO (n= 7)
01 0 2 0
0
20
40
60
% Original tumor volume
Control Trametinib Control Trametinib
p-ERK1/2
ERK1/2
p-p90RSK
p90RSK
Cyclin D1
SPRY4
GAPDH
0 0.5 1 24 82
Time (hour):
Trametinib (10 nM)
0 0.5 1 2 8 24 0 0.5 1 2 8 24 0 0.5 1 2 8 24 0 0.5 1 2 8 24 0 0.5 1 2 8 24
0
20
40
60
% Ki67 positive cells
Control Tram Control Tram
GNA11Q209L BAP1KO BRafV600E BAP1KO
A375
OMM1.3
MEL202
MEL270
OMM1.3
UPMD2
A375
A2058
COLO800
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
GAPDH

Figure 4. Reduced MEK Sensitivity in Gα11/q-Driven Tumors
(A) H&E and Ki-67 IHC of GNA11Q209L Bap1KO and BRAFV600E Bap1KO tumors treated with trametinib or vehicle.
(B) Immunoblots for MAPK in explanted control and trametinib-treated GNA11Q209L Bap1KO and BRAFV600E Bap1KO tumors.
(C) Quantification of Ki67 in explanted control and trametinib-treated GNA11Q209L Bap1KO and BRAFV600E Bap1KO tumors, shown as Tukey box-and-whisker
plots. Outliers are shown as dots. p < 0.0001.
(D) Tumor growth of grafted GNA11Q209LBap1KO or BRAFV600E Bap1KO tumors in SCID mice with treatment as indicated. n = tumors per group. Error bars, SEM. p < 0.001 for BRAFV600E Bap1KO treatment.
(E) Immunoblot of Gα11/q mutant UM and BRAF mutant CM cell lines treated with 10 nM trametinib.
and 6E). Therefore, UM cells require RasGRP3 for Ras activation and cellular proliferation.

**RasGRP3 is Required for Gα11/q-Mediated Growth**

Human UM cells harboring Gα11/q mutations selectively require RasGRP3 for growth and MAPK activation, suggesting Gα11/q-mediated oncogenesis might require RasGRP3. To explore this hypothesis, we determined the requirement of RasGRP3 in an immortalized mouse melanocytic cell line, melan-a. Melan-a cells require phorbol esters, such as the DAG analog TPA (12-O-tetradecanoylphorbol-13-acetate), that can activate PKC for growth, and they can become TPA independent upon the expression of oncogenic mutations (Wellbrock et al., 2004). We transduced melan-a cells with GNAQQ209L, BRAFV600E, and KRASG12V, and we cultured the cells in the absence of TPA to establish oncogene-dependent growth. At baseline, melan-a cells with TPA-dependent growth expressed endogenous Rasgrp3. While GNAQQ209L-dependent cells retained Rasgrp3, BRAF V600E- and KRASG12V-dependent cells lost Rasgrp3 (Figure S7C). BRAF V600E- and KRASG12V-dependent cells lost pigmentation and expression of melanocyte lineage proteins, while GNAQQ209L-dependent cells retained pigmentation and melanocyte lineage proteins (Figure S7C). Re-introduction of TPA to the media of BRAFV600E- and KRASG12V-dependent cells failed to re-establish Rasgrp3 expression (Figure S7D). The difference in melanocyte lineage commitment between Gα11/q and RAS/RAF-driven transformed melanocytes is consistent with observations in our GEMMs and patient tumors.

To determine the potential role of RasGRP3 in Gα11/q-, BRAF-, and KRAS-mediated tumorigenesis, we performed shRNA-mediated knockdown of Rasgrp3 (shRasgrp3-1 and shRasgrp3-2). Knockdown of Rasgrp3 significantly reduced cell growth in GNAQQ209L-dependent, but not in BRAFV600E-, or KRASG12V-dependent, melan-a cells (Figures 7A and S7E). Depletion of Rasgrp3 in GNAQQ209L melan-a cells reduced Ras-GTP and the phosphorylation of ERK and P90RSK (Figure 7B). Therefore, RasGRP3 is specifically required for Gα11/q-mediated oncogenic growth.

**DISCUSSION**

UMs, LMNs, and blue nevi harbor activating mutations along the CYSLTR2-Gα11/q-PLCβ pathway, and they can have mutually...
exclusive cooperating mutations in \textit{BAP1}, \textit{SF3B1}, and \textit{EIF1AX} that convey poor, intermediate, and favorable risk, respectively (de la Fouchardière et al., 2015; Goldman-Levy et al., 2016; Küsters-Vandevelde et al., 2016). This distinct molecular profile is observed in a small subset of CMs and 10% of mucosal melanomas (Sheng et al., 2016). Pathologically, UMs are characterized by their retention of the melanocyte lineage program, including pigmentation (Rothermel et al., 2016).

No proven effective therapies exist for UM. As with Ras, it is difficult to target G-proteins with competitive inhibitors to the nucleotide-binding site due to the high cellular concentrations of GTP. Since \( \text{G}_{\alpha11/q} \) signaling activates PKC and the MAPK pathway, via PLC\( _b \), many groups have studied the role of PKC and MAPK in UM. Cells with \( \text{G}_{\alpha11/q} \) mutations were modestly sensitive to MEK inhibition and combination treatment of PKC and MEK inhibitors (Chen et al., 2014). Unfortunately, PKC targeting is limited by toxicity, and a completed phase 3 trial with selumetinib showed no clinical benefit (Komatsubara et al., 2016). In addition to PLC\( _b \), \( \text{G}_{\alpha q} \) directly interacts with the Trio family of Rho-GEFs (Trio, p63-RhoGEF, and Kalirin) to activate

Figure 6. RASGRP3 Is Required for Growth and ras Activation in UM Cells

(A and B) Growth curves of UM (A) or CM (B) cells with shSCR, shRASGRP3-1, or shRASGRP3-2, shown as relative luminescence units (RLUs). Error bars, means ± SEM from six technical replicates, \( p < 0.001 \) (GNAQ\( ^{209L} \)) and \( p = \text{ns} \) (BRAF\( ^{600E} \) and KRAS\( ^{G12V} \)) for reduction in growth. (B) Immunoblots of RASGRP3 and MAPK pathway following RASGRP3 depletion. See also Figure S7.

Figure 7. Rasgrp3 Is Required for \( \text{G}_{\alpha 11/q} \)-Mediated Growth and MAPK Activation in Melan-a Cells

(A) Growth curves of GNAQ\( ^{209L} \), BRAF\( ^{600E} \), or KRAS\( ^{G12V} \) melan-a cells, grown in the absence of TPA and expressing shSCR, shRasgrp3-1, or shRasgrp3-2, shown as RLUs. Error bars, means ± SEM from six technical replicates, \( p < 0.001 \) (GNAQ\( ^{209L} \)) and \( p = \text{ns} \) (BRAF\( ^{600E} \) and KRAS\( ^{G12V} \)) for reduction in growth. (B) Immunoblots of Rasgrp3 and MAPK pathway following Rasgrp3 depletion. See also Figure S7.
Rac and Rho and downstream YAP, and this pathway may represent a therapeutic target (Feng et al., 2014; Yu et al., 2014). Another promising target is ARF6, a GTPase involved in vesicle trafficking and required for proper shuffling of activated Gαq to cytoplasmic vesicles, where downstream signaling to both PLC and Rho were localized (Yoo et al., 2016). In addition to Gα11/q signaling, another therapeutic strategy is targeting the melanocyte lineage. IMCgp100 is a bispecific antibody that binds gp100 (PMEL) on tumor cells and CD3 on T cells (Carvajal et al., 2014, J. Stem Cell Res. Ther., abstract). A phase 1 trial in melanoma showed a disease control rate of 21% and 57% in CM and UM, respectively, and an expanded study in UM showed a similar disease control rate with some durable responses (Lams et al., 2017).

Here we sought to generate a clinically relevant GEMM of aggressive Gα11-driven melanoma, combining GNA11Q209L and BAP1 loss, and we compared it to an isogenic BrafV600E model to identify Gα11-driven phenotypes and vulnerabilities. We found GNA11Q209L drove neoplastic growth in cutaneous and many non-cutaneous sites whereas BrafV600E only promotes CM. While the lung and lymph nodes are the preferential sites of metastasis in the Tyr-CreERT2; BrafCA/+; Ptenflox/flox mouse (Dankort et al., 2009) and in a transgenic mouse of Tyr-driven SV40 T-antigen (Bradl et al., 1991), one limitation in our mouse model was the inability to specifically activate GNA11Q209L in defined melanocytic subsets, and this hampers the ability to definitively assign metastasis (Gibson et al., 2010; Klein-Szanto et al., 1991).

GNA11Q209L-driven tumors were highly pigmented compared to BrafV600E, consistent with clinical observation that Gαq-driven primary blue nevi, UM, and UM metastases retain pigmentation (Emley et al., 2011; Rothermel et al., 2016). Therefore, Gα2/q1 signaling drives lineage commitment, and targeting the lineage, such as IMCgp100, is a promising therapeutic strategy (Carvajal et al., 2014, J. Stem Cell Res. Ther., abstract). Bap1 loss in our GEMM accelerated skin melanoma growth, consistent with the clinical observation that BAP1 loss is found in transformed, but not benign, blue nevi (Griewank et al., 2017). Yet, there was no significant change of uveal pathology, highlighting a limitation of our model.

Cross-species comparison between representative GEMM models with human disease can identify critical mediators of tumorigenesis (Johnson et al., 2010). By cross-referencing the RNA-seq data from the GEMM and human disease data, we identified a Ras-GEF, RasGRP3, as a required signaling node for UM. Consistent with a recently published study (Chen et al., 2017), we observed RasGRP3 is highly upregulated in UM and is required for proliferation. We additionally showed engineered cells driven by mutant Gαq specifically require RasGRP3 for Ras activation and growth. RasGRP3 expression is tissue specific and, among cancers, constrained to Gα11/q-driven melanomas, leukemias, and lymphomas, suggesting RasGRP3 is a specific vulnerability in Gα11/q-driven tumors and potentially a therapeutic target. The interaction between G-proteins and their GEFs is a viable drug target, as exemplified by the antibiotic brefedolin A, which blocks interaction between ARF1 and its GEF Sec7 (Mossessova et al., 2003), and RasGRP3 may be similarly targeted.

**EXPERIMENTAL PROCEDURES**

Further details and an outline of the resources used in this work can be found in the Supplemental Experimental Procedures.

**Mouse Experiments**

All animal studies were performed in accordance with the MSKCC IACUC (11-12-023). For GEMM studies, three cohorts of mice, Tyr-CreERT2; GNA11Q209L; Brapflox/flox, Tyr-CreERT2; GNA11Q209L; Bap1lox/lox, and Tyr-CreERT2; Brapflox/flox; Bap1lox/lox, were administered with intraperitoneal tamoxifen at 4 weeks of age with no regard to the sex of the animals, and histology was similar between males and females. Mice developed tumors in situ after tamoxifen injection. Mice were euthanized in response but not limited to the following: tumors larger than 1 cm³, tumor ulceration, tumors located too close to the trunk of the mice to impede movement and blood flow, and tumor burden, and time of euthanization was used for Kaplan-Meier survival analysis. For allograft studies, GEMM-derived tumors were grafted into 6- to 8-week-old female CB17-SCID mice and treated with vehicle or trametinib via oral gavage.

**Histology, Immunohistochemistry, and Immunofluorescence**

All tissues were fixed at 4°C overnight in 4% paraformaldehyde. Tissue processing, embedding, sectioning, H&E staining, and H&E staining with melanin bleaching were performed by Histoserv. Skull sections were performed following decalcification.

**RNA-Seq**

Total RNA was extracted from fresh-frozen tissue or cell lines using QIAGEN’s RNeasy Mini Kit. The isolated RNA was processed for RNA-seq by the Integrated Genomics Core Facility at MSKCC.

**Cell Lines**

Melan-a cells were provided by D. Bennett (Bennett et al., 1987); MEL202, MEL270, OMM1 3, COLO800, UPMJM3, A375, and A2058 cells were submitted for short tandem repeat (STR) profiling and MSK-IMPACT (integration muta- tion profiling of actionable cancer targets) for mutational status at MSKCC to confirm their authenticity.

**Statistics**

Boxplots represent 25th and 75th percentiles with midline indicating the median; whiskers extend to the lowest/highest value within 1.5 times the inter-quartile range. Outliers are shown as dots. Comparisons for growth curves and xenograft experiments between two groups were performed using a two-tailed parametric t-test. All statistics were performed using GraphPad Prism 6.0 software.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the data reported in this paper is GEO: GSE97225.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.081.

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AUTHOR CONTRIBUTIONS

Project Planning and Experimental Design, P.C., Y.C., A.R.M., L.R., K.G.G., and A.N.S.; Pathology Review, R.M. and S.M.; Bioinformatics, Y.C. and A.R.M.; Cellular Assays, A.R.M., L.R., Y.G., and T.D.H.; Mice, A.R.M., J.J.S., and E.G.W.; Expression Vectors, A.R.M., Y.G., J.Q.Z., C.H., and T.W.; Manuscript Writing, A.R.M., P.C., and Y.C.; Review of the Final Manuscript, all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

GNA11 Q209L Mouse Model Reveals RasGRP3 as an Essential Signaling Node in Uveal Melanoma

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Genetically Engineered Mice

We cloned in human GNA11 Q209L with an internal glu-glu tag (Van Raamsdonk et al., 2010) into a modified pBTG (Murtaugh et al., 2003) (Addgene plasmid 268) with LoxP sites reversed to remove the forward ATG codon within LoxP site and into Rosa26 locus as previously described (Addgene 15036) (Chen et al., 2013).

Gene targeting was performed at the Rockefeller University Gene Targeting Resource Center (Head: Chingwen Yang). The targeting plasmid was electrophoresed into albino C57BL/6J ES cells and G418 resistant clones were isolated by standard procedures. The clones were screened by Southern blotting. Two positive clones were injected into C57BL/6J blastocysts by the MSKCC Mouse Genetics Core Facility (Head: Willie Mark) and chimeras were mated with albino C57BL/6J females. Germline transmission was confirmed in albino offspring using Southern blotting. For subsequent generations, GNA11 Q209L mouse genotyping was performed by qPCR of genomic DNA using primers listed in Table S2.

Bap1\textsuperscript{flox/flox} (C57BL/6N-Bap1tm1c(EUCOMM)Hmgu/Wtsi) mice were purchased from EUCOMM (LaFave et al., 2015). Tyrosinase-CreERT2 and B RAF\textsuperscript{V600E} mice were obtained from Jackson Labs [B6.Cg-Tg(Tyr-cre/ERT2)13Bos/J (012328); B6.129P2(Cg)-Braftm1Mmcm/J (017837)](Bosenberg et al., 2006). CAG-LSL-EYFP (also known as Ai3) mice (Madisen et al., 2010) were obtained from Jackson Labs [B6.Cg-Gt(Rosa)26Sortm3(CAG-EYFP)Hze/J (007903)]. Standard PCR was performed for genotyping of Tyr-CreERT2, B RAF\textsuperscript{V600E}, Bap1\textsuperscript{flox/flox} are listed in Table S2.

Mouse survival was determined under the recommendation of MSKCC veterinary services or upon encountering visible discomfort and in accordance to the MSKCC Institutional Animal Care and Use Committee (IACUC 11-12-029). In accordance to our animal protocol, mice were euthanized in response but not limited to the following: tumors larger than 1cm\textsuperscript{3}, tumor ulceration, tumors located too close to the trunk of the mice to impeded movement and blood flow and tumor burden.

Intraperitoneal injection of tamoxifen was performed at 4-weeks of age with no regard to the sex of the animals and histology was similar performed between both males and females.

Allograft and Treatment

Tumors from Tyr-CreERT2;B RAF\textsuperscript{V600E};Bap1\textsuperscript{K0} and Tyr-CreERT2;GNA11 Q209L;Bap1\textsuperscript{K0} mice were expanded in 6-8 week old C.B17-scid mice (C.B-Igh-1\textsuperscript{b}/IcrTac-Prkdc\textsuperscript{scid}, Taconic) and then serial grafted bilaterally using equal size tumors. The experimental cohort of mice 6-8 week old female C.B17-scid mice (Taconic) of 8 tumors for the vehicle and trametinib treated Tyr-CreERT2;GNA11 Q209L;Bap1\textsuperscript{K0} and 24 tumors for the trametinib treated arm and 7 tumors for the control arm for Tyr-CreERT2;B RAF\textsuperscript{V600E};Bap1\textsuperscript{K0} tumors. The size of each cohort was determined on the basis of previous experiments without specific statistical methods. Mice we treated with 3mg kg\textsuperscript{-1} trametinib (Active Biochem) dissolved in trametinib solvent (30% PEG-400, 0.5% TWEEN\textsuperscript{®} 80, 5% propylene glycol in PBS) once daily for 5 days a week. Tumors were measured with calipers every 2 or 3 days for up to 25 days with trametinib treatment and were actively measured for at least 10 days before treatment. Tumor growth curves were visualized with Prism GraphPad 6.0. Tumor volume was calculated using the formula: \( \text{volume} = \frac{\pi \text{length} \times \text{width} \times \text{height}}{6} \).

Histology and immunohistochemistry
Prior to immunohistochemistry, melanin bleaching was performed using a delicate melanin bleach kit (Polysciences Inc.) according to the manufacturer’s instructions. Immunohistochemistry for HMB45, DT101, BC199 melanoma cocktail (Abcam, ab732, 1:50), MITF (Cell Signaling Technology, 12590, 1:50), Ki-67 (Abcam, ab16667, 1:100) antibodies were diluted in SignalStain antibody diluent (Cell Signaling Technology). Staining was performed using a standard multimer/diaminobenzidine (DAB) detection protocol for MITF and Ki67 and red chromogen staining (alkaline phosphatase; Ventana; UltraMap Red) for the melanoma cocktail following epitope retrieval with citrate (Ventana; CC1), hematoxylin counterstain on a Discovery Ultra system (Roche/Ventana) with appropriate negative and positive controls. Immunohistochemistry for each tissue shown was performed on tissues harvested from at least 5 animals.

**Immunofluorescence of eye and skin**

All tissues were fixed at room temperature for 30min in 4% paraformaldehyde (Electron Microscopy Sciences). Tissues were then incubated in 30% sucrose in PBS at 4°C overnight, washed once with PBS, embed in OCT, flash frozen and cut into 5 µM sections using a cryostat. Tissue sections were blocked for 1 hour using 5% goat serum, incubated with Gp100 antibody at 4 °C overnight and secondary antibody for 2 hours at room temperature. Slides were mounted using PBS with 1ug ml⁻¹ DAPI for direct visualization of YFP and Gp100. Images were taken on a Nikon Eclipse TE2000-E microscope using a Photometric Coolsnap HQ camera. Images were taken with ×20 (numerical aperture 0.75) objectives. Monochrome images taken with DAPI, YFP and Texas Red filter sets were pseudo-colored blue, green and red, respectively, and merged using ImageJ.

Intraperitoneal injection of tamoxifen was administered in both sexes of the animals and histology was similar between both males and females. At least 45 animals for each genotype (GNA11Q209L, GNA11Q209L; Bap1lox/lox, Bap1lox/lox, BRafV600E; Bap1lox/lox) were injected with tamoxifen and observed. At least 14 animals for each genotype underwent a full necropsy and were tissues evaluated by H&E.

**RNA-seq**

For mouse tumors, total RNA was extracted from fresh-frozen tissue or cell lines using Qiagen’s RNeasy Mini Kit (Qiagen). The isolated RNA was processed for RNA-sequencing by the Integrated Genomics Core Facility at MSKCC. The libraries were sequenced on an Illumina HiSeq-2500 platform with 51 bp paired-end reads to obtain a minimum yield of 40 million reads per sample. The sequence data were mapped to the mouse reference genome (mm9) and the number of reads was quantified using STAR v2.3.30 (Dobin et al., 2013).

For UPMM3 cells, the libraries were sequenced on an Illumina HiSeq-2500 platform with 51 bp single-end reads to obtain a minimum yield of 40 million reads per sample. The sequence data were mapped to the human reference genome (hg19) and the number of reads was quantified using Cufflinks (Roberts et al., 2011). GSEA was performed using JAVA GSEA 2.0 program (Subramanian et al., 2005). The gene sets used for analysis were the Broad Molecular Signatures Database gene sets c2 (curated gene sets), c5 (gene ontology gene sets), c6 (oncogenic signatures). GSEA enrichment sets are shown in Table S1.

Level 3 RNA-seqV2 data for TCGA uveal melanoma (n=80) and cutaneous melanoma (SKCM; n=471) were downloaded from NIH TCGA server. We merged the samples and annotated them by mutational status (BRafV600E, GNAQ or GNA11 at R183 or Q209) and by tissue source (primary or metastatic). Differential gene expression was performed using ANOVA analysis with the Partek Genomics Suite 6.6 in both the TCGA and genetically engineered mouse model datasets. Expression of RASGR3 from pan-TCGA cancers was downloaded from cBioportal (Cerami et al., 2012) and from Gene Expression across Normal and Tumor tissue (GENT) (Shin et al., 2011) was downloaded from [http://medicalgenome.kribb.re.kr/GENT/](http://medicalgenome.kribb.re.kr/GENT/).
**Ki-67 quantification**

Following immunohistochemistry for Ki-67, 10 randomly selected field images at 40 x magnifications were taken. These images were converted to greyscale, threshold adjusted, and particles were analyzed using ImageJ v1.60 (NIH) automated counting to determine the number of cells per field. The same parameters were used throughout quantification. To determine the percent of cells that were positive for Ki-67, we performed identical procedures while optimizing the threshold to include only cells with positive DAB staining. The same parameters were used throughout quantification.

**PCR for Bap1 lox/lox excision**

DNA from Bap1 mice was isolated using DNeasy Blood & Tissue Kit (Qiagen). DNA from whole cutaneous tumors and dissected uveal tracts were used for the PCR. Control tissues were isolated from mouse toes. PCR of genomic DNA was performed using primers listed in Table S2.

**Cell lines**

Melan-a cells were provided by D. Bennett (St. George’s Hospital, University of London, London, UK) (Bennett et al., 1987) and were grown in RPMI with 200nM 12-O-Tetradecanoylphorbol-13-Acetate (TPA; Sigma-Aldrich) before stable expression. Melan-a cells stably expressing GNAQQ209L, BRAFV600E and KRASG12V were propagated in the absence of TPA. MEL202, MEL270, OMM1.3, COLO800 cells were grown in RPMI. UPMD1, UPMD2 and UPMM3 cells were grown in Ham’s F12 media. A375 and A2058 cells were grown in DMEM media. 293T cells were used for retrovirus and lentivirus production and maintained in DMEM media. All cell culture media contained 10% FBS, penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹), L-glutamine (2mM). All cells were grown in a humidified incubator at 37 °C with 5% CO₂ and were tested regularly for mycoplasma contamination. All cell lines used were negative for mycoplasma. All uveal melanoma cell lines were validated for mutations status by MSK-IMPACT.

**Proliferation and survival assays**

Growth curves were performed following shRNA infection and around 1,000–3,000 cells were plated in a 96-well plate 24 hours following infection. Every 48 hours following infection, the number of cells was determined using a CelltiterGlo assay (Promega). IC₅₀ assays were performed by plating 1,000–3,000 cells in a 96-well plate, allowed to adhere overnight, and then incubated with either fresh media containing trametinib. After 5 days, the number of cells was determined using a CelltiterGlo assay. Representative experiments are shown. Cell growth was assessed in three independent experiments, each in quadruplet.

**Immunoblotting**

Whole cell lysates were prepared in cell lysis buffer (Cell Signaling Technology) containing freshly added protease and phosphatase inhibitors (PhosSTOP, Roche, cOmplete EDTA-Free, Roche). Tumor lysates were generated by homogenization of flash-frozen tissue in cell lysis buffer (Cell Signaling Technology) containing freshly added protease and phosphatase inhibitors (PhosSTOP, Roche, cOmplete EDTA-Free, Roche). Equal amounts of protein, as measured by BCA protein assay (Thermo Scientific), were resolved on NuPAGE Novex 4-12% Bis-Tris Protein Gels (Life Technologies) and transferred electrophoretically onto a 0.45μm nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 hour at room temperature in StartingBlock TBS (Thermo Scientific) or Odyssey blocking buffer (LI-COR) before being incubated overnight at 4°C with the primary antibodies diluted at 1:1000 unless otherwise noted in either StartingBlock or Odyssey blocking buffer. The following primary antibodies were used (Cell Signaling unless noted otherwise): phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; 4370), p44/42 MAPK (Erk1/2;4695), phospho-P90_RSK(Ser380;12032), RSK1/2/3 (9355), RasGRP3 (3334), Ras (8832), CyclinD1 (2922), SPRY4 (Santa Cruz Biotechnology; sc-30051), BAP1 (Santa Cruz Biotechnology; sc-28383), HA-tag (Roche; 3F10), GNAQ (abcam; ab199533), GAPDH (1:5,000
dilution; Applied Biological Materials, G041), β-actin (1:5,000 dilution; abcam, AC15). The Ras-GTP assay was performed according to manufactures instructions (Cell Signaling; 8821).

**shRNA and inhibitors**

For shRNA mediated knockdown of RASGRP3 the following hairpins were used with sequences listed in Table S3. Human shRASGRP3-1 and shRASGRP3-2 were obtained from the MSKCC RNAi Core in pLKO.1 and were subcloned into Tet-pLKO-puro (Wiederschain et al., 2009) (Addgene plasmid 21915). Mouse shRasgr3p-1 and shRasgrp3-2 were obtained from the MSKCC RNAi Core in pLKO.1. Stable expressing Tet-ON shRasGRP3 were induced with doxycycline 1µg ml⁻¹ (Research Products International) and cells were harvested for protein 72 hours post-doxycycline addition. Melan-a cells were treated with the indicated dose of trametinib (SelleckChem). Cell growth for the half-maximal inhibitory concentration (IC₅₀) was assessed using CellTiter-Glo (Promega) five days post trametinib treatment.

**Exogenous gene expression**

Melan-a cells were stably transduced with: BRAF V600E, MSCV-HA-FLAG-BRAF-V600E. MSCV-HA-FLAG-V600E was cloned using the gateway method using pDONOR-BRAF (Addgene plasmid 70300) and subcloned into MSCV-HA-FLAG-puro-Dest. Site directed mutagenesis was performed using QuikChange (Agilent) to wild-type BRAF to introduce a p.Val600Glu mutation to give BRAF-V600E. KRAS G12V: MSCV-GFP-KRAS G12V, KRAS G12V (Addgene plasmid 31200) (Yang et al., 2011) was subcloned into MSCV-IREs-GFP-Dest using the gateway method. GNAQ Q209L: MSCV-puro-GNAQ Q209L, cDNA for the wild-type human GNAQ was obtained from Origene (sc128110) and cloned into MSCV-puro (Addgene plasmid 24828)(Olive et al., 2009). Site directed mutagenesis was performed using QuikChange (Agilent) to wild-type GNAQ to introduce a p.Gln209Leu mutation to give GNAQ Q209L. QuikChange primers are listed in Table S2. The sequences of all constructs were confirmed by Sanger sequencing.

**Resource Table**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rabbit monoclonal anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) | Cell Signaling Technology | Cat#4370; RRID: AB_2315112 |
| Rabbit monoclonal anti-p44/42 MAPK (Erk1/2) | Cell Signaling Technology | Cat#4695; RRID: AB_390779 |
| Rabbit monoclonal anti-Phospho-p90RSK (Ser380) | Cell Signaling Technology | Cat#:12032 |
| Rabbit monoclonal anti-RSK1/RSK2/RSK3 | Cell Signaling Technology | Cat#9355; RRID: AB_659900 |
| Rabbit monoclonal anti-RASGRP3 | Cell Signaling Technology | Cat#3334; RRID: AB_2269292 |
| Mouse monoclonal anti-RAS | Cell Signaling Technology | Cat#8832 |
| Rat monoclonal anti-HA (hemagglutinin) | Roche | Cat#3F10; RRID: AB_2314622 |
| Rabbit monoclonal anti-GNAQ | Abcam | Cat#ab199533 |
| Mouse monoclonal anti-BAP1 (C-4) | Santa Cruz Biotechnology | Cat# sc-28383; RRID:AB_626723 |
| Rabbit monoclonal anti-Ki67 [SP6] | Abcam | Cat#ab16667, RRID:AB_302459 |
| Rabbit monoclonal anti-MITF | Cell Signaling Technology | Cat# 12590, RRID:AB_2616024 |
| Rabbit polyclonal anti-SPRY4 | Santa Cruz Biotechnology | Cat# sc-30051 RRID:AB_2195449 |
| Rabbit monoclonal anti-CyclinD1 | Cell Signaling Technology | Cat# 2922 RRID:AB_2228523 |
| Rabbit monoclonal anti-Melanoma gp100 | Abcam | Cat#ab137078 |
|----------------------------------------|-------|--------------|
| Mouse monoclonal anti-Melanoma antibody [HMB45 + DT101 + BC199] | Abcam | Cat# ab732, RRID:AB_305844 |

**Biological Samples**

| Genetically engineered mouse tissues | This paper | N/A |

**Chemicals, Peptides, and Recombinant Proteins**

| Trametinib (GSK1120212; mouse treatment) | Active Biochem | Cat#: A-1258; CAS: 871700-17-3 |
|----------------------------------------|---------------|-------------------------------|
| Trametinib (GSK1120212; cell treatment) | Selleck Chemicals | S2673; CAS: 871700-17-3 |

**Critical Commercial Assays**

| Ras-GTP | Cell Signaling Technology | Cat#8821 |
|----------------------------------------|---------------|------------|
| CellTiterGlo | Promega | G9242 |

**Deposited Data**

| Raw and analyzed RNA-sequencing data | This paper | GEO: GSE97225 |
|----------------------------------------|------------|--------------|
| Mendeley raw data | This paper | doi:10.17632/g2v849vzzm |
| | | doi:10.17632/my5g8mdrkg |
| | | doi:10.17632/8r58nb2ttc |

**Experimental Models: Cell Lines**

| Mouse: Melan-a | (Bennett et al., 1987) | Wellcome Trust Functional Genomics Cell Bank; RRID:CVCL_4624 |
|----------------------------------------|---------------|-------------------------------|
| Human: MEL202 | (Griewank et al., 2012) | RRID:CVCL_C301 |
| Human: MEL270 | (Griewank et al., 2012) | RRID:CVCL_C302 |
| Human: UPM2 | (Griewank et al., 2012) | RRID:CVCL_C298 |
| Human: OMM1.3 | (Griewank et al., 2012) | RRID:CVCL_C306 |
| Human: UPM3 | (Griewank et al., 2012) | RRID:CVCL_C295 |
| Human: UPM1 | (Griewank et al., 2012) | RRID:CVCL_C297 |
| Human: A375 | ATCC | CRL-1619; RRID:CVCL_0132 |
| Human: A2058 | ATCC | CRL-11147; RRID:CVCL_1059 |
| Human: COL800 | Sigma-Aldrich | 93051123; RRID:CVCL_1135 |

**Experimental Models: Organisms/Strains**

| Mouse: Tyr::CreER^{12}: B6.Cg-Tg(Tyr-cre/ERT2)13Box/J | The Jackson Laboratory | RRID:IMSR_JAX:012328 |
|----------------------------------------------------------|------------------------|----------------------|
| Mouse: Braf\(^{∆V600E}\): B6.129P2(Cg-Braf\^{∆V600E})\_\_\_J | The Jackson Laboratory | RRID:IMSR_JAX:017837 |
| Mouse: Bap1\(^{tm1a(EUCOMM)Hmgu}\)\_\_\_J | (LaFave et al., 2015) | RRID:MGI:5550605 |
| Mouse: GNA11\(^{Q209L}\) (R26-LSL-GNA11_\_\_Q209L) | This paper | N/A |
| Mouse: CB17-SCID: C.B-Igh-2\(^{b}\)\_\_\_J | Taconic | RRID:IMSR_TAC:cb17sc |
| Mouse: R26-LSL-EYFP (B6.Cg-Gt(Rosa)26Sor\^{tm1(CAG-EYFP)Lzr}) | The Jackson Laboratory | RRID: IMR_JAX: 007903 |

**Oligonucleotides**
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Figure S1, related to Figure 1. GNA11^{Q209L} mouse targeting

(A) Targeting strategy for conditional GNA11^{Q209L} allele in the Rosa26 locus. Upon Cre-mediated recombination of the loxP, the Neo-STOP cassette is excised and GNA11^{Q209L} is expressed. Southern probe and EcoRV sites are indicated and targeting cassette introduces a new EcoRV site.

(B) Southern blot analysis using EcoRV and probing for 5’Rosa26. ES cell clones 56, 52 are positive for GNA11^{Q209L} allelic targeting.

(C) EYFP fluorescence, Gp100 immunofluorescence and bright-field images of uveal tract and skin in Tyr-CreER^T2; CAG-LSL-EYFP and control Cre-negative; CAG-LSL-EYFP mice 7 days after tamoxifen injection. Melanocytes are marked by Gp100-positivity as well as strong pigment readily appreciable on bright-field.
Figure S2, related to Figure 1. GNA11Q209L and control mice phenotypes.
(A) Photographs of Tyr-CreERT2 negative control and GNA11Q209L mice 1 month after tamoxifen injection. Boxes highlight the pigmentation differences in the ears and tail. Arrow highlights the eye bulging.
(B-C) Representative H&E images of the skin (B) and eye (C) of a Rosa26-LSL-GNA11Q209L (Cre-negative) mouse 12 months after tamoxifen injection.
(D) Immunohistochemistry of skin from a Tyr-CreERT2; Rosa26-LSL-GNA11Q209L mouse 3 months after tamoxifen injection for melanoma cocktail (HMB45, DT101, BC199) using Red Chromogen (red) staining.
(E-F) Representative H&E images of the skin (E) and eye (F) of a 12-month tamoxifen treated BRafV600E mouse.
(G) Tukey plot of uveal width in mice of the indicated genotype 6 months after tamoxifen injection. Unpaired T-test.
Figure S3, related to Figures 2. GNA11Q209L CNS phenotype and controls.

(A) Representative images of whole brains showing focal pigmentation of the leptomeninges in GNA11Q209L (arrow).

(B) Sequential coronal skull sections (top), magnified images of select brain regions (i) olfactory bulb, (ii) choroid plexus of the third ventricle, (iii) meninges of the longitudinal fissure. Scale bar 2000μm (top), 500μm (bottom).

(C) Representative H&E image of heart and lung Tyr-CreERT2; BRafV600E mice.

(D) Magnification of heart valve; box and gray arrowhead in (C). Scale bar 500μm.

(E) Prevalence of lung lesions in Tyr-CreERT2; R26-LSL-GNA11Q209L and Tyr-CreERT2; R26-LSL-GNA11Q209L mice by age post-tamoxifen injection.
Figure S4, related to Figure 3. Bap1KO mouse confirmation and histology

Representative H&E images of haired skin and subcutis (A) and eye (B) of Tyr-CreERT2 Bap1KO mice 20-months post-tamoxifen injection. Scale bar 500μm.

(C) PCR for Bap1 lox/lox excision, controls and BAP1 KO tumors. Lox/lox amplification 327 bp and Bap1 KO (excision) 451 bp.

(D-E) Representative images of a GNA11Q209L Bap1KO mouse liver. Scale bar 500μm (D) and 50μm (E).

(F) Representative image of a GNA11Q209L Bap1KO mouse lung and heart. Scale bar 2000μm.

(G) Selected image of lung lesions. Image refers to box in (F). Scale bar 50 μm

(H) Quantification of lung tumor area (μm²) in two mice per group (GNA11Q209L and GNA11Q209L-Bap1KO). Shown as scatter-dot plot; each dot represents the quantification of one tumor. Error bars; means ± s.e.m from all tumors present. P = ns.
(A) RNA-seq quantification of GNA11<sup>Q209L</sup> positive mouse cutaneous tumors for Rosa26- GNA11<sup>Q209L</sup> and endogenous Gnaq and Gna11.  (B) Representative IGV view of RNA-seq reads at Bapi for GNA11<sup>Q209L</sup> Bapi<sup>WT</sup> and GNA11<sup>Q209L</sup> Bapi<sup>KO</sup> tumors showing deletion of exons 6-12. (C) IGV view of RNA-seq reads at BAPI for UPMM3 showing frameshift deletion (*). (D) Immunoblot of BAP1 for UPMM3 cells EGFP, BAP1 WT, BAP1 C91W and BAP1 A95P. (E) Hierarchical clustering of differential gene expression upon introduction of EGFP, BAP1 WT, BAP1 C91W and BAP1 A95P in duplicate. (F) Representative cell cycle signature (WHITFIELD_CELL_CYCLE) and (G) metastatic signature (WINNEPENNINCKX_MELANOMA_METASTASIS_UP) from GSEA analysis of each of three BAPI datasets (mouse model, TCGA, and UPMM3 cell line).

Figure S5, related to Figure 3. Bap1 RNA-sequencing reads in GNA11<sup>Q209L</sup> tumors
Figure S6, related to Figure 5. *RASGRP3* expression in Affymetrix U133Plus2 datasets

(A) Gene expression of *RASGRP3* across tumor types from the GENT (Gene Expression across Normal and Tumor tissue) U133Plus2 microarray database. Outliers shown in red dots. Uveal melanoma (red) and cutaneous melanoma (blue).
Figure S7, related to Figure 6 & 7. Depletion of RasGRP3 in panel of uveal and cutaneous melanoma cell lines

(A) Protein levels of RasGRP3 at day 3 of shRNA mediated knockdown of growth curve shown in Figure 6A-B. (B) Competition assay of empty vector-GFP rescue in the presence of RasGRP3 shRNA-mediated depletion. Uveal and cutaneous melanoma cells stably expressing doxycycline-inducible scrambled shRNA (shSCR) or shRNA targeting RASGRP3 (shRASGRP3-1, shRASGRP3-2) in the presence of KRASG12V-GFP. Percentage of GFP analyzed via flow cytometry over time. (C) Protein levels of RasGRP3 and melanocyte makers (Trp2, C-Kit and Gp100) in transduced melan-a cells with GNAQ<sup>G209L</sup>, BRAF<sup>V600E</sup> and KRAS<sup>G12V</sup>. (D) Protein levels of RasGRP3 by immunoblot at upon addition of TPA to the media of GNAQ<sup>G209L</sup>, BRAF<sup>V600E</sup> and KRAS<sup>G12V</sup> cells for 1- and 3-weeks, respectively. (E) Protein levels of RasGRP3 by immunoblot at day 3 of shRNA mediated knockdown of Rasgrp3 corresponding to the growth curve shown in Figure 7A.
Table S2. Mouse genotyping primers and Quickchange primers. Related to Figures 1-3, 6-7.

| Genotype primer | Forward | Reverse | Additional |
|-----------------|---------|---------|------------|
| Tyr-Cre         | CAGGGTGTTATAAGCAATCCC | CCTGGAAATGCTTCTGTCCG | |
| Bap1 lox/lox    | CCACAACGGGTTCTTTCTGTT | ACTGCAAGGAATGCTGACCTG | GAAAAGGTCTGACCACAGATCA |
| BRAF CA         | TGAGTATTTTTTGGCAAACGTG | CTCTGCTGGAAAAGCGGC | |
| GNA11 Q290L     | CGTGGAAGAGGTGACCACC | GATCCACTTCCCTCGCTC | |
| Rosa26          | TCCCGACAAAAACCGAAAATC | AAGCACGTT TCCGACTTGAG | |

| Quikchange primer | Forward | Reverse |
|-------------------|---------|---------|
| BRAF V600E         | ccactccatgcagatctctgtagagcaaat | atgtggctagctacagagaaatctgatgg | |
| GNAQ Q209L         | cttctctctgacctagacccctacatga | tcgatgtaggctgaagagctagag | |

| BAP1 excision primer | Forward | Reverse |
|----------------------|---------|---------|
| Bap1 KO              | GCCACTGCATGGTATCTGGT | CAGGTGGCCTCCTCTACTCT | TCTTTCCGCCTACTGCGAC |
Table S3. shRNA sequences and RNAi Consortium ShRNA Library numbers. Related to Figures 6-7.

|                         | Sequence                                      | The RNAi Consortium ShRNA Library # |
|-------------------------|-----------------------------------------------|------------------------------------|
| Human shRASGRP3-1       | GCTGCAATGAATTTGATTAA                         | TRCN0000048113                     |
| Human shRASGRP3-2       | GCCTCAGTCATAGTCCATT                           | TRCN0000048114                     |
| Mouse shRasgrp3-1       | GCCTGCCTCTTTATTTGACCATA                      | TRCN0000022732                     |
| Mouse shRasgrp3-2       | GCTGGGTGGATGTTGTAGATA                        | TRCN0000022733                     |